ISSN 2150-5330 (online)

World Journal of *Gastrointestinal Pathophysiology*

World J Gastrointest Pathophysiol 2016 February 15; 7(1): 1-198

Published by Baishideng Publishing Group Inc

WJGP World Journal of Gastrointestinal Pathophysiology

A peer-reviewed, online, open-access journal of gastrointestinal pathophysiology

Editorial Board

2016-2019

The *World Journal of Gastrointestinal Pathophysiology* Editorial Board consists of 503 members, representing a team of worldwide experts in gastrointestinal pathophysiology. They are from 45 countries, including Argentina (2), Australia (13), Austria (2), Belgium (7), Brazil (9), Brunei Darussalam (1), Canada (21), China (30), Croatia (1), Czech Repoublic (2), Denmark (3), Egypt (1), Estonia (1), Finland (1), France (8), Germany (24), Greece (7), Hungary (5), India (12), Indonesia (1), Iran (2), Ireland (2), Israel (8), Italy (38), Japan (45), Lebanon (3), Malaysia (1), Mexico (2), Netherlands (7), Norway (1), Poland (4), Portugal (1), Romania (1), Russia (1), Singapore (4), South Korea (12), Spain (24), Sweden (11), Switzerland (3), Thailand (2), Turkey (7), Ukraine (1), United Kingdom (10), United States (161) and Venezuela (1).

Zhan-Ju Liu, *Shanghai*
Shi Liu, *Wuhan*
Zhen-Ning Wang, *Shenyang*
Xiao-Hong Wang, *Beijing*
Wei Wei, *Hefei*
Dong-Ping Xie, *Shanghai*
Wen-Xie Xu, *Shanghai*
Xiao Yang, *Beijing*
Hua Yang, *Chongqing*
Wei-Zhen Zhang, *Beijing*
Hua-Chuan Zheng, *Shenyang*
Min-Sheng Zhu, *Nanjing*
Jinxia Zhu, *Beijing*
Da-Ling Zhu, *Harbin*
Yong-Liang Zhu, *Hangzhou*

Croatia

Alen Protic, *Rijeka*

Czech Republic

Pavel Hladik, *Semily*
Martin Vokurka, *Prague*

Denmark

Lars Arendt-Nielsen, *Aalborg*
Frank V Schiodt, *Copenhagen*
Jonas Worsoe, *Aarhus*

Egypt

Mahmoud Aboelneen Khattab, *Minia*

Estonia

Enn Seppet, *Tartu*

Finland

Pauli Antero Puolakkainen, *Turku*

France

Bruno Bonaz, *Grenoble*
Pierre M Dechelotte, *Rouen*
Jean-Paul Lallès, *Saint-Gilles*
Charles-Henri Malbert, *Saint-Gilles*
Thierry Piche, *Nice*
Pascale Plaisancié, *Lyon*
Michelina Plateroti, *Lyon*
Véronique Vitton, *Marseille*

Germany

Hans G Beger, *Ulm*
Carsten Bergmann, *Ingelheim*
Elke Cario, *Essen*
Arno J Dormann, *Koln*
Nikolaus Gassler, *Aachen*
Andreas Geier, *Wuerzburg*
Werner Hartwig, *Heidelberg*
Marion Hewicker-Trautwein, *Hannover*
Jens Hoeppner, *Freiburg*
Tobias Keck, *Freiburg*
Jorg Kleeff, *Munich*
Peter Malfertheiner, *Magdeburg*
Oliver Mann, *Hamburg*
Christoph W Michalski, *Munich*
Andreas K Nussler, *Munich*
Christian Pehl, *Vilsbiburg*
Peter Schemmer, *Heidelberg*
Manuel A Silva, *Penzberg*
Marc Stemmler, *Freiburg*
Frank Tacke, *Aachen*
Sya N Ukena, *Braunschweig*
Brigitte Vollmar, *Rostock*
Thomas M Wex, *Magdeburg*
Margot Zoller, *Heidelberg*

Greece

Stelios F Assimakopoulos, *Patras*
George N Dalekos, *Larissa*
Alkiviadis Efthymiou, *thessaloniki*
Maria Gazouli, *Athens*
Ioannis E Koutroubakis, *Heraklion*
Gerassimos J Mantzaris, *Athens*
George V Papatheodoridis, *Athens*

Hungary

Mária Bagyánszki, *Szeged*
Mihály Boros, *Szeged*
Laszlo Czako, *Szeged*
Pal Miheller, *Budapest*
Zoltan Rakonczay, *Szeged*

India

Anil K Agarwal, *Delhi*
Uday Bandyopadhyay, *Kolkata*
Sriparna Basu, *Varanasi*
Chandra K Chakraborti, *Rourkela*
Nilesh M Dagia, *Mumbai*
Rajeev Garg, *Punjab*
Akhtar Mahmood, *Chandigarh*
Veerareddy P Reddy, *Andhra Pradesh*
Chandra P Sharma, *Thiruvananthapuram*
Shailesh V Shrikhande, *Mumbai*
Virendra Singh, *Chandigarh*
Nicholas J Skill, *Indianapolis*

Indonesia

Laurentius A Lesmana, *Jakarta*

Iran

Gholamreza Roshandel, *Gorgan*
Shahram Shahabi, *Urmia*

Ireland

Billy Bourke, *Dublin*
Stephen J Keely, *Dublin*

Israel

Yosefa Avraham, *Jerusalem*
Yaron Bar-Dayan, *Holon*
Shomron Ben-Horin, *Tel-Hashomer*
Boris Kirshtein, *Beer Sheva*
Stephen Malnick, *Rehovot*
Yaakov Maor, *Tel-Hashomer*
Rifaat Safadi, *Jerusalem*
Nachum Vaisman, *Tel Aviv*

Italy

Rosaria Acquaviva, *Catania*
Alessandro Antonelli, *Pisa*
Giacosa Attilio, *Genova*
Salavtore Auricchio, *Naples*
Guido Basilisco, *Milan*
Antonio Basoli, *Rome*
Massimo Bellini, *Pisa*
Luigi Bonavina, *Milano*
Alfio Brogna, *Catania*
Giuseppe Calamita, *Bari*
Raffaele Capasso, *Naples*
Ignazio Castagliuolo, *Padova*
Francesco Cresi, *Torino*
Rosario Cuomo, *Napoli*
Salvatore Cuzzocrea, *Gazzi*
Mario M D'Elios, *Florence*
Cinzia Domeneghini, *Milan*
Luca Elli, *Milano*
Walter Fries, *Messina*
Eugenio Gaudio, *Rome*
Marco Gobbetti, *Bari*
Fabio Grizzi, *Rozzano*
Enzo Grossi, *Milanese*
Enzo Ierardi, *Foggia*
Pietro Invernizzi, *Monza*
Angelo A Izzo, *Naples*
Anna Kohn, *Rome*
Giovanni Latella, *L'Aquila*
Massimo Marignani, *Rome*
Sergio Morini, *Rome*
Raffaele Pezzilli, *Bologna*
Cristiano Rumio, *Milan*
Giovanni Sarnelli, *Naples*
Edoardo V Savarino, *Padua*
Pierpaolo Sileri, *Roma*
Annamaria Staiano, *Naples*
Giacomo C Sturniolo, *Padua*
Claudio Tiribelli, *Trieste*

Japan

Hirotada Akiho, *Fukuoka*
Akihiro Asakawa, *Kagoshima*
Hisashi Aso, *Sendai*
Yasu-Taka Azuma, *Osaka*
Shotaro Enomoto, *Wakayama*
Mikihiro Fujiya, *Hokkaido*
Takahisa Furuta, *Hamamatsu*
Akira Hokama, *Nishihara*
Ryota Hokari, *Saitama*
Yuichi Hori, *Hyogo*
Masahiro Iizuka, *Akita*
Shoji Ikuo, *Kobe*
Motohiro Imano, *Osaka*

Hajime Isomoto, *Nagasaki*
Tatehiro Kagawa, *Isehara*
Haruki Kitazawa, *Sendai*
Shigeru BH Ko, *Tokyo*
Xiao-Kang Li, *Tokyo*
Noriaki Manabe, *Okayama*
Atsushi Masamune, *Sendai*
Hiroyuki Matsubayashi, *Suntogun*
Kazuyuki Matsushita, *Inohana Chuo-ku*
Reiko Miyazawa, *Gunma*
Kazunari Murakami, *Oita*
Hikaru Nagahara, *Tokyo*
Yuji Naito, *Kyoto*
Atsushi Nakajima, *Yokohama*
Shoji Natsugoe, *Kagoshima City*
Tsutomu Nishida, *Suita*
Koji Nomoto, *Tokyo*
Shouji Shimoyama, *Tokyo*
Goshi Shiota, *Yonago*
Hidekazu Suzuki, *Tokyo*
Hitoshi Takagi, *Gunma*
Toru Takahashi, *Okayama*
Yoshihisa Takahashi, *Tokyo*
Kan Uchiyama, *Chiba*
Yoshiyuki Ueno, *Sendai*
Takato Ueno, *Fukuoka*
Hisayuki Uneyama, *Kwasaki*
Mitsunori Yamakawa, *Yamagata*
Takayuki Yamamoto, *Mie*
Yutaka Yata, *Maebashi-city*
Naohisa Yoshida, *Kyoto*
Hitoshi Yoshiji, *Kashihara*

Lebanon

Costantine F Daher, *Byblos*
Assaad M Soweid, *Beirut*
Julnar Usta, *Beirut*

Malaysia

Andrew CS Boon, *Ipoh*

Mexico

José M de la Roca-Chiapas, *Leon*
Maria-Raquel Huerta-Franco, *Leon*

Netherland

Wouter J de Jonge, *Amsterdam*
Aldo Grefhorst, *Groningen*
Daniel Keszthelyi, *Maastricht*
Cornelis FM Sier, *ZA Leiden*
Pieter J Tanis, *AZ Amsterdam*
Luc JW van der Laan, *Rotterdam*
Sander van der Marel, *Leiden*

Norway

Anne M Bakke, *Oslo*

Poland

Stanistaw A Hac, *Gdansk*
Stanislaw J Kowalski, *Krakow*
Agata Mulak, *Wroclaw*
Napoleon Waszkiewicz, *Choroszcz*

Portugal

Ricardo Marcos, *Porto*

Romania

Mihai Ciocirlan, *Bucharest*

Russia

Ludmila Filaretova, *Petersburg*

Singapore

Madhav Bhatia, *Singapore*
Brian K Goh, *Singapore*
Khek-Yu Ho, *Singapore*
Cliff KS Ong, *Singapore*

South Korea

Jae Hee Cheon, *Seoul*
Myung-Haing Cho, *Seoul*
Ki-Baik Hahm, *Seongnam*
HO Jae Han, *Gwangju*
Chang-Duk Jun, *Gwangju*
Sang Geon Kim, *Seoul*
Hong Joo Kim, *Seoul*
Jin-Kyung Kim, *Gyeongsan-Si*
Won-Jae Lee, *Seoul*
Sung-Joo Park, *Iksan*
Seung Ha Park, *Gangwon Do*
Kwan-Kyu Park, *Daegu*

Spain

Raquel Abalo, *Madrid*
Juan G Abraldes, *Barcelona*
Dario Acuna-Castroviejo, *Armilla*
Agustin Albillos, *Madrid*
Maria-Angeles Aller, *Madrid*
Fernando Azpiroz, *Barcelona*
Ramón Bataller, *Barcelona*
Marco Bustamante-Balén, *Valencia*
Andres Cardenas, *Barcelona*
Joan Claria, *Barcelona*
Pere Clavé, *Barcelona*
Manuel B de Acosta, *Santiago*
Manuel Giner, *Madrid*
Borja Hernandez-Breijo, *Madrid*
Angel I Lanas, *Zaragoza*
Maite T Martin, *Barcelona*
Vicente Martinez, *Bellaterra*
Jose M Mates, *Malaga*
Julio Mayol, *Madrid*
Marcal Pastor-Anglada, *Barcelona*
María E Sáez, *Seville*
Yolanda Sanz, *Burjassot*
Carlos Taxonera, *Madrid*
Maria D Yago, *Granada*

Sweden

Marco Del Chiaro, *Stockholm*
Frida Fak, *Gothenburg*
Gunnar FA Flemstrom, *Uppsala*
Evangelos Kalaitzakis, *Gothenburg*
Kristina Lamas, *Umea*
Bob R Olsson, *Goteborg*
Sara M Regnér, *Malmo*
Peter T Schmidt, *Stockholm*
Xiao-Feng Sun, *Linkoping*
Henrik Thorlacius, *Malmo*
Curt Tysk, *Orebro*

Switzerland

Jyrki J Eloranta, *Zurich*
Remy Meier, *Liestal*
Catherine M Pastor, *Geneva*

Thailand

Thawatchai Akaraviputh, *Bangkok*
Weekitt Kittisupamongkol, *Bangkok*

Turkey

Mehmet Bektas, *Ankara*
Ugur Duman, *Bursa*
Mukaddes Esrefoglu, *Istanbul*
Ahmet Guven, *Ankara*
Muammer Karadeniz, *Bornova-Izmir*
Elvan Ozbek, *Sakarya*
Ilhami Yuksel, *Ankara*

Ukraine

Oksana S Zayavhkivska, *Lviv*

United Kingdom

Geoffrey Burnstock, *London*
Janice E Drew, *Aberdeen*
Girish L Gupte, *Birmingham*
David C Hay, *Edinburgh*
Nusrat Husain, *Manchester*
Michael L Lucas, *Glasgow*
Jamie Murphy, *London*
Vadim Sumbayev, *Chatham Maritime*
Wing-Kin Syn, *Birmingham*
Andrea Varro, *Liverpool*

United States

Sami R Achem, *Florida*
Tauseef Ali, *Oklahoma City*
Shrikant Anant, *Oklahoma*
Mohammed S Anwer, *North Grafton*
Andrew Aronsohn, *Chicago*
Toms Augustin, *Sayre*

WJGP

World Journal of Gastrointestinal Pathophysiology

Contents

Quarterly Volume 7 Number 1 February 15, 2016

EDITORIAL

DIAGNOSTIC ADVANCES

REVIEW

MINIREVIEWS

Contents

World Journal of Gastrointestinal Pathophysiology
Volume 7 Number 1 February 15, 2016

World Journal of Gastrointestinal Pathophysiology
Volume 7 Number 1 February 15, 2016

Contents

NAME OF JOURNAL
World Journal of Gastrointestinal Pathophysiology

ISSN
ISSN 2150-5330 (online)

LAUNCH DATE
April 15, 2010

Frequency
Quarterly

Room 903, Building D, Ocean International Center,
No. 62 Dongsihuan Zhonglu, Chaoyang District,
Beijing 100025, China
Telephone: +86-10-85381891
Fax: +86-10-85381893
E-mail: editorialoffice@wjgnet.com
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
http://www.wjgnet.com

PUBLISHER
Baishideng Publishing Group Inc
8226 Regency Drive,
Pleasanton, CA 94588, USA
Telephone: +1-925-223-8242
Fax: +1-925-223-8243
E-mail: bpgoffice@wjgnet.com
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
http://www.wjgnet.com

PUBLICATION DATE
February 15, 2016

World Journal of Gastrointestinal Pathophysiology

Submit a Manuscript: http://www.wjgnet.com/esps/
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.4291/wjgp.v7.i1.1

World J Gastrointest Pathophysiol 2016 February 15; 7(1): 1-16
ISSN 2150-5330 (online)

EDITORIAL

Mucosal healing in inflammatory bowel disease: Maintain or de-escalate therapy

Marcello Cintolo, Giuseppe Costantino, Socrate Pallio, Walter Fries

Marcello Cintolo, Giuseppe Costantino, Walter Fries, Clinical Unit for Chronic Bowel Disorders, Department of Clinical and Experimental Medicine, University of Messina, 98125 Messina, Italy

Socrate Pallio, Digestive Endoscopy Unit, Department of Human Pathology, University of Messina, 98125 Messina, Italy

Author contributions: Cintolo M and Fries W drafted and wrote the manuscript; Costantino G and Pallio S critically reviewed the manuscript; all authors approved the final version of the article.

Conflict-of-interest statement: Marcello Cintolo, Giuseppe Costantino and Socrate Pallio have no conflict of interests to declare. Walter Fries, MSD, AbbVie, Mundipharma advisory board; Zambon, Mundipharma: speaker fees; MSD unrestricted research grant.

Correspondence to: Walter Fries, MD, Clinical Unit for Chronic Bowel Disorders, Department of Clinical and Experimental Medicine, University of Messina, Via Consolare Valeria, 1, 98125 Messina, Italy. fwalter@unime.it
Telephone: +39-090-2212380
Fax: +39-090-2213538

Received: July 29, 2015
Peer-review started: August 24, 2015
First decision: October 13, 2015
Revised: November 16, 2015
Accepted: December 7, 2015
Article in press: December 8, 2015
Published online: February 15, 2016

Abstract

In the past decade, thanks to the introduction of biologic therapies, a new therapeutic goal, mucosal healing (MH), has been introduced. MH is the expression of an arrest of disease progression, resulting in minor hospitalizations, surgeries, and prolonged clinical remission. MH may be achieved with several therapeutic strategies reaching success rates up to 80% for both, ulcerative colitis (UC) and Crohn's disease (CD). Various scoring systems for UC and for the transmural CD, have been proposed to standardize the definition of MH. Several attempts have been undertaken to de-escalate therapy once MH is achieved, thus, reducing the risk of adverse events. In this review, we analysed the available studies regarding the achievement of MH and the subsequent treatment de-escalation according to disease type and administered therapy, together with non-invasive markers proposed as predictors for relapse. The available data are not encouraging since de-escalation after the achievement of MH is followed by a high number of clinical relapses reaching up to 50% within one year. Unclear is also another question, in case of combination therapies, which drug is more appropriate to stop, in order to guarantee a durable remission. Predictors of unfavourable outcome such as disease extension, perianal disease, or early onset disease appear to be inadequate to foresee behaviour of disease. Further studies are warranted to investigate the role of histologic healing for the further course of disease.

Key words: De-escalation; Mucosal healing; Biological therapy; Deep remission; Discontinuation; Ulcerative colitis; Crohn's disease; Immunosuppressors

Core tip: Mucosal healing is achieved in a discrete number of patients with immunomodulators, biologics

or combined therapies. Attempts to de-escalate therapy, thus permitting a drug holiday, are disappointing. Clinical predictors to identify patients at risk for early relapse after drug withdrawal are still insufficient. Further investigations are needed to prospectively evaluate the validity of histologic healing and to validate an appropriate scoring system for histology in ulcerative colitis and in Crohn's disease.

Cintolo M, Costantino G, Pallio S, Fries W. Mucosal healing in inflammatory bowel disease: Maintain or de-escalate therapy. *World J Gastrointest Pathophysiol* 2016; 7(1): 1-16 Available from: URL: http://www.wjgnet.com/2150-5330/full/v7/i1/1.htm DOI: http://dx.doi.org/10.4291/wjgp.v7.i1.1

INTRODUCTION

Inflammatory bowel diseases (IBD) are a group of diseases of growing importance in the Western world, due to the steady increase in terms of incidence and prevalence[1]. IBD are characterized by gut mucosal inflammation and a chronic relapsing behaviour[2], therefore, it is necessary to ensure a long-term therapeutic strategy for patients, avoiding surgery, and achieving a good level of quality of life[3].

In the last few years, the goals of therapy have changed: Thanks to the introduction of anti-TNFα drugs, in monotherapy or in combination with immunomodulators, there are higher rates of response, also in more complicated cases of both ulcerative colitis (UC) and Crohn's disease (CD). Thus, the target of therapy has become not only clinical remission but also mucosal healing (MH), *i.e.*, "deep remission". According to available evidence, deep remission is associated with less flares, lower surgery rates and less hospitalizations[4].

Although the role of these drugs in achieving clinical remission and MH has been recognized, their prolonged use, above all when in combination with immunomodulators, exposes the patient to a higher risk of infection and adverse events[5] especially with increasing patient's age or in the presence of comorbidities[6]. Since IBD are lifelong diseases, it becomes important to spare years of immunosuppressive therapy for patients, whenever possible, without the risk of undertreatment, thus minimizing the risk of infection or malignancies[7]. While on the one hand, the importance of reaching endoscopic remission is now generally accepted, on the other hand, there are no clear indications in current guidelines regarding how long to continue immunosuppressive therapies after reaching MH and, in the case of therapy reduction, which drugs to use as maintenance therapy[8,9].

ENDOSCOPIC SCORES AND DEFINITION OF MH

Several endoscopic scores are available to assess disease activity in UC and CD. For CD, the first endoscopic score was CD Endoscopic Index of Severity (CDEIS), a score based on the evaluation of: (1) Presence and absence of ulcers (superficial or deep); (2) presence or absence of stenosis (ulcerated or non ulcerated); and (3) measurement of the surface extension of disease activity, evaluating five intestinal segments (terminal ileum, right, transverse, and left colon and rectum) with a final numerical rating between 0 and 44[10]. The newer Simple Endoscopic Score-CD (SES-CD) was created subsequently by Daperno *et al*[11]; this score is obtained by evaluating: (1) The surface affected by ulcers; (2) the surface affected by other lesions; (3) the presence of ulcers; and (4) the presence of narrowing in five gut segments (terminal ileum, right colon, transverse, left colon and rectum). Each variable can be quantified with a score from 0 to 3, reaching a final score between 0 and 60. Finally, for the assessment of recurrence of disease after resective surgery, the Rutgeerts score is used; this score is based on a rating between 0 and 4; i0: No recurrence, i1: < 5 aphtous lesions, i2: > 5 severe aphtous lesions, i3: Diffuse inflammation with diffuse ulcers, i4: Nodules and/or narrowing[12].

In UC, several scores have been proposed; the Truelove and Witts score evaluated just hyperemia and granularity[13]. Subsequently, the Baron score, based on bleeding and friability, with a range from 0 to 4, was developed[14]. The Sutherland score and the Powell-Tuck score are both sigmoidoscopic scores and consider only bleeding features[15,16]. The first score to assess not only bleeding and hyperaemia but also ulcers, granularity and erosions was the Rachmilewitz Endoscopic score, based on the evaluation of bleeding (by contact or spontaneous), mucosal disease (ulcers, erosions and presence of mucus), granularity and vascular pattern[17]. Severity is assessed with a range from 0 to 12.

The Mayo score is currently the most used score to evaluate clinical activity in UC, and consists of four subscores: (1) Stool frequency; (2) rectal bleeding; (3) endoscopic findings; and (4) physician's global assessment. Each one of these subscores ranges from 0 to 3, arriving at a final score between 0 and 12 (⩽ 2 remission, 3-5 mild disease, 6-10 moderate disease, 11-12 severe disease)[18]. The endoscopic subscore divides the endoscopic findings into four degrees of severity: 0 remission; 1 mild disease with erythema and mild friability; 2 moderate disease with presence of marked erythema, friability, erosions and absence of vascular pattern; 3 severe disease with spontaneous bleeding and diffuse ulcerations. A recent endoscopic score, is the UC Endoscopic Index of Severity (UCEIS); this score evaluates three variables: Vascular pattern, ranged with a score between 0 and 3 points, while bleeding and presence of erosions/ulcers (divided into superficial and deep) are evaluated in a range between 0 and 4 points. Compared with the Mayo score, this score better evaluates the depth of the ulcers, but it is not yet widely used[19].

In 2013, a new score, the UC Colonoscopy Index of

Severity was validated; it is calculated on four variables: Vascular pattern, granularity, ulceration, bleeding/friability and severity of damage in each colon segment. The evaluation of damage is based on a four-points scale and on a 10 cm-visual scale[20].

The major drawback of all UC scores is that they do not consider the extension of disease since they are all based on the worst appearing segment explored by endoscopy and only the most recent modified Mayo endoscopic score seems to overcome this issue[21]. In CD, endoscopic scores are limited to the appearance of gut mucosa in a transmural disease.

DEFINITION OF MH

MH is now defined, in most of the more recent UC and CD trials, as the complete absence of ulcers and inflammatory lesions (Mayo score 0, SES-CD 0, CDEIS 0). Nevertheless, in many UC trials, the definition of MH includes the presence of friability and hyperaemia at endoscopic examination, without ulcers and erosions (Mayo score 1).

The most important controversies about MH regard the weight of mucosal remission assessed by endoscopic examination in CD; many authors consider this an inadequate parameter to evaluate a progressive, full thickness disease of the bowel wall, characterized during its natural history by the presence of fistulas, strictures, abscesses and surgical resections. These features are well evaluated by the Lémann score, recently created to combine the characteristics already considered by previous endoscopic scores, with the new concept of "cumulative bowel damage"[22]. The Lémann score combines upper endoscopy and ileocolonoscopy findings with the radiological findings obtained by computed tomography enterography and magnetic resonance enterography (MRE). This score, for each gastrointestinal segment (divided into upper digestive tract, small bowel, colon or rectum and anal or perianal), ranges between 0 and 3 according to severity of the disease[23]. The overall score is obtained by adding the above subscores up to 10 points. The Lémann score, however, is still rarely used, for its complexity and poor practicality.

MRE today represents the gold standard technique to study the small bowel; it assesses wall thickness, presence of edema, deep ulcers and/or strictures, together with the evaluation of surrounding tissues, with high accuracy. In 2011, the Magnetic Resonance Index of Activity (MaRia) score was proposed; this score measures all the above parameters and is calculated according to the formula MaRia = [1.5 × wall thickness (mm)] + (0.02 × relative contrast enhancement) + (5 × edema) + (10 × ulcer). In a prospective, multicentre study, Ordás *et al*[24] demonstrated that the MaRia score well correlates with CDEIS and with endoscopic findings. MRE is a useful tool to assess disease activity in CD and may be a good alternative to endoscopy in clinical practice and trials.

NON-INVASIVE BIOMARKERS OF INFLAMMATION

Several biomarkers have been studied in the last few years, to find an inexpensive and non-invasive way to assess the presence or absence of gut mucosal inflammation and, thus for the follow-up in IBD patients. A Norwegian group recently reported a significantly higher mucosal gene expression of TNF, IL17A and FOXP3 in CD patients who relapsed within six months after anti-TNF withdrawal[25]. The dimeric isoform of M2-pyruvate kinase (M2-PK) was elevated in IBD patients, both in active and in inactive disease[26]. This latter marker was even higher in faeces of pediatric IBD patients with a good response to corticosteroids[27], and elevated serum and mucosal levels of the long pentraxin (PTX3) were found in patients with active UC[28]. A complete revision of every marker investigated at this moment is however beyond the scope of this editorial (for review see[29,30]). In the present review we focus on two well-known and largely used biomarkers, C-reactive protein (CRP) and fecal calprotectin (FC), and on the neutrophil gelatinase-associated lipocalin (NGAL) and matrix metalloproteinase 9 (NGAL-MMP-9) complex, a novel marker of mucosal inflammation, recently investigated in CD and UC.

CRP

CRP is an acute-phase protein with a circular, pentameric conformation synthesized by the liver; its serum levels increase in response to inflammation and in particular to IL-6 secretion; its physiological role is to bind lysophosphatidylcholine present on the surface of dying cells or bacteria, to activate the complement system[31]. Blood CRP levels rise in several cases like infections, sepsis, inflammation, neoplastic processes, cardiovascular diseases and infarction.

The role of CRP in the diagnosis and follow-up of IBD is well known. Determination is non-invasive and cheap, yet few studies have confirmed its reliability in the assessment of mucosal inflammation, mainly because of its poor specificity and the fact that a percentage of around 25% of patients with CD, and up to 80% with distal UC, do not have a CRP-positive inflammatory response[32,33]. CRP seems to be less reliable in reflecting endoscopic inflammation, compared to stool markers, like calprotectin or lactoferrin, while the combination of stool markers, CRP and the clinical scores, can improve the diagnostic accuracy, especially in UC[34,35].

FC

FC is a heterodimer composed of two subunits, S100A8 and S100A9, representing almost 60% of neutrophilic cytosolic soluble proteins; it can bind calcium and *in vitro* it showed mild antifungal and bacteriostatic activity. It is released by neutrophils during their activation or death and, being highly represented in the luminal side of the enterocytes, it is easily measurable in faeces. Measurement correlates with gut inflammatory activity

with good accuracy, and several studies have shown a significantly higher level of FC in subjects with IBD compared to normal controls[36].

To date, FC is considered a useful tool in the IBD diagnostic work-up, with a sensitivity of 95%-100% and a specificity of 35%-50%, according to different studies[35,37]. However, considering adjusted cut-offs, FC specificity increased, especially compared to other non-invasive markers, like polymorphonuclear-elastase or lactoferrin, though the latter has been proven to have slightly higher sensibility in UC[35]. In clinical practice, FC is increasingly used also in the follow-up of IBDs, to guide clinical and therapeutic choices, such as optimization or discontinuation of treatment[37].

In former studies, FC has proven to have a good correlation with endoscopic findings and scores, both in UC[35] and CD[38], and in a very recent paper, a cut-off level of 192 mg/kg of FC identified patients with MH assessed by the Mayo endoscopic subscore and UCEIS with negative predictive values of 0.90 and 0.93, respectively. Moreover, a cut-off level of 171 mg/kg identified patients with histological healing[39].

NGAL-MMP-9 complex

MMP-9 is a zinc-dependent peptidase, belonging to the bigger family of MMPs, involved in the degradation of extracellular matrix, in angiogenesis, in remodelling of tissues, and wound healing. MMP activity is regulated by tissue inhibitors of metalloproteinases that bind MMPs in order to balance the process of matrix degradation and synthesis. Another protein involved in this process is NGAL, mostly contained in secondary granules of neutrophils. This marker, measured in the urine, has been shown to promptly respond to Infliximab (IFX) infusion[40]. MMP-9 and NGAL blood levels are both increased in active IBDs. Recent studies have assessed that NGAL binds MMP-9 to avoid degradation of the latter. A dosage of NGAL-MMP-9 complex has been reported to be a sensitive marker of MH. In a recent study, serum NGAL-MMP-9 complex was measured in UC patients before and after treatment with IFX; at the endoscopic check, MH was defined as Mayo 1 or Mayo 0 endoscopic subscore. The serum NGAL-MMP-9 complex was higher in UC patients in comparison to healthy controls; a cut-off level of 97.7 ng/mL identified patients with MH at endoscopy[41]. Similar findings have now been reported also in CD[42].

HOW TO ACHIEVE MH

Almost every kind of therapy has been described to achieve MH and the choice of treatment depends on the severity of the disease. In the classical step-up model of therapy, the first choice is mesalazine (limited to UC) followed by low bioavailability steroids, systemic steroids, immunomodulators and, finally, biologics. We hereafter briefly review the available data on treatment success in terms of MH with the different therapies in UC and in CD.

UC

Salicylates

Although most studies concerning mesalamine (5-aminosalicylic acid, 5-ASA) had been carried out before the introduction of the new paradigm of MH, there are several studies that evaluated efficacy of 5-ASA or newer formulations to induce MH. Vecchi *et al*[43], comparing oral 5-ASA 4 g daily *vs* oral 5-ASA 2 g + 2 g daily + enema in UC patients, demonstrated the achievement of MH, respectively in 58% and 71% of patients at week 6, assessed by the Rachmilewitz score (Table 1). Mansfield *et al*[44] compared Balsalazide 6.75 g/d *vs* Sulfasalazine 3 g/d; at week 8, MH rate was similar in both groups of UC patients, 27% and 25%.

In 2003, Kruis *et al*[45] compared the efficacy in UC of three different doses of oral 5-ASA: 0.5 g t.i.d., 1 g t.i.d. and 1.5 g t.i.d.; MH was achieved respectively in 53%, 84% and 70% of patients, assessed at week 8 by the Rachmilewitz score and considering as MH an improvement of the Histological Activity Index (HAI; a score that assesses the degree of mucosal inflammation, ranged between 0 and 3) of at least one point. In 2009, the same author investigated the use of fractionated doses of oral 5-ASA (1 g t.i.d.), with the administration of a single dose (3 g once/day), finding no difference in terms of MH between the two groups of patients (respectively 71% *vs* 70%)[46]. In the ASCEND I study, Hanauer *et al*[47] evaluated two doses of oral delayed-release 5-ASA in mild to moderate UC: 4.8 g/d *vs* 2.4 g/d. Efficacy was assessed by Inflammatory Bowel Disease Questionnaire and by patient's global assessment (PGA) a four-point score, based on stool frequency, rectal bleeding, endoscopic findings, patient's functional assessment and the investigator's clinical assessment. At sigmoidoscopy performed at week 3 and 6, no differences were found between the two groups in terms of MH. Nevertheless, considering only the subgroup of patients with moderate disease, PGA and sigmoidoscopy scores improved significantly in the group on 4.8 g/d compared with 2.4 g/d (84% *vs* 67%). In another trial with 5-ASA multi matrix system (MMX), Kamm *et al*[48] compared 5-ASA MMX 4.8 g/d, 2.4 g/d and placebo; an endoscopic follow-up, scheduled at week 8, showed the achievement of MH, defined by a modified Sutherland score < 1, in 77%, 69% and 46% of the patients, respectively.

Steroids

Most trials using corticosteroids have clinical outcomes; this is due to the fact that only recently greater emphasis has been put on mucosal and histological healing.

In 2011, Ardizzone *et al*[49] published a prospective study, based on the observation of 157 patients with moderate to severe UC, needing their first systemic steroid course (40 mg to 60 mg of oral prednisone or parenteral methylprednisolone) within 12 mo from diagnosis. The endoscopic check, scheduled at month

Table 1 Studies concerning the achievement of mucosal healing

Ref.	Design	No. of patients	Drugs (dose)	Time of endoscopy	Endoscopic index	Definition of MH	Results
5-ASA							
Vecchi *et al*[43]	RCT, mc	130 UC	5-ASA 4 g p.o. *vs* 2 + 2 g and enema	6 wk	Rachmilewitz	< 4	58% *vs* 71%
Mansfield *et al*[44]	RCT, db, mc	50 UC	Balsalazide 6.75 g *vs* SASP 3 g	8 wk	4 point score	Score 0	27% *vs* 25%
Kruis *et al*[45]	RCT, db, mc	321 UC	5-ASA 0.5 g × 3 *vs* 1 g × 3 *vs* 1.5 g × 3	8 wk	Rachmilewitz	Histology improvement	53% *vs* 84% *vs* 70%
Hanauer *et al*[47] ASCEND 1 and 2	RCT, db, mc	391 UC	Asacol 4.8 g *vs* 2.4 g	6 wk	No score	Normal endoscopy	84% *vs* 67% (in moderate UC)
Kamm *et al*[48] MMX	RCT, db, mc	343 UC	Mesalamine MMX 4.8 g *vs* 2.4 g *vs* plc	8 wk	Mod. Sutherland index	< 1	77% *vs* 69% *vs* 46%
Kruis *et al*[46]	RCT, db, mc	380 UC	5-ASA 3 g *vs* 1 g × 3	8 wk	Rachmilewitz	< 4	71% *vs* 70%
Steroids							
Ardizzone *et al*[49]	RA, sc	157 UC	Systemic steroids 40-60 mg	3 mo	Mod. Baron score	Score 0	38%
Sandborn *et al*[50] CORE	RTC, db, mc	672 UC	Budesonide MMX 9 mg *vs* 6 mg *vs* plc	8 wk	UCDAI mucosal appearance	0	27% *vs* 16% *vs* 17%
Van Assche *et al*[51]	RTC, db, mc	282 UC	BDP 5 mg/d *vs* PD 40 mg/d (tap.)	4 wk	DAI subscore	0	23% *vs* 21%
Immunomodulators							
D'Haens *et al*[60]	PA, sc	15 CD	AZA 2 mg/kg	26 wk	Rutgeerts score	Ri 0	40%
Ardizzone *et al*[52]	RCT, sc	72 UC	5-ASA *vs* AZA	3 mo and 6 mo	Baron score	Improving mean Baron index	At 3 mo: 2.3 *vs* 1.1 at 6 mo: 2.2. *vs* 0.9
Mantzaris *et al*[61]	RCT, sc	57 CD	AZA 2-2.5 mg/kg *vs* budesonide 6-9 mg	52 wk	CDEIS	CDEIS < 4	83% *vs* 24%
Laharie *et al*[62]	RTC, sc	51 CD	MTX 15-25 mg/wk *vs* AZA 2-3 mg/kg *vs* IFX 5 mg/kg		CDEIS	CDEIS < 4	11% *vs* 50% *vs* 60%
Rispo *et al*[53]	PA, sc	104 UC	AZA or 6-MP	104 wk	Mayo	Mayo 0-1	36%
Biologics							
Colombel *et al*[63] SONIC	RCT, db, mc	508 CD	AZA 2.5 mg/kg *vs* IFX 5 mg/kg *vs* AZA 2.5 mg/kg + IFX 5 mg/kg	26 wk	No score	Absence of ulcers	16% *vs* 30% *vs* 44%
Reinisch *et al*[54] ULTRA 1	RCT, db, mc	390 UC	ADA 160/80/40 mg *vs* 80/40 mg *vs* plc	8 wk	Mayo	Mayo 0-1	47% *vs* 37% *vs* 41%
Sandborn *et al*[55] ULTRA 2	RCT, db, mc	518 UC	ADA 160/80/40 mg *vs* plc	8 wk and 52 wk	Mayo	Mayo 0-1	18% *vs* 10% (Sustained MH)
Rutgeerts *et al*[64] EXTEND	RCT, db, mc	135 CD	ADA only induction (plc in maintenance) *vs* ADA continuous	12 wk and 52 wk	CDEIS	CDEIS 0	Baseline CDEIS ⩽ 9: Continuous at 12 wk 40%, at 52 wk 30%. Baseline CDEIS < 9: Continuous at 12 wks 16% at 52 wk 19%
Laharie *et al*[58]	RA, mc	63 UC	IFX 5 mg/kg	6-52 wk	Mayo	Mayo 0-1	48%
Feagan *et al*[56] GEMINI	RCT, db, mc	746 UC	Vedolizumab every 8 wk *vs* Vedolizumab every 4 wk *vs* plc	6 wk and 52 wk	Mayo	Mayo 0-1	6 wk: VDZ 41% *vs* placebo 24%; 52 wk: 56% *vs* 51% *vs* 20%
Sandborn *et al*[59] PURSUIT	RCT, db, mc	774 UC	Golimumab 400/200 mg *vs* 200/100 mg *vs* plc	6 wk	Mayo	Mayo 0-1	17.9% *vs* 17.8% *vs* 6.4%

RCT: Randomized controlled trial; MH: Mucosal healing; 5-ASA: 5-aminosalicylic acid; mc: Multicenter; db: Double-blind; sc: Single-centre; RA: Retrospective analysis; AZA: Azathioprine; CDEIS: Crohn's Disease Endoscopic Index of Severity; UC: Ulcerative colitis; CD: Crohn's disease; MMX: Multi Matrix System; IFX: Infliximab; MTX: Methotrexate; PA: Prospective analysis; BDF: Beclomethasone dipropionate.

3, showed MH in 38% of patients assessed by the modified Baron score.

Sandborn *et al*[50] compared the use of Budesonide MMX 9 mg/d, 6 mg/d and placebo in 672 UC patients; at week 8, considering a UCDAI score of 0 for MH, 27% of patients achieved MH with Budesonide MMX 9 mg/d. No differences were found between patients on Budesonide MMX 6 mg/d and placebo[50].

Van Assche *et al*[51] have recently realized a trial, randomizing 282 UC patients, to receive beclomethasone dipropionate (BDP)-prolonged release tablets 5 mg once daily for 4 wk, and then on alternate days for an additional 4 wk, or oral prednisone (PD) 40 mg once daily tapered by 10 mg every 2 wk during the 8 wk of observation. After 4 wk of treatment, the two cohorts of patients, BDP and PD, shows similar rates of endoscopic remission, respectively of 23% and 21%, while 45% and 60% showed mild mucosal activity. No statistical differences were reported between the two groups in terms of MH[51].

Immunomodulators

Ardizzone *et al*[52] compared the use of 5-ASA *vs* azathioprine (AZA) in 72 steroid-dependent UC patients, with a follow-up at 3 and 6 mo. They showed a highly significant superior mean Baron index in patients treated with 5-ASA compared to those on AZA, both at month 3 (2.3 *vs* 1.1) and at month 6 (2.2 *vs* 0.9)[52].

In a recent Italian study, analyzing prospective data from 205 steroid-dependent IBD patients who received a 2-year maintenance treatment with thiopurines (AZA or 6-mercaptopurine, 6-MP), good MH rates were seen, particularly in UC compared to CD: 36% *vs* 16%[53].

Biological treatments

At the beginning of this decade, many trials regarding the use of biological drugs, both in UC and CD, were published and most of them had MH as primary outcome in accordance with the latest knowledge on the importance of achieving this aim, in terms of maintenance of clinical remission, reduction of hospitalization and improvement of quality of life.

In 2011, a large trial, ULTRA 1, was published on the use of Adalimumab (ADA) in UC. In this study, Reinisch *et al*[54] compared three different schedules of induction: The first group of patients received ADA 160 mg/80/40 mg at weeks 0, 2, 4 and 6. The second group of patients was randomized to a second induction protocol with subcutaneous ADA 80/40 mg at weeks 0, 2 and thereafter every 2 wk. The last group of patients was randomized to placebo. At week 8, a colonoscopy was performed: MH (defined by a Mayo score of 0-1) was achieved in almost 47% of the first group of patients, compared to 37% of the second group, and 41% of the placebo group. This unusual data was not statistically nor clinically significant. One year later, Sandborn *et al*[55], in the ULTRA 2 trial, randomized 518 UC patients to subcutaneous ADA 40 mg/every 2 wk (induction with 160/80/40 mg) and to placebo. Endoscopy control was performed at weeks 8, 32 and 52. The percentage of patients who maintained a sustained MH (Mayo 0-1) at all endoscopic checks was about 18% compared to 10% for the placebo group.

In 2012, Feagan *et al*[56] published the results of the GEMINI 1 trial, a very large study involving 746 UC patients. During the induction phase, 225 patients received Vedolizumab at a dose of 300 mg, or placebo, intravenously at weeks 0 and 2; 521 patients received open-label Vedolizumab at weeks 0 and 2. In the maintenance phase, Vedolizumab was administered every 8 wk or every 4 wk. MH was achieved at week 6 in 41% of patients who received Vedolizumab compared to 24% of patients who received placebo. At week 52, MH was achieved in 56% of patients who received Vedolizumab every 4 wk, in 51% of patients who received Vedolizumab every 8 wk, and in only 20% of patients who received placebo.

The same study for CD patients (GEMINI 2) considered only clinical outcome and did not include endoscopic assessment[57].

A small French study on UC patients reported an MH rate of 48% in subjects treated with IFX, with an endoscopic check carried out between 6 and 52 wk after treatment start[58].

Sandborn *et al*[59], in the PURSUIT trial, investigated the efficacy of Golimumab with three different induction protocols in 774 patients with moderate-to-severe UC. At weeks 0 and 2, the first cohort received placebo, the second cohort received subcutaneous Golimumab at a dose of 200 mg/100 mg, and the third received subcutaneous Golimumab at a dose of 400 mg/200 mg; at the 6th week, MH, defined by the endoscopic Mayo subscore of 0-1, was achieved respectively in 6%, 17.8% and 17.9% of patients[59].

CD

Immunomodulators

In 1997, D'Haens *et al*[60] evaluated the use of AZA (at a dose of 2 mg/kg per day) in CD patients who underwent surgery and subsequently developed severe recurrences; at endoscopy, scheduled at week 26, they showed MH in 40% of cases, rated by the Rutgeerts score equal to i0.

Mantzaris *et al*[61] compared the use of AZA 2-2.5 mg/kg per day to Budesonide 6-9 mg/d in CD patients; at endoscopic check at week 52, 83% of patients on AZA achieved MH compared with 24% of patients on Budesonide. MH was defined by a CDEIS score < 4.

In another recent trial on CD patients, Laharie *et al*[62] evaluated methotrexate (MTX) 15-25 mg/wk, AZA 2-3 mg/kg per day, and IFX 5 mg/kg. The endoscopic control, performed according to clinical needs (median follow-up 13.2 mo), showed achievement of MH in 11%, 50% and 60% of patients respectively; MH corresponded to a CDAI score of less than 4.

Biological treatment

In 2010, the SONIC trial[63] compared treatment with AZA, IFX and combination therapy (IFX plus AZA) in 508 patients with CD, naive both to biologics and immunomodulators. The first group of patients was randomized to AZA 2.5 mg/kg per day, the second group to intravenous IFX 5 mg/kg per day and the third group to a combination therapy with intravenous IFX 5 mg/kg and AZA 2.5 mg/kg. The results in terms of MH were clear: The combination therapy was more effective than others in inducing MH (defined by the absence of mucosal ulceration), achieving it in 44% of patients at the 26th week, compared to 30% of patients treated with only IFX and 16% of patients treated with only immunosuppressor. In 2012, the results from the EXTEND trial[64] were published; in this study, 129 patients with CD were divided into two groups: The first group was randomized to subcutaneous ADA only during induction and placebo for maintenance, the second group to a maintenance treatment with ADA. Endoscopic checks were performed at baseline and at weeks 12 and 52. The results were stratified according to the baseline

CDEIS in two groups: Patients with baseline CDEIS ⩽ 9 and patients with baseline CDEIS > 9. Patients with ADA maintenance achieved MH (considered as CDEIS 0) at week 12 in 40% (CDEIS ⩽ 9) and 16% (CDEIS > 9); the MH rate dropped to 30% (CDEIS ⩽ 9) and 19% (CDEIS > 9) at week 52. The second group of patients (ADA induction only) achieved MH at the 12th week in 13% (CDEIS ⩽ 9) and 14% (CDEIS > 9); at the 52nd week no patient maintained MH.

WHAT TO DO AFTER ACHIEVING CLINICAL REMISSION OR MH?

No clear indications are available regarding the correct timing of drug withdrawal in IBD. Excluding studies on clinical remission achieved by 5-ASA monotherapy and subsequent dose reduction or withdrawal, the following studies investigated the relapse rate starting from patients in clinical remission, with or without endoscopic assessment.

THIOPURINE DISCONTINUATION

In 2002, a large retrospective study, (Fraser *et al*[65]), analyzed 346 UC patients and 272 CD patients in treatment with AZA; during the observation period, 517 of these patients needed to stop AZA for several reasons (clinical remission or side effects) (Table 2). Authors compared the cumulative remission rate over the years in those who continued and in those who discontinued: At the first year, 95% who continued remained in clinical remission (defined as Harvey Bradshaw Index, HBI < 4) *vs* 63% of patients who stopped AZA; at the second year, the remission rate was respectively 90% *vs* 44%, at the third 69% *vs* 34% and 62% *vs* 25%, at the fifth year. Predictors for a lower risk of relapse, while the patients were still on treatment, were a minimum leucocyte count less than 5000 el/mmc, an age of more than 36 years at start of treatment and male gender, the latter only for CD. There was no difference in terms of maintenance of remission, according to the duration of previous treatment with AZA. Similar results have been reported examining 61 UC patients taking 6-MP; among these patients, 22 persons discontinued 6-MP. The median time to relapse was 58 wk in patients that continued therapy, and 24 wk in those who discontinued. On multivariate analysis, the authors did not find any significant differences between the two groups in terms of age, gender, extent/duration of disease and duration of treatment with 6-MP before achieving remission[66].

In a French study, the cumulative recurrence rate after an 18 mo follow-up was higher in CD patients in clinical remission who had stopped AZA (21%), compared to patients who had continued (8%), although according to their results it did not reach statistical significance[67]. CRP levels > 20 mg/L, time steroid-free < 50 mo and a hemoglobin level < 12 g/dL, on multivariate analysis, were all risk factors for relapse.

In 2009, Cassinotti *et al*[68], retrospectively analyzed data from 127 UC patients in therapy with AZA for at least 3 mo and in steroid-free remission. Patients who continued therapy with AZA were included in the first group, while in the second group, patients who discontinued it electively, mainly for adverse events, were included. In the withdrawal group, the cumulative relapse-free survival was 65% in the first year, 51% in the second, 41% in the third, 39% and 35%, respectively in the fourth and fifth year. Stratifying patients for the duration of AZA treatment, authors observed a higher relapse-free survival in patients with longer treatment duration before discontinuation[68].

A similar study has been published by Treton *et al*[69] on 66 CD patients with long standing remission while on AZA. Cumulative relapse rates after AZA withdrawal were 14% in the first year, 52% in the third and 62% in the fifth. According to their data, a CRP level ⩾ 20 mg/L, neutrophil count ⩾ 4000/mmc, hemoglobin level < 12 g/dL were risk factors associated with a higher probability of relapse[69].

More recently, Kennedy *et al*[70] have studied a large cohort of IBD patients (129 CD, 108 UC) in deep remission with thiopurines after drug withdrawal. In the first 12 mo, 22% of CD patients had a moderate to severe relapse, *vs* 12% in UC patients (only moderate, none severe). At 2 years, the relapse rate grew to 39% in CD and 25% in UC. Elevated CRP levels at thiopurine withdrawal were associated with higher relapse rates at 12 mo in CD, while elevated WBC counts were predictive for relapse in UC[70]. A Spanish group studied the withdrawal of thiopurines after a treatment duration of at least 6 mo and a sustained steroid-free remission of at least 6 mo. After a median follow-up of 27 mo (IQR, 9-75), the cumulative percentages of clinical relapse were 18.8% after one year, 36.5% at year 3, and 43% at the fifth year. Predictive factors for relapse were biological remission, thiopurine treatment duration, pancolitis, time from diagnosis until start of thiopurines, number of relapses before the withdrawal[71]. Very recently, Qiu *et al*[72] have reported on 109 CD patients after discontinuation of thiopurines with a median follow-up of 46 mo. Endoscopic flares occurred in 45% of patients during follow-up, clinical flares in 37%, surgery was necessary in 16%, and hospitalizations in 23%. Independent risk factors for flare were prior bowel complication (HR 1.74), perianal disease at diagnosis (HR 2.24) and CRP > 3 mg/L (HR 4.05)[72].

ANTI-TNF DISCONTINUATION

Van Assche *et al*[73] randomized 80 CD patients to receive a combined treatment, for more than 6 mo, with IFX and an immunosuppressant (AZA, 6-MP or MTX) (Con), or to stop immunosuppressants receiving only *i.v.* IFX maintenance (Dis) (Table 3). They considered several clinical and endoscopic outcomes during a scheduled follow-up of 104 wk; the need to change or to stop

Table 2 Studies concerning withdrawal of immunomodulators

Ref.	Design	Disease n. patients	Intervention	Surveillance	Evaluation	Main outcome	Results	Predictive factors
Fraser *et al*[65]	RA, sc	272 CD 346 UC	Continue AZA *vs* discontinue AZA	-	Clinical assessment	Cumulative remission rate	At 1 yr 95% *vs* 63%, at 2 yr 90% *vs* 44%, at 3 yr 69% *vs* 34%, at 4 yr 63% *vs* 28%, at 5 yr 62% *vs* 25%	Risk factors for relapse: Female sex (only CD) and higher WBC; no differences for treatment duration of AZA
Lobel *et al*[66]	RA, sc	61 UC	Continue 6-MP *vs* discontinue 6-MP	Median f-u: 40 mo (range 4-344)	Clinical and endoscopic assessment	Median time to relapse (wk)	58 wk *vs* 24 wk Relapse at 1 yr: 43% *vs* 77%	No significant risk factors for relapse were found
Lémann *et al*[67]	RCT, db, mc	83 CD	Continue AZA *vs* placebo	18 mo	Clinical assessment	Relapse rate	8% *vs* 21%	Risk factors for relapse: CRP > 20 mg/L, time steroid-free < 50 mo, Hb < 12 g/dL
Van Assche *et al*[73]	RCT, db, mc	80 CD	Continue IFX + IS *vs* IFX + stop IS	104 wk	Clinical and endoscopic assessment	Median CRP; Median TL; Median SES-CD; AE rate; 12-mo relapse	1.6 mg/L *vs* 2.8 mg/L; 2.8 μg/mL *vs* 1.6 μg/mL; 1 *vs* 2.5 7.5% *vs* 7.5%; 58% *vs* 72.7%	Not significant *P*-value for endoscopic features in either groups
Cassinotti *et al*[68]	RA, mc	127 UC	AZA discontinuation	Median f-u: 55 mo (range 1-182)	Clinical assessment	Cumulative relapse rate	At 1 yr 35%, at 2 yr 49%, at 3 yr 59%, at 4 yr 61%, at 5 yr 65%	Risk factors for relapse: Short treatment duration of AZA
Treton *et al*[69]	PA, mc	66 CD	AZA discontinuation	Median f-u: 54 mo (IQR 20-69)	Clinical assessment	Cumulative relapse rate	At 1 yr 14%, at 3 yr 53%, at 5 yr 62%	Risk factors for relapse: Higher WCB count
Kennedy *et al*[70]	RA, mc	129 CD 108 UC	Thiopurine discontinuation	12 mo and 24 mo	Clinical assessment	Cumulative relapse rate	CD at 12 mo: Severe 8.5%, moderate 14%; at 24 mo: Severe 12%, moderate 27%. UC at 12 mo: Severe 0%, moderate 12%; at 24 mo: Severe 3%, moderate 22%	Risk factors for relapse: Elevated CRP (only in CD), higher WBC count (only in UC)
Moreno-Rincón *et al*[71]	RA, mc	102 UC	Thiopurine discontinuation	Median f-u: 27 mo (IRQ 9-75)	Clinical assessment	Cumulative relapse rate	At 1 yr: 18.8%, at 3 yr: 36.5%, at 5 yr: 43%	Risk factors for relapse: Biological remission, thiopurine treatment duration, pancolitis, time from diagnosis until the starting of thiopurines, number of relapse before the withdrawal
Qiu *et al*[72]	PA, sc	109 CD	Thiopurine discontinuation	Median f-u: 46 mo (IQR 27-67)	Clinical and endoscopic assessment	Cumulative relapse rate	45% endoscopic flare, 37% clinical flare, 16% surgery, 23% hospitalization	Risk factors for relapse: Prior bowel complication, perianal disease at diagnosis, CRP > 3 mg/L

RCT: Randomized controlled trial; UC: Ulcerative colitis; CD: Crohn's disease; mc: Multicenter; db: Double-blind; sc: Single-centre; AZA: Azathioprine; RA: Retrospective analysis; PA: Prospective analysis; IRQ: Interquartile range; CRP: C-reactive protein.

IFX dosing (primary endpoint) was seen in 60% in the continuation group and in 55% of the discontinuation group (P = 0.65). As secondary endpoints, median CRP levels were lower in the first group (Con), whereas the median IFX trough levels (TL) were higher compared with the discontinuation group. Median SES-CD was 1 (range 0-14) in patients who continued combination therapy and 2.5 (range 0-13) in patients who discontinued immunosuppressants. Endoscopic healing was reached in 64% in the first cohort *vs* 61% in the second one; there was no difference in terms of adverse events (7.5% *vs* 7.5%). Authors concluded affirming that combined therapy (IFX plus immunosuppressants) was not superior to IFX monotherapy, despite the increased median levels of CRP and lower TL of patients treated with only IFX. The higher CRP level and the decrease of TL, after immunosuppressant withdrawal, could be useful as early predictors of loss of response.

In 2010, Waugh *et al*[74] observed 48 CD patients in clinical remission in maintenance therapy with IFX every 8 wk. These patients discontinued therapy for reasons other than loss of response or an inadequate follow-up.

Table 3 Studies concerning withdrawal of biologic therapies

Ref.	Design	Disease n. patients	Drugs and intervention	Surveillance	Evaluation	Main outcome	Main findings	Predictive factors
Waugh *et al*[74]	PA, mc	48 CD	IFX discontinuation	Median f-u: 4.1 yr (IQR 0.5-6.7)	Clinical assessment	Cumulative relapse rate	50% relapse rate at a median of 477 d; 35% remain in remission without treatment	Probably 35% in deep remission are different genetic-kind of CD
Louis *et al*[75]	PA, mc	115 CD	IFX + IMM (IFX discontinuation)	30 mo after withdrawal	Clinical and endoscopic assessment	Cumulative relapse rate	At 1 yr: 44%, at 2 yr: 52%	Risk factors for relapse: Male sex, absence of surgical resections, CDEIS > 0, IFX TL > 2 mg/L, CS use between 6 and 12 mo before baseline, WBC count > 6000/mmc, Hb ≤ 14.5 g/dL, CRP ≥ 5 mg/L and fecal calprotectin ≥ 300 μg/g
Steenholdt *et al*[76]	RA, sc	53 CD 28 UC	IFX discontinuation	1 yr and 2 yr	Clinical assessment	Cumulative remission rate (no need to restart IFX, no need of CS, no surgery)	at 1 yr: 61% CD, 75% UC at 2 yr: 20% CD, 40% UC	Risk factors for relapse: Long disease duration (only in CD) (univariate)
Molnár *et al*[77]	PA, mc	121 CD	Anti-TNF discontinuation	1 yr	Clinical assessment	Cumulative relapse rate	45%	Risk factors for relapse: Smoking, using CS at the start of anti-TNF, previous biological therapy, elevated CRP level at the start of anti-TNF and a dose intensification in the first yr of anti-TNF (univariate)
Farkas *et al*[78]	PA, mc	51 UC	IFX discontinuation	1 yr	Clinical assessment	Cumulative relapse rate (need to restart IFX)	35%	Risk factors for relapse: Previous biological therapy
Rismo *et al*[25]	PA, sc	37 CD	Anti-TNF discontinuation	1-44 mo (range)	Clinical assessment	Cumulative relapse rate	74%	Risk factors for relapse: Elevated mucosal TNF and IL17 expression
Molander *et al*[79]	PA, sc	17 CD 30 UC 5 IBDU	Anti-TNF discontinuation	12 mo	Clinical and endoscopic assessment	Cumulative remission rate	67% clinical remission, 85% endoscopic remission	No significant risk factors for relapse were found
Brooks *et al*[80]	PA, mc	86 CD	Anti-TNF discontinuation	Median f-u: 495 d (365-2160)	Clinical, endoscopic and radiologic assessment	Whole cohort relapse rate; Endoscopic cohort	At 90 d: 4.7%, at 180 d: 18.6%, at 365 d: 36%; 6.3%, 12.5%, 31.3%	Risk factors for relapse: Montreal location and previous anti-TNF therapy
Chauvin *et al*[81]	RA, sc	92 CD	IFX + IMM (IFX discontinuation)	Median f-u: 47.1 mo (4.4-110.2)	Clinical assessment	Cumulative relapse rate	Cumulative: 72%, at 1 yr: 30%, at 2 yr: 48%	Risk factors for relapse: Smoking, previous antimetabolite failure, perianal disease (multivariate)
Dai *et al*[82]	PA, sc	109 CD 107 UC	IFX discontinuation	12 mo	Clinical and endoscopic assessment	Cumulative relapse rate (need to restart IFX)	Pt achieved clinical remission: 13.9% Pt achieved full remission: 6.5% Pt achieved MH: 10%	No significant risk factors for relapse were found. MH doesn't not predict sustained clinical remission
Ben-Horin *et al*[83]	RA, mc	30 CD 18 UC	Anti-TNF discontinuation	Median f-u: 12 mo	Clinical and endoscopic assessment	Cumulative relapse rate	Detectable drug: 80%, undetectable drug: 30%	Risk factors for relapse: Detectable drug levels

Papamichael *et al*[84]	PA, sc	100 CD	IFX discontinuation	Median f-u: 9.7 yr	Clinical assessment	Cumulative remission rate	Cumulative: 52%, at 1 yr 96%, at 2 yr 93%, at 3 yr 88%, at 4 yr 80%, at 5 yr 73%	At the univariate analysis were associated with a SCR: Age at diagnosis ⩾ 25 yr, disease duration from diagnosis to start of IFX < 1 years, MH at the IFX dis., IFX TC < 6 mg/mL at the IFX dis., positive serum VCAM-1 at the IFX dis.
Bortlik *et al*[85]	PA, sc	61 CD	IFX discontinuation	Median f-u: 28 mo (7-47)	Clinical assessment	Cumulative relapse rate	At 6 mo 18%, at 12 mo 41%, at 24 mo 49%. With MH: 18%, 36%, 60%	Risk factors for relapse: Ileal disease
Monterubbianesi *et al*[86]	RA, sc	58 CD	Anti-TNF discontinuation	5 yr	Clinical and endoscopic assessment	Cumulative relapse rate	At 1 yr 31%, at 2 yr 48% at 5 yr 65%	MH doesn't not predict sustained clinical remission
Bodini *et al*[87]	RCT, sc	9 CD 6 UC	Anti-TNF discontinuation; maintenance with AZA *vs* 5-ASA	Median f-u: 48 wk (20-78)	Clinical assessment	Cumulative relapse rate	AZA 0% *vs* 5-ASA 30%	Patients in therapy with 5-ASA have an earlier recurrence
Ampuero *et al*[88]	RA, sc	75 CD	IFX + IMM (IFX dis *vs* IMM dis.)	12 mo	Clinical and endoscopic assessment	Cumulative relapse rate	30.9% *vs* 20%	Risk factors for relapse: CRP > 5 mg/L, younger age at diagnosis

RCT: Randomized controlled trial; UC: Ulcerative colitis; CD: Crohn's disease; mc: Multicenter; db: Double-blind; sc: Single-centre; AZA: Azathioprine; RA: Retrospective analysis; PA: Prospective analysis; IRQ: Interquartile range; CRP: C-reactive protein; IFX: Infliximab; 5-ASA: 5-aminosalicylic acid; MH: Mucosal healing; TL: Trough level; CS: Costicosteroid; TNF: Tumor necrosis factor; IL: Interleukin; IMM: Immunomodulator.

Fifty percent of relapse was reached at a median interval of 477 d from discontinuation; interestingly, 35% of patients remained in long-term remission beyond follow-up (median follow-up 4.1 years, IQR 0.5-6.7)[74]; authors concluded that patients still in remission 5 years later might have a genetically different type of CD.

In 2012, an observational study on 115 CD patients in combined therapy with IFX and an antimetabolite (AZA, 6-MP or MTX), reported cumulative relapse rates after IFX discontinuation as high as 44% in the first year and 52% in the second year, with a median follow-up of 28 ± 2 mo. At multivariate analysis, relapse increased with an increasing number of unfavorable factors, such as male sex, absence of surgical resections, CDEIS > 0, IFX trough level > 2 mg/L, corticosteroid use between 6 and 12 mo before baseline, WBC count > 6000/mmc, Hb ⩽ 14.5 g/dL, CRP ⩾ 5 mg/L and FC ⩾ 300 μg/g[75].

A retrospective study evaluated the cumulative remission rate in 81 IBD patients, with a primary response to IFX and in steroid-free remission, following IFX discontinuation; in the first year, 61% and 75%, respectively in CD and UC, were still in remission; at the second year, the percentage dropped to 20% and 40%. Long disease duration was the unique risk factor for relapse only in CD patients[76].

In a prospective study, 121 CD patients who had achieved clinical remission after one year of anti-TNF therapy were followed after withdrawal of biologics. In 45% of patients, a restart of biological therapy was necessary within one year because of clinical relapse. On univariate analysis, smoking, the use of corticosteroids at the start of anti-TNF therapy, previous biological therapies, elevated serum CRP levels at start of anti-TNF therapy and a dose intensification in the first year of biological treatment were all significantly associated with the need to restart anti-TNF. At multivariate analysis, only male gender and a previous biological treatment were independently associated with relapse[77].

In another prospective trial that involved several IBD centers in Hungary, 51 UC patients who stopped IFX while in clinical remission were analyzed. At follow-up, 35% of patients needed to restart biologic therapy within the first year. Only previous biological therapy was associated to the need to restart biologics[78].

Rismo *et al*[25] observed 37 CD patients who stopped the anti-TNF drugs after achieving MH; before discontinuation, biopsies from the healed mucosa were taken in order to evaluate mucosal gene expression of inflammatory cytokines. According to data, at the end of follow-up (range 1-44 mo), 74% of patients experienced a relapse. Gene expression of TNF, IL17A and FOXP3 were significantly higher in patients who relapsed before six months. Normalization of the latter was associated with long-term remission[25].

In the same year, Molander *et al*[79] included 52 IBD patients who had achieved clinical and endoscopic remission and suspended the anti-TNF treatment in a retrospective study; in the first year after withdrawal, 65% of the patients maintained clinical remission and 85% of these were still in endoscopic remission. No significant risk factors predictive for relapse were found[79].

In 2014, Brooks *et al*[80] published a well-designed, prospective trial on 86 CD patients in clinical and/or endoscopic remission with anti-TNF drugs. They evaluated clinical, endoscopic and radiologic relapse after anti-TNF discontinuation. The follow-up was scheduled at 90, 180 and 365 d: In the whole cohort, relapse rates

were respectively 4% (at 90 d), 18% (at 180 d) and 36% (at 365 d); in patients assessed endoscopically, the rates were 6%, 12%, and 31%, respectively. Ileocolonic localization at diagnosis (OR 3.1) and previous anti-TNF therapy (OR 8.9) were found to predict relapse at any point of follow-up[80].

In a retrospective analysis, 92 CD patients were investigated after stopping IFX coming from a combined therapy with an immunomodulator (thiopurines or MTX) and IFX. After a median follow-up of 47 mo, the cumulative relapse rate was 72%. In the first year, 30% of the patients relapsed, while 48% of patients had a relapse in the second year. Based on multivariate analysis, the risk factors for relapse were active smoking, previous antimetabolite failure and perianal disease[81].

In a large trial, Dai *et al*[82] investigated relapse rates, defined as the need to restart IFX, in 109 CD patients and in 107 UC patients after discontinuation of IFX, after 1 year of continuous therapy. Need to restart IFX was observed in 13.9% of patients in clinical remission and in 6.5% of patients in deep remission. The Kaplan-Maier analysis did not show differences between clinical remission and MH concerning time to restart IFX (flare), in neither CD nor in UC[82]. A good response rate to retreatments with IFX was reported (78% in CD and 66% in UC). An interesting result came from a study conducted by Ben-Horin *et al*[83] on 48 IBD patients (30 CD and 18 UC) in remission who discontinued anti-TNF: During 12 mo median follow-up a higher incidence of relapse (80%) was observed in patients with measurable TL compared with patients (30%) who had undetectable levels (P = 0.002). Probably, the patients with undetectable TL were in remission independently of anti-TNF therapy[83].

Papamichael *et al*[84] performed a long-term retrospective observation of 100 CD patients from the moment of IFX discontinuation due to clinical remission. After a median follow-up of 9.7 years, the cumulative relapse rate was 48%; at univariate analysis, age at diagnosis $\geqslant$ 25 years, disease duration from diagnosis to start of IFX < 1 years, complete MH at the time of IFX cessation, IFX TL < 6 mg/mL at the time of IFX cessation and positive serum VCAM-1 at the time of IFX cessation were significantly associated with a sustained clinical remission (SCR). At multivariate analysis, only age at diagnosis $\geqslant$ 25 years remained associated with SCR[84].

Very recently, Bortlik *et al*[85] have analyzed the cumulative relapse rate after IFX discontinuation in 61 CD patients in clinical remission (median time of follow-up 28 mo) in a prospective analysis. At 6 mo of follow-up, 18% of patients relapsed, at one year 41%, and after two years of follow-up almost half of the patients. Surprisingly, among those patients who achieved MH, the cumulative relapse rates were similar: 18% at 6 mo, 36% at one year and even 60% after two years. Ileal localization of disease was the only risk factor for relapse[85].

Monterubbianesi *et al*[86] studied 58 CD patients in therapy for $\geqslant$ 12 mo with IFX or ADA who had stopped treatment because of deep remission. They observed a cumulative recurrence rate after discontinuation of anti-TNF drugs equal to 31% at one year, 48% at the second year and 65% at the third. They concluded saying that achieving MH before discontinuation did not predict a prolonged clinical remission[86].

In a controlled trial, Bodini *et al*[87], randomized 15 IBD patients (6 UC and 9 CD) to AZA 2 mg/kg per day or 5-ASA 2.4 g/die in UC and 3 g/d in CD, after stopping an anti-TNF drug. During the entire follow-up (median f-u time 48 wk), 100% of patients on AZA remained in remission, unlike patients on 5-ASA who relapsed in 30% of cases. Patients on maintenance therapy with 5-ASA showed, moreover, an earlier relapse compared to the other group[87]. Finally, in a retrospective analysis, CD patients in combined treatment continued with either IFX or immunomodulator. The 1-year relapse rate was not significantly different between the two groups, being 20% for those who continued IFX, and 30.9% for those on immunomodulators[88].

CONCLUSION

Thanks to the advent of biological drugs, MH has become an important aim to achieve, in order to stop progress of IBD and avoid related complications. New kinds of drugs will be introduced over the coming years and will be available for physicians in the hope to get better long-term remission rates. Nevertheless, the issue of saving treatment years, introducing drug holidays[89], will always be of central importance to ensure the least possible exposure to biologics and immunosuppressants, in an attempt to limit, as much as possible, adverse events and opportunistic infections. Another important aim is to reduce costs and ensure a sustainable future for National Health Services, in the management of a growing problem[90]. The use of immunosuppressive drugs in general (immunomodulators and biologics) has certainly changed over time, assuming greater importance, also in light of growing evidence from literature, and they have earned several indications: Early treatment in patients with more aggressive disease, use in first instance of combination therapy (the top-down strategy) in patients with steroid-resistant or steroid-dependent disease, and in the prevention of recurrences in patients who have undergone surgical resection. Important progress was also made regarding perianal disease and the importance of using the correct timing of biological therapies and surgery. Finally, the use of rescue-therapy with anti-TNF drugs or cyclosporine in severe UC has been well established.

Although the importance of achieving MH has been well documented, several recent studies have shown that maintaining or de-escalating therapy did not change the outcome significantly (Table 4), in terms of clinical and endoscopic relapse. Conversely, in a

Table 4 Synopsis on relapse rates with or without withdrawal of immunomodulator or biologics

Drugs	Overall patients number	Relapse at 1 yr	Relapse at 2 yr	Relapse at 5 yr
Immunomodulator	659 CD, 744 UC			
Maintenance	Mixed	6% (range 5-43)[65-67]	10%[65]	48%[65]
Median (range)				
	Only CD	6%[67]		
	Only UC	43%[66]		
De-escalation	Mixed	20.50% (range 12-37)[65,68-71]	44% (range 25-56)[65,68,70]	63.50% (range 43-75)[65,68-71]
Median (range)				
	Only CD	18% (range 14-22)[69,70]	39%[70]	62%[69]
	Only UC	19% (range 12-35)[68,70,71]	37% (range 25-49)[68,70]	54% (range 43-65)[68,71]
Anti-TNF	605 CD, 216 UC			
Maintenance	Mixed	49% (range 23-75)[58,95]	72% (range 36-83)[58,73,95]	
Median (range)				
	Only CD	75%[95]	77.5% (range 72-83)[73,95]	
	Only UC	23%[58]	36%[58]	
De-escalation	Mixed	35% (range 4-45)[76-80,82,84-86]	48.5% (range 7-80)[76,84-86]	46% (range 27-65)[84,86]
Median (range)				
	Only CD	37.5% (range 4-45)[76,77,80,84-86]	48.50% (range 7-80)[76,84-86]	46% (range 27-65)[84,86]
	Only UC	30% (range 25-35)[76,78]	60%[76]	
Combination therapy	362 CD			
Anti-TNF de-escalation	262 CD	31% (range 30-44)[75,81,88]	50% (range 48-52)[75,81]	
Immunomodulator de-escalation	100 CD	20%[88]	58%[73]	

UC: Ulcerative colitis; CD: Crohn's disease.

recent report, a lower rate of colectomy in a 10-year follow-up period was reported in patients that reached Mayo score 0 compared to score 1[91]. These conflicting data makes the physician's choice in this moment even more difficult. Looking at the evidence related to discontinuation in patients treated with thiopurines, a higher WBC count, elevated CRP serum levels and short duration of treatment, seem to be adverse factors, capable to predict an unfavorable disease course after drug discontinuation.

For patients on treatment with anti-TNF therapy, risk factors for relapse, such as elevated WBC count or serum CRP, seem to have a weaker influence; neither does the achievement of MH seem to predict a better course of disease.

FC could be a useful tool to assess inflammatory activity of colonic disease and it correlates well with MH[80], thus it should be dosed before withdrawal, to assess the degree of inflammation. Another important issue to be developed is histological healing, but at this moment no standardized score is available for either UC or CD[92,93]. At present, there is only one report concerning the superiority of histological over endoscopic healing in UC in terms of hospitalizations and steroid use[94]. It seems that histological healing will become an essential therapeutic target to ensure optimal disease control and less progression of organ damage, but new randomized controlled trials are needed to better define the real weight of histology in decision making, especially in transmural CD, on withdrawal of an immunomodulator or biologic drug. This lack of knowledge and evidence could probably explain the poor correlation between achievement of MH and maintenance of remission.

At this moment, drug withdrawal in the presence of mild mucosal lesions and of concomitant unfavorable features of disease, or positive markers of inflammation (like serum CRP or FC) seems to be unreasonable.

REFERENCES

1 **Molodecky NA**, Soon IS, Rabi DM, Ghali WA, Ferris M, Chernoff G, Benchimol EI, Panaccione R, Ghosh S, Barkema HW, Kaplan GG. Increasing incidence and prevalence of the inflammatory bowel diseases with time, based on systematic review. *Gastroenterology* 2012; **142**: 46-54.e42; quiz e30 [PMID: 22001864 DOI: 10.1053/j.gastro.2011.10.001]

2 **Peyrin-Biroulet L**, Loftus EV, Colombel JF, Sandborn WJ. The natural history of adult Crohn's disease in population-based cohorts. *Am J Gastroenterol* 2010; **105**: 289-297 [PMID: 19861953 DOI: 10.1038/ajg.2009.579]

3 **Sandborn WJ**, Hanauer S, Van Assche G, Panés J, Wilson S, Petersson J, Panaccione R. Treating beyond symptoms with a view to improving patient outcomes in inflammatory bowel diseases. *J Crohns Colitis* 2014; **8**: 927-935 [PMID: 24713173 DOI: 10.1016/j.crohns.2014.02.021]

4 **Rutgeerts P**, Vermeire S, Van Assche G. Mucosal healing in inflammatory bowel disease: impossible ideal or therapeutic target? *Gut* 2007; **56**: 453-455 [PMID: 17369375 DOI: 10.1136/gut.2005.088732]

5 **Marehbian J**, Arrighi HM, Hass S, Tian H, Sandborn WJ. Adverse events associated with common therapy regimens for moderate-to-severe Crohn's disease. *Am J Gastroenterol* 2009; **104**: 2524-2533 [PMID: 19532125 DOI: 10.1038/ajg.2009.322]

6 **Cottone M**, Kohn A, Daperno M, Armuzzi A, Guidi L, D'Inca R, Bossa F, Angelucci E, Biancone L, Gionchetti P, Ardizzone S, Papi C, Fries W, Danese S, Riegler G, Cappello M, Castiglione F, Annese V, Orlando A. Advanced age is an independent risk factor for severe infections and mortality in patients given anti-tumor necrosis factor therapy for inflammatory bowel disease. *Clin Gastroenterol Hepatol* 2011; **9**: 30-35 [PMID: 20951835 DOI: 10.1016/j.cgh.2010.09.026]

7 **Toruner M**, Loftus EV, Harmsen WS, Zinsmeister AR, Orenstein R, Sandborn WJ, Colombel JF, Egan LJ. Risk factors for opportunistic infections in patients with inflammatory bowel disease. *Gastroenterology* 2008; **134**: 929-936 [PMID: 18294633 DOI: 10.1053/j.gastro.2008.01.012]

8 **Dignass A**, Van Assche G, Lindsay JO, Lémann M, Söderholm J, Colombel JF, Danese S, D'Hoore A, Gassull M, Gomollón F, Hommes DW, Michetti P, O'Morain C, Oresland T, Windsor A, Stange EF, Travis SP. The second European evidence-based Consensus on the diagnosis and management of Crohn's disease: Current management. *J Crohns Colitis* 2010; **4**: 28-62 [PMID: 21122489 DOI: 10.1016/j.crohns.2009.12.002]

9 **Dignass A**, Lindsay JO, Sturm A, Windsor A, Colombel JF, Allez M, D'Haens G, D'Hoore A, Mantzaris G, Novacek G, Oresland T, Reinisch W, Sans M, Stange E, Vermeire S, Travis S, Van Assche G. Second European evidence-based consensus on the diagnosis and management of ulcerative colitis part 2: current management. *J Crohns Colitis* 2012; **6**: 991-1030 [PMID: 23040451 DOI: 10.1016/j.crohns.2012.09.002]

10 **Mary JY**, Modigliani R. Development and validation of an endoscopic index of the severity for Crohn's disease: a prospective multicentre study. Groupe d'Etudes Thérapeutiques des Affections Inflammatoires du Tube Digestif (GETAID). *Gut* 1989; **30**: 983-989 [PMID: 2668130 DOI: 10.1136/gut.30.7.983]

11 **Daperno M**, D'Haens G, Van Assche G, Baert F, Bulois P, Maunoury V, Sostegni R, Rocca R, Pera A, Gevers A, Mary JY, Colombel JF, Rutgeerts P. Development and validation of a new, simplified endoscopic activity score for Crohn's disease: the SES-CD. *Gastrointest Endosc* 2004; **60**: 505-512 [PMID: 15472670 DOI: 10.1016/S0016-5107(04)01878-4]

12 **Rutgeerts P**, Geboes K, Vantrappen G, Beyls J, Kerremans R, Hiele M. Predictability of the postoperative course of Crohn's disease. *Gastroenterology* 1990; **99**: 956-963 [PMID: 2394349]

13 **Truelove SC**, Witts LJ. Cortisone in ulcerative colitis; final report on a therapeutic trial. *Br Med J* 1955; **2**: 1041-1048 [PMID: 13260656 DOI: 10.1159/000199983]

14 **Baron JH**, Connell AM, Lennard-Jones JE. Variation between observers in describing mucosal appearances in proctocolitis. *Br Med J* 1964; **1**: 89-92 [PMID: 14075156 DOI: 10.1136/bmj.1.5375.8]

15 **Sutherland LR**, Martin F, Greer S, Robinson M, Greenberger N, Saibil F, Martin T, Sparr J, Prokipchuk E, Borgen L. 5-Aminosalicylic acid enema in the treatment of distal ulcerative colitis, proctosigmoiditis, and proctitis. *Gastroenterology* 1987; **92**: 1894-1898 [PMID: 3569765 DOI: 10.1007/BF01312466]

16 **Powell-Tuck J**, Bown RL, Lennard-Jones JE. A comparison of oral prednisolone given as single or multiple daily doses for active proctocolitis. *Scand J Gastroenterol* 1978; **13**: 833-837 [PMID: 364626]

17 **Rachmilewitz D**. Coated mesalazine (5-aminosalicylic acid) versus sulphasalazine in the treatment of active ulcerative colitis: a randomised trial. *BMJ* 1989; **298**: 82-86 [PMID: 2563951 DOI: 10.1136/bmj.298.6666.82]

18 **Schroeder KW**, Tremaine WJ, Ilstrup DM. Coated oral 5-aminosalicylic acid therapy for mildly to moderately active ulcerative colitis. A randomized study. *N Engl J Med* 1987; **317**: 1625-1629 [PMID: 3317057 DOI: 10.1056/NEJM198712243172603]

19 **Travis SP**, Schnell D, Krzeski P, Abreu MT, Altman DG, Colombel JF, Feagan BG, Hanauer SB, Lémann M, Lichtenstein GR, Marteau PR, Reinisch W, Sands BE, Yacyshyn BR, Bernhardt CA, Mary JY, Sandborn WJ. Developing an instrument to assess the endoscopic severity of ulcerative colitis: the Ulcerative Colitis Endoscopic Index of Severity (UCEIS). *Gut* 2012; **61**: 535-542 [PMID: 21997563 DOI: 10.1136/gutjnl-2011-300486]

20 **Samuel S**, Bruining DH, Loftus EV, Thia KT, Schroeder KW, Tremaine WJ, Faubion WA, Kane SV, Pardi DS, de Groen PC, Harmsen WS, Zinsmeister AR, Sandborn WJ. Validation of the ulcerative colitis colonoscopic index of severity and its correlation with disease activity measures. *Clin Gastroenterol Hepatol* 2013; **11**: 49-54.e1 [PMID: 22902762 DOI: 10.1016/j.cgh.2012.08.003]

21 **Lobatón T**, Bessissow T, De Hertogh G, Lemmens B, Maedler C, Van Assche G, Vermeire S, Bisschops R, Rutgeerts P, Bitton A, Afif W, Marcus V, Ferrante M. The Modified Mayo Endoscopic Score (MMES): A New Index for the Assessment of Extension and Severity of Endoscopic Activity in Ulcerative Colitis Patients. *J Crohns Colitis* 2015; **9**: 846-852 [PMID: 26116558]

22 **Pariente B**, Mary JY, Danese S, Chowers Y, De Cruz P, D'Haens G, Loftus EV, Louis E, Panés J, Schölmerich J, Schreiber S, Vecchi M, Branche J, Bruining D, Fiorino G, Herzog M, Kamm MA, Klein A, Lewin M, Meunier P, Ordas I, Strauch U, Tontini GE, Zagdanski AM, Bonifacio C, Rimola J, Nachury M, Leroy C, Sandborn W, Colombel JF, Cosnes J. Development of the Lémann index to assess digestive tract damage in patients with Crohn's disease. *Gastroenterology* 2015; **148**: 52-63.e3 [PMID: 25241327 DOI: 10.1053/j.gastro.2014.09.015]

23 **Pariente B**, Cosnes J, Danese S, Sandborn WJ, Lewin M, Fletcher JG, Chowers Y, D'Haens G, Feagan BG, Hibi T, Hommes DW, Irvine EJ, Kamm MA, Loftus EV, Louis E, Michetti P, Munkholm P, Oresland T, Panés J, Peyrin-Biroulet L, Reinisch W, Sands BE, Schoelmerich J, Schreiber S, Tilg H, Travis S, van Assche G, Vecchi M, Mary JY, Colombel JF, Lémann M. Development of the Crohn's disease digestive damage score, the Lémann score. *Inflamm Bowel Dis* 2011; **17**: 1415-1422 [PMID: 21560202 DOI: 10.1002/ibd.21506]

24 **Ordás I**, Rimola J, Rodríguez S, Paredes JM, Martínez-Pérez MJ, Blanc E, Arévalo JA, Aduna M, Andreu M, Radosevic A, Ramírez-Morros AM, Pinó S, Gallego M, Jauregui-Amezaga A, Ricart E, Panés J. Accuracy of magnetic resonance enterography in assessing response to therapy and mucosal healing in patients with Crohn's disease. *Gastroenterology* 2014; **146**: 374-382.e1 [PMID: 24177375 DOI: 10.1053/j.gastro.2013.10.055]

25 **Rismo R**, Olsen T, Cui G, Paulssen EJ, Christiansen I, Johnsen K, Florholmen J, Goll R. Normalization of mucosal cytokine gene expression levels predicts long-term remission after discontinuation of anti-TNF therapy in Crohn's disease. *Scand J Gastroenterol* 2013; **48**: 311-319 [PMID: 23302000 DOI: 10.3109/00365521.201]

26 **Chung-Faye G**, Hayee B, Maestranzi S, Donaldson N, Forgacs I, Sherwood R. Fecal M2-pyruvate kinase (M2-PK): a novel marker of intestinal inflammation. *Inflamm Bowel Dis* 2007; **13**: 1374-1378 [PMID: 17577247 DOI: 10.1002/ibd.20214]

27 **Turner D**, Leach ST, Mack D, Uusoue K, McLernon R, Hyams J, Leleiko N, Walters TD, Crandall W, Markowitz J, Otley AR, Griffiths AM, Day AS. Faecal calprotectin, lactoferrin, M2-pyruvate kinase and S100A12 in severe ulcerative colitis: a prospective multicentre comparison of predicting outcomes and monitoring response. *Gut* 2010; **59**: 1207-1212 [PMID: 20801771 DOI: 10.1136/gut.2010.211755]

28 **Kato S**, Ochiai M, Sakurada T, Ohno S, Miyamoto K, Sagara M, Ito M, Takeuchi K, Imaki J, Itoh K, Yakabi K. Increased expression of long pentraxin PTX3 in inflammatory bowel diseases. *Dig Dis Sci* 2008; **53**: 1910-1916 [PMID: 17990107 DOI: 10.1007/s10620-007-0075-z]

29 **Daperno M**, Castiglione F, de Ridder L, Dotan I, Färkkilä M, Florholmen J, Fraser G, Fries W, Hebuterne X, Lakatos PL, Panés J, Rimola J, Louis E. Results of the 2nd part Scientific Workshop of the ECCO. II: Measures and markers of prediction to achieve, detect, and monitor intestinal healing in inflammatory bowel disease. *J Crohns Colitis* 2011; **5**: 484-498 [PMID: 21939926 DOI: 10.1016/j.crohns.2011.07.003]

30 **Florholmen J**, Fries W. Candidate mucosal and surrogate biomarkers of inflammatory bowel disease in the era of new technology. *Scand J Gastroenterol* 2011; **46**: 1407-1417 [PMID: 22040230 DOI: 10.3109/00365521.2011.627449]

31 **Thompson D**, Pepys MB, Wood SP. The physiological structure

of human C-reactive protein and its complex with phosphocholine. *Structure* 1999; **7**: 169-177 [PMID: 10368284 DOI: 10.1016/S0969-2126(99)80023-9]

32 **Liu S**, Ren J, Xia Q, Wu X, Han G, Ren H, Yan D, Wang G, Gu G, Li J. Preliminary case-control study to evaluate diagnostic values of C-reactive protein and erythrocyte sedimentation rate in differentiating active Crohn's disease from intestinal lymphoma, intestinal tuberculosis and Behcet's syndrome. *Am J Med Sci* 2013; **346**: 467-472 [PMID: 23689052 DOI: 10.1097/MAJ.0b013e3182959a18]

33 **Henriksen M**, Jahnsen J, Lygren I, Stray N, Sauar J, Vatn MH, Moum B. C-reactive protein: a predictive factor and marker of inflammation in inflammatory bowel disease. Results from a prospective population-based study. *Gut* 2008; **57**: 1518-1523 [PMID: 18566104 DOI: 10.1136/gut.2007.146357]

34 **Zubin G**, Peter L. Predicting Endoscopic Crohn's Disease Activity Before and After Induction Therapy in Children: A Comprehensive Assessment of PCDAI, CRP, and Fecal Calprotectin. *Inflamm Bowel Dis* 2015; **21**: 1386-1391 [PMID: 25851564 DOI: 10.1097/MIB.0000000000000388]

35 **Langhorst J**, Elsenbruch S, Koelzer J, Rueffer A, Michalsen A, Dobos GJ. Noninvasive markers in the assessment of intestinal inflammation in inflammatory bowel diseases: performance of fecal lactoferrin, calprotectin, and PMN-elastase, CRP, and clinical indices. *Am J Gastroenterol* 2008; **103**: 162-169 [PMID: 17916108 DOI: 10.1111/j.1572-0241.2007.01556.x]

36 **Tibble J**, Teahon K, Thjodleifsson B, Roseth A, Sigthorsson G, Bridger S, Foster R, Sherwood R, Fagerhol M, Bjarnason I. A simple method for assessing intestinal inflammation in Crohn's disease. *Gut* 2000; **47**: 506-513 [PMID: 10986210 DOI: 10.1136/gut.47.4.506]

37 **Voiosu T**, Benguş A, Dinu R, Voiosu AM, Bălănescu P, Băicuş C, Diculescu M, Voiosu R, Mateescu B. Rapid fecal calprotectin level assessment and the SIBDQ score can accurately detect active mucosal inflammation in IBD patients in clinical remission: a prospective study. *J Gastrointestin Liver Dis* 2014; **23**: 273-278 [PMID: 25267955 DOI: 10.15403/jgld.2014.1121.233.thv]

38 **Sipponen T**, Kärkkäinen P, Savilahti E, Kolho KL, Nuutinen H, Turunen U, Färkkilä M. Correlation of faecal calprotectin and lactoferrin with an endoscopic score for Crohn's disease and histological findings. *Aliment Pharmacol Ther* 2008; **28**: 1221-1229 [PMID: 18752630 DOI: 10.1111/j.1365-2036.2008.03835]

39 **Theede K**, Holck S, Ibsen P, Ladelund S, Nordgaard-Lassen I, Nielsen AM. Level of Fecal Calprotectin Correlates With Endoscopic and Histologic Inflammation and Identifies Patients With Mucosal Healing in Ulcerative Colitis. *Clin Gastroenterol Hepatol* 2015; **13**: 1929-1936.e1 [PMID: 26051392 DOI: 10.1016/j.cgh.2015.05.038]

40 **Bolignano D**, Della Torre A, Lacquaniti A, Costantino G, Fries W, Buemi M. Neutrophil gelatinase-associated lipocalin levels in patients with crohn disease undergoing treatment with infliximab. *J Investig Med* 2010; **58**: 569-571 [PMID: 20061984 DOI: 10.231/JIM.0b013e3181ccc20c]

41 **de Bruyn M**, Arijs I, Wollants WJ, Machiels K, Van Steen K, Van Assche G, Ferrante M, Rutgeerts P, Vermeire S, Opdenakker G. Neutrophil gelatinase B-associated lipocalin and matrix metalloproteinase-9 complex as a surrogate serum marker of mucosal healing in ulcerative colitis. *Inflamm Bowel Dis* 2014; **20**: 1198-1207 [PMID: 24871805 DOI: 10.1097/MIB.0000000000000068]

42 **de Bruyn M**, Arijs I, De Hertogh G, Ferrante M, Van Assche G, Rutgeerts P, Vermeire S, Opdenakker G. Serum Neutrophil Gelatinase B-associated Lipocalin and Matrix Metalloproteinase-9 Complex as a Surrogate Marker for Mucosal Healing in Patients with Crohn's Disease. *J Crohns Colitis* 2015; **pii**: jjv148 [PMID: 26351381]

43 **Vecchi M**, Meucci G, Gionchetti P, Beltrami M, Di Maurizio P, Beretta L, Ganio E, Usai P, Campieri M, Fornaciari G, de Franchis R. Oral versus combination mesalazine therapy in active ulcerative colitis: a double-blind, double-dummy, randomized multicentre study. *Aliment Pharmacol Ther* 2001; **15**: 251-256 [PMID: 11148445 DOI: 10.1046/j.1365-2036.2001.00913]

44 **Mansfield JC**, Giaffer MH, Cann PA, McKenna D, Thornton PC, Holdsworth CD. A double-blind comparison of balsalazide, 6.75 g, and sulfasalazine, 3 g, as sole therapy in the management of ulcerative colitis. *Aliment Pharmacol Ther* 2002; **16**: 69-77 [PMID: 11856080 DOI: 10.1046/j.1365-2036.2002.01151]

45 **Kruis W**, Bar-Meir S, Feher J, Mickisch O, Mlitz H, Faszczyk M, Chowers Y, Lengyele G, Kovacs A, Lakatos L, Stolte M, Vieth M, Greinwald R. The optimal dose of 5-aminosalicylic acid in active ulcerative colitis: a dose-finding study with newly developed mesalamine. *Clin Gastroenterol Hepatol* 2003; **1**: 36-43 [PMID: 15017515 DOI: 10.1053/jcgh.2003.50006]

46 **Kruis W**, Kiudelis G, Rácz I, Gorelov IA, Pokrotnieks J, Horynski M, Batovsky M, Kykal J, Boehm S, Greinwald R, Mueller R. Once daily versus three times daily mesalazine granules in active ulcerative colitis: a double-blind, double-dummy, randomised, non-inferiority trial. *Gut* 2009; **58**: 233-240 [PMID: 18832520 DOI: 10.1136/gut.2008.154302]

47 **Hanauer SB**, Sandborn WJ, Dallaire C, Archambault A, Yacyshyn B, Yeh C, Smith-Hall N. Delayed-release oral mesalamine 4.8 g/day (800 mg tablets) compared to 2.4 g/day (400 mg tablets) for the treatment of mildly to moderately active ulcerative colitis: The ASCEND I trial. *Can J Gastroenterol* 2007; **21**: 827-834 [PMID: 18080055 DOI: 10.1053/j.gastro.2009.08.069]

48 **Kamm MA**, Sandborn WJ, Gassull M, Schreiber S, Jackowski L, Butler T, Lyne A, Stephenson D, Palmen M, Joseph RE. Once-daily, high-concentration MMX mesalamine in active ulcerative colitis. *Gastroenterology* 2007; **132**: 66-75; quiz 432-433 [PMID: 17241860 DOI: 10.1053/j.gastro.2006.10.011]

49 **Ardizzone S**, Cassinotti A, Duca P, Mazzali C, Penati C, Manes G, Marmo R, Massari A, Molteni P, Maconi G, Porro GB. Mucosal healing predicts late outcomes after the first course of corticosteroids for newly diagnosed ulcerative colitis. *Clin Gastroenterol Hepatol* 2011; **9**: 483-489.e3 [PMID: 21195796 DOI: 10.1016/j.cgh.2010.12.028]

50 **Sandborn WJ**, Travis S, Moro L, Jones R, Gautille T, Bagin R, Huang M, Yeung P, Ballard ED. Once-daily budesonide MMX ® extended-release tablets induce remission in patients with mild to moderate ulcerative colitis: results from the CORE I study. *Gastroenterology* 2012; **143**: 1218-1226.e1-2 [PMID: 22892337 DOI: 10.1053/j.gastro.2012.08.003]

51 **Van Assche G**, Manguso F, Zibellini M, Cabriada Nuño JL, Goldis A, Tkachenko E, Varoli G, Kleczkowski D, Annese V, D'Heygere F, Balzano A. Oral prolonged release beclomethasone dipropionate and prednisone in the treatment of active ulcerative colitis: results from a double-blind, randomized, parallel group study. *Am J Gastroenterol* 2015; **110**: 708-715 [PMID: 25869389 DOI: 10.1038/ajg.2015.114]

52 **Ardizzone S**, Maconi G, Russo A, Imbesi V, Colombo E, Bianchi Porro G. Randomised controlled trial of azathioprine and 5-aminosalicylic acid for treatment of steroid dependent ulcerative colitis. *Gut* 2006; **55**: 47-53 [PMID: 15972298 DOI: 10.1136/gut.2005.068809]

53 **Rispo A**, Testa A, De Palma GD, Donetto S, Diaferia M, Musto D, Nardone O, Maione F, Caporaso N, Castiglione F. Different Profile of Efficacy of Thiopurines in Ulcerative Colitis and Crohn's Disease. *Inflamm Bowel Dis* 2015; **21**: 2570-2575 [PMID: 26222340 DOI: 10.1097/MIB.0000000000000538]

54 **Reinisch W**, Sandborn WJ, Hommes DW, D'Haens G, Hanauer S, Schreiber S, Panaccione R, Fedorak RN, Tighe MB, Huang B, Kampman W, Lazar A, Thakkar R. Adalimumab for induction of clinical remission in moderately to severely active ulcerative colitis: results of a randomised controlled trial. *Gut* 2011; **60**: 780-787 [PMID: 21209123 DOI: 10.1136/gut.2010.221127]

55 **Sandborn WJ**, van Assche G, Reinisch W, Colombel JF, D' Haens G, Wolf DC, Kron M, Tighe MB, Lazar A, Thakkar RB. Adalimumab induces and maintains clinical remission in patients with moderate-to-severe ulcerative colitis. *Gastroenterology* 2012; **142**: 257-65.e1-e3 [PMID: 22062358 DOI: 10.1053/j.gastro.2011.1

0.032]
56 **Feagan BG**, Rutgeerts P, Sands BE, Hanauer S, Colombel JF, Sandborn WJ, Van Assche G, Axler J, Kim HJ, Danese S, Fox I, Milch C, Sankoh S, Wyant T, Xu J, Parikh A. Vedolizumab as induction and maintenance therapy for ulcerative colitis. *N Engl J Med* 2013; **369**: 699-710 [PMID: 23964932 DOI: 10.1056/NEJMoa1215734]
57 **Sandborn WJ**, Feagan BG, Rutgeerts P, Hanauer S, Colombel JF, Sands BE, Lukas M, Fedorak RN, Lee S, Bressler B, Fox I, Rosario M, Sankoh S, Xu J, Stephens K, Milch C, Parikh A. Vedolizumab as induction and maintenance therapy for Crohn's disease. *N Engl J Med* 2013; **369**: 711-721 [PMID: 23964933 DOI: 10.1056/NEJMoa1215739]
58 **Laharie D**, Filippi J, Roblin X, Nancey S, Chevaux JB, Hébuterne X, Flourié B, Capdepont M, Peyrin-Biroulet L. Impact of mucosal healing on long-term outcomes in ulcerative colitis treated with infliximab: a multicenter experience. *Aliment Pharmacol Ther* 2013; **37**: 998-1004 [PMID: 23521659 DOI: 10.1111/apt.12289]
59 **Sandborn WJ**, Feagan BG, Marano C, Zhang H, Strauss R, Johanns J, Adedokun OJ, Guzzo C, Colombel JF, Reinisch W, Gibson PR, Collins J, Järnerot G, Hibi T, Rutgeerts P. Subcutaneous golimumab induces clinical response and remission in patients with moderate-to-severe ulcerative colitis. *Gastroenterology* 2014; **146**: 85-95; quiz e14-15 [PMID: 23735746 DOI: 10.1053/j.gastro.2013.05.048]
60 **D'Haens G**, Geboes K, Ponette E, Penninckx F, Rutgeerts P. Healing of severe recurrent ileitis with azathioprine therapy in patients with Crohn's disease. *Gastroenterology* 1997; **112**: 1475-1481 [PMID: 9136824 DOI: 10.1016/S0016-5085(97)70027-1]
61 **Mantzaris GJ**, Christidou A, Sfakianakis M, Roussos A, Koilakou S, Petraki K, Polyzou P. Azathioprine is superior to budesonide in achieving and maintaining mucosal healing and histologic remission in steroid-dependent Crohn's disease. *Inflamm Bowel Dis* 2009; **15**: 375-382 [PMID: 19009634 DOI: 10.1002/ibd.20777]
62 **Laharie D**, Reffet A, Belleannée G, Chabrun E, Subtil C, Razaire S, Capdepont M, de Lédinghen V. Mucosal healing with methotrexate in Crohn's disease: a prospective comparative study with azathioprine and infliximab. *Aliment Pharmacol Ther* 2011; **33**: 714-721 [PMID: 21235604 DOI: 10.1111/j.1365-2036.2010.04569]
63 **Colombel JF**, Sandborn WJ, Reinisch W, Mantzaris GJ, Kornbluth A, Rachmilewitz D, Lichtiger S, D'Haens G, Diamond RH, Broussard DL, Tang KL, van der Woude CJ, Rutgeerts P. Infliximab, azathioprine, or combination therapy for Crohn's disease. *N Engl J Med* 2010; **362**: 1383-1395 [PMID: 20393175 DOI: 10.1056/NEJMoa0904492]
64 **Rutgeerts P**, Van Assche G, Sandborn WJ, Wolf DC, Geboes K, Colombel JF, Reinisch W, Kumar A, Lazar A, Camez A, Lomax KG, Pollack PF, D'Haens G. Adalimumab induces and maintains mucosal healing in patients with Crohn's disease: data from the EXTEND trial. *Gastroenterology* 2012; **142**: 1102-1111.e2 [PMID: 22326435 DOI: 10.1053/j.gastro.2012.01.035]
65 **Fraser AG**, Orchard TR, Jewell DP. The efficacy of azathioprine for the treatment of inflammatory bowel disease: a 30 year review. *Gut* 2002; **50**: 485-489 [PMID: 11889067 DOI: 10.1136/gut.50.4.485]
66 **Lobel EZ**, Korelitz BI, Xuereb MA, Panagopoulos G. A search for the optimal duration of treatment with 6-mercaptopurine for ulcerative colitis. *Am J Gastroenterol* 2004; **99**: 462-465 [PMID: 15056086 DOI: 10.1111/j.1572-0241.2004.04104]
67 **Lémann M**, Mary JY, Colombel JF, Duclos B, Soule JC, Lerebours E, Modigliani R, Bouhnik Y. A randomized, double-blind, controlled withdrawal trial in Crohn's disease patients in long-term remission on azathioprine. *Gastroenterology* 2005; **128**: 1812-1818 [PMID: 15940616 DOI: 10.1053/gast.1996.v110.pm8608877]
68 **Cassinotti A**, Actis GC, Duca P, Massari A, Colombo E, Gai E, Annese V, D'Albasio G, Manes G, Travis S, Porro GB, Ardizzone S. Maintenance treatment with azathioprine in ulcerative colitis: outcome and predictive factors after drug withdrawal. *Am J Gastroenterol* 2009; **104**: 2760-2767 [PMID: 19623172 DOI: 10.1038/ajg.2009.410]
69 **Treton X**, Bouhnik Y, Mary JY, Colombel JF, Duclos B, Soule JC, Lerebours E, Cosnes J, Lemann M. Azathioprine withdrawal in patients with Crohn's disease maintained on prolonged remission: a high risk of relapse. *Clin Gastroenterol Hepatol* 2009; **7**: 80-85 [PMID: 18849016 DOI: 10.1016/j.cgh.2008.08.028]
70 **Kennedy NA**, Kalla R, Warner B, Gambles CJ, Musy R, Reynolds S, Dattani R, Nayee H, Felwick R, Harris R, Marriott S, Senanayake SM, Lamb CA, Al-Hilou H, Gaya DR, Irving PM, Mansfield J, Parkes M, Ahmad T, Cummings JR, Arnott ID, Satsangi J, Lobo AJ, Smith M, Lindsay JO, Lees CW. Thiopurine withdrawal during sustained clinical remission in inflammatory bowel disease: relapse and recapture rates, with predictive factors in 237 patients. *Aliment Pharmacol Ther* 2014; **40**: 1313-1323 [PMID: 25284134 DOI: 10.1111/apt.12980]
71 **Moreno-Rincón E**, Benítez JM, Serrano-Ruiz FJ, Vázquez-Morón JM, Pallarés-Manrique H, Herrera-Justiniano JM, Leo-Carnerero E, Gómez-García MR, Cabello-Tapia MJ, Castro-Fernández M, Rojas-Feria M, Castro-Laria L, Argüelles-Arias F, Camargo-Camero R, Alcaín-Martínez G, Iglesias-Flores E, García-Sánchez V. Prognosis of Patients with Ulcerative Colitis in Sustained Remission After Thiopurines Withdrawal. *Inflamm Bowel Dis* 2015; **21**: 1564-1571 [PMID: 26070002 DOI: 10.1097/MIB.0000000000000400]
72 **Qiu Y**, Mao R, Chen BI, Y. He, Zeng ZR, Chen MH. DOP064 Predictors of disease relapse of patients with Crohn's disease in Deep Remission: Who and when can withdraw thiopurine maintenance therapy? Proceedings of 10th Congress of ECCO. Barcelona, Spain, 2015 Feb 18-21. Available from: URL: https://www.ecco-ibd.eu/index.php/publications/congress-abstract-s/abstracts-2015/item/dop064-predictors-of-disease-relapse-of-patients-with-crohnaposs-disease-in-deep-remission-who-and-when-can-withdraw-thiopurine-maintenance-therapy.html
73 **Van Assche G**, Magdelaine-Beuzelin C, D'Haens G, Baert F, Noman M, Vermeire S, Ternant D, Watier H, Paintaud G, Rutgeerts P. Withdrawal of immunosuppression in Crohn's disease treated with scheduled infliximab maintenance: a randomized trial. *Gastroenterology* 2008; **134**: 1861-1868 [PMID: 18440315 DOI: 10.1053/j.gastro.2008.03.004]
74 **Waugh AW**, Garg S, Matic K, Gramlich L, Wong C, Sadowski DC, Millan M, Bailey R, Todoruk D, Cherry R, Teshima CW, Dieleman L, Fedorak RN. Maintenance of clinical benefit in Crohn's disease patients after discontinuation of infliximab: long-term follow-up of a single centre cohort. *Aliment Pharmacol Ther* 2010; **32**: 1129-1134 [PMID: 20807218 DOI: 10.1111/j.1365-2036.2010.04446]
75 **Louis E**, Mary JY, Vernier-Massouille G, Grimaud JC, Bouhnik Y, Laharie D, Dupas JL, Pillant H, Picon L, Veyrac M, Flamant M, Savoye G, Jian R, Devos M, Porcher R, Paintaud G, Piver E, Colombel JF, Lemann M. Maintenance of remission among patients with Crohn's disease on antimetabolite therapy after infliximab therapy is stopped. *Gastroenterology* 2012; **142**: 63-70.e5; quiz e31 [PMID: 21945953 DOI: 10.1053/j.gastro.2011.09.034]
76 **Steenholdt C**, Molazahi A, Ainsworth MA, Brynskov J, Østergaard Thomsen O, Seidelin JB. Outcome after discontinuation of infliximab in patients with inflammatory bowel disease in clinical remission: an observational Danish single center study. *Scand J Gastroenterol* 2012; **47**: 518-527 [PMID: 22375898 DOI: 10.3109/00365521.2012.660541]
77 **Molnár T**, Lakatos PL, Farkas K, Nagy F, Szepes Z, Miheller P, Horváth G, Papp M, Palatka K, Nyári T, Bálint A, Lőrinczy K, Wittmann T. Predictors of relapse in patients with Crohn's disease in remission after 1 year of biological therapy. *Aliment Pharmacol Ther* 2013; **37**: 225-233 [PMID: 23181359 DOI: 10.1111/apt.12160]
78 **Farkas K**, Lakatos PL, Nagy F, Szepes Z, Miheller P, Papp M, Palatka K, Bálint A, Bor R, Wittmann T, Molnár T. Predictors of relapse in patients with ulcerative colitis in remission after one-year of infliximab therapy. *Scand J Gastroenterol* 2013; **48**: 1394-1398 [PMID: 24131338 DOI: 10.3109/00365521.2013.845906]
79 **Molander P**, Färkkilä M, Salminen K, Kemppainen H, Blomster T, Koskela R, Jussila A, Rautiainen H, Nissinen M, Haapamäki J, Arkkila P, Nieminen U, Kuisma J, Punkkinen J, Kolho KL, Mustonen H, Sipponen T. Outcome after discontinuation of TNFα-blocking therapy in patients with inflammatory bowel disease in

deep remission. *Inflamm Bowel Dis* 2014; **20**: 1021-1028 [PMID: 24798636 DOI: 10.1097/MIB.0000000000000052]

80 **Brooks AJ**, Sebastian S, Cross SS, Robinson K, Warren L, Wright A, Marsh AM, Tsai H, Majeed F, McAlindon ME, Preston C, Hamlin PJ, Lobo AJ. Outcome of elective withdrawal of anti-tumour necrosis factor-α therapy in patients with Crohn's disease in established remission. *J Crohns Colitis* 2015; **pii**: jjv000 [PMID: 25311864]

81 **Chauvin A**, Le Thuaut A, Belhassan M, Le Baleur Y, Mesli F, Bastuji-Garin S, Delchier JC, Amiot A. Infliximab as a bridge to remission maintained by antimetabolite therapy in Crohn's disease: A retrospective study. *Dig Liver Dis* 2014; **46**: 695-700 [PMID: 24893686 DOI: 10.1016/j.dld.2014.04.012]

82 **Dai C**, Liu WX, Jiang M, Sun MJ. Mucosal healing did not predict sustained clinical remission in patients with IBD after discontinuation of one-year infliximab therapy. *PLoS One* 2014; **9**: e110797 [PMID: 25330148 DOI: 10.1371/journal.pone.0110797]

83 **Ben-Horin S**, Chowers Y, Ungar B, Kopylov U, Loebstein R, Weiss B, Eliakim R, Del Tedesco E, Paul S, Roblin X. Undetectable anti-TNF drug levels in patients with long-term remission predict successful drug withdrawal. *Aliment Pharmacol Ther* 2015; **42**: 356-364 [PMID: 26032402 DOI: 10.1111/apt.13268]

84 **Papamichael K**, Vande Casteele N, Gils A, Tops S, Hauenstein S, Singh S, Princen F, Van Assche G, Rutgeerts P, Vermeire S, Ferrante M. Long-term outcome of patients with Crohn's disease who discontinued infliximab therapy upon clinical remission. *Clin Gastroenterol Hepatol* 2015; **13**: 1103-1110 [PMID: 25478919 DOI: 10.1016/j.cgh.2014.11.026]

85 **Bortlik M**, Duricová D, Machkova N, Hruba V, Lukas M, Mitrova K, Romanko I, Bina V, Lukas M. Deep remission in Crohn's disease does not prevent disease relapse after withdrawal of anti-TNFa therapy. *J Crohns Colitis* 2015; **9** (suppl 1): S4

86 **Monterubbianesi R**, Papi C, Kohn A. Maintenance of clinical remission in Crohn's disease patients after discontinuation of long term reatment with Infliximab: results of a single centre cohort. *Dig Liver Dis* 2015; **47** (Suppl 2): e137-e138

87 **Bodini G**, Savarino V, Dulbecco P, Baldissarro I, Savarino E. IBD recurrence after stopping anti-TNF-alpha therapy: a prospective randomized controlled study comparing mesalamine and azathioprine – Preliminary results. *Dig Liver Dis* 2015; **47** (Suppl 3): e100

88 **Ampuero J**, Rojas-Feria M, Castro-Fernández M, Millán-Lorenzo M, Guerrero-Jiménez P, Romero-Gómez M. Remission maintained by monotherapy after biological + immunosuppressive combination for Crohn's Disease in clinical practice. *J Gastroenterol Hepatol* 2015; Epub ahead of print [PMID: 26173493 DOI: 10.1111/jgh.13039]

89 **Papamichael K**, Vermeire S. Withdrawal of anti-tumour necrosis factor α therapy in inflammatory bowel disease. *World J Gastroenterol* 2015; **21**: 4773-4778 [PMID: 25944990 DOI: 10.3748/wjg.v21.i16.4773]

90 **Rogler G**, Bernstein CN, Sood A, Goh KL, Yamamoto-Furusho JK, Abbas Z, Fried M. Role of biological therapy for inflammatory bowel disease in developing countries. *Gut* 2012; **61**: 706-712 [PMID: 21997549 DOI: 10.1136/gutjnl-2011-300613]

91 **Manginot C**, Baumann C, Peyrin-Biroulet L. An endoscopic Mayo score of 0 is associated with a lower risk of colectomy than a score of 1 in ulcerative colitis. *Gut* 2015; **64**: 1181-1182 [PMID: 25550182 DOI: 10.1136/gutjnl-2014-308839]

92 **Bressenot A**, Salleron J, Bastien C, Danese S, Boulagnon-Rombi C, Peyrin-Biroulet L. Comparing histological activity indexes in UC. *Gut* 2015; **64**: 1412-1418 [PMID: 25246423 DOI: 10.1136/gutjnl-2014-307477]

93 **Bryant RV**, Winer S, Travis SP, Riddell RH. Systematic review: histological remission in inflammatory bowel disease. Is 'complete' remission the new treatment paradigm? An IOIBD initiative. *J Crohns Colitis* 2014; **8**: 1582-1597 [PMID: 25267173 DOI: 10.1016/j.crohns.2014.08.011]

94 **Bryant RV**, Burger DC, Delo J, Walsh AJ, Thomas S, von Herbay A, Buchel OC, White L, Brain O, Keshav S, Warren BF, Travis SP. Beyond endoscopic mucosal healing in UC: histological remission better predicts corticosteroid use and hospitalisation over 6 years of follow-up. *Gut* 2015; **pii**: gutjnl-2015-309598 [PMID: 25986946 DOI: 10.1136/gutjnl-2015-309598]

95 **D'Haens G**, Baert F, van Assche G, Caenepeel P, Vergauwe P, Tuynman H, De Vos M, van Deventer S, Stitt L, Donner A, Vermeire S, Van de Mierop FJ, Coche JC, van der Woude J, Ochsenkühn T, van Bodegraven AA, Van Hootegem PP, Lambrecht GL, Mana F, Rutgeerts P, Feagan BG, Hommes D. Early combined immunosuppression or conventional management in patients with newly diagnosed Crohn's disease: an open randomised trial. *Lancet* 2008; **371**: 660-667 [PMID: 18295023 DOI: 10.1016/S0140-6736(08)60304-9]

P- Reviewer: Castiglione F, Nguyen H
S- Editor: Ji FF **L- Editor**: A **E- Editor**: Jiao XK

World Journal of Gastrointestinal Pathophysiology

Submit a Manuscript: http://www.wjgnet.com/esps/
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.4291/wjgp.v7.i1.17

World J Gastrointest Pathophysiol 2016 February 15; 7(1): 17-26
ISSN 2150-5330 (online)

EDITORIAL

Adipose tissue-liver axis in alcoholic liver disease

Zhi-Gang Wang, Xiao-Bing Dou, Zhan-Xiang Zhou, Zhen-Yuan Song

Zhi-Gang Wang, College of Medical Laboratory Science and Technology, Harbin Medical University at Daqing, Daqing 163319, Heilongjiang Province, China

Xiao-Bing Dou, Zhen-Yuan Song, College of Life Science, Zhejiang Chinese Medical University, Hangzhou 310053, Zhejiang Province, China

Zhan-Xiang Zhou, Department of Nutrition, Center for Translational Biomedical Research, University of North Carolina at Greensboro, Kannapolis, NC 28081, United States

Zhen-Yuan Song, Department of Pathology, University of Illinois, Medical Center, Chicago, IL 60612, United States

Zhen-Yuan Song, Department of Kinesiology and Nutrition, University of Illinois at Chicago, Chicago, IL 60612, United States

Author contributions: All authors contributed to this paper.

Supported by National Institutes of Health NIAAA, No. R01 AA017442.

Conflict-of-interest statement: The authors declare no conflict of interest.

Correspondence to: Zhen-Yuan Song, PhD, Associate Professor, Department of Kinesiology and Nutrition, University of Illinois at Chicago, 1919 W Taylor Street, RM 627, Chicago, IL 60612, United States. song2008@uic.edu
Telephone: +1-312-9967892
Fax: +1-312-4130319

Received: May 26, 2015
Peer-review started: May 28, 2015
First decision: August 25, 2015
Revised: October 28, 2015
Accepted: November 24, 2015
Article in press: November 25, 2015
Published online: February 15, 2016

Abstract

Alcoholic liver disease (ALD) remains an important health problem worldwide. The disease spectrum is featured by early steatosis, steatohepatitis (steatosis with inflammatory cells infiltration and necrosis), with some individuals ultimately progressing to fibrosis/cirrhosis. Although the disease progression is well characterized, no effective therapies are currently available for the treatment in humans. The mechanisms underlying the initiation and progression of ALD are multifactorial and complex. Emerging evidence supports that adipose tissue dysfunction contributes to the pathogenesis of ALD. In the first part of this review, we discuss the mechanisms whereby chronic alcohol exposure contributed to adipose tissue dysfunction, including cell death, inflammation and insulin resistance. It has been long known that aberrant hepatic methionine metabolism is a major metabolic abnormality induced by chronic alcohol exposure and plays an etiological role in the pathogenesis of ALD. The recent studies in our group documented the similar metabolic effect of chronic alcohol drinking on methionine in adipose tissue. In the second part of this review, we also briefly discuss the recent research progress in the field with a focus on how abnormal methionine metabolism in adipose tissue contributes to adipose tissue dysfunction and liver damage.

Key words: Alcohol; Lipolysis; Adipose; Methylation; Methionine; Liver

Core tip: Alcoholic liver disease (ALD) remains an important health problem worldwide. The disease spectrum is

featured by early steatosis, steatohepatitis (steatosis with inflammatory cells infiltration and necrosis), with some individuals ultimately progressing to fibrosis/cirrhosis. Although the disease progression is well characterized, no effective therapies are currently available for the treatment in humans. The mechanisms underlying the initiation and progression of ALD are multifactorial and complex. Emerging evidence supports that adipose tissue dysfunction contributes to the pathogenesis of ALD. In this review, we discuss the mechanisms whereby chronic alcohol exposure contributed to adipose tissue dysfunction, as well as their contribution to ALD.

Wang ZG, Dou XB, Zhou ZX, Song ZY. Adipose tissue-liver axis in alcoholic liver disease. *World J Gastrointest Pathophysiol* 2016; 7(1): 17-26 Available from: URL: http://www.wjgnet.com/2150-5330/full/v7/i1/17.htm DOI: http://dx.doi.org/10.4291/wjgp.v7.i1.17

ALCOHOLIC LIVER DISEASE

Sustained and excessive alcohol consumption is often accompanied with pathological changes in the liver, termed alcoholic liver disease (ALD). The spectrum of ALD encompasses steatosis, steatohepatitis (steatosis with inflammatory cells infiltration and hepatocyte necrosis), with some individuals ultimately progressing to fibrosis/cirrhosis, leading to increased risk of hepatocellular carcinoma[1-3]. The early-stage ALD, including steatosis and early steatohepatitis, is clinically reversible after termination of alcohol drinking, although the latter takes longer time for the recovery. The pathomechanism implicated in the development of ALD is complex and believed to involve multiple pathogenic factors. Although much progress has been made over last three decades of research on the mechanisms underlying the disease, ALD remains an important health problem worldwide. It ranks among the major causes of morbidity and mortality in the world, and affects millions of patients worldwide each year and there is currently no Food and Drug Administration-approved therapy available to halt or reverse this process in humans.

PATHOGENESIS OF ALD

Steatosis

Hepatic steatosis, characterized by the excessive accumulation of fat in hepatocytes, is the most common and earliest response of the liver to chronic alcohol consumption. Although "pure" steatosis is clinically considered to be a benign condition, excessive fat accumulation makes hepatocytes vulnerable to the attack of "the second hit", such as proinflammatory cytokines and oxidative stress, leading to the progression to steatohepatitis[4,5]. The mechanisms involved in the development of alcohol-induced hepatic steatosis are multifactorial and remain to be fully elucidated. Sterol regulatory element binding proteins (SREBP)-1c, a master transcription factor controlling *de novo* lipogenesis, is upregulated in the liver of mice chronically exposed to ethanol-containing diet[6]. Importantly, liver-specific knockout of SREBP-1c protected mice against alcohol-induced fatty liver and liver damage[7], supporting the notion that enhanced hepatic *de novo* lipogenic process plays a pivotal role in alcohol-triggered fat accumulation in the liver. Moreover, chronic alcohol exposure is associated with impaired fatty acid β-oxidation, contributing to fat accumulation in hepatocytes. Suppressions of both adenosine monophosphate-activated protein kinase (AMPK) and peroxisome proliferator-activated receptor-alpha (PPAR-α), two regulatory proteins of fatty acids oxidation, are mechanistically involved in this process[8,9]. Furthermore, long-term alcohol consumption is reported to enhance uptakes of free fatty acids (FFAs) and triglyceride-rich lipoproteins by hepatocytes[10,11] and impair hepatic very-low-density lipoprotein secretion *via* suppressing microsomal triglyceride transfer protein activity[12], thereby contributing to fatty liver after chronic alcohol exposure.

Steatohepatitis

Steatohepatitis is characterized by fatty liver, hepatic neutrophil infiltration, and hepatocyte cell death. The stage is a prerequisite for progression to fibrosis and cirrhosis[13]. The molecular mechanism for the progression from steatosis to steatohepatitis involve complicated interactions between the direct effects of toxic ethanol metabolites on different cell types in the liver, overproduction of reactive oxygen species (ROS), and overactivated inflammatory responses[14-21]. Acetaldehyde, the major product of ethanol metabolism in the liver, plays an important role in the development of alcoholic steatohepatitis. Acetaldehyde is a reactive compound and highly toxic to hepatocytes. It binds to both proteins and DNA, leading to not only their functional changes but also activation of adaptive immune system and immune cell infiltration to the damaged liver[22,23]. Moreover, acetaldehyde also impair mitochondrial integrity and function, leading to oxidative stress and cell death[24-26]. Oxidative stress, derived from an imbalance between ROS production and cellular antioxidant capability, is believed to play a critical role in the transition from simple steatosis to steatohepatitis[27]. Many pathways have been suggested to contribute to the occurrence of oxidative stress in response to chronic ethanol exposure. In hepatocytes, the cytochrome P450 2E1 (CYP2E1) activation and mitochondria dysfunction seem to play central roles in inducing cellular oxidative stress state. CYP2E1 is highly inducible and has high catalytic activity for ethanol. During its catalytic circle, CYP2E1 generate significant amount of ROS, which can subsequently leads to cellular injury, lipid peroxidation, and mitochondrial damage[28-31]. Chronic alcohol consumption is associated with increased CYP2E1 expression

and activity[32], partially resulting from increased protein stability due to decreased proteasomal degradation[33,34]. CYP2E1 activity correlates with ethanol-induced liver injury and lipid peroxidation, which was reduced by the inhibition of CYP2E1 using either chemical inhibitors or genetic knockout of *CYP2E1* gene[35-38]. The detrimental effects of chronic alcohol consumption on liver mitochondria have been well documented. Long-term alcohol exposure is associated with reduced activity of key enzymes in mitochondrial respiratory chain and decreased mitochondria oxygen utilization[39,40]. Alcohol also leads to disruption between complex Ⅰ and complex Ⅲ of the mitochondrial electron transport chain, leading to elevated superoxide anion production[41]. Furthermore, chronic alcohol drinking is associated with damaged mitochondrial membrane integrity, possibly due to acetaldehyde accumulation, leading to defective mitochondrial GSH uptake, which sensitizes hepatocytes to TNF-α-induced cell death[42,43]. In addition to hepatocytes, accumulated evidence identified Kupffer cells (KCs) activation to be a central element in the development of steatohepatitis. Chronic alcohol exposure not only results in intestinal gram-negative bacterial overgrowth but also increases gut permeability, leading to the translocation of bacteria-derived LPS from the gut lumen to the blood[44-46]. The increased circulating LPS induces inflammatory actions in KCs in the liver *via* interacting with toll-like receptor (TLR)-4, resulting in production of oxidative stress and proinflammatory cytokines, including TNF-α, which plays a pivotal role in alcohol-induced hepatocyte cell death[47-50].

Fibrosis

Liver fibrogenesis is a wound-healing response to chronic liver injury. It is featured by excessive extracellular deposition of collagen and other extracellular matrix proteins, mainly derived from activated hepatic stellate cells (HSCs)[51-53]. The major stimuli for HSCs activation during chronic alcohol consumption include acetaldehyde[54,55], the main ethanol metabolite, and proinflammatory cytokines produced by KCs in response to gut-derived products *via* LPS-TLR4 interactions[56,57].

ADIPOSE TISSUE REGULATES WHOLE BODY LIPID HOMEOSTASIS

Adipose tissue plays a central role in regulating whole body lipid and energy homeostasis. The modern concept viewed adipose tissue as a complex, essential, and highly active metabolic and endocrine organ, not only as a reservoir for energy storage[58,59]. Adipose tissue communicates with other tissues and organs, including the liver, to integrate total body lipid homeostasis *via* both controlling circulating FFAs levels and synthesizing and releasing a host of secreted molecules, collectively designated as adipokines, including leptin, adiponectin, resistin, to name a few[58,60]. Adipose tissue stores excess energy in the form of triglycerides (TGs) and releases it in the form of FFAs, a process called lipolysis, to meet other tissues or organs' energy requirements[61]. Under physiological conditions, lipid storage and release are both coordinated and tightly regulated so that lipid fuels are stored during postprandial periods and released during fasting states. When the regulation of TG storage and FFAs release by adipose tissue is perturbed, particularly when release of FA becomes dissociated from energy requirements in extra-adipose tissues, plasma FA levels are elevated and excessive storage of TGs in these tissues, such as the liver, ensues, leading to hepatic steatosis (fatty liver). The critical role of adipose tissue in regulating hepatic lipid homeostasis can be best manifested by the facts that both long-term fasting and lipodystrophy (adipose tissue deficiency) results in fatty liver[62-64]. Insulin plays a dominant role in suppressing adipose tissue lipolysis during postprandial periods[65,66]. Therefore, adipose tissue insulin resistance is associated with elevated circulating FFAs levels due to uncontrolled lipolysis.

ADIPOSE TISSUE DYSFUNCTION IN ALD

Although it has been well-established that chronic alcohol consumption exerts a detrimental effect on hepatic fat synthesis and disposal, leading to the development of hepatic steatosis, emerging evidence supports that adipose tissue dysfunction also plays an important role in the pathogenesis of ALD. In the clinic setting, it has been reported that visceral fat accumulation is positively related to the onset of alcoholic liver damage and body mass index represents an independent risk factor for fibrosis in alcoholic patients[67-70]. Moreover, adipose tissue inflammation is correlated with the severity of pathological features in the liver of patients with ALD[69]. Experimentally, long-term alcohol consumption is associated with adipose tissue oxidative stress, insulin resistance, inflammation, adipocyte cell death, and adiponectin decline[71-75]. Chronic alcohol feeding results in hyper-lipolysis (degradation of TGs) in adipose tissue, leading to elevated circulation FFAs concentrations and a significant loss of white adipose tissue[10]. A recent study demonstrated that moderate obesity and alcohol synergistically induced steatohepatitis[76], further supporting the critical role of adipose tissue (dys)/function in the development of ALD. Importantly, both rosiglitazone (a PPAR-γ agonist mainly targeting adipocytes)[77] and recombinant adiponectin (an adipokine exclusively secreted by adipocytes)[78] improved ALD, suggesting that improving adipose tissue function represents a potential therapeutic approach for ALD.

EFFECTS OF CHRONIC ALCOHOL CONSUMPTION ON ADIPOSE TISSUE FUNCTION

Oxidative stress, cell death, and inflammation

Although the liver is the major organ for ethanol me-

tabolism, accumulated evidence supports that chronic alcohol feeding is also associated with increased oxidative stress, cell death, and inflammatory response in adipose tissue. Upregulation of CYP2E1 seems to play an important role in these events. Chronic ethanol feeding of rats causes increased expression and enzymatic activity of CYP2E1 in adipocytes/adipose tissue[72,79], leading to oxidative stress induction, which is evidenced by elevated 4-HNE production and protein carbonyls. When fully-differentiated 3T3-L1 adipocytes with CYP2E1 overexpression were exposed to ethanol-containing medium, increased oxidative stress was observed[72]. In contrast, overexpression of antisense CYP2E1 in adipocytes prevented ethanol-triggered oxidative stress[72]. Adipocyte cell death plays a pivotal role in triggering adipose tissue inflammatory responses[80,81]. Chronic alcohol consumption results in CYP2E1/Bid cascade-dependent adipocyte cell death in adipose tissue of rats, which contributes to adipose tissue inflammation in response to chronic alcohol exposure in that both Cyp2e1 and Bid knockout mice are protected from adipose tissue inflammation[72]. Interestingly, C1q, a component of the classical pathway of complement, seems to represent a critical link between cell death and inflammation in the setting of chronic alcohol consumption[72].

Adipokines

Alcohol consumption is known to disrupt adipokine release from adipose tissue[82,83]. Adiponectin and leptin are key adipokines that modulate hepatic lipid homeostasis. Reduced circulating leptin and adiponectin levels are observed in chronic alcohol exposed rodents, which contribute to the development of ALD[72,84-86]. *Via* binding with adiponectin receptor on hepatocytes, adiponectin activates AMPK pathway to stimulate FA oxidation, leading to reduced fat accumulation in the liver[87,88]. Indeed, either exogenous adiponectin administration or endogenously stimulating adiponectin production attenuate alcohol induced fatty liver in mice[78,84,89,90]. Mechanisms underlying alcohol-triggered adiponectin decline are multifactorial, including oxidative stress[79,91], hyperhomocysteinemia (HHcy)[84], and heme-oxygenase-1 dependent pathway[92]. Other than adiponectin, leptin is another adipokine reported to be affected by chronic alcohol consumption and contribute to ALD. Animal studies showed that chronic alcohol consumption decreases circulating leptin levels[80,93,94], which is associated with adipose tissue mass reduction. Importantly, exogenous leptin administration restored plasma leptin reduction triggered by chronic alcohol feeding and alleviated hepatic steatosis[95]. It is noteworthy here that clinical investigations support that moderate alcohol consumption is associated with increased adiponectin levels[95,96].

Lipolysis

Uncontrolled adipose tissue lipolysis, mainly due to insulin resistance, plays an etiological role in the development of obesity-related non-alcoholic fatty liver disease[97-100]. Insulin signaling in adipocytes plays a central role in controlling FFAs release by adipose tissue *via* lipolysis[65,66]. Peripheral insulin resistance derived from obesity compromises the suppressive effect of insulin on lipolysis, leading to increased exposure of the liver to circulating FFAs with subsequent development of fatty liver. The effect of chronic alcohol consumption on adipose tissue insulin sensitivity remains to be fully characterized; however, existing evidence supports that chronic alcohol consumption is associated with adipose tissue insulin resistance. In rats, chronic ethanol feeding results in impaired insulin-stimulated glucose transport in adipocytes, which is associated with a disruption of insulin-mediated Cbl/TC10 signaling and actin polymerization[97]. Moreover, chronic ethanol feeding compromises the suppression of the anti-lipolytic effects of insulin in adipocytes in both rats and mice, leading to enhanced triglyceride degradation in adipose tissue[10,73]. Interestingly, in comparison to subcutaneous fat, visceral white adipose tissue seems to be more susceptible to alcohol-induced lipolysis enhancement, which may involve increased acetaldehyde production[100]. In contrast to adipose tissue insulin resistance in obese animals which is associate with an increase in body weight and adipocyte size, chronic alcohol consumption leads to reduced adipose tissue mass and adipocyte size[10], indicating that distinct mechanism(s) are involved in the initiation of adipose tissue insulin resistance. Adipose triglyceride lipase and hormone sensitive lipase (HSL) are two critical enzymes catalyzing fatty acids release from the adipose tissue. Both enzymes are found to be activated in mice chronically fed with alcohol. Interestingly, chronic alcohol feeding has no effect on plasma catecholamine and insulin levels, while PTEN and SOC3, two negative regulators of insulin signaling pathway, are up-regulated, leading to insulin resistance[10].

ABERRANT METHIONINE METABOLISM AND ALCOHOL-INDUCED ADIPOSE TISSUE DYSFUNCTION

Methionine metabolism abnormality in ALD: Aberrant hepatic methionine metabolism is a major metabolic abnormality induced by chronic alcohol exposure and plays an etiological role in the pathogenesis of ALD[101-104]. As illustrated in Figure 1, intracellular methionine metabolism involves two major pathways, transmethylation reaction and transsulfuration reaction. The first step in methionine metabolism is the formation of S-adenosylmethionine (SAM) in a reaction catalyzed by methionine adenosyltransferase. Under physiological conditions, most of the SAM generated per day is used in transmethylation reactions in which methyl groups are added to a vast number of molecules, including DNA, RNA, phospholipids, histones, and other proteins, *via* specific methyltransferases. In this process, SAM is

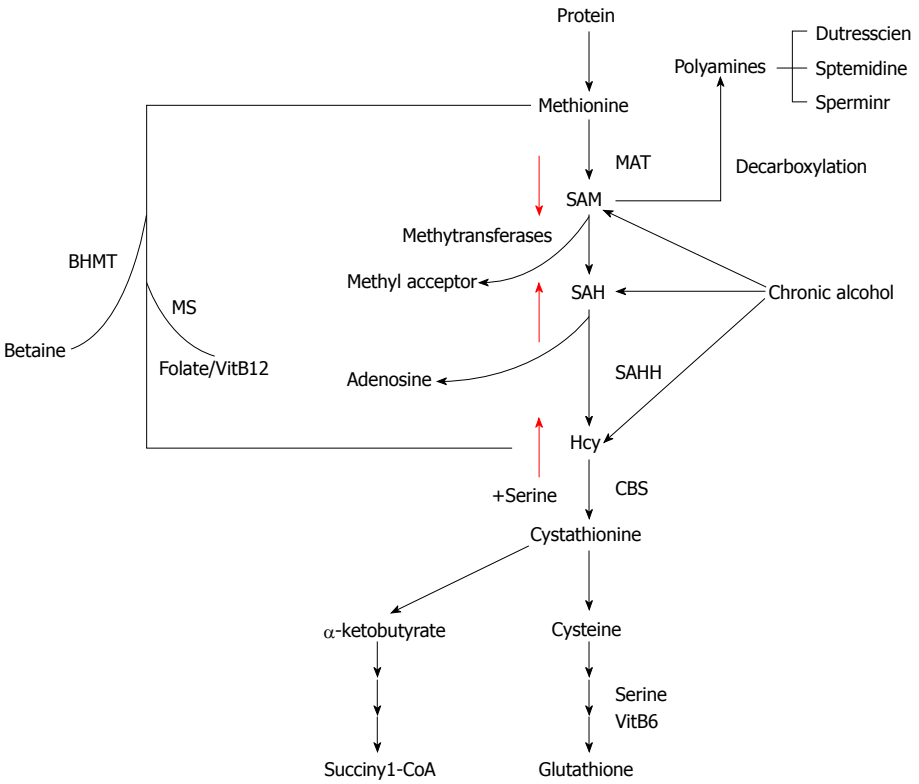


Figure 1 Intracellular methionine metabolism. Chronic alcohol consumption causes SAM deficiency, but enhancement of homocysteine and SAH. MAT: Methionine adenosyl-transferase; SAM: S-adenosylmethionine; SAH: S-adenosylhomocysteine; Hcy: Homocysteine; CBS: Cystathionine beta synthase; SAHH: S-adensylmonocysteine hydrolase; MS: Methionine synthase; BHMT: Betaine-homocysteine methyltransferase.

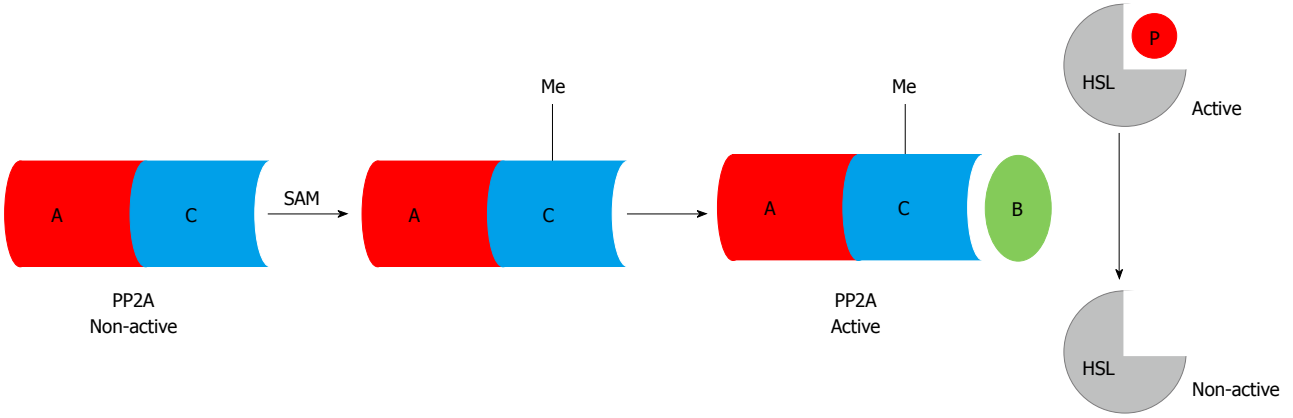


Figure 2 S-adenosylmethionine-dependent methylation reactions are required for protein phosphatase 2A activation, which dephosphorylates (inhibits) hormone sensitive lipase. Chronic alcohol consumption induces intracellular hypomethylation status in adipocytes, which suppresses PP2A activity, leading to uncontrolled HSL activation. SAM: S-adenosylmethionine; Me: Methyl group; PP2A: Protein phosphatase 2A; HSL: Hormone sensitive lipase.

converted to S-adenosylhomocysteine (SAH), followed by homocysteine (Hcy) and cysteine, a precursor for glutathione biosynthesis, *via* transsulfuration pathway. SAH is a potent competitive inhibitor of most methyltransferases studied and decreased SAM:SAH ratio has been widely employed as an indicator of suppressed transmethylation reactions[105,106]. While chronic alcohol exposure leads to hepatic SAM deficiency, both SAH and Hcy are increased in the liver in response to alcohol[101,104,107]. HHcy is associated with ER stress induction, leading to hepatocyte dysfunction[108], while increased intracellular SAH level enhances the sensitivity of hepatocytes to TNF-α-induced hepatotoxicity[104], both contributing to the pathogenesis of ALD.

Aberrant adipose methionine metabolism and hyper-lipolytic response in adipose tissue: Studies using both genetic and dietary animal models demonstrated that HHcy is associated with adipose tissue dysfunction[71,108-112], suggesting that methionine metabolism regulates adipose tissue function. We are the first to report that, similar to its effect on the liver, chronic alcohol feeding induces methionine metabolism

abnormality in adipose tissue, which is characterized by SAM deficiency, and accumulation of Hcy and SAH[71,113], leading to significant decrease of the SAM/SAH ratio, a strong indicator of inhibitory transmethylation reactions (hypomethylation). HSL is considered a rate-limiting lipase for adipose tissue lipolysis[114]. Upon lipolytic hormone stimulation, such as with catecholamine, cAMP/PKA-mediated-phosphorylations in certain serine residues activate HSL[115]. In contrast, protein phosphatase 2A -catalyzed dephosphorylation at Ser660 leads to HSL inactivation[116,117]. PP2A is a heterotrimeric protein phosphatase. The catalytic and scaffold subunits of PP2A are ubiquitously expressed and have remarkable sequence conservation within eukaryotes. Interestingly, accumulating evidence reveals that PP2A activation is under control of intracellular methylation status[118,119]. Carboxyl methylation of the PP2A catalytic subunit, catalyzed by PP2A-specific methyltransferaseleucine carboxyl methyltransferase-1, plays a critical role in regulating holoenzyme assembly[120]. These previous studies provide rational for us to posit that altered intracellular methylation status in adipocytes may affect adipose tissue lipolytic response. In a very recent study, we provided evidence supporting that intracellular hypomethylation status in adipocytes in the setting of chronic alcohol feeding contributes to adipose tissue hyper-lipolytic response in ALD *via* suppressing PP2A activity, leading to HSL overactivation[114] (Figure 2). Our data support that rectification of methionine metabolism through dietary supplementation of betaine protects against alcohol-induced liver damage, at least partially *via* improving adipose tissue function. Taken together, the recent research in our group suggest that aberrant methionine metabolism in adipocytes contributes to alcohol-elicited adipose tissue dysfunction and liver damage.

CONCLUSION

Despite its high prevalence, ALD has received limited attention during past decades and no major breakthrough in terms of its clinical management. Emerging evidence shows that adipose tissue plays an important role in both initiation and progression of liver damage induced by chronic alcohol consumption. However, the exact underlying cellular/molecular mechanisms involved in adipose tissue dysfunction in ALD remain to be fully elucidated. Future efforts in identifying the common factors that promote dysfunctions in both adipose tissue and the liver in response to chronic alcohol consumption will pave the way for the discovery of new therapeutic approach.

REFERENCES

1 **Adachi M**, Brenner DA. Clinical syndromes of alcoholic liver disease. *Dig Dis* 2005; **23**: 255-263 [PMID: 16508290 DOI: 10.1159/000090173]

2 **Tilg H**, Day CP. Management strategies in alcoholic liver disease. *Nat Clin Pract Gastroenterol Hepatol* 2007; **4**: 24-34 [PMID: 17203086 DOI: 10.1038/ncpgasthep0683]

3 **Grant BF**, Dufour MC, Harford TC. Epidemiology of alcoholic liver disease. *Semin Liver Dis* 1988; **8**: 12-25 [PMID: 3283941 DOI: 10.1055/s-2008-1040525]

4 **Stewart S**, Jones D, Day CP. Alcoholic liver disease: new insights into mechanisms and preventative strategies. *Trends Mol Med* 2001; **7**: 408-413 [PMID: 11530336 DOI: 10.1016/S1471-4914(01)02096-2]

5 **Reddy JK**, Rao MS. Lipid metabolism and liver inflammation. II. Fatty liver disease and fatty acid oxidation. *Am J Physiol Gastrointest Liver Physiol* 2006; **290**: G852-G858 [PMID: 16603729 DOI: 10.1152/ajpgi.00521.2005]

6 **You M**, Fischer M, Deeg MA, Crabb DW. Ethanol induces fatty acid synthesis pathways by activation of sterol regulatory element-binding protein (SREBP). *J Biol Chem* 2002; **277**: 29342-29347 [PMID: 12036955 DOI: 10.1074/jbc.M202411200]

7 **Ji C**, Chan C, Kaplowitz N. Predominant role of sterol response element binding proteins (SREBP) lipogenic pathways in hepatic steatosis in the murine intragastric ethanol feeding model. *J Hepatol* 2006; **45**: 717-724 [PMID: 16879892 DOI: 10.1016/j.jhep.2006.05.009]

8 **You M**, Matsumoto M, Pacold CM, Cho WK, Crabb DW. The role of AMP-activated protein kinase in the action of ethanol in the liver. *Gastroenterology* 2004; **127**: 1798-1808 [PMID: 15578517 DOI: 10.1053/j.gastro.2004.09.049]

9 **Fischer M**, You M, Matsumoto M, Crabb DW. Peroxisome proliferator-activated receptor alpha (PPARalpha) agonist treatment reverses PPARalpha dysfunction and abnormalities in hepatic lipid metabolism in ethanol-fed mice. *J Biol Chem* 2003; **278**: 27997-28004 [PMID: 12791698]

10 **Zhong W**, Zhao Y, Tang Y, Wei X, Shi X, Sun W, Sun X, Yin X, Sun X, Kim S, McClain CJ, Zhang X, Zhou Z. Chronic alcohol exposure stimulates adipose tissue lipolysis in mice: role of reverse triglyceride transport in the pathogenesis of alcoholic steatosis. *Am J Pathol* 2012; **180**: 998-1007 [PMID: 22234172 DOI: 10.1016/j.ajpath.2011.11.017]

11 **Wang Z**, Dou X, Li S, Zhang X, Sun X, Zhou Z, Song Z. Nuclear factor (erythroid-derived 2)-like 2 activation-induced hepatic very-low-density lipoprotein receptor overexpression in response to oxidative stress contributes to alcoholic liver disease in mice. *Hepatology* 2014; **59**: 1381-1392 [PMID: 24170703 DOI: 10.1002/hep.26912]

12 **Sugimoto T**, Yamashita S, Ishigami M, Sakai N, Hirano K, Tahara M, Matsumoto K, Nakamura T, Matsuzawa Y. Decreased microsomal triglyceride transfer protein activity contributes to initiation of alcoholic liver steatosis in rats. *J Hepatol* 2002; **36**: 157-162 [PMID: 11830326]

13 **Bataller R**, Rombouts K, Altamirano J, Marra F. Fibrosis in alcoholic and nonalcoholic steatohepatitis. *Best Pract Res Clin Gastroenterol* 2011; **25**: 231-244 [PMID: 21497741 DOI: 10.1016/j.bpg.2011.02.010]

14 **Lieber CS**. Alcoholic fatty liver: its pathogenesis and mechanism of progression to inflammation and fibrosis. *Alcohol* 2004; **34**: 9-19 [PMID: 15670660 DOI: 10.1016/j.alcohol.2004.07.008]

15 **Cederbaum AI**, Lu Y, Wu D. Role of oxidative stress in alcohol-induced liver injury. *Arch Toxicol* 2009; **83**: 519-548 [PMID: 19448996 DOI: 10.1007/s00204-009-0432-0]

16 **Leung TM**, Nieto N. CYP2E1 and oxidant stress in alcoholic and non-alcoholic fatty liver disease. *J Hepatol* 2013; **58**: 395-398 [PMID: 22940046 DOI: 10.1016/j.jhep.2012.08.018]

17 **Nassir F**, Ibdah JA. Role of mitochondria in alcoholic liver disease. *World J Gastroenterol* 2014; **20**: 2136-2142 [PMID: 24605012 DOI: 10.3748/wjg.v20.i9.2136]

18 **Fernández-Checa JC**, Kaplowitz N, García-Ruiz C, Colell A. Mitochondrial glutathione: importance and transport. *Semin Liver Dis* 1998; **18**: 389-401 [PMID: 9875556 DOI: 10.1055/s-2007-1007172]

19 **Mandrekar P**, Ambade A, Lim A, Szabo G, Catalano D. An essential role for monocyte chemoattractant protein-1 in alcoholic

liver injury: regulation of proinflammatory cytokines and hepatic steatosis in mice. *Hepatology* 2011; **54**: 2185-2197 [PMID: 21826694 DOI: 10.1002/hep.24599]

20 **Wheeler MD**, Kono H, Yin M, Nakagami M, Uesugi T, Arteel GE, Gäbele E, Rusyn I, Yamashina S, Froh M, Adachi Y, Iimuro Y, Bradford BU, Smutney OM, Connor HD, Mason RP, Goyert SM, Peters JM, Gonzalez FJ, Samulski RJ, Thurman RG. The role of Kupffer cell oxidant production in early ethanol-induced liver disease. *Free Radic Biol Med* 2001; **31**: 1544-1549 [PMID: 11744328 DOI: 10.1016/S0891-5849(01)00748-1]

21 **Uesugi T**, Froh M, Arteel GE, Bradford BU, Thurman RG. Toll-like receptor 4 is involved in the mechanism of early alcohol-induced liver injury in mice. *Hepatology* 2001; **34**: 101-108 [PMID: 11431739 DOI: 10.1053/jhep.2001.25350]

22 **Svegliati-Baroni G**, Baraona E, Rosman AS, Lieber CS. Collagen-acetaldehyde adducts in alcoholic and nonalcoholic liver diseases. *Hepatology* 1994; **20**: 111-118 [PMID: 7912686 DOI: 10.1002/hep.1840200118]

23 **You M**, Crabb DW. Recent advances in alcoholic liver disease II. Minireview: molecular mechanisms of alcoholic fatty liver. *Am J Physiol Gastrointest Liver Physiol* 2004; **287**: G1-G6 [PMID: 15194557 DOI: 10.1152/ajpgi.00056.2004]

24 **Zhou Z**, Sun X, Kang YJ. Ethanol-induced apoptosis in mouse liver: Fas- and cytochrome c-mediated caspase-3 activation pathway. *Am J Pathol* 2001; **159**: 329-338 [PMID: 11438480 DOI: 10.1016/S0002-9440(10)61699-9]

25 **Bradham CA**, Plümpe J, Manns MP, Brenner DA, Trautwein C. Mechanisms of hepatic toxicity. I. TNF-induced liver injury. *Am J Physiol* 1998; **275**: G387-G392 [PMID: 9724248]

26 **Román J**, Colell A, Blasco C, Caballeria J, Parés A, Rodés J, Fernández-Checa JC. Differential role of ethanol and acetaldehyde in the induction of oxidative stress in HEP G2 cells: effect on transcription factors AP-1 and NF-kappaB. *Hepatology* 1999; **30**: 1473-1480 [PMID: 10573527 DOI: 10.1002/hep.510300623]

27 **Meagher EA**, Barry OP, Burke A, Lucey MR, Lawson JA, Rokach J, FitzGerald GA. Alcohol-induced generation of lipid peroxidation products in humans. *J Clin Invest* 1999; **104**: 805-813 [PMID: 10491416 DOI: 10.1172/jci5584]

28 **Porter TD**, Coon MJ. Cytochrome P-450. Multiplicity of isoforms, substrates, and catalytic and regulatory mechanisms. *J Biol Chem* 1991; **266**: 13469-13472 [PMID: 1856184]

29 **Rendic S**, Di Carlo FJ. Human cytochrome P450 enzymes: a status report summarizing their reactions, substrates, inducers, and inhibitors. *Drug Metab Rev* 1997; **29**: 413-580 [PMID: 9187528 DOI: 10.3109/03602539709037591]

30 **Guengerich FP**. Uncommon P450-catalyzed reactions. *Curr Drug Metab* 2001; **2**: 93-115 [PMID: 11469727 DOI: 10.2174/1389200013338694]

31 **Lewis DF**, Pratt JM. The P450 catalytic cycle and oxygenation mechanism. *Drug Metab Rev* 1998; **30**: 739-786 [PMID: 9844808 DOI: 10.3109/03602539808996329]

32 **Lieber CS**. Cytochrome P-4502E1: its physiological and pathological role. *Physiol Rev* 1997; **77**: 517-544 [PMID: 9114822 DOI: 10.1007/978-3-540-47648-1_1463]

33 **Gonzalez FJ**. The 2006 Bernard B. Brodie Award Lecture. Cyp2e1. *Drug Metab Dispos* 2007; **35**: 1-8 [PMID: 17020953 DOI: 10.1124/dmd.106.012492]

34 **Roberts BJ**, Song BJ, Soh Y, Park SS, Shoaf SE. Ethanol induces CYP2E1 by protein stabilization. Role of ubiquitin conjugation in the rapid degradation of CYP2E1. *J Biol Chem* 1995; **270**: 29632-29635 [PMID: 8530344]

35 **Bai J**, Cederbaum AI. Adenovirus-mediated expression of CYP2E1 produces liver toxicity in mice. *Toxicol Sci* 2006; **91**: 365-371 [PMID: 16549397 DOI: 10.1093/toxsci/kfj165]

36 **Bardag-Gorce F**, Yuan QX, Li J, French BA, Fang C, Ingelman-Sundberg M, French SW. The effect of ethanol-induced cytochrome p4502E1 on the inhibition of proteasome activity by alcohol. *Biochem Biophys Res Commun* 2000; **279**: 23-29 [PMID: 11112412 DOI: 10.1006/bbrc.2000.3889]

37 **Bell LN**, Temm CJ, Saxena R, Vuppalanchi R, Schauer P, Rabinovitz M, Krasinskas A, Chalasani N, Mattar SG. Bariatric surgery-induced weight loss reduces hepatic lipid peroxidation levels and affects hepatic cytochrome P-450 protein content. *Ann Surg* 2010; **251**: 1041-1048 [PMID: 20485142 DOI: 10.1097/SLA.0b013e3181dbb572]

38 **Butura A**, Nilsson K, Morgan K, Morgan TR, French SW, Johansson I, Schuppe-Koistinen I, Ingelman-Sundberg M. The impact of CYP2E1 on the development of alcoholic liver disease as studied in a transgenic mouse model. *J Hepatol* 2009; **50**: 572-583 [PMID: 19157621 DOI: 10.1016/j.jhep.2008]

39 **Bailey SM**, Cunningham CC. Contribution of mitochondria to oxidative stress associated with alcoholic liver disease. *Free Radic Biol Med* 2002; **32**: 11-16 [PMID: 11755312 DOI: 10.1016/S0891-5849(01)00769-9]

40 **Bailey SM**, Pietsch EC, Cunningham CC. Ethanol stimulates the production of reactive oxygen species at mitochondrial complexes I and III. *Free Radic Biol Med* 1999; **27**: 891-900 [PMID: 10515594 DOI: 10.1016/S0891-5849(99)00138-0]

41 **Hoek JB**, Cahill A, Pastorino JG. Alcohol and mitochondria: a dysfunctional relationship. *Gastroenterology* 2002; **122**: 2049-2063 [PMID: 12055609 DOI: 10.1053/gast.2002.33613]

42 **Lluis JM**, Colell A, García-Ruiz C, Kaplowitz N, Fernández-Checa JC. Acetaldehyde impairs mitochondrial glutathione transport in HepG2 cells through endoplasmic reticulum stress. *Gastroenterology* 2003; **124**: 708-724 [PMID: 12612910 DOI: 10.1053/gast.2003.50089]

43 **Colell A**, García-Ruiz C, Miranda M, Ardite E, Marí M, Morales A, Corrales F, Kaplowitz N, Fernández-Checa JC. Selective glutathione depletion of mitochondria by ethanol sensitizes hepatocytes to tumor necrosis factor. *Gastroenterology* 1998; **115**: 1541-1551 [PMID: 9834283 DOI: 10.1016/S0016-5085(98)70034-4]

44 **Enomoto N**, Schemmer P, Ikejima K, Takei Y, Sato N, Brenner DA, Thurman RG. Long-term alcohol exposure changes sensitivity of rat Kupffer cells to lipopolysaccharide. *Alcohol Clin Exp Res* 2001; **25**: 1360-1367 [PMID: 11584157 DOI: 10.1111/j.1530-0277.2001.tb02359.x]

45 **Enomoto N**, Ikejima K, Yamashina S, Hirose M, Shimizu H, Kitamura T, Takei Y, Sato And N, Thurman RG. Kupffer cell sensitization by alcohol involves increased permeability to gut-derived endotoxin. *Alcohol Clin Exp Res* 2001; **25**: 51S-54S [PMID: 11410742 DOI: 10.1111/j.1530-0277.2001.tb02418.x]

46 **Tsukamoto H**, Takei Y, McClain CJ, Joshi-Barve S, Hill D, Schmidt J, Deaciuc I, Barve S, Colell A, Garcia-Ruiz C, Kaplowitz N, Fernandez-Checa JC, Yokoyama H, Okamura Y, Nakamura Y, Ishii H, Chawla RK, Barve S, Joshi-Barve S, Watson W, Nelson W, Lin M, Ohata M, Motomura K, Enomoto N, Ikejima K, Kitamura T, Oide H, Hirose M, Bradford BU, Rivera CA, Kono H, Peter S, Yamashina S, Konno A, Ishikawa M, Shimizu H, Sato N, Thurman R. How is the liver primed or sensitized for alcoholic liver disease? *Alcohol Clin Exp Res* 2001; **25**: 171S-181S [PMID: 11391068 DOI: 10.1111/j.1530-0277.2001.tb02393.x]

47 **Miller AM**, Horiguchi N, Jeong WI, Radaeva S, Gao B. Molecular mechanisms of alcoholic liver disease: innate immunity and cytokines. *Alcohol Clin Exp Res* 2011; **35**: 787-793 [PMID: 21284667 DOI: 10.1111/j.1530-0277.2010.01399.x]

48 **Szabo G**, Mandrekar P, Petrasek J, Catalano D. The unfolding web of innate immune dysregulation in alcoholic liver injury. *Alcohol Clin Exp Res* 2011; **35**: 782-786 [PMID: 21284666 DOI: 10.1111/j.1530-0277.2010.01398.x]

49 **Szabo G**. Gut-liver axis in alcoholic liver disease. *Gastroenterology* 2015; **148**: 30-36 [PMID: 25447847 DOI: 10.1053/j.gastro.2014.10.042]

50 **Mandrekar P**, Szabo G. Signalling pathways in alcohol-induced liver inflammation. *J Hepatol* 2009; **50**: 1258-1266 [PMID: 19398236 DOI: 10.1016/j.jhep.2009.03.007]

51 **Fujii H**, Kawada N. Fibrogenesis in alcoholic liver disease. *World J Gastroenterol* 2014; **20**: 8048-8054 [PMID: 25009376 DOI: 10.3748/wjg.v20.i25.8048]

52 **Suh YG**, Jeong WI. Hepatic stellate cells and innate immunity in alcoholic liver disease. *World J Gastroenterol* 2011; **17**: 2543-2551

[PMID: 21633659 DOI: 10.3748/wjg.v17.i20.2543]
53 **Mormone E**, George J, Nieto N. Molecular pathogenesis of hepatic fibrosis and current therapeutic approaches. *Chem Biol Interact* 2011; **193**: 225-231 [PMID: 21803030 DOI: 10.1016/j.cbi.2011.07.001]
54 **Reyes-Gordillo K**, Shah R, Arellanes-Robledo J, Hernández-Nazara Z, Rincón-Sánchez AR, Inagaki Y, Rojkind M, Lakshman MR. Mechanisms of action of acetaldehyde in the up-regulation of the human α2(I) collagen gene in hepatic stellate cells: key roles of Ski, SMAD3, SMAD4, and SMAD7. *Am J Pathol* 2014; **184**: 1458-1467 [PMID: 24641900 DOI: 10.1016/j.ajpath.2014.01.020]
55 **Szuster-Ciesielska A**, Plewka K, Daniluk J, Kandefer-Szerszeń M. Zinc supplementation attenuates ethanol- and acetaldehyde-induced liver stellate cell activation by inhibiting reactive oxygen species (ROS) production and by influencing intracellular signaling. *Biochem Pharmacol* 2009; **78**: 301-314 [PMID: 19376089 DOI: 10.1016/j.bcp.2009.04.009]
56 **Paik YH**, Schwabe RF, Bataller R, Russo MP, Jobin C, Brenner DA. Toll-like receptor 4 mediates inflammatory signaling by bacterial lipopolysaccharide in human hepatic stellate cells. *Hepatology* 2003; **37**: 1043-1055 [PMID: 12717385 DOI: 10.1053/jhep.2003.50182]
57 **Gobejishvili L**, Barve S, Breitkopf-Heinlein K, Li Y, Zhang J, Avila DV, Dooley S, McClain CJ. Rolipram attenuates bile duct ligation-induced liver injury in rats: a potential pathogenic role of PDE4. *J Pharmacol Exp Ther* 2013; **347**: 80-90 [PMID: 23887098 DOI: 10.1124/jpet.113.204933]
58 **Ahima RS**, Flier JS. Adipose tissue as an endocrine organ. *Trends Endocrinol Metab* 2000; **11**: 327-332 [PMID: 10996528 DOI: 10.1016/S1043-2760(00)00301-5]
59 **Frühbeck G**, Gómez-Ambrosi J, Muruzábal FJ, Burrell MA. The adipocyte: a model for integration of endocrine and metabolic signaling in energy metabolism regulation. *Am J Physiol Endocrinol Metab* 2001; **280**: E827-E847 [PMID: 11350765]
60 **Piya MK**, McTernan PG, Kumar S. Adipokine inflammation and insulin resistance: the role of glucose, lipids and endotoxin. *J Endocrinol* 2013; **216**: T1-T15 [PMID: 23160966 DOI: 10.1530/JOE-12-0498]
61 **Suganami T**, Tanaka M, Ogawa Y. Adipose tissue inflammation and ectopic lipid accumulation. *Endocr J* 2012; **59**: 849-857 [PMID: 22878669 DOI: 10.1507/endocrj.EJ12-0271]
62 **Gan SK**, Watts GF. Is adipose tissue lipolysis always an adaptive response to starvation?: implications for non-alcoholic fatty liver disease. *Clin Sci* (Lond) 2008; **114**: 543-545 [PMID: 18181765 DOI: 10.1042/CS20070461]
63 **Safar Zadeh E**, Lungu AO, Cochran EK, Brown RJ, Ghany MG, Heller T, Kleiner DE, Gorden P. The liver diseases of lipodystrophy: the long-term effect of leptin treatment. *J Hepatol* 2013; **59**: 131-137 [PMID: 23439261 DOI: 10.1016/j.jhep.2013.02.007]
64 **Reue K**, Phan J. Metabolic consequences of lipodystrophy in mouse models. *Curr Opin Clin Nutr Metab Care* 2006; **9**: 436-441 [PMID: 16778573 DOI: 10.1097/01.mco.0000232904.82038.db]
65 **Chakrabarti P**, Kandror KV. Adipose triglyceride lipase: a new target in the regulation of lipolysis by insulin. *Curr Diabetes Rev* 2011; **7**: 270-277 [PMID: 21644917 DOI: 10.2174/157339911796397866]
66 **Frühbeck G**, Méndez-Giménez L, Fernández-Formoso JA, Fernández S, Rodríguez A. Regulation of adipocyte lipolysis. *Nutr Res Rev* 2014; **27**: 63-93 [PMID: 24872083 DOI: 10.1017/S095442241400002X]
67 **Hart CL**, Morrison DS, Batty GD, Mitchell RJ, Davey Smith G. Effect of body mass index and alcohol consumption on liver disease: analysis of data from two prospective cohort studies. *BMJ* 2010; **340**: c1240 [PMID: 20223873 DOI: 10.1136/bmj.c1240]
68 **Tsai J**, Ford ES, Zhao G, Li C, Greenlund KJ, Croft JB. Co-occurrence of obesity and patterns of alcohol use associated with elevated serum hepatic enzymes in US adults. *J Behav Med* 2012; **35**: 200-210 [PMID: 21626151 DOI: 10.1007/s10865-011-9353-5]
69 **Loomba R**, Bettencourt R, Barrett-Connor E. Synergistic association between alcohol intake and body mass index with serum alanine and aspartate aminotransferase levels in older adults: the Rancho Bernardo Study. *Aliment Pharmacol Ther* 2009; **30**: 1137-1149 [PMID: 19737152 DOI: 10.1111/j.1365-2036.2009.04141.x]
70 **Shen Z**, Li Y, Yu C, Shen Y, Xu L, Xu C, Xu G. A cohort study of the effect of alcohol consumption and obesity on serum liver enzyme levels. *Eur J Gastroenterol Hepatol* 2010; **22**: 820-825 [PMID: 19829121 DOI: 10.1097/MEG.0b013e3283328b86]
71 **Sebastian BM**, Roychowdhury S, Tang H, Hillian AD, Feldstein AE, Stahl GL, Takahashi K, Nagy LE. Identification of a cytochrome P4502E1/Bid/C1q-dependent axis mediating inflammation in adipose tissue after chronic ethanol feeding to mice. *J Biol Chem* 2011; **286**: 35989-35997 [PMID: 21856753 DOI: 10.1074/jbc.M111.254201]
72 **Song Z**, Zhou Z, Deaciuc I, Chen T, McClain CJ. Inhibition of adiponectin production by homocysteine: a potential mechanism for alcoholic liver disease. *Hepatology* 2008; **47**: 867-879 [PMID: 18167065 DOI: 10.1002/hep.22074]
73 **Kang L**, Chen X, Sebastian BM, Pratt BT, Bederman IR, Alexander JC, Previs SF, Nagy LE. Chronic ethanol and triglyceride turnover in white adipose tissue in rats: inhibition of the anti-lipolytic action of insulin after chronic ethanol contributes to increased triglyceride degradation. *J Biol Chem* 2007; **282**: 28465-28473 [PMID: 17686776 DOI: 10.1074/jbc.M705503200]
74 **Chen X**, Sebastian BM, Nagy LE. Chronic ethanol feeding to rats decreases adiponectin secretion by subcutaneous adipocytes. *Am J Physiol Endocrinol Metab* 2007; **292**: E621-E628 [PMID: 17047161 DOI: 10.1152/ajpendo.00387.2006]
75 **Tang H**, Sebastian BM, Axhemi A, Chen X, Hillian AD, Jacobsen DW, Nagy LE. Ethanol-induced oxidative stress via the CYP2E1 pathway disrupts adiponectin secretion from adipocytes. *Alcohol Clin Exp Res* 2012; **36**: 214-222 [PMID: 21895711 DOI: 10.1111/j.1530-0277.2011.01607.x]
76 **Xu J**, Lai KK, Verlinsky A, Lugea A, French SW, Cooper MP, Ji C, Tsukamoto H. Synergistic steatohepatitis by moderate obesity and alcohol in mice despite increased adiponectin and p-AMPK. *J Hepatol* 2011; **55**: 673-682 [PMID: 21256905 DOI: 10.1016/j.jhep.2010.12.034]
77 **Sun X**, Tang Y, Tan X, Li Q, Zhong W, Sun X, Jia W, McClain CJ, Zhou Z. Activation of peroxisome proliferator-activated receptor-γ by rosiglitazone improves lipid homeostasis at the adipose tissue-liver axis in ethanol-fed mice. *Am J Physiol Gastrointest Liver Physiol* 2012; **302**: G548-G557 [PMID: 22173916 DOI: 10.1152/ajpgi.00342.2011]
78 **West M**. Dead adipocytes and metabolic dysfunction: recent progress. *Curr Opin Endocrinol Diabetes Obes* 2009; **16**: 178-182 [PMID: 19306530 DOI: 10.1097/MED.0b013e3283292327]
79 **Lafontan M**. Adipose tissue and adipocyte dysregulation. *Diabetes Metab* 2014; **40**: 16-28 [PMID: 24139247 DOI: 10.1016/j.diabet.2013.08.002]
80 **Rogers CQ**, Ajmo JM, You M. Adiponectin and alcoholic fatty liver disease. *IUBMB Life* 2008; **60**: 790-797 [PMID: 18709650 DOI: 10.1002/iub.124]
81 **Nicolás JM**, Fernández-Solà J, Fatjó F, Casamitjana R, Bataller R, Sacanella E, Tobías E, Badía E, Estruch R. Increased circulating leptin levels in chronic alcoholism. *Alcohol Clin Exp Res* 2001; **25**: 83-88 [PMID: 11198718 DOI: 10.1111/j.1530-0277.2001.tb02130.x]
82 **You M**, Considine RV, Leone TC, Kelly DP, Crabb DW. Role of adiponectin in the protective action of dietary saturated fat against alcoholic fatty liver in mice. *Hepatology* 2005; **42**: 568-577 [PMID: 16108051 DOI: 10.1002/hep.20821]
83 **Otaka M**, Konishi N, Odashima M, Jin M, Wada I, Matsuhashi T, Ohba R, Watanabe S. Effect of alcohol consumption on leptin level in serum, adipose tissue, and gastric mucosa. *Dig Dis Sci* 2007; **52**: 3066-3069 [PMID: 17406835 DOI: 10.1007/s10620-006-9635-x]
84 **Tan X**, Sun X, Li Q, Zhao Y, Zhong W, Sun X, Jia W, McClain CJ, Zhou Z. Leptin deficiency contributes to the pathogenesis of alcoholic fatty liver disease in mice. *Am J Pathol* 2012; **181**: 1279-1286 [PMID: 22841822 DOI: 10.1016/j.ajpath.2012.06.013]

85 **You M**, Rogers CQ. Adiponectin: a key adipokine in alcoholic fatty liver. *Exp Biol Med* (Maywood) 2009; **234**: 850-859 [PMID: 19491377 DOI: 10.3181/0902-MR-61]

86 **Marra F**, Bertolani C. Adipokines in liver diseases. *Hepatology* 2009; **50**: 957-969 [PMID: 19585655 DOI: 10.1002/hep.23046]

87 **Shen Z**, Liang X, Rogers CQ, Rideout D, You M. Involvement of adiponectin-SIRT1-AMPK signaling in the protective action of rosiglitazone against alcoholic fatty liver in mice. *Am J Physiol Gastrointest Liver Physiol* 2010; **298**: G364-G374 [PMID: 20007851 DOI: 10.1152/ajpgi.00456.2009]

88 **Ajmo JM**, Liang X, Rogers CQ, Pennock B, You M. Resveratrol alleviates alcoholic fatty liver in mice. *Am J Physiol Gastrointest Liver Physiol* 2008; **295**: G833-G842 [PMID: 18755807 DOI: 10.1152/ajpgi.90358.2008]

89 **Zhang X**, Wang Z, Li J, Gu D, Li S, Shen C, Song Z. Increased 4-hydroxynonenal formation contributes to obesity-related lipolytic activation in adipocytes. *PLoS One* 2013; **8**: e70663 [PMID: 23940618 DOI: 10.1371/journal.pone.0070663]

90 **Mandal P**, Pritchard MT, Nagy LE. Anti-inflammatory pathways and alcoholic liver disease: role of an adiponectin/interleukin-10/heme oxygenase-1 pathway. *World J Gastroenterol* 2010; **16**: 1330-1336 [PMID: 20238399 DOI: 10.3748/wjg.v16.i11.1330]

91 **Santolaria F**, Pérez-Cejas A, Alemán MR, González-Reimers E, Milena A, de la Vega MJ, Martínez-Riera A, Gómez-Rodríguez MA. Low serum leptin levels and malnutrition in chronic alcohol misusers hospitalized by somatic complications. *Alcohol Alcohol* 2004; **38**: 60-66 [PMID: 12554610 DOI: 10.1093/alcalc/agg015]

92 **Calissendorff J**, Brismar K, Röjdmark S. Is decreased leptin secretion after alcohol ingestion catecholamine-mediated? *Alcohol Alcohol* 2004; **39**: 281-286 [PMID: 15208157 DOI: 10.1093/alcalc/agh054]

93 **Joosten MM**, Witkamp RF, Hendriks HF. Alterations in total and high-molecular-weight adiponectin after 3 weeks of moderate alcohol consumption in premenopausal women. *Metabolism* 2011; **60**: 1058-1063 [PMID: 21353262 DOI: 10.1016/j.metabol.2011.01.001]

94 **Joosten MM**, Beulens JW, Kersten S, Hendriks HF. Moderate alcohol consumption increases insulin sensitivity and ADIPOQ expression in postmenopausal women: a randomised, crossover trial. *Diabetologia* 2008; **51**: 1375-1381 [PMID: 18504547 DOI: 10.1007/s00125-008-1031-y]

95 **Jou J**, Choi SS, Diehl AM. Mechanisms of disease progression in nonalcoholic fatty liver disease. *Semin Liver Dis* 2008; **28**: 370-379 [PMID: 18956293 DOI: 10.1055/s-0028-1091981]

96 **Jacome-Sosa MM**, Parks EJ. Fatty acid sources and their fluxes as they contribute to plasma triglyceride concentrations and fatty liver in humans. *Curr Opin Lipidol* 2014; **25**: 213-220 [PMID: 24785962 DOI: 10.1097/MOL.0000000000000080]

97 **Donnelly KL**, Smith CI, Schwarzenberg SJ, Jessurun J, Boldt MD, Parks EJ. Sources of fatty acids stored in liver and secreted via lipoproteins in patients with nonalcoholic fatty liver disease. *J Clin Invest* 2005; **115**: 1343-1351 [PMID: 15864352 DOI: 10.1172/jci200523621]

98 **Combs TP**, Marliss EB. Adiponectin signaling in the liver. *Rev Endocr Metab Disord* 2014; **15**: 137-147 [PMID: 24297186 DOI: 10.1007/s11154-013-9280-6]

99 **Sebastian BM**, Nagy LE. Decreased insulin-dependent glucose transport by chronic ethanol feeding is associated with dysregulation of the Cbl/TC10 pathway in rat adipocytes. *Am J Physiol Endocrinol Metab* 2005; **289**: E1077-E1084 [PMID: 16105861 DOI: 10.1152/ajpendo.00296.2005]

100 **Zhang W**, Zhong W, Sun X, Sun Q, Tan X, Li Q, Sun X, Zhou Z. Visceral white adipose tissue is susceptible to alcohol-induced lipodystrophy in rats: role of acetaldehyde. *Alcohol Clin Exp Res* 2015; **39**: 416-423 [PMID: 25703837 DOI: 10.1111/acer.12646]

101 **Barak AJ**, Beckenhauer HC, Kharbanda KK, Tuma DJ. Chronic ethanol consumption increases homocysteine accumulation in hepatocytes. *Alcohol* 2001; **25**: 77-81 [PMID: 11747976 DOI: 10.1016/S0741-8329(01)00168-9]

102 **Mato JM**, Cámara J, Fernández de Paz J, Caballería L, Coll S, Caballero A, García-Buey L, Beltrán J, Benita V, Caballería J, Solà R, Moreno-Otero R, Barrao F, Martín-Duce A, Correa JA, Parés A, Barrao E, García-Magaz I, Puerta JL, Moreno J, Boissard G, Ortiz P, Rodés J. S-adenosylmethionine in alcoholic liver cirrhosis: a randomized, placebo-controlled, double-blind, multicenter clinical trial. *J Hepatol* 1999; **30**: 1081-1089 [PMID: 10406187 DOI: 10.1016/S0168-8278(99)80263-3]

103 **Lu SC**, Huang ZZ, Yang H, Mato JM, Avila MA, Tsukamoto H. Changes in methionine adenosyltransferase and S-adenosylmethionine homeostasis in alcoholic rat liver. *Am J Physiol Gastrointest Liver Physiol* 2000; **279**: G178-G185 [PMID: 10898761]

104 **Song Z**, Zhou Z, Uriarte S, Wang L, Kang YJ, Chen T, Barve S, McClain CJ. S-adenosylhomocysteine sensitizes to TNF-alpha hepatotoxicity in mice and liver cells: a possible etiological factor in alcoholic liver disease. *Hepatology* 2004; **40**: 989-997 [PMID: 15382170 DOI: 10.1002/hep.20412]

105 **Mato JM**, Alvarez L, Ortiz P, Pajares MA. S-adenosylmethionine synthesis: molecular mechanisms and clinical implications. *Pharmacol Ther* 1997; **73**: 265-280 [PMID: 9175157 DOI: 10.1016/S0163-7258(96)00197-0]

106 **Chiang PK**, Gordon RK, Tal J, Zeng GC, Doctor BP, Pardhasaradhi K, McCann PP. S-Adenosylmethionine and methylation. *FASEB J* 1996; **10**: 471-480 [PMID: 8647346]

107 **Ji C**, Kaplowitz N. Betaine decreases hyperhomocysteinemia, endoplasmic reticulum stress, and liver injury in alcohol-fed mice. *Gastroenterology* 2003; **124**: 1488-1499 [PMID: 12730887 DOI: 10.1016/S0016-5085(03)00276-2]

108 **Gupta S**, Kruger WD. Cystathionine beta-synthase deficiency causes fat loss in mice. *PLoS One* 2011; **6**: e27598 [PMID: 22096601 DOI: 10.1371/journal.pone.0027598]

109 **Mikael LG**, Wang XL, Wu Q, Jiang H, Maclean KN, Rozen R. Hyperhomocysteinemia is associated with hypertriglyceridemia in mice with methylenetetrahydrofolate reductase deficiency. *Mol Genet Metab* 2009; **98**: 187-194 [PMID: 19560954 DOI: 10.1016/j.ymgme.2009.05.011]

110 **Li Y**, Zhang H, Jiang C, Xu M, Pang Y, Feng J, Xiang X, Kong W, Xu G, Li Y, Wang X. Hyperhomocysteinemia promotes insulin resistance by inducing endoplasmic reticulum stress in adipose tissue. *J Biol Chem* 2013; **288**: 9583-9592 [PMID: 23417716 DOI: 10.1074/jbc.M112.431627]

111 **Li Y**, Jiang C, Xu G, Wang N, Zhu Y, Tang C, Wang X. Homocysteine upregulates resistin production from adipocytes in vivo and in vitro. *Diabetes* 2008; **57**: 817-827 [PMID: 18192543 DOI: 10.2337/db07-0617]

112 **Wang Z**, Dou X, Yao T, Song Z. Homocysteine inhibits adipogenesis in 3T3-L1 preadipocytes. *Exp Biol Med* (Maywood) 2011; **236**: 1379-1388 [PMID: 22114064 DOI: 10.1258/ebm.2011.011234]

113 **Dou X**, Xia Y, Chen J, Qian Y, Li S, Zhang X, Song Z. Rectification of impaired adipose tissue methylation status and lipolytic response contributes to hepatoprotective effect of betaine in a mouse model of alcoholic liver disease. *Br J Pharmacol* 2014; **171**: 4073-4086 [PMID: 24819676 DOI: 10.1111/bph.12765]

114 **Holm C**, Kirchgessner TG, Svenson KL, Fredrikson G, Nilsson S, Miller CG, Shively JE, Heinzmann C, Sparkes RS, Mohandas T. Hormone-sensitive lipase: sequence, expression, and chromosomal localization to 19 cent-q13.3. *Science* 1988; **241**: 1503-1506 [PMID: 3420405 DOI: 10.1126/science.3420405]

115 **Anthonsen MW**, Rönnstrand L, Wernstedt C, Degerman E, Holm C. Identification of novel phosphorylation sites in hormone-sensitive lipase that are phosphorylated in response to isoproterenol and govern activation properties in vitro. *J Biol Chem* 1998; **273**: 215-221 [PMID: 9417067 DOI: 10.1074/jbc.273.1.215]

116 **Wood SL**, Emmison N, Borthwick AC, Yeaman SJ. The protein phosphatases responsible for dephosphorylation of hormone-sensitive lipase in isolated rat adipocytes. *Biochem J* 1993; **295** (Pt 2): 531-535 [PMID: 8240253 DOI: 10.1042/bj2950531]

117 **Kinney BP**, Qiao L, Levaugh JM, Shao J. B56alpha/protein phosphatase 2A inhibits adipose lipolysis in high-fat diet-induced obese mice. *Endocrinology* 2010; **151**: 3624-3632 [PMID: 20534721

DOI: 10.1210/en.2010-0245]

118 **Sontag JM**, Nunbhakdi-Craig V, Sontag E. Leucine carboxyl methyltransferase 1 (LCMT1)-dependent methylation regulates the association of protein phosphatase 2A and Tau protein with plasma membrane microdomains in neuroblastoma cells. *J Biol Chem* 2013; **288**: 27396-27405 [PMID: 23943618 DOI: 10.1074/jbc.M113.490102]

119 **Jackson JB**, Pallas DC. Circumventing cellular control of PP2A by methylation promotes transformation in an Akt-dependent manner. *Neoplasia* 2012; **14**: 585-599 [PMID: 22904676]

120 **Stanevich V**, Jiang L, Satyshur KA, Li Y, Jeffrey PD, Li Z, Menden P, Semmelhack MF, Xing Y. The structural basis for tight control of PP2A methylation and function by LCMT-1. *Mol Cell* 2011; **41**: 331-342 [PMID: 21292165 DOI: 10.1016/j.molcel.2010.12.030]

P- Reviewer: Abenavoli L, Gonzalez-Reimers E, Mattner J, Takahashi T, Wang JS, Xu CF
S- Editor: Ji FF **L- Editor**: A **E- Editor**: Jiao XK

World Journal of Gastrointestinal Pathophysiology

Submit a Manuscript: http://www.wjgnet.com/esps/
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.4291/wjgp.v7.i1.27

World J Gastrointest Pathophysiol 2016 February 15; 7(1): 27-37
ISSN 2150-5330 (online)

Current application of proteomics in biomarker discovery for inflammatory bowel disease

Patrick PY Chan, Valerie C Wasinger, Rupert W Leong

Patrick PY Chan, Valerie C Wasinger, Bioanalytical Mass Spectrometry Facility, Mark Wainwright Analytical Centre, the University of NSW Australia, Kensington, NSW 2052, Australia

Rupert W Leong, Gastroenterology and Liver Services, Concord Repatriation General Hospital, Concord, NSW 2137, Australia

Author contributions: Chan PPY wrote the paper; Wasinger VC and Leong RW reviewed and edited the draft for submission.

Conflict-of-interest statement: No potential conflicts of interest. No financial support.

Correspondence to: Rupert W Leong, MD, MBBS, Gastroenterology and Liver Services, Concord Repatriation General Hospital, Hospital Road, Concord, NSW 2137, Australia. rupert.leong@sswahs.nsw.gov.au
Telephone: +61-2-97676111
Fax: +61-2-97676767

Received: August 31, 2015
Peer-review started: September 2, 2015
First decision: September 29, 2015
Revised: November 13, 2015
Accepted: December 29, 2015
Article in press: January 4, 2016
Published online: February 15, 2016

Abstract

Recently, the field of proteomics has rapidly expanded in its application towards clinical research with objectives ranging from elucidating disease pathogenesis to discovering clinical biomarkers. As proteins govern and/or reflect underlying cellular processes, the study of proteomics provides an attractive avenue for research as it allows for the rapid identification of protein profiles in a biological sample. Inflammatory bowel disease (IBD) encompasses several heterogeneous and chronic conditions of the gastrointestinal tract. Proteomic technology provides a powerful means of addressing major challenges in IBD today, especially for identifying biomarkers to improve its diagnosis and management. This review will examine the current state of IBD proteomics research and its use in biomarker research. Furthermore, we also discuss the challenges of translating proteomic research into clinically relevant tools. The potential application of this growing field is enormous and is likely to provide significant insights towards improving our future understanding and management of IBD.

Key words: Proteomics; Inflammatory bowel disease; Biomarkers; Molecular diagnostic techniques; Mass spectrometry

Core tip: Proteomic methods provide a powerful tool that can be applied to the discovery of disease markers, allowing for rapid identification and quantification of proteins. Inflammatory bowel disease (IBD) currently faces many challenges, ranging from the elucidation of its pathophysiology to the accurate diagnosis in patients. Proteomics has been widely employed in many disease in the search of biomarkers, particularly cancer proteins. It has great potential to improve both our understanding and clinical management of IBD. Our review summarises the current application of proteomics to IBD and discusses challenges relating to translation into clinical practice.

Chan PPY, Wasinger VC, Leong RW. Current application of proteomics in biomarker discovery for inflammatory bowel disease. *World J Gastrointest Pathophysiol* 2016; 7(1): 27-37 Available from: URL: http://www.wjgnet.com/2150-5330/full/v7/i1/27.htm DOI: http://dx.doi.org/10.4291/wjgp.v7.i1.27

INTRODUCTION

Inflammatory bowel disease (IBD) encompasses a group of conditions characterised by chronic gastrointestinal inflammation, with the two major subtypes being Crohn's disease (CD) and ulcerative colitis (UC). Differentiating between subtypes of IBD sometimes has a degree of uncertainty due to overlapping clinical and pathological features[1]. Despite clinical evaluation, radiological, endoscopic and histopathological testing by expert physicians, up to 20% of IBD cases are classified as "indeterminate colitis" or "IBD undifferentiated"[2,3]. However, accurate classification of IBD is essential as response to medication, surgical indications and prognosis can vary between UC and CD[4]. The field of proteomics is a rapidly expanding area of research that has been employed in many diseases such as cancer[5,6], exploring everything from understanding disease pathways to discovering diagnostic markers[7-9]. This review examines the current state of biomarkers in IBD, with particular reference to the application of proteomics.

CURRENT BIOMARKERS IN IBD

Biomarkers are measureable substances that can objectively evaluate either physiological processes or therapeutic outcomes[10] and could potentially play a pivotal role in IBD as cheap and non-invasive alternatives to endoscopy[11]. Different biomarkers could be beneficial across all aspects of IBD (illustrated in Figure 1)[12]. The major commercially available biomarkers are summarised below based on their application in Table 1. Whilst some of these biomarkers demonstrate high diagnostic accuracy, they are currently unable to replace endoscopy entirely and limited only to being adjuncts[11,13]. Therefore, there is a prevailing need for the development of additional non-invasive biomarkers that are sufficiently sensitive and specific in the diagnosis and prognosis of IBD.

PROTEOMICS

The term "proteome" was initially defined as the total protein complement encoded by a given genome[14] but now also encompasses any isoforms, post-translational modifications, interactions and effectively anything "post-genomic"[15]. The study of proteomics involves large scale detection, identification and characterisation of proteins, making it highly promising for biomarker discovery across many diseases[16]. The most common method applied is a combination of two-dimensional electrophoresis (2-DE) and mass-spectrometry. 2-DE provides a powerful tool isolating proteins that differ in abundance between cases and controls[17]. Mass spectrometry can then identify proteins utilising techniques such as "surface enhanced laser desorption/ionisation time-of-flight" (SELDI-TOF) or "matrix-assisted laser desorption/ionisation time-time-of-flight" (MALDI-TOF). Both these technique involve fragmentation of proteins into peptides, determining their mass-to-charge ratio based on their "time-of-flight" within an electric field and comparing their peptide mass signatures to a database of known proteins to identify the original protein.

Although mass spectrometry is not inherently quantitative, many methods have been developed to achieve accurate quantitative data[17,18]. The crux of selecting candidate biomarkers in proteomic studies rely detecting differences in abundances between cases and controls; therefore quantitative proteomics is an essential aspect. Multiple reaction monitoring (MRM) is a quantitative technique that achieves absolute quantitation and has a relatively high sensitivity when detecting peptides in low abundance, suiting it towards application in proteomic biomarker studies[19].

APPLYING PROTEOMICS TO IBD

The process leading up to clinical implementation of a novel proteomic biomarker can be divided into three major stages of a pipeline: Discovery, verification and validation, which all vary in both aim and study design (Figure 2)[20]. At present, the application of proteomics in IBD (and many other diseases) remains largely in its infancy in the initial discovery phase. This stage involves the rapid analysis of entire protein profiles within a target sample (*e.g.,* plasma from an IBD patient), to screen for proteins that have relative differences in abundance compared to control samples[21]. The main disadvantage however, is that these discovery experiments do not provide absolute quantification and are labour intensive (and therefore typically have small sample sizes). The "verification" and "validation" stages addresses these issues by confirming the presence of and quantifying candidate markers in larger populations to assess their value in clinical usage.

Biomarker discovery studies

Proteomic studies involving IBD biomarkers have been divided into those relating to diagnosis and those pertaining to disease characteristics.

The most common approach towards biomarker discovery in proteomics involves assessing relative differences in proteins between cases and controls, for example, identifying which protein is differentially expressed between IBD patients and healthy controls. Furthermore, with the common objective of developing a clinically relevant assay, many groups have analysed

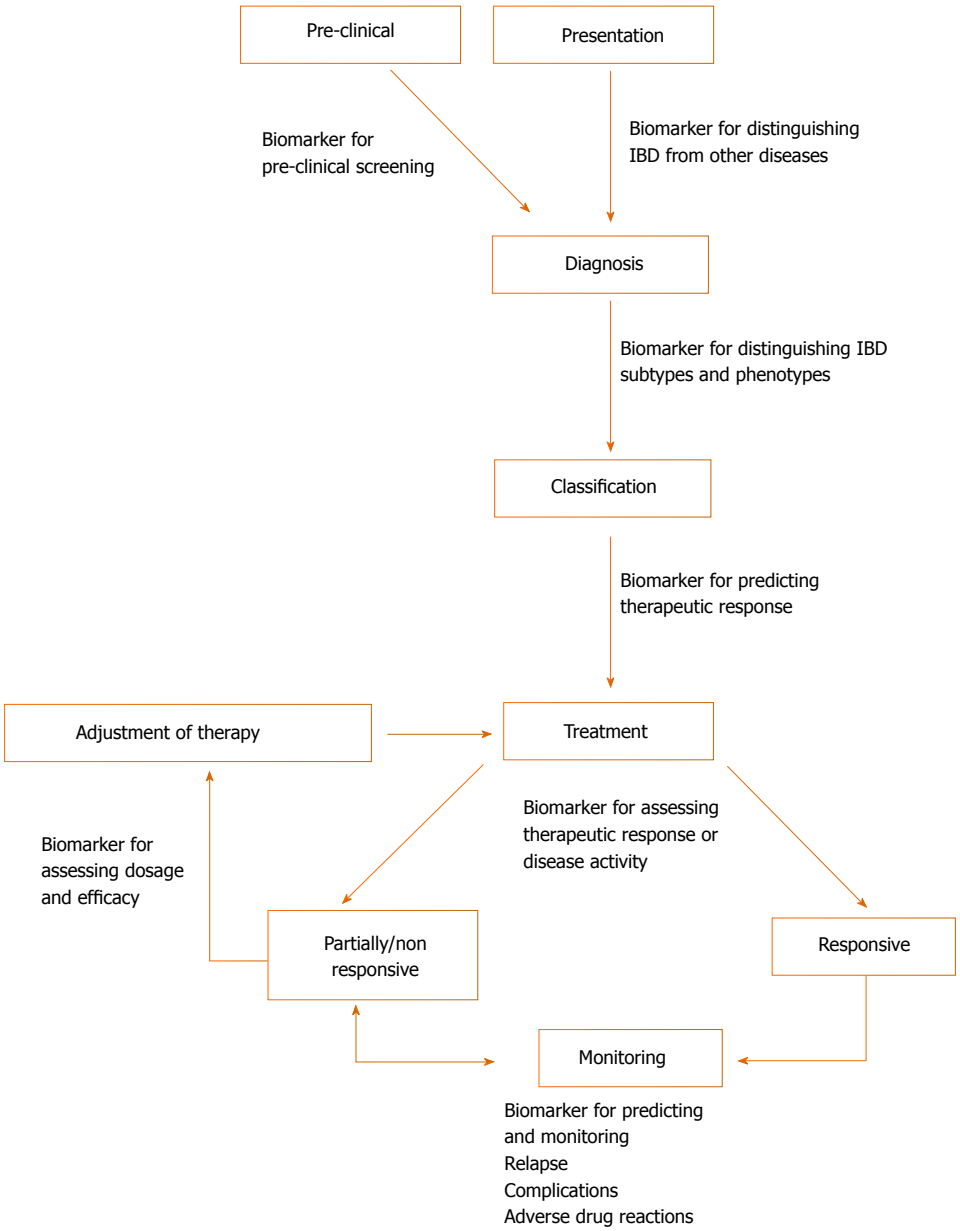


Figure 1 Potential application of biomarkers in inflammatory bowel disease in different stages of clinical management. When presenting clinically, one important use of biomarkers could be in the diagnosis of IBD, as well as differentiating subtypes (*e.g.*, UC *vs* CD) and phenotypes (*e.g.*, fistulising). Whilst not currently part of management, preclinical screening for IBD may be a possibility. Biomarkers can also be used to predict response to therapy and objectively measure therapeutic response and disease severity. Due to the relapsing and remitting course of IBD, monitoring is necessary for assessing relapse, adverse outcomes and complications (*e.g.*, strictures, fistulas and colorectal cancer). Most of these aspects necessitate endoscopic procedures and would benefit from biomarker substitutes. CD: Crohn's disease; UC: Ulcerative colitis; IBD: Inflammatory bowel disease.

plasma/serum for candidate markers (summarised in Table 2).

In 2007, Meuwis *et al*[22] reported a proteomic profile detected with SELDI-TOF MS that could discriminate active UC and CD with a high sensitivity and specificity, performing similarly or better than current ANCA and ASCA serology. From the protein spectra detected, platelet factor 4, myeloid related protein 8, fibrinopeptide A and haptoglobin α2 were considered diagnostically important. Kanmura *et al*[23] examined UC serum samples using SELDI-TOF MS and identified that human neutrophil peptide (HNP) 1-3 was differentially expressed. HNP 1-3 was confirmed by ELISA to differentiate active UC from inactive UC, all CD cases and controls, but not colorectal cancer. Similar studies using variants of mass spectrometry have yielded similar results where protein profiles could accurately distinguish between selected UC and CD cases[24-27]. A recent study by Vaiopoulou *et al*[28] sought to investigate pediatric biomarkers for CD by comparing the proteomic profile between adult and pediatric CD patients. 3 proteins (ceruloplasmin, clusterin and apolipoprotein B-100) were shown to be significantly different between the two cohorts. Whilst the plasma proteome is the most comprehensive collection of proteins, potential biomarkers are more difficult to detect as they exist in

Table 1 Current biomarkers and their utility in inflammatory bowel disease management[12]

Application	Biomarker	Utility
Diagnosis of IBD	Fecal calprotectin[69]	Sensitivity: 89%-98%, specificity: 81%-91%
	Fecal lactoferrin[70]	Sensitivity: 80%, specificity: 82%
	Fecal 100A12[71] (differentiating from IBS)	Sensitivity: 86%, specificity: 96%
	CRP[72-74]	Sensitivity: Approximately equal 100% in CD, approximately equal 50% in UC poor specificity
Distinguishing UC and CD	ASCA[75]	Sensitivity: 40%-50%, specificity: > 90% in CD
	pANCA[75]	Sensitivity: 57%, specificity: 92%
	Escherichia coli antibodies (Anti-OmpC, Anti-I2, Anti-CBir1)[76]	Sensitivity: 18%-55%, specificity: 76%-93%[76]
Marker of disease activity	Fecal lactoferrin[77,78]	Sensitivity: 66%-80% Specificity: 60%-100%
	Fecal calprotectin[77,78]	Sensitivity: 70%-100% Specificity: 44%-100%
	CRP[78]	Sensitivity: 48% Specificity: 91%
Assessing mucosal healing	Fecal calprotectin	Several studies demonstrate significant reduction in biomarker in the presence of mucosal healing with treatment
Predicting disease course	Fecal lactoferrin[77]	May be associated with complications including; structuring or fistulising disease, and small bowel disease
	ASCA pANCA Anti-I2, Anti-OmpC[12]	pANCA may predict aggressive UC and pouchitis following surgery[79]
Predicting Relapse within 12 mo	Fecal calprotectin[80,81]	Sensitivity: 69%-90% Specificity: 69%-82% Positive predictive value: 81%/87% (UC/CD) Negative predictive value: 90%/43% (UC/CD)
	Fecal lactoferrin[81]	Sensitivity: 62% Specificity: 65%
Predicting therapeutic response	pANCA[82]	Conflicting reports, possible lower response rate to infliximab in patients with a positive serology
	Anti-I2[83]	94% responded to fecal diversion

CD: Crohn's disease; UC: Ulcerative colitis; IBD: Inflammatory bowel disease; ASCA: Anti-Saccharomyces cerevisiae; pANCA: Perinuclear antineutrophil cytoplasmic antibodies; CRP: C-Reactive protein; PF4: Platelet factor 4.

significantly lower concentrations compared to other proteins such as albumin[20,29,30]. The alternative approach that has also become popular involves sampling "proximal fluid", as any biological material directly sampled from the site of disease is likely to contain greater concentrations of potential biomarkers relative to plasma[20,30-32]. Employing a similar rationale, direct sampling of diseased tissue in IBD (a far simpler task compared to other diseases due to routine endoscopic biopsies) has been utilised for proteomic experiments (Table 2). Shkoda *et al*[33] reported the first proteomic study of intestinal tissue, identifying nine statistically significant proteins delineating inflamed IBD tissue from non-inflamed controls. Furthermore, 40 proteins were further detected between inflamed and non-inflamed UC tissue, although only two pairs of patient samples were analysed. Similarly, Han *et al*[34] identified a large number of differentially expressed proteins (37 relevant for CD, 27 for UC and 11 associated with general IBD) that were seen as candidate biomarkers. M'koma and colleagues conducted two studies that identified spectral peaks representing unknown protein profiles and reported being able to accurately distinguish between the UC and CD using an algorithm[35,36]. These tissue findings however are likely to require validation in plasma samples as the aim involves develop a clinical assay such as a blood test.

A similar demand for objective biomarkers exists across all aspects of IBD patient management, as such these markers have been investigated in a number of studies (summarised in Table 3). Han *et al*[34] identified 16 additional proteins that were expressed differently between active and inactive CD. Kanmura *et al*[23] associated a higher level of HNP 1-3 with a positive response following induction of corticosteroid therapy, whilst non-responders had lower HNP 1-3 levels. Meuwis *et al*[37] published a second report which identified a serum protein profile which correlated with infliximab response. Gazouli *et al*[38] performed a similar study using MALDI-TOF MS, identifying 15 proteins that were differentially expressed amongst patients that responded differently to infliximab. They were however, unable to confirm the findings by Meuwis *et al*[37].

Most recently, Wasinger *et al*[39] reported a panel of protein markers that were progressed into the "validation" stage using MRM. Two proteins [phosphoprotein 24 (SPP24) and α-1 microglobulin], were reported to be able to differentiate IBD patients and health controls whilst guanylin and secretogranin-1 differentiated UC and CD. Furthermore, three of these proteins (secre-

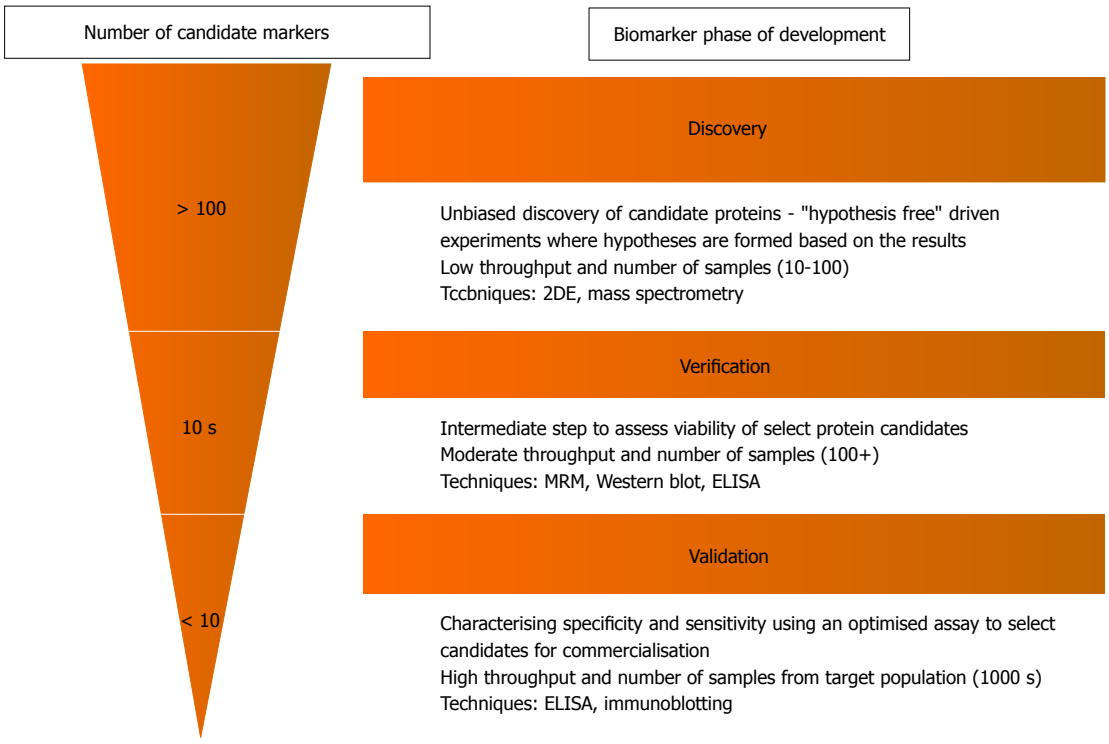


Figure 2 Biomarker "Pipeline" indicating the various stages from biomarker discovery to clinical application[20]. The number of candidate proteins (rough estimate of numbers indicated in figure) is narrowed down significantly in each step, selecting only the best candidates for further assessment and characterisation in a larger sample. The methodology also varies between the different phases. The early discovery phase uses low throughput methods such as 2-DE and mass spectrometry to screen large numbers of proteins in a low number of samples. Verification and validation require much more accurate quantitative methods as candidate proteins are narrowed down from the discovery phase and are assessed for their clinical utility in a large target population. This requires higher throughput methods such as MRM and immunoassays such as ELISA. CD: Crohn's disease; UC: Ulcerative colitis; IBD: Inflammatory bowel disease; MRM: Multiple reaction monitoring.

togranin-1, SPP24 and α-1 microglobulin), were able to distinguish between active and quiescent disease in UC and CD.

An important consideration when investigating IBD biomarkers is that a single protein may not provide the clinical utility desired, but rather a panel of markers governed by a scoring index or algorithm[40]. An existing example is the Brignola score which predicts relapse risk in asymptomatic Crohn's patients by measures erythrocyte sedimentation rate, white blood cell count, hemoglobin, albumin, alpha 2-globulin, serum iron, C-reactive protein, alpha 1-glycoprotein, and alpha 2-antitrypsin[41]. This has been hinted at in several IBD proteomic studies which differentiated UC and CD using protein profiles rather than discrete markers[22,24]. The role of multiple biomarkers is highlighted by OVA1, the first Food and Drug Administration approved proteomic panel of biomarkers, consisting of 5 markers as a multivariate index assay. This assay combines multiple variables in an algorithm that produces a single diagnostic result[42]. These markers were identified using SELDI[43] and predicts the probability of a malignancy in a woman undergoing surgery for an adnexal mass[44]. Similarly, Plevy *et al*[45] used a combined panel of 8 serological markers, 4 genetic markers and 5 inflammatory aimed at discriminating CD from UC. The utility of this test however still requires validation in a prospective cohort. Furthermore, as it was a North American cross-sectional study, this warrants additional investigation into its validity when considering factors such as stability of markers over time[45] and ethnical variations[46].

An area that has yet to be addressed relates to the influence of IBD medications on protein abundance levels. Schreiber *et al*[47] reported the possible link between high dose 5-aminosalicylic acid (5-ASA) and modulated urinary protein concentrations. However, other groups have suggested that these urinary proteins reflect renal extra-intestinal manifestations rather than 5-ASA toxicity[48,49]. Derici *et al*[50] identified an association between similar urinary proteins and disease activity in UC, however none of these have been conclusive. Similarly, Mishima *et al*[51] detected elevated plasma levels of osteopontin in IBD patients, whilst Lorenzen *et al*[52] suggested a possible association between increased urinary osteopontin expression and steroid induced nephrotic syndrome. Whilst the relation between medications and their effect on protein expression is currently unclear, there are a number of implications in the context of biomarker discovery. Depending on the clinical question, the influence of medications would require strict experimental design and patient selection to avoid confounders. Additionally, biomarkers predicting or identifying adverse drug

Table 2 Proteomic studies for discovering diagnostic inflammatory bowel disease biomarkers

Ref.	Bio-sample	Sample size	Proteomic technique	Results
Meuwis *et al*[22]	Serum	CD: 30 UC: 30 Inflammatory control: 30 Healthy controls: 30	SELDI-TOF	4 candidate proteins selected for high diagnostic value; PF4, MRP8, FIBA, Hpα 2. PF4 and Hpα 2 were also confirmed and correlated using ELISA and immunoblotting
Kanmura *et al*[23]	Blood	CD: 22 UC: 48 Colorectal Cancer: 5 Infectious colitis: 6 Healthy controls: 13	SELDI-TOF	Plasma concentrations of HNP1, 2 and 3 were significantly higher in active UC compared to inactive UC, CD and control patients
Hatsugai *et al*[24]	Blood	CD: 13 UC: 17 Healthy controls: 17	2-DE MALDI-TOF	Multivariate analysis of peripheral blood mononuclear cells protein profile 58 protein) allowed for accurate discrimination between UC and CD
Nanni *et al*[25]	Blood	Healthy controls: 48 CD: 15	Liquid chromatography quadrupole-TOF SELDI-TOF	Exopeptidase activity may distinguish CD from UC. Label free method developed could accurately distinguish synthetic spiked samples of serum
Sumramanian *et al*[26]	Serum	CD: 48 UC: 62		Protein signature of 12 mass: Charge peaks could classify CD with approximately equal 95% sensitivity/specificity 4 proteins identified as clinically useful
Nanni *et al*[27]	Serum	Healthy controls: 48 CD: 15 UC: 26	Solid-phase extraction MALDI-TOF	20 protein signals could be used to accurately classify IBD patients
Vaiopoulou *et al*[28]	Serum	CD: 24 (12 adults, 12 children)	2-DE MALDI-TOF	Clusterin was found to be overexpressed in adult CD. Ceruloplasmin and apolipoprotein B-100 was over-expressed in children
Han *et al*[34]	Intestinal biopsy	CD: 3 UC: 4 Inflammatory polyps: 2 Normal colon: 3	Liquid chromatography quadrupole-TOF	Increased in UC: TTBK2, SYNE2, SUCLG2, POSTN Up-regulated in CD: ANXA2, EPX, LAP3, RDX Up-regulated in IBD: S100A8, MPO, DEFA1B Up-regulated in CD ($P < 0.05$ AND > 2x increase): PRG2, LCP1, PSME1
M'koma *et al*[35]	Colon samples	CD: 27 UC: 24	Histology directed MALDI-TOF	5 m:z peaks were identified and cross-validated for the differentiation of UC and CD
Seeley *et al*[36]	Colon samples	CD: 26 UC: 36	Histology directed MALDI-TOF	Using a support vector machine and 25 m:z peaks, UC and CD cases were predicted in 93.3% and 60.4% respectively. A lower spectral accuracy cut-off increased sensitivity
Wasinger *et al*[39]	Serum	UC: 27 CD: 56 Controls: 14 RA controls: 12	MRM	SPP24 differentiated IBD patients from healthy controls α-1-microglobulin distinguished patients with UC in remission from healthy controls

CD: Crohn's disease; UC: Ulcerative colitis; IBD: Inflammatory bowel disease; MRM: Multiple reaction monitoring; PF4: Platelet factor 4; MRP8: Myeloid related protein 8; FIBA: Fibrinopeptide A; Hpα2: Haptoglobin α2.

reactions introduces an additional area of research as IBD often requires lifelong medical therapy.

It is clear that proteomics could play a potentially significant role towards improving the clinical management of IBD. Despite this, the value of these studies and their findings remain unknown and require validation in future studies.

FUTURE CONSIDERATIONS FOR IBD PROTEOMICS

Current limitations

Despite significant advancements in discovery-phase technologies and protocols, the rate at which new diagnostic protein assays are being introduced remains static, averaging 1.5/year[29]. The stagnation occurs at the verification stage, effectively obstructing any progression towards the development of a clinical assay[53,54]. This is clearly evident by the inundation of IBD discovery-phase experiments published in the recent decade with little to no candidate proteins undergoing validation.

One common criticism of many proteomic studies is the lack of strict experimental design, resulting in questionable results that cannot be reproduced; in particular, a small sample size and insufficient statistical power biomarker discovery[40,55]. This issue holds true across the aforementioned studies in IBD as out of 19 bio-sample based discovery experiments, 8 studies used $\leqslant$ 6 cases and controls[33,34,38,56-59]. In an effort to address this issue, Skates *et al*[55] designed a statistical model that estimates the statistical power of discovery and verification studies in tissue and plasma. Statistical power is estimated using 5 parameters: Biospecimen used (serum/tissue/proximal fluid), number of candidate proteins selected during discovery, number of cases/controls, percentage of cases where the biomarker is expressed and the difference in standard deviation between the biomarker signal in cases compared to controls. In addition, biomarkers typically occur in low abundance and may randomly exceed machine

Table 3 Proteomic studies for discovering inflammatory bowel disease management biomarkers

Ref.	Bio-sample	Sample size	Proteomic technique	Results
Disease activity biomarkers				
Han *et al*[34]	Intestinal tissue	CD: 3 UC: 4 Inflammatory Polyps: 2 Normal colon: 3	LC-QTOF	16 proteins distinguishing active/inactive CD 4 proteins distinguishing active/inactive UC
Wasinger *et al*[39]	Serum	UC: 27 CD: 56 Controls: 14 RA controls: 12	MRM	SPP24 was able to differentiate active and quiescent disease in both UC and CD
Prognostic biomarkers				
May *et al*[57]	Intestinal epithelial cells	Non-dysplastic tissue from non-progressors: 5 Non-dysplastic tissue from progressors: 5 Highly dysplastic tissue from UC progressors: 5	High-performance liquid chromatography quadrupole -TOF	155 candidate proteins were expressed differentially by > 2x between dysplastic/cancerous and non-dysplastic UC tissue. They were identified as mitochondrial, cytoskeletal, apoptotic and RAS superfamily proteins
Response to therapy biomarkers				
Meuwis *et al*[37]	Serum	Infliximab responders: 40 Infliximab non-responders: 40	SELDI-TOF	3 proteins (PF4, sCD40L and IL-6) were identified infliximab non-responders, although PF4 and sCD40L could not be confirmed or correlated with ELISA
Kanmura *et al*[23]	Blood samples	CD: 22 UC: 48 Colorectal cancer: 5 Infectious colitis: 6 Healthy controls: 13	SELDI-TOF	Plasma concentration of HNP1, 2 and 3 decreased following successful corticosteroid therapy compared to non-responders
Gazouli *et al*[38]	Serum	Infliximab responders: 6 Infliximab non-responders: 6 Infliximab partial responders: 6	2-DE, MALDI-TOF	7 proteins were increased in CD patients who did not achieve remission on infliximab. 4 were increased in patients who achieved remission. 3 proteins were lower in remission patients

CD: Crohn's disease; UC: Ulcerative colitis; IBD: Inflammatory bowel disease; MRM: Multiple reaction monitoring.

sensitivity limits, resulting in artificial differences between samples. This combined with inherent biological variations between patient samples further emphasises the importance of achieving sufficient statistical power[60,61]. It has already been noted that the concentration of candidate IBD biomarkers may be more concentrated in the intestinal tissue compared to serum, potentially reducing the chance of false discoveries. This highlights one advantage of analysing tissue samples over serum, although it is unknown which would yield better results[20]. Significant efforts have been made to address such limitations including: Recent requirements on reporting, inclusion of standards, and superior methods. These all aim to improve accuracy and reliability and will all contribute to translatable proteomic markers for disease[62,63].

Hanash *et al*[30] identified a number of confounding factors that could contribute to variations and false discoveries when identifying potential biomarkers. Patient factors include genetic variations, metabolic state, acute phase reactants and non-specific changes such as cell death. The use of model systems such as cell cultures and animal models, provides an alternative approach that could control for confounding environmental and genetic factors[20,30]. At least 66 different animal models of IBD exist, however these may not accurately reflect the true pathophysiology of IBD. Differences in methodology that could produce artificial differences include: Sample collection and preparation, improper characterisation and randomisation, and sample/statistical analysis. Zhang *et al*[64] hypothesize that many are likely site specific, suggesting that "multisite sampling" may suffice in the absence of careful prospective sample collection and randomization. This would theoretically reduce the impact of these factors and improve the likelihood of clinically useful candidate biomarkers being detected[64].

The issues highlighted above demonstrate the requirement for standardisation of protocols in large-scale proteomics experiments or at least stringent experimental design to increase the chances of discovering valid biomarkers.

Towards verification and validation

The process of validation differs significantly from the initial discovery stage as candidate proteins are tested in thousands of samples. This phase uses reliable high throughput methods (*e.g.*, immunoassays) in order to evaluate the biomarker's utility in the target population. Unfortunately this phase requires significant financial investment and produces a major barrier to validating the numerous proteins identified as "candidate biomarkers"[20,53,54]. Consequently, many potential markers are identified in the literature but require further investigation.

The gap between the inherent inaccuracies of the

discovery phase and the prohibitive cost of validation gave rise to the notion of an intermediate "verification" stage, aimed towards bridging this gap. This is achieved by quantification of selected candidate biomarkers in a larger sample that better represents the target population[55]. Ideally this is performed using reliable and established immunoassays, however, commercial antibodies are unavailable for the majority of protein targets, especially novel candidate markers. Assays must then be developed specifically for testing of the biomarker, an extremely costly endeavour when considering the large numbers of biomarkers[54]. Mass spectrometry can be further utilised here through quantitative techniques. Methods such as MRM have emerged as a viable alternatives towards cost-effectively triaging proteins of interest for further validation[20,53,54,65] and has been published for a number of biomarkers in other diseases[66-68].

CONCLUSION

The field and application of proteomics has expanded greatly in recent years and could have profound implications on the clinical diagnosis and management of IBD through the discovery of novel biomarkers. Many groups have already begun the "discovery" process and have identified many potential candidates. Although the transition into clinical validation is challenging, the tremendous potential of proteomics has garnered great interest and success in other diseases and further investigation into IBD proteomics should certainly be pursued.

REFERENCES

1 **Bernstein CN**, Fried M, Krabshuis JH, Cohen H, Eliakim R, Fedail S, Gearry R, Goh KL, Hamid S, Khan AG, LeMair AW, Malfertheiner Q, Rey JF, Sood A, Steinwurz F, Thomsen OO, Thomson A, Watermeyer G. World Gastroenterology Organization Practice Guidelines for the diagnosis and management of IBD in 2010. *Inflamm Bowel Dis* 2010; **16**: 112-124 [PMID: 19653289 DOI: 10.1002/ibd.21048]

2 **Price AB**. Overlap in the spectrum of non-specific inflammatory bowel disease--'colitis indeterminate'. *J Clin Pathol* 1978; **31**: 567-577 [PMID: 670413 DOI: 10.1136/jcp.31.6.567]

3 **Theodossi A**, Spiegelhalter DJ, Jass J, Firth J, Dixon M, Leader M, Levison DA, Lindley R, Filipe I, Price A. Observer variation and discriminatory value of biopsy features in inflammatory bowel disease. *Gut* 1994; **35**: 961-968 [PMID: 8063225 DOI: 10.1136/gut.35.7.961]

4 **Farmer M**, Petras RE, Hunt LE, Janosky JE, Galandiuk S. The importance of diagnostic accuracy in colonic inflammatory bowel disease. *Am J Gastroenterol* 2000; **95**: 3184-3188 [PMID: 11095339 DOI: 10.1016/s0002-9270(00)01992-4]

5 **Chen L**, Fang B, Giorgianni F, Gingrich JR, Beranova-Giorgianni S. Investigation of phosphoprotein signatures of archived prostate cancer tissue specimens via proteomic analysis. *Electrophoresis* 2011; **32**: 1984-1991 [PMID: 21739434 DOI: 10.1002/elps.201100101]

6 **Glen A**, Gan CS, Hamdy FC, Eaton CL, Cross SS, Catto JW, Wright PC, Rehman I. iTRAQ-facilitated proteomic analysis of human prostate cancer cells identifies proteins associated with progression. *J Proteome Res* 2008; **7**: 897-907 [PMID: 18232632 DOI: 10.1021/pr070378x]

7 **Alaiya AA**, Franzén B, Auer G, Linder S. Cancer proteomics: from identification of novel markers to creation of artifical learning models for tumor classification. *Electrophoresis* 2000; **21**: 1210-1217 [PMID: 10786893 DOI: 10.1002/(SICI)1522-2683(20000401)21:6<1210::AID-ELPS1210>3.0.CO;2-S]

8 **Celis JE**, Gromov P. Proteomics in translational cancer research: toward an integrated approach. *Cancer Cell* 2003; **3**: 9-15 [PMID: 12559171 DOI: 10.1016/S1535-6108(02)00242-8]

9 **Jungblut PR**, Zimny-Arndt U, Zeindl-Eberhart E, Stulik J, Koupilova K, Pleissner KP, Otto A, Müller EC, Sokolowska-Köhler W, Grabher G, Stöffler G. Proteomics in human disease: cancer, heart and infectious diseases. *Electrophoresis* 1999; **20**: 2100-2110 [PMID: 10451122 DOI: 10.1002/(sici)1522-2683(19990701)20:10<2100::aid-elps2100>3.0.co;2-d]

10 **Wagner JA**, Williams SA, Webster CJ. Biomarkers and surrogate end points for fit-for-purpose development and regulatory evaluation of new drugs. *Clin Pharmacol Ther* 2007; **81**: 104-107 [PMID: 17186007 DOI: 10.1038/sj.clpt.6100017]

11 **Vermeire S**, Van Assche G, Rutgeerts P. Laboratory markers in IBD: useful, magic, or unnecessary toys? *Gut* 2006; **55**: 426-431 [PMID: 16474109 DOI: 10.1136/gut.2005.069476]

12 **Lewis JD**. The utility of biomarkers in the diagnosis and therapy of inflammatory bowel disease. *Gastroenterology* 2011; **140**: 1817-1826.e2 [PMID: 21530748 DOI: 10.1053/j.gastro.2010.11.058]

13 **Langhorst J**, Elsenbruch S, Koelzer J, Rueffer A, Michalsen A, Dobos GJ. Noninvasive markers in the assessment of intestinal inflammation in inflammatory bowel diseases: performance of fecal lactoferrin, calprotectin, and PMN-elastase, CRP, and clinical indices. *Am J Gastroenterol* 2008; **103**: 162-169 [PMID: 17916108 DOI: 10.1111/j.1572-0241.2007.01556.x]

14 **Wasinger VC**, Cordwell SJ, Cerpa-Poljak A, Yan JX, Gooley AA, Wilkins MR, Duncan MW, Harris R, Williams KL, Humphery-Smith I. Progress with gene-product mapping of the Mollicutes: Mycoplasma genitalium. *Electrophoresis* 1995; **16**: 1090-1094 [PMID: 7498152 DOI: 10.1002/elps.11501601185]

15 **Ferreri AJ**, Illerhaus G, Zucca E, Cavalli F. Flows and flaws in primary central nervous system lymphoma. *Nat Rev Clin Oncol* 2010; **7**: 193-197 [PMID: 20700952 DOI: 10.1038/nrclinonc.2010.9-c1]

16 **Vaiopoulou A**, Gazouli M, Theodoropoulos G, Zografos G. Current advantages in the application of proteomics in inflammatory bowel disease. *Dig Dis Sci* 2012; **57**: 2755-2764 [PMID: 22740064 DOI: 10.1007/s10620-012-2291-4]

17 **Ong SE**, Mann M. Mass spectrometry-based proteomics turns quantitative. *Nat Chem Biol* 2005; **1**: 252-262 [PMID: 16408053 DOI: 10.1038/nchembio736]

18 **Bachi A**, Bonaldi T. Quantitative proteomics as a new piece of the systems biology puzzle. *J Proteomics* 2008; **71**: 357-367 [PMID: 18640294 DOI: 10.1016/j.jprot.2008.07.001]

19 **Wasinger VC**, Zeng M, Yau Y. Current status and advances in quantitative proteomic mass spectrometry. *Int J Proteomics* 2013; **2013**: 180605 [PMID: 23533757 DOI: 10.1155/2013/180605]

20 **Rifai N**, Gillette MA, Carr SA. Protein biomarker discovery and validation: the long and uncertain path to clinical utility. *Nat Biotechnol* 2006; **24**: 971-983 [PMID: 16900146 DOI: 10.1038/nbt1235]

21 **Srinivas PR**, Verma M, Zhao Y, Srivastava S. Proteomics for cancer biomarker discovery. *Clin Chem* 2002; **48**: 1160-1169 [PMID: 12142368]

22 **Meuwis MA**, Fillet M, Geurts P, de Seny D, Lutteri L, Chapelle JP, Bours V, Wehenkel L, Belaiche J, Malaise M, Louis E, Merville MP. Biomarker discovery for inflammatory bowel disease, using proteomic serum profiling. *Biochem Pharmacol* 2007; **73**: 1422-1433 [PMID: 17258689 DOI: 10.1016/j.bcp.2006.12.019]

23 **Kanmura S**, Uto H, Numata M, Hashimoto S, Moriuchi A, Fujita H, Oketani M, Ido A, Kodama M, Ohi H, Tsubouchi H. Human neutrophil peptides 1-3 are useful biomarkers in patients with active ulcerative colitis. *Inflamm Bowel Dis* 2009; **15**: 909-917 [PMID:

19107772 DOI: 10.1002/ibd.20854]
24 **Hatsugai M**, Kurokawa MS, Kouro T, Nagai K, Arito M, Masuko K, Suematsu N, Okamoto K, Itoh F, Kato T. Protein profiles of peripheral blood mononuclear cells are useful for differential diagnosis of ulcerative colitis and Crohn's disease. *J Gastroenterol* 2010; **45**: 488-500 [PMID: 20049485]
25 **Nanni P**, Levander F, Roda G, Caponi A, James P, Roda A. A label-free nano-liquid chromatography-mass spectrometry approach for quantitative serum peptidomics in Crohn's disease patients. *J Chromatogr B Analyt Technol Biomed Life Sci* 2009; **877**: 3127-3136 [PMID: 19683480 DOI: 10.1016/j.jchromb.2009.08.003]
26 **Subramanian V**, Subramanian D, Pollok RC. S1182 Serum Protein Signatures Determined By Mass Spectrometry (SELDI-ToF) Accurately Distinguishes Crohn's Disease (CD) from Ulcerative Colitis (UC). *Gastroenterology* 2008; **134**: A-196 [DOI: 10.1016/S0016-5085(08)60904-X]
27 **Nanni P**, Parisi D, Roda G, Casale M, Belluzzi A, Roda E, Mayer L, Roda A. Serum protein profiling in patients with inflammatory bowel diseases using selective solid-phase bulk extraction, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry and chemometric data analysis. *Rapid Commun Mass Spectrom* 2007; **21**: 4142-4148 [PMID: 18022963 DOI: 10.1002/rcm.3323]
28 **Vaiopoulou A**, Gazouli M, Papadopoulou A, Anagnostopoulos AK, Karamanolis G, Theodoropoulos GE, M'Koma A, Tsangaris GT. Serum protein profiling of adults and children with Crohn disease. *J Pediatr Gastroenterol Nutr* 2015; **60**: 42-47 [PMID: 25250685 DOI: 10.1097/MPG.0000000000000579]
29 **Anderson NL**. The clinical plasma proteome: a survey of clinical assays for proteins in plasma and serum. *Clin Chem* 2010; **56**: 177-185 [PMID: 19884488 DOI: 10.1373/clinchem.2009.126706]
30 **Hanash SM**, Pitteri SJ, Faca VM. Mining the plasma proteome for cancer biomarkers. *Nature* 2008; **452**: 571-579 [PMID: 18385731 DOI: 10.1038/nature06916]
31 **Kennedy S**. Proteomic profiling from human samples: the body fluid alternative. *Toxicol Lett* 2001; **120**: 379-384 [PMID: 11323197 DOI: 10.1016/S0378-4274(01)00269-7]
32 **Teng PN**, Bateman NW, Hood BL, Conrads TP. Advances in proximal fluid proteomics for disease biomarker discovery. *J Proteome Res* 2010; **9**: 6091-6100 [PMID: 21028795 DOI: 10.1021/pr100904q]
33 **Shkoda A**, Werner T, Daniel H, Gunckel M, Rogler G, Haller D. Differential protein expression profile in the intestinal epithelium from patients with inflammatory bowel disease. *J Proteome Res* 2007; **6**: 1114-1125 [PMID: 17330946 DOI: 10.1021/pr060433m]
34 **Han NY**, Choi W, Park JM, Kim EH, Lee H, Hahm KB. Label-free quantification for discovering novel biomarkers in the diagnosis and assessment of disease activity in inflammatory bowel disease. *J Dig Dis* 2013; **14**: 166-174 [PMID: 23320753 DOI: 10.1111/1751-2980.12035]
35 **M'Koma AE**, Seeley EH, Washington MK, Schwartz DA, Muldoon RL, Herline AJ, Wise PE, Caprioli RM. Proteomic profiling of mucosal and submucosal colonic tissues yields protein signatures that differentiate the inflammatory colitides. *Inflamm Bowel Dis* 2011; **17**: 875-883 [PMID: 20806340 DOI: 10.1002/ibd.21442]
36 **Seeley EH**, Washington MK, Caprioli RM, M'Koma AE. Proteomic patterns of colonic mucosal tissues delineate Crohn's colitis and ulcerative colitis. *Proteomics Clin Appl* 2013; **7**: 541-549 [PMID: 23382084 DOI: 10.1002/prca.201200107]
37 **Meuwis MA**, Fillet M, Lutteri L, Marée R, Geurts P, de Seny D, Malaise M, Chapelle JP, Wehenkel L, Belaiche J, Merville MP, Louis E. Proteomics for prediction and characterization of response to infliximab in Crohn's disease: a pilot study. *Clin Biochem* 2008; **41**: 960-967 [PMID: 18489908 DOI: 10.1016/j.clinbiochem.2008.04.021]
38 **Gazouli M**, Anagnostopoulos AK, Papadopoulou A, Vaiopoulou A, Papamichael K, Mantzaris G, Theodoropoulos GE, Anagnou NP, Tsangaris GT. Serum protein profile of Crohn's disease treated with infliximab. *J Crohns Colitis* 2013; **7**: e461-e470 [PMID: 23562004 DOI: 10.1016/j.crohns.2013.02.021]
39 **Wasinger VC**, Yau Y, Duo X, Zeng M, Campbell B, Shin S, Luber R, Redmond D, Leong RW. Low mass blood peptides discriminative of inflammatory bowel disease (IBD) severity: A quantitative proteomic perspective. *Mol Cell Proteomics* 2016; **15**: 256-265 [PMID: 26530476 DOI: 10.1074/mcp.M115.055095]
40 **Mischak H**, Apweiler R, Banks RE, Conaway M, Coon J, Dominiczak A, Ehrich JH, Fliser D, Girolami M, Hermjakob H, Hochstrasser D, Jankowski J, Julian BA, Kolch W, Massy ZA, Neususess C, Novak J, Peter K, Rossing K, Schanstra J, Semmes OJ, Theodorescu D, Thongboonkerd V, Weissinger EM, Van Eyk JE, Yamamoto T. Clinical proteomics: A need to define the field and to begin to set adequate standards. *Proteomics Clin Appl* 2007; **1**: 148-156 [PMID: 21136664 DOI: 10.1002/prca.200600771]
41 **Brignola C**, Campieri M, Bazzocchi G, Farruggia P, Tragnone A, Lanfranchi GA. A laboratory index for predicting relapse in asymptomatic patients with Crohn's disease. *Gastroenterology* 1986; **91**: 1490-1494 [PMID: 3770373]
42 **Zhang Z**. An In Vitro Diagnostic Multivariate Index Assay (IVDMIA) for Ovarian Cancer: Harvesting the Power of Multiple Biomarkers. *Rev Obstet Gynecol* 2012; **5**: 35-41 [PMID: 22582125]
43 **Rai AJ**, Zhang Z, Rosenzweig J, Shih IeM, Pham T, Fung ET, Sokoll LJ, Chan DW. Proteomic approaches to tumor marker discovery. *Arch Pathol Lab Med* 2002; **126**: 1518-1526 [PMID: 12456215]
44 **Ueland F**, Desimone C, Seamon L, Ware R, Goodrich S, Podzielinski I, Smith A, Santoso J, Van Nagell J, Zhang Z. The OVA1 test improves the preoperative assessment of ovarian tumors. *Gynecol Oncol* 2010; **116**: S23
45 **Plevy S**, Silverberg MS, Lockton S, Stockfisch T, Croner L, Stachelski J, Brown M, Triggs C, Chuang E, Princen F, Singh S. Combined serological, genetic, and inflammatory markers differentiate non-IBD, Crohn's disease, and ulcerative colitis patients. *Inflamm Bowel Dis* 2013; **19**: 1139-1148 [PMID: 23518807 DOI: 10.1097/MIB.0b013e318280b19e]
46 **Prideaux L**, De Cruz P, Ng SC, Kamm MA. Serological antibodies in inflammatory bowel disease: a systematic review. *Inflamm Bowel Dis* 2012; **18**: 1340-1355 [PMID: 22069240 DOI: 10.1002/ibd.21903]
47 **Schreiber S**, Hämling J, Zehnter E, Howaldt S, Daerr W, Raedler A, Kruis W. Renal tubular dysfunction in patients with inflammatory bowel disease treated with aminosalicylate. *Gut* 1997; **40**: 761-766 [PMID: 9245930]
48 **Fraser JS**, Muller AF, Smith DJ, Newman DJ, Lamb EJ. Renal tubular injury is present in acute inflammatory bowel disease prior to the introduction of drug therapy. *Aliment Pharmacol Ther* 2001; **15**: 1131-1137 [PMID: 11472315 DOI: 10.1046/j.1365-2036.2001.01041.x]
49 **Mahmud N**, O'Toole D, O'Hare N, Freyne PJ, Weir DG, Kelleher D. Evaluation of renal function following treatment with 5-aminosalicylic acid derivatives in patients with ulcerative colitis. *Aliment Pharmacol Ther* 2002; **16**: 207-215 [PMID: 11860403 DOI: 10.1046/j.1365-2036.2002.01155.x]
50 **Derici U**, Tuncer C, Ebinç FA, Mutluay R, Yakaryilmaz F, Kulaksizoglu S, Soylemezoglu O, Sindel S. Does the urinary excretion of alpha1-microglobulin and albumin predict clinical disease activity in ulcerative colitis? *Adv Ther* 2008; **25**: 1342-1352 [PMID: 19002407 DOI: 10.1007/s12325-008-0109-8]
51 **Mishima R**, Takeshima F, Sawai T, Ohba K, Ohnita K, Isomoto H, Omagari K, Mizuta Y, Ozono Y, Kohno S. High plasma osteopontin levels in patients with inflammatory bowel disease. *J Clin Gastroenterol* 2007; **41**: 167-172 [PMID: 17245215 DOI: 10.1097/MCG.0b013e31802d6268]
52 **Lorenzen J**, Shah R, Biser A, Staicu SA, Niranjan T, Garcia AM, Gruenwald A, Thomas DB, Shatat IF, Supe K, Woroniecki RP, Susztak K. The role of osteopontin in the development of albuminuria. *J Am Soc Nephrol* 2008; **19**: 884-890 [PMID: 18443355 DOI: 10.1681/ASN.2007040486]
53 **Makawita S**, Diamandis EP. The bottleneck in the cancer biomarker

pipeline and protein quantification through mass spectrometry-based approaches: current strategies for candidate verification. *Clin Chem* 2010; **56**: 212-222 [PMID: 20007861 DOI: 10.1373/clinchem.2009.127019]

54 **Whiteaker JR**, Lin C, Kennedy J, Hou L, Trute M, Sokal I, Yan P, Schoenherr RM, Zhao L, Voytovich UJ, Kelly-Spratt KS, Krasnoselsky A, Gafken PR, Hogan JM, Jones LA, Wang P, Amon L, Chodosh LA, Nelson PS, McIntosh MW, Kemp CJ, Paulovich AG. A targeted proteomics-based pipeline for verification of biomarkers in plasma. *Nat Biotechnol* 2011; **29**: 625-634 [PMID: 21685906 DOI: 10.1038/nbt.1900]

55 **Skates SJ**, Gillette MA, LaBaer J, Carr SA, Anderson L, Liebler DC, Ransohoff D, Rifai N, Kondratovich M, Težak Ž, Mansfield E, Oberg AL, Wright I, Barnes G, Gail M, Mesri M, Kinsinger CR, Rodriguez H, Boja ES. Statistical design for biospecimen cohort size in proteomics-based biomarker discovery and verification studies. *J Proteome Res* 2013; **12**: 5383-5394 [PMID: 24063748 DOI: 10.1021/pr400132j]

56 **Fogt F**, Jian B, Krieg RC, Wellmann A. Proteomic analysis of mucosal preparations from patients with ulcerative colitis. *Mol Med Rep* 2008; **1**: 51-54 [PMID: 21479377 DOI: 10.3892/mmr.1.1.51]

57 **May D**, Pan S, Crispin DA, Lai K, Bronner MP, Hogan J, Hockenbery DM, McIntosh M, Brentnall TA, Chen R. Investigating neoplastic progression of ulcerative colitis with label-free comparative proteomics. *J Proteome Res* 2011; **10**: 200-209 [PMID: 20828217 DOI: 10.1021/pr100574p]

58 **Nanni P**, Mezzanotte L, Roda G, Caponi A, Levander F, James P, Roda A. Differential proteomic analysis of HT29 Cl.16E and intestinal epithelial cells by LC ESI/QTOF mass spectrometry. *J Proteomics* 2009; **72**: 865-873 [PMID: 19168159 DOI: 10.1016/j.jprot.2008.12.010]

59 **Shkoda A**, Ruiz PA, Daniel H, Kim SC, Rogler G, Sartor RB, Haller D. Interleukin-10 blocked endoplasmic reticulum stress in intestinal epithelial cells: impact on chronic inflammation. *Gastroenterology* 2007; **132**: 190-207 [PMID: 17241871 DOI: 10.1053/j.gastro.2006.10.030]

60 **Alex P**, Gucek M, Li X. Applications of proteomics in the study of inflammatory bowel diseases: Current status and future directions with available technologies. *Inflamm Bowel Dis* 2009; **15**: 616-629 [PMID: 18844215 DOI: 10.1002/ibd.20652]

61 **Araki K**, Mikami T, Yoshida T, Kikuchi M, Sato Y, Oh-ishi M, Kodera Y, Maeda T, Okayasu I. High expression of HSP47 in ulcerative colitis-associated carcinomas: proteomic approach. *Br J Cancer* 2009; **101**: 492-497 [PMID: 19603022 DOI: 10.1038/sj.bjc.6605163]

62 **Carr SA**, Abbatiello SE, Ackermann BL, Borchers C, Domon B, Deutsch EW, Grant RP, Hoofnagle AN, Hüttenhain R, Koomen JM, Liebler DC, Liu T, MacLean B, Mani DR, Mansfield E, Neubert H, Paulovich AG, Reiter L, Vitek O, Aebersold R, Anderson L, Bethem R, Blonder J, Boja E, Botelho J, Boyne M, Bradshaw RA, Burlingame AL, Chan D, Keshishian H, Kuhn E, Kinsinger C, Lee JS, Lee SW, Moritz R, Oses-Prieto J, Rifai N, Ritchie J, Rodriguez H, Srinivas PR, Townsend RR, Van Eyk J, Whiteley G, Wiita A, Weintraub S. Targeted peptide measurements in biology and medicine: best practices for mass spectrometry-based assay development using a fit-for-purpose approach. *Mol Cell Proteomics* 2014; **13**: 907-917 [PMID: 24443746 DOI: 10.1074/mcp.M113.036095]

63 **Percy AJ**, Chambers AG, Smith DS, Borchers CH. Standardized protocols for quality control of MRM-based plasma proteomic workflows. *J Proteome Res* 2013; **12**: 222-233 [PMID: 23245390 DOI: 10.1021/pr300893w]

64 **Zhang Z**, Chan DW. The road from discovery to clinical diagnostics: lessons learned from the first FDA-cleared in vitro diagnostic multivariate index assay of proteomic biomarkers. *Cancer Epidemiol Biomarkers Prev* 2010; **19**: 2995-2999 [PMID: 20962299 DOI: 10.1158/1055-9965]

65 **Meng Z**, Veenstra TD. Targeted mass spectrometry approaches for protein biomarker verification. *J Proteomics* 2011; **74**: 2650-2659 [PMID: 21540133 DOI: 10.1016/j.jprot.2011.04.011]

66 **Cho CK**, Drabovich AP, Batruch I, Diamandis EP. Verification of a biomarker discovery approach for detection of Down syndrome in amniotic fluid via multiplex selected reaction monitoring (SRM) assay. *J Proteomics* 2011; **74**: 2052-2059 [PMID: 21624510 DOI: 10.1016/j.jprot.2011.05.025]

67 **Drabovich AP**, Jarvi K, Diamandis EP. Verification of male infertility biomarkers in seminal plasma by multiplex selected reaction monitoring assay. *Mol Cell Proteomics* 2011; **10**: M110.004127 [PMID: 21933954 DOI: 10.1074/mcp.M110.004127]

68 **Kim K**, Kim SJ, Han D, Jin J, Yu J, Park KS, Yu HG, Kim Y. Verification of multimarkers for detection of early stage diabetic retinopathy using multiple reaction monitoring. *J Proteome Res* 2013; **12**: 1078-1089 [PMID: 23368427 DOI: 10.1021/pr3012073]

69 **von Roon AC**, Karamountzos L, Purkayastha S, Reese GE, Darzi AW, Teare JP, Paraskeva P, Tekkis PP. Diagnostic precision of fecal calprotectin for inflammatory bowel disease and colorectal malignancy. *Am J Gastroenterol* 2007; **102**: 803-813 [PMID: 17324124 DOI: 10.1111/j.1572-0241.2007.01126.x]

70 **Gisbert JP**, McNicholl AG, Gomollon F. Questions and answers on the role of fecal lactoferrin as a biological marker in inflammatory bowel disease. *Inflamm Bowel Dis* 2009; **15**: 1746-1754 [PMID: 19363798 DOI: 10.1002/ibd.20920]

71 **Kaiser T**, Langhorst J, Wittkowski H, Becker K, Friedrich AW, Rueffer A, Dobos GJ, Roth J, Foell D. Faecal S100A12 as a non-invasive marker distinguishing inflammatory bowel disease from irritable bowel syndrome. *Gut* 2007; **56**: 1706-1713 [PMID: 17675327 DOI: 10.1136/gut.2006.113431]

72 **Beattie RM**, Walker-Smith JA, Murch SH. Indications for investigation of chronic gastrointestinal symptoms. *Arch Dis Child* 1995; **73**: 354-355 [PMID: 7492204 DOI: 10.1136/adc.73.4.354]

73 **Poullis AP**, Zar S, Sundaram KK, Moodie SJ, Risley P, Theodossi A, Mendall MA. A new, highly sensitive assay for C-reactive protein can aid the differentiation of inflammatory bowel disorders from constipation- and diarrhoea-predominant functional bowel disorders. *Eur J Gastroenterol Hepatol* 2002; **14**: 409-412 [PMID: 11943955 DOI: 10.1097/00042737-200204000-00013]

74 **Shine B**, Berghouse L, Jones JE, Landon J. C-reactive protein as an aid in the differentiation of functional and inflammatory bowel disorders. *Clin Chim Acta* 1985; **148**: 105-109 [PMID: 3995779 DOI: 10.1016/0009-8981(85)90219-0]

75 **Ruemmele FM**, Targan SR, Levy G, Dubinsky M, Braun J, Seidman EG. Diagnostic accuracy of serological assays in pediatric inflammatory bowel disease. *Gastroenterology* 1998; **115**: 822-829 [PMID: 9753483 DOI: 10.1016/S0016-5085(98)70252-5]

76 **Peyrin-Biroulet L**, Standaert-Vitse A, Branche J, Chamaillard M. IBD serological panels: facts and perspectives. *Inflamm Bowel Dis* 2007; **13**: 1561-1566 [PMID: 17636565 DOI: 10.1002/ibd.20226]

77 **Sipponen T**, Björkesten CG, Färkkilä M, Nuutinen H, Savilahti E, Kolho KL. Faecal calprotectin and lactoferrin are reliable surrogate markers of endoscopic response during Crohn's disease treatment. *Scand J Gastroenterol* 2010; **45**: 325-331 [PMID: 20034360 DOI: 10.3109/00365520903483650]

78 **Sipponen T**, Savilahti E, Kolho KL, Nuutinen H, Turunen U, Färkkilä M. Crohn's disease activity assessed by fecal calprotectin and lactoferrin: correlation with Crohn's disease activity index and endoscopic findings. *Inflamm Bowel Dis* 2008; **14**: 40-46 [PMID: 18022866 DOI: 10.1002/ibd.20312]

79 **Vecchi M**, Gionchetti P, Bianchi MB, Belluzzi A, Meucci G, Campieri M, de Franchis R. p-ANCA and development of pouchitis in ulcerative colitis patients after proctocolectomy and ileoanal pouch anastomosis. *Lancet* 1994; **344**: 886-887 [PMID: 7916418 DOI: 10.1016/S0140-6736(94)92859-2]

80 **Costa F**, Mumolo MG, Ceccarelli L, Bellini M, Romano MR, Sterpi C, Ricchiuti A, Marchi S, Bottai M. Calprotectin is a stronger predictive marker of relapse in ulcerative colitis than in Crohn's disease. *Gut* 2005; **54**: 364-368 [PMID: 15710984 DOI: 10.1136/gut.2004.043406]

81 **Gisbert JP**, Bermejo F, Pérez-Calle JL, Taxonera C, Vera I,

McNicholl AG, Algaba A, López P, López-Palacios N, Calvo M, González-Lama Y, Carneros JA, Velasco M, Maté J. Fecal calprotectin and lactoferrin for the prediction of inflammatory bowel disease relapse. *Inflamm Bowel Dis* 2009; **15**: 1190-1198 [PMID: 19291780 DOI: 10.1002/ibd.20933]

82 **Dubinsky MC**, Mei L, Friedman M, Dhere T, Haritunians T, Hakonarson H, Kim C, Glessner J, Targan SR, McGovern DP, Taylor KD, Rotter JI. Genome wide association (GWA) predictors of anti-TNFalpha therapeutic responsiveness in pediatric inflammatory bowel disease. *Inflamm Bowel Dis* 2010; **16**: 1357-1366 [PMID: 20014019 DOI: 10.1002/ibd.21174]

83 **Spivak J**, Landers CJ, Vasiliauskas EA, Abreu MT, Dubinsky MC, Papadakis KA, Ippoliti A, Targan SR, Fleshner PR. Antibodies to I2 predict clinical response to fecal diversion in Crohn's disease. *Inflamm Bowel Dis* 2006; **12**: 1122-1130 [PMID: 17119386 DOI: 10.1097/01.mib.0000235833.47423.d7]

P- Reviewer: Bonaz BL, Ciccone MM, Gazouli M, Maric I, Naito Y, Sorrentino D, Tandon R, Vecchi M
S- Editor: Qiu S **L- Editor**: A **E- Editor**: Jiao XK

World Journal of Gastrointestinal Pathophysiology

Submit a Manuscript: http://www.wjgnet.com/esps/
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.4291/wjgp.v7.i1.38

World J Gastrointest Pathophysiol 2016 February 15; 7(1): 38-47
ISSN 2150-5330 (online)

REVIEW

Amniotic fluid: Source of trophic factors for the developing intestine

Soham Dasgupta, Shreyas Arya, Sanjeev Choudhary, Sunil K Jain

Soham Dasgupta, Shreyas Arya, Sunil K Jain, Department of Pediatrics, University of Texas Medical Branch, Galveston, TX 77555, United States

Sanjeev Choudhary, Department of Endocrinology, University of Texas Medical Branch, Galveston, TX 77555, United States

Sanjeev Choudhary, Department of Internal Medicine, University of Texas Medical Branch, Galveston, TX 77555, United States

Sunil K Jain, Department of Neonatology, University of Texas Medical Branch, Galveston, TX 77555, United States

Author contributions: Dasgupta S, Arya S, Choudhary S and Jain SK contributed equally to this work; Dasgupta S and Arya S were involved in the drafting of the manuscript, creation of figures, analyzing the manuscript and results and reviewing it as well; Choudhary S and Jain SK were involved in drafting the manuscript, reviewing and revising the manuscript and figures and also analyzing the results.

Conflict-of-interest statement: All of the authors do not have any conflict of interest to disclose.

Correspondence to: Sunil K Jain, MD, Department of Neonatology, University of Texas Medical Branch, 301 University Blvd., Galveston, TX 77555, United States. skjain@utmb.edu
Telephone: +1-713-3059772
Fax: +1-409-7720747

Received: August 27, 2015
Peer-review started: August 30, 2015
First decision: September 28, 2015
Revised: December 22, 2015
Accepted: January 5, 2016

Article in press: January 7, 2016
Published online: February 15, 2016

Abstract

The gastrointestinal tract (GIT) is a complex system, which changes in response to requirements of the body. GIT represents a barrier to the external environment. To achieve this, epithelial cells must renew rapidly. This renewal of epithelial cells starts in the fetal life under the influence of many GIT peptides by swallowing amniotic fluid (AF). Development and maturation of GIT is a very complex cascade that begins long before birth and continues during infancy and childhood by breast-feeding. Many factors like genetic preprogramming, local and systemic endocrine secretions and many trophic factors (TF) from swallowed AF contribute and modulate the development and growth of the GIT. GIT morphogenesis, differentiation and functional development depend on the activity of various TF in the AF. This manuscript will review the role of AF borne TF in the development of GIT.

Key words: Amniotic; Fluid; Gastrointestinal; Factors; Tract; Trophic; Development

Core tip: The gastrointestinal tract (GIT) is a complex system with a combination of factors being responsible for its development. Trophic factors (TF) in amniotic fluid (AF) represent an important component that affects the development and maturation of the GIT during fetal life. We highlight the various phases of GIT development, the formation/circulation of AF, various TF in AF and the respective roles they play in fetal GIT development. We also emphasize that much remains to be known about the milieu of TF within AF. We hope this article provides an insight of what is known about

such TF and what we hope to discover in the future.

Dasgupta S, Arya S, Choudhary S, Jain SK. Amniotic fluid: Source of trophic factors for the developing intestine. *World J Gastrointest Pathophysiol* 2016; 7(1): 38-47 Available from: URL: http://www.wjgnet.com/2150-5330/full/v7/i1/38.htm DOI: http://dx.doi.org/10.4291/wjgp.v7.i1.38

INTRODUCTION

There is a combination of many factors which are responsible for the growth and development of the gastrointestinal tract (GIT) like genetic programming, trophic factors (TF) in amniotic fluid (AF), endocrine factors like corticosteroids, growth hormone and insulin and paracrine growth factors[1-4]. Development and maturation of GIT is a continuum and a very complex process which finally results in a mature GIT.

As a barrier to the external environment, gut epithelium must be renewed rapidly and repeatedly[1]. Growth and renewal of gut epithelial cells is dependent on controlled cell stimulation and proliferation by a number of signaling processes and TF[1]. The importance of AF in fetal nutrition is an acknowledged fact[5]. It has been documented that swallowing of AF enhanced fetal gastrointestinal (GI) development[5] in rabbits. Experiments performed in sheep showed that esophageal ligation decreases fetal intestinal growth and re-establishment of swallowing promoted growth[5]. Surana *et al*[6] studied the effect of AF on fetal intestinal growth in humansand and found that proximal obstruction of intestines led to more growth impairment as compared to a distal obstruction. Hirai *et al*[7] examined the trophic effects of AF, human milk and several recombinant growth factors on a human fetal small intestine cell line. They discovered that AF and human milk promoted cell growth equally[7]. They also noted that growth factors stimulated cell growth but enhancement was less than that of AF alone[7]. All of these suggest that AF is a source of TF for the development and maturation of intestine.

But do we know what a mature GIT is? How can we clinically differentiate immature and mature gut? What affects the maturation and development of GIT during fetal life and after birth? To answer these questions we will start with the development of fetal GIT.

FETAL GIT DEVELOPMENT

Development and maturation of GIT is a continuous process which starts during early fetal life and continues well into infancy and childhood. Now the question is what a mature GIT is and what leads to a mature GIT? To answer this question, we need to know the fetal and neonatal GIT development and maturation and what is responsible for such processes. There are five phases of fetal GIT development (Table 1 and Figure 1).

Phase 1 (embryonic phase)

This is a phase of organogenesis which begins immediately after conception and quickly leads to phase 2[1]. In phase 1, there is formation of primitive gut. GIT undergoes very rapid growth from the week 5 of gestation onwards[8].

Phase 2

There is selective growth and apoptosis that allows the formation of the rudimentary gut tube[1]. The GIT undergoes rapid growth with the formation of villi and microvilli[8-9]. During this phase an entrance and an exit site is formed (future mouth and anus respectively) and fetus starts swallowing AF, which has both physical and trophic effects on the development of GIT (Figure 2).

Phase 3 (Late gestational age)

This comprises active differentiation during late gestation when the GIT prepares for extra-uterine life[1]. The intestinal cells actively divide causing cells to migrate up the villus and ultimately forming actively absorbing cells[1]. This phase is also characterized by selective apoptosis at the tips and crypts of villi[10]. After about 96 h, the villous epithelial cells slough off in the lumen of intestine where they mix with mucous and bile to become meconium[9].

Phase 4 (Neonatal phase)

This phase begins after birth when exposure to enteral nutrition leads to more rapid mucosal differentiation and development of GIT for the extra uterine adaptation[1]. Premature infants lack this developmental phase, which occurs during the third trimester. This is why this process is more prolonged in premature infants. This is also the phase that has the largest antigenic load presented to it in the form of dietary proteins and pathogens[1]. During this time the gut develops the ability to distinguish between foreign pathogens and safe nutrient proteins[11,12].

Phase 5 (Weaning phase)

This is the last phase of gut development and occurs in late infancy/early childhood[1] as the child transitions from milk based diet to complementary diet. During this time, there is a second phase of mucosal expansion which is associated with epithelial hyperplasia that renders the gut functionally more mature. During this phase, the intestinal mucosal immunity develops the ability to differentiate between foreign pathogen and nutrient proteins[12]. Preterm infants lack this process due to lack of exposure to AF borne TF due to premature birth. That is why premature infants are at increased risk of proliferation of pathogenic bacteria in the lumen of intestine and subsequent mucosal invasion of pathogenic microorganisms[13]. This may significantly impact the GIT function as well systemic immune functions[13].

Table 1 Phases of mammalian gastrointestinal development (adapted from reference 1)

Phase	Development
Phase 1	Embryonic phase of organogenesis Forms primitive GIT
Phase 2	Entrance and exit sites of GIT form Formation of rudimentary primitive GIT Formation of mouth and anus Fetal swallowing of amniotic fluid begins
Phase 3	Active differentiation Increase in cell number in crypts Cells from crypts start migrating up to the villi GIT growth is more rapid than the fetal body as a whole Growth accompanied by selective apoptosis
Phase 4	After birth, exposure to enteral nutrition Breast milk feeding – rapid mucosal differentiation and development Infancy – mucosal growth continues with deepening crypts, increasing villi (increasing width and number) and appearance of sub-mucosal folds Development of GIT mucosal immunity due to exposure to dietary antigens Mucosal immune system can distinguish between foreign pathogens and safe nutrient proteins and commensal organisms
Phase 5 (Weaning)	Late infancy – early childhood. Transition from milk feeding to solid foods. This is second phase of mucosal immunity with epithelial hyperplasia with maturation of gut functions similar to older children.

GIT: Gastrointestinal tract.

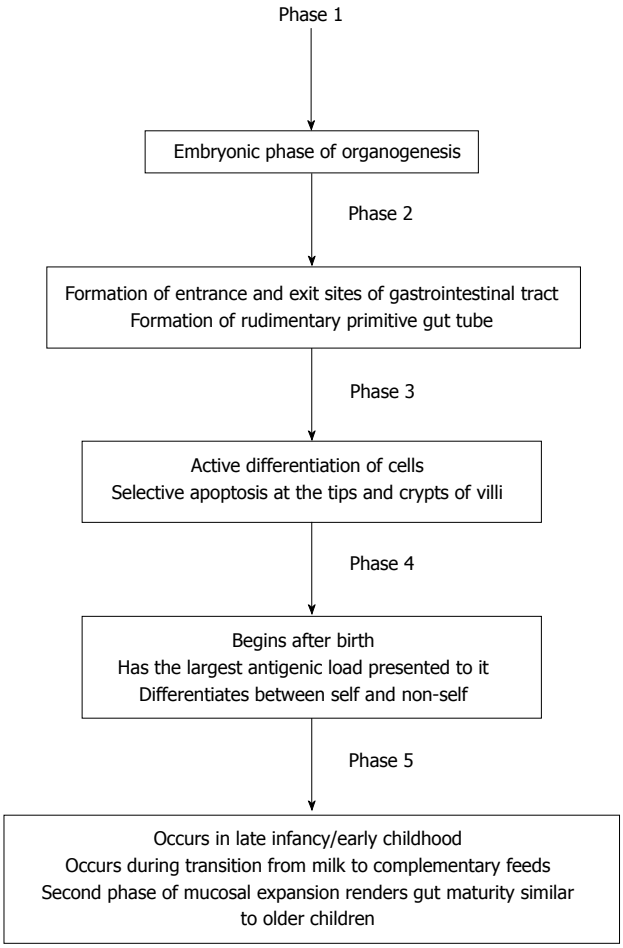


Figure 1 Various phases of mammalian gut development.

FORMATION OF AF

The first fluid to enter the developing fetal GIT is the AF[1]. It is a bioactive medium containing proteins, lipids and phospholipids, urea and electrolytes that is actively secreted by cells lining the amniotic cavity[1]. During early gestation (organogenesis), AF volume increases by getting water from the mother's plasma and is transported to the fetus through fetal membranes depending on the hydrostatic and osmotic pressure gradients. AF volume increases from 10 mL at 10 wk to around 400 mL at 20 wk gestational age. Around 8-10 wk gestation, the skin is not keratinized hence there is a bi-directional fluid diffusion between the fetus and the AF. During early gestation, AF volume and fetal size are related in a linear manner. Around this time, fetus starts passing urine and fetal swallowing also begins. Keratinization of fetal skin starts around 20 wk of gestation and is completed around 25 wk. After skin keratinization, the relationship of fetal size and AF volume is no longer linear. Around 28 wk gestation, AF volume increases to its maximum volume (about 800 mL) where it plateaus near term gestation and thereafter AF volume starts declining to 400 mL at 42 wk. Fetus swallows around 250 mL/kg/d of AF, which is the main source of AF removal[14]. The chemical composition of AF changes with increasing gestational age. AF is not the main source of fetal nutrition; it contributes only 15% of fetal nutritional requirements[15,16] but has a very important role in the development and maturation of gut[1]. The important nutritional components of AF are summarized in Table 2. In the second half of pregnancy, sodium, chloride and osmolality decrease whereas urea and creatinine concentrations increase. AF composition is more regulated than the AF volume. (Refer to reference 18 for further details)[17].

AF CIRCULATION

Figure 3 shows 5 pathways of exchange and AF circulation that have been identified[18]. Excretion of urine

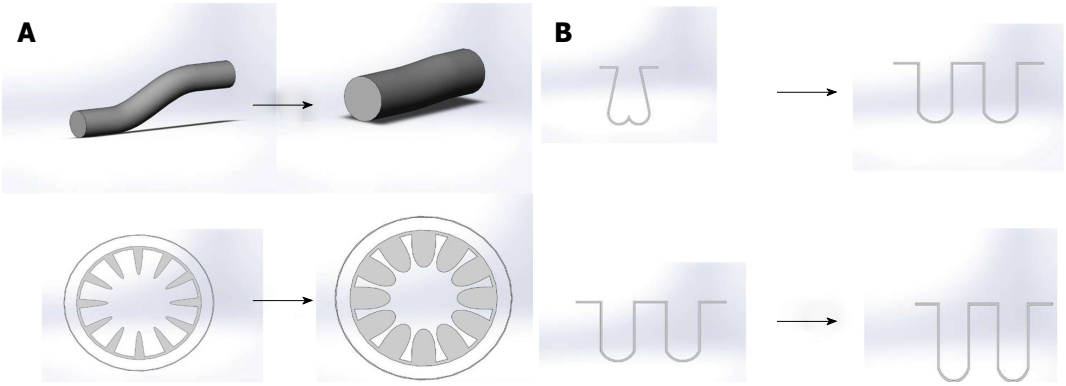


Figure 2 Two patterns of the growth of small intestine. A: Cylindrical organ growth in length and diameter; B: Luminal mucosal growth with amplification of the internal surface area by submucosal folds and villi.

Table 2 Important nutritional components of amniotic fluid

Component	Most important examples
Amino acids	Glutamine, arginine
Proteins	Lactoferrin
Minerals	Zinc, iron
Hormones	Growth hormone, prolactin
Growth factors	IGF-1, EGF

IGF-1: Insulin like growth factor-1; EGF: Epidermal growth factor.

Table 3 Amniotic fluid volume changes with increasing gestational age

Gestational Age	Volume of AF
10 wk	25 mL
20 wk	400 mL
28 wk	800 mL
Term gestation	Plateau in volume of AF
42 wk	400 mL

AF: Amniotic fluid.

and the secretion of oral, nasal, tracheal and pulmonary fluids predominantly accomplish production of AF[19]. Fetal breathing movements also lead to the efflux of lung fluid into the AF but this effect is minimal[18]. Removal of AF is predominantly accomplished by fetal swallowing. Also a significant intramembranous pathway transfers fluid and solutes from the amniotic cavity to the fetal circulation across the amniotic membranes[20]. The trans-membranous pathway, which is the movement of AF across the fetal membranes into the maternal circulation, affects the AF volume only minimally[18]. All of these pathways together maintain the relative stability of the AF in spite of large fluid shifts[18].

VOLUME CHANGES OF AF WITH INCREASING GESTATIONAL AGE

Table 3 summarized the volume changes of AF with increasing gestational age[18].

Ten weeks of gestation: 25 mL (AF and fetal size related in a linear fashion in this early period[18]. Fetal kidneys start making urine by 8 wk gestation and swallowing begins soon after).

Twenty weeks of gestation: 400 mL (Keratinization of skin is complete by 25 wk gestation and AF and fetal size lose their linear relationship).

Twenty-eight weeks of gestation: 800 mL.

Term gestation: Plateau near term gestation.

Forty-two weeks of gestation: 400 mL.

AF: SOURCE OF TF

It is important to note that AF is the first fluid which bathes the GIT secondary to swallowing of AF (450 mL/d to 1000 mL/d at term gestation)[21]. Table 4 summarized the roles of various TF found in AF in intestinal development and the location of their receptors. AF serves as the physical barrier to the external environment. In the 1970's, Chochinov *et al*[22] correlated AF borne growth hormone concentration with the development and maturation of fetal kidney. Mulvihill *et al*[16] *in vitro* studies showed that AF and fetal bovine serum had equivalent stimulating effect on fetal gastric epithelial cells. Further Barka *et al*[23] confirmed the trophic properties of AF. More recently, Maheshwari[24] described the role of cytokines in AF and their role in the development of GIT. In an *in vitro* study, Hirai *et al*[7] demonstrated the trophic effects of AF and further showed that trophic effects of AF were equivalent to breast milk. The growth of intestine occurs by duplication of intestinal crypts, which leads to cylindrical growth of small intestine (Figure 2A). During growth, the crypts gradually divide longitudinally into two daughter crypts (Figure 2B). This process is promoted by various TF like epidermal growth factor (EGF), keratinocyte growth factor, and many other TF which are present in the AF. Over the years, different investigators have found many TF in the AF. Different TF present in AF work in concert to provide bioactivity. Wagner *et al*[25] used fetal small intestinal cells (Fhs74) and showed a synergistic relationship of EGF and transforming growth factor-α

Table 4 Roles of various trophic factors found in amniotic fluid in intestinal development and the location of their receptors

Trophic factor	Location of receptors	Role in intestinal growth
EGF	Basolateral intestinal membrane	Stimulates cell mitosis and differentiation Stimulates intestinal epithelial cell proliferation
HGF	Intestinal crypt epithelial cells and in the muscle layers of the intestine	Intestinal cell proliferation *in vitro* and has been demonstrated to induce intestinal growth in rats
TGF-α and TGF-β	Basolateral intestinal membrane	Primary role may be intestinal mucosal repair
IGF-1	Crypt cells, basolateral membrane and in the distal intestine	Primary mediator of both intrauterine and postnatal growth in mammals May be important for growth of muscle growth of distal small intestine
EPO	Apical surface of intestinal epithelial cells	Increased villus height, villus area, crypt depth and crypt epithelia cell proliferation in rat pups. *In vitro*, recombinant EPO has been shown to protect cells against mucosal injury
G-CSF	Apical regions of the intestine	Role in epithelial cell maintenance
IL family	Intestinal epithelial cells	Enhances intestinal epithelial cell restitution. Enhances the integrity of the intestinal epithelial cell junctions. Intestinal epithelial cell proliferation and increased nutrient uptake

IGF-1: Insulin like growth factor-1; EGF: Epidermal growth factor; TGF: Transforming growth factor; HGF: Hepatocyte growth factor; EPO: Erythropoietin; G-CSF: Granulocyte colony stimulating factor; IL: Interleukin.

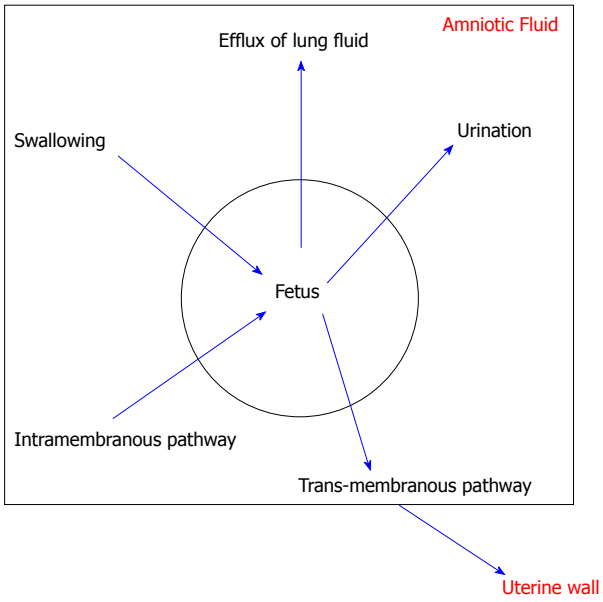


Figure 3 Various pathways of amniotic fluid circulation.

(TGF-α), which was greater than individual effect of recombinant EGF (rEGF), or TGF-α alone. Booth *et al*[26] also showed that no single growth factor increased cell proliferation of rat intestinal epithelial cells but when rEGF, TGF-α, insulin like growth factor-1 (IGF-1) and platelet derived growth factors were combined, epithelial cell proliferation was increased significantly. How these TF in AF work- whether they work in concert or separately- is only directed by *in vitro* studies. The interplay of these TF in AF *in vivo* is not well understood. In this review, we will focus on different TF in the AF and their role in the development and maturation of the GIT.

EGF

In 1962, a growth factor was discovered from mouse saliva which could induce premature eruption of the teeth and opening of eyelids - that is why it was called EGF[27]. EGF is a family of peptides that share structure and affinity to the EGF receptor. The salivary glands and Brunner's glands of duodenum in the GIT continuously secrete EGF. The EGF receptor (erbB1) is found in fetal as well adult GIT, liver and pancreas. EGF receptor levels increase in intestinal pathology like ulceration of rat oxyntic mucosa[28]. It is a small peptide which functions as a luminal surveillance peptide that can attach to the EGF receptor on the basolateral membrane when the luminal barrier is damaged[29]. As GIT is an important barrier to outside noxious substances, there is quick healing of injured epithelial lining by epithelial migration and proliferation, called restitution[30]. EGF stimulates restitution of the superficial epithelial lining of GIT. It stimulates cell mitosis and differentiation, decreases acid secretion, increases bicarbonate, mucus secretions and GIT blood flow and helps in digestion by increasing amylase secretions and by increasing gastric motility. EGF is also a cytoprotective molecule that can stabilize GIT epithelial cells from agents like ethanol or non-steroidal anti-inflammatory drugs[31]. EGF has two main physiological functions: (1) Involved in mucosal protection and healing of damaged epithelial lining; and (2) involved in digestion, absorption and transportation of nutrients.

EGF is found is significant quantities in human AF and it increases with progression of pregnancy[32]. The suggested site of production of EGF is either the lungs or the amniotic membranes[5]. EGF has been shown to increase DNA and glycoprotein synthesis in cultured human fetal gastric cells[33]. The impact of EGF in AF on fetal intestinal growth is an area of active research. EGF receptors are mainly expressed on the basolateral intestinal membrane[24]. It is largely resistant to gastric proteolysis in the preterm infant and thus remains bioavailable in the intestine[24,34]. All in all, EGF is a potent stimulus to intestinal epithelial cell proliferation[15] (Table 5).

In summary, EGF has mitogenic as well as nonmitogenic roles in GIT function. Further understanding of its role is required before we use it in the settings of

Table 5 Effects of epidermal growth factor on the gastrointestinal tract

Increased effect on	Possible secondary message
Proliferation	-
Bicarbonate secretion	Prostaglandins
NaCl and glucose uptake	Na^+- glucose cotransporter, lipids
Mucus secretion	Prostaglandins
GI blood flow	Beta-adrenergic NO prostaglandins
Longitudinal smooth muscle contraction	Prostaglandins
Circular smooth muscle contraction	Desensitizes (not prostaglandins)
Restitution	Cell-migration prostaglandins
Permeability	-
Mucosal protection	Proliferation, polyamines, mucus, trefoil peptides
Decreased effect on	Possible secondary message
Gastric acid secretion	Protein kinase C, cAMP
Gastric emptying	-
Increased and decreased effect on	Possible secondary message
Chloride secretion	Phosphatidylinositol 3-kinase
Pancreatic amylase (3.2.1.1) secretion	cAMP phospholipase C

GI: Gastrointestinal; NaCl: Sodium chloride; cAMP: Cyclic adenosine monophosphate.

inflammation of mucosal damage such as necrotizing enterocolitis.

Hepatocyte growth factor

It is present in AF and human milk and is expressed in embryonic and fetal intestinal tissue[24]. Hepatocyte growth factor (HGF) and C-met mRNA are expressed in the fetal intestine[24]. HGF receptor, C-met - a proto-oncogene, is present on intestinal crypt epithelial cells although it is also expressed in the muscle layers of the intestine[15]. HGF stimulates intestinal cell proliferation *in vitro* and has been demonstrated to induce intestinal growth in rats when administered in pharmacologic doses[24]. In an animal model of NEC, we showed that oral supplementation of AF is protective against experimental NEC in a rat model of NEC (hypoxia and hypothermia model) which was mediated, at least partly, by HGF.

TGF-α

Detectable in the human GIT at 15 wk gestation[24]. It has a structure similar to EGF and binds to the same receptor. It is found in AF and human milk. Recombinant TGF-α has been shown to elicit a synergistic trophic response on cultured intestinal cells when combined with EGF, IGF-1, FGF and HGF[7]. However it was noted that this trophic response was not as strong as that seen with AF or human milk alone. Its primary role is believed to be in mucosal repair[24].

TGF-beta

It belongs to a family of signaling peptides that influences the distribution of intestinal stem cells. It is found in human AF only during the late stages of gestation[5]. It is believed to induce terminal differentiation of intestinal epithelial cells and to accelerate the rate of healing of intestinal wounds by stimulating cell migration[5]. A role in the prevention of necrotizing enterocolitis has been suggested as well[34]. We showed that TGFβ, especially TGFβ2, suppresses macrophage inflammatory responses in the developing intestine and protects against mucosal inflammatory injury. We further showed that enteral feeding of TGFβ2 protected mice from experimental NEC-like injury[35].

Insulin like IGF-1

It is the primary mediator of both intrauterine and postnatal growth in mammals. Experiments have shown that enterally infused IGF-1 in sheep that have undergone esophageal ligation led to an increase in somatic growth and bowel wall thickness[36]. The concentration of IGF-1 in human AF can reach as high as 20 ng/mL. This means that fetal swallowing near term may lead to 20 mcg of IGF-1 being ingested daily[37].

Insulin like Growth Factor-2

It is a major modulator of early embryonic and 2nd trimester fetal growth[5]. It is synthesized by fetal lung and likely exits the fetal lung *via* the efflux of fetal lung fluid[5]. Insulin like growth factor-2 (IGF-Ⅱ) peaks in AF at 19 wk gestation, declining thereafter[38]. Deletion of the *IGF-Ⅱ* gene expressed in the placental trophoblast led to reduced placental growth followed several days later by fetal growth restriction[39].

Receptors to IGF-Ⅰ and IGF-Ⅱ are expressed on crypt cells, on the basolateral membrane and in the distal intestine[24]. Ingested IGF is likely to remain bioactive in the intestine secondary to its relative stability and presence of milk borne protease inhibitors. Effects similar to the effects on sheep bowel described previously have been observed in human duodenal explants[24].

Fibroblast Growth Factor

Activity has been demonstrated in AF and human milk[7]. A study by Hirai *et al*[7] showed that inhibition of fibroblast growth factor (FGF) activity in AF caused a 58% reduction in AF induced intestinal epithelial cell proliferation[4], suggesting an important role in the

Table 6 Important trophic factors involved in gut development and the most relevant reference articles

Trophic factor	Ref.	*n* of references cited
EGF	Maheshwari (2004)[24]	36
	Underwood (2005)[18]	63
	Playford (1996)[29]	23
	Cummins (2002)[15]	108
HGF	Maheshwari (2004)[24]	36
	Underwood (2005)[18]	63
	Cummins (2002)[15]	108
TGF-α and TGF-β	Maheshwari (2004)[24]	36
	Seare (1998)[56]	32
	Underwood (2005)[18]	63
	Cummins (2002)[15]	108
IGF-1	Maheshwari (2004)[24]	36
	Underwood (2005)[18]	63
	Seare (1998)[56]	32
	Cummins (2002)[15]	108
Cytokines	Maheshwari (2004)[24]	36
	Seare (1998)[56]	32

IGF-1: Insulin like growth factor-1; TGF: Transforming growth factor; EGF: Epidermal growth factor; HGF: Hepatocyte growth factor.

development of the GIT.

Erythropoietin

It is present in AF and human milk and its receptor is present on the apical surface of intestinal epithelial cells[24]. It resists degradation and remains bioactive in the intestine. It has been shown that administration of recombinant erythropoietin (EPO) increased villus height, villus area, crypt depth and crypt epithelial cell proliferation in rat pups[24]. *In vitro*, recombinant EPO has been shown to protect cells against mucosal injury and there are reports that the incidence of necrotizing enterocolitis is lower in neonates who had received recombinant EPO[40].

Granulocyte colony stimulating factor

Significant concentrations of granulocyte colony stimulating factor (G-CSF) are present in AF and human milk. Similar to EPO, it resists simulated neonatal digestion[24]. Its receptors are mostly present in the apical regions of the intestine starting at 10 wk gestation. There is evidence that G-CSF may play a role in epithelial cell maintenance[41].

Interleukin family

Interleukin (IL)-2 is present in AF and is believed to enhance intestinal epithelial cell restitution. There is evidence that IL-2 plays a crucial role in mucosal healing because IL-2 knockout mice develop colitis similar to human ulcerative colitis[42]. IL-4, which is also present in AF, is believed to enhance the integrity of the intestinal epithelial cell junctions[24]. IL-6 which is detectable in AF as early as 7 wk of gestation, may play a role in protecting intestinal cells against apoptosis secondary to hypoxia or other severe insults[43]. IL-1 found in AF, leads to intestinal epithelial cell proliferation and increased nutrient uptake[24]. IL-1 also induces expression of decay accelerating factor, which is responsible for the degradation of activated complement and thus may play a protective role against complement activation[15].

Other factors such as vascular endothelial growth factor, IL-8 and the trefoil factor family need more in-depth evaluation in terms of their respective roles in the development of the human intestine. Table 6 summarizes the important TF involved in gut development and their important references.

GIT morphogenesis, functional development and differentiation are regulated by various TF present in the AF[44]. In contrast to adult enterocytes, fetal enterocytes hyper-react to microbial challenges hence the defense responses are inadequate[45]. AF borne TF compensates for immaturity of enterocytes by modulating exaggerated responses of the enterocytes and by promoting immune maturation[46]. Enterocytes are not fully mature at birth and breast milk continues to provide TF and immunomodulatory molecules which promote enterocyte development.

EVIDENCE OF AF AS A SOURCE OF TF

Recently, there have been few studies done to show the role of AF in protecting against intestinal mucosal injuries. Good *et al*[47] showed administration of AF into fetal intestine reduced LPS-mediated signaling within the fetal intestinal mucosa by reducing expression of EGF receptor. The reduced expression of EGF receptor occurs by inhibiting toll-like receptor-4 signaling within the fetal intestine and hence attenuates experimental NEC. Sigger *et al*[48], in a pig model of NEC showed decreased NEC by enteral administration of AF by suppressing pro-inflammatory intestinal responses. Zani *et al*[49] demonstrated that intra peritoneal administration of AF stem cells improves survival and reduced NEC like injury in a rat model of NEC. They further showed that

pre-treatment with AF stem cells resulted in improved intestinal function, decreased intestinal inflammation and increased enterocyte proliferation. We also showed oral administration of AF protected rat pups against NEC-like mucosal injury, which is partly mediated by HGF[50]. There is more research going in our and other laboratories mainly focusing on the role of each individual TF present in AF by blocking different TF. In summary, there is definite evidence of protective effect of AF against NEC. But still many more questions need to be answered before we can use AF in human infants.

PERSPECTIVE

GIT epithelial biology research has changed to a dynamic interface which is able to sense environmental changes and immune responses. GIT epithelial cells can differentiate even if the stimuli from the gut lumen are harmful or not. This depends upon the underlying mechanisms like immunological tolerance. These functions depend upon the developmental status of the enterocytes. The AF during fetal life and breast milk after birth, compensate for the developmental immaturity to assure the maturation of enterocytes. Understanding of the interaction between fetal AF and exposure to various TF is not only theoretical but is also beginning to modulate the clinical management of premature infants. The use of EGF and other TF present in AF, that target GIT epithelial lining, has expanded a new horizon in the management of premature infants.

APUD (Amine Precursor Uptake and Decarboxylation) cells are responsible for synthesis of biological amines and peptides and accumulation of them in the cytoplasm in the form of secretory granules of "neuroendocrine type"[51]. Originally all APUD cells were believed to be derived from the neural crest. However recently it has been proposed that APUD cells of the GIT originate from neuroendocrine-programmed ectoblast[52]. Andrew *et al*[53] in 1975 had found APUD cells in the intestinal groove of the chick, disappearing on the day 3 but reappearing by the day 12 onwards. It was hypothesized that the early arising APUD cells are precursors to one or more islet types. It was confirmed in 2007 that AF harbors a heterogeneous mixture of these multipotent stem cells[54]. APUD cells harvested from AF may very well prove to be helpful in the repair of damaged intestinal epithelial cells following disease processes.

LIFE AFTER AF: HUMAN MILK AND THE GIT ADAPTATION

After fetal maturation, human milk is essential for the neonate's development. Human milk also has the potential to stimulate cell growth and repair and improve immune-competence[55,56]. Human milk contains growth factors, which are similar in nature to that contained in AF. It thus stimulates cells to grow and undergo reparative processes[1]. The neonatal GIT undergoes rapid growth and maturational changes after birth. The tight junctions of the GIT mature and there is selective entrance of nutrients and exclusion of pathogens[57,58]. There is development of an effective mucosal barrier, immaturity of which may result in clinical disease states such as NEC. This intestinal mucous provides a protective interface between the internal and external milieu[1]. Thus a functionally and developmentally mature gut has effective barrier function, epithelial integrity and also adequate mucous production. The role of AF as a source of TF for this above-mentioned development cannot be underscored.

UNANSWERED QUESTIONS

As discussed above, there are so many TF present in AF but the functions and significance of each individual TF remain incompletely understood. Fetal swallowing of AF is very important for the development and maturation of GIT. But some infants are born with esophageal atresia and other conditions leading to malabsorption while other infants have normal functioning GIT. This means there is a way other than just swallowing of AF by which the intestine is exposed to these TF. There is a strong theoretical possibility that TF in AF may protect the preterm infants against NEC and or increase intestinal recovery during the healing phase of NEC[50,59]. Could early feeding of harvested human AF or simulated AF be used in preterm infants at risk for NEC or during recovery phases of NEC? Sullivan *et al*[60] showed that enteral feeding of "simulated AF" containing G-CSF and EPO was well tolerated in preterm infants and the same group further showed[61] that simulated AF was also tolerated by infants who were recovering from NEC.

Do individual TF work separately or work together in concert? What is the most important TF in AF? Can enough AF be harvested at the time of elective cesarean section deliveries? Would harvested AF remain sterile after storage and safe to be used in preterm infants? Could harvested AF be pasteurized without inactivation of TF? Porter *et al*[62] have shown stability of TF in AF after storage. As in utero, fetus can absorb large volumes of AF but would infants with short gut syndrome or gastroschisis be able to tolerate enteral AF infusion which will prevent or decrease villous atrophy? Early trophic feeding in preterm infants is well established. Extremely low birth weight infants are not fed for the first few days. Would feeding AF during this time be beneficial to these infants? So there are many questions which are not well understood regarding how AF borne TF work together and lead to the development of a mature GIT.

FUTURE DIRECTIONS

Many questions about AF remain unanswered. The functions and significance of individual growth factors in human AF remain incompletely described[18]. There

is also much to be learned about the immunoprotective properties of AF. Future directions include research into the use of synthetic or harvested AF as enteral infusions to promote the development of the GI tract in premature infants or infants recovering from NEC[18]. Which TF should be included in the simulated AF? The important question of whether TF would survive the process of storage and freezing is yet to be answered. Although many TF have been elicited and described in human AF, many remain to be understood completely and even more remain to be discovered. More *in vivo* studies are required with individual TF as well with a combination of different TF. At the same time, *in vivo* studies are required to see the role of individual growth factors in AF by blocking different TF. At present, AF remains a potential fluid with possible benefits to extremely premature infants until a definitive beneficial role is confirmed by more research and until its safety is proven.

REFERENCES

1 **Wagner CL**, Taylor SN, Johnson D. Host factors in amniotic fluid and breast milk that contribute to gut maturation. *Clin Rev Allergy Immunol* 2008; **34**: 191-204 [PMID: 18330727 DOI: 10.1007/s12016-007-8032-3]

2 **Grand RJ**, Watkins JB, Torti FM. Development of the human gastrointestinal tract. A review. *Gastroenterology* 1976; **70**: 790-810 [PMID: 770227]

3 **Klein RM**, McKenzie JC. The role of cell renewal in the ontogeny of the intestine. I. Cell proliferation patterns in adult, fetal, and neonatal intestine. *J Pediatr Gastroenterol Nutr* 1983; **2**: 10-43 [PMID: 6886934 DOI: 10.1097/00005176-198302010-00004]

4 **Klein RM**, McKenzie JC. The role of cell renewal in the ontogeny of the intestine. II. Regulation of cell proliferation in adult, fetal, and neonatal intestine. *J Pediatr Gastroenterol Nutr* 1983; **2**: 204-228 [PMID: 6223992 DOI: 10.1097/00005176-198302020-00002]

5 **Underwood MA**, Sherman M. Nutritional Characteristics of Amniotic Fluid. *NeoReviews* 2006; **7**: e310-e316 [DOI: 10.1542/neo.7-6-e310]

6 **Surana R**, Puri P. Small intestinal atresia: effect on fetal nutrition. *J Pediatr Surg* 1994; **29**: 1250-1252 [PMID: 7807360 DOI: 10.1016/0022-3468(94)90816-8]

7 **Hirai C**, Ichiba H, Saito M, Shintaku H, Yamano T, Kusuda S. Trophic effect of multiple growth factors in amniotic fluid or human milk on cultured human fetal small intestinal cells. *J Pediatr Gastroenterol Nutr* 2002; **34**: 524-528 [PMID: 12050579 DOI: 10.1097/00005176-200205000-00010]

8 **Weaver LT**, Walker WA. Epidermal growth factor and the developing human gut. *Gastroenterology* 1988; **94**: 845-847 [PMID: 3257453]

9 **Neu J**, Li N. The neonatal gastrointestinal tract: developmental anatomy, physiology, and clinical implications. *NeoReviews* 2003; **4**: e7-e13 [DOI: 10.1542/neo.4-1-e7]

10 **Godlewski MM**, Słupecka M, Woliński J, Skrzypek T, Skrzypek H, Motyl T, Zabielski R. Into the unknown--the death pathways in the neonatal gut epithelium. *J Physiol Pharmacol* 2005; **56** Suppl 3: 7-24 [PMID: 16077193]

11 **Brandtzaeg P**. The secretory immunoglobulin system: Regulation and biological significance. In: Davis M, Isaacs C (eds). Integrating population outcomes, biological mechanisms and research methods in the study of human milk and lactation. New York: Springer 2002; **503**: 1-16 [DOI: 10.1007/978-1-4615-0559-4_1]

12 **van Odijk J**, Kull I, Borres MP, Brandtzaeg P, Edberg U, Hanson LA, Høst A, Kuitunen M, Olsen SF, Skerfving S, Sundell J, Wille S. Breastfeeding and allergic disease: a multidisciplinary review of the literature (1966-2001) on the mode of early feeding in infancy and its impact on later atopic manifestations. *Allergy* 2003; **58**: 833-843 [PMID: 12911410 DOI: 10.1034/j.1398-9995.2003.00264.x]

13 **Sherman P**, Forstner J, Roomi N, Khatri I, Forstner G. Mucin depletion in the intestine of malnourished rats. *Am J Physiol* 1985; **248**: G418-G423 [PMID: 2580445]

14 **Brace RA**, Wolf EJ. Normal amniotic fluid volume changes throughout pregnancy. *Am J Obstet Gynecol* 1989; **161**: 382-388 [PMID: 2764058 DOI: 10.1016/0002-9378(89)90527-9]

15 **Cummins AG**, Thompson FM. Effect of breast milk and weaning on epithelial growth of the small intestine in humans. *Gut* 2002; **51**: 748-754 [PMID: 12377819 DOI: 10.1136/gut.51.5.748]

16 **Mulvihill SJ**, Stone MM, Debas HT, Fonkalsrud EW. The role of amniotic fluid in fetal nutrition. *J Pediatr Surg* 1985; **20**: 668-672 [PMID: 4087096 DOI: 10.1016/S0022-3468(85)80021-X]

17 **Wintour EM**, Shandley L. Effects of fetal fluid balance on amniotic fluid volume. *Semin Perinatol* 1993; **17**: 158-172 [PMID: 8378800]

18 **Underwood MA**, Gilbert WM, Sherman MP. Amniotic fluid: not just fetal urine anymore. *J Perinatol* 2005; **25**: 341-348 [PMID: 15861199 DOI: 10.1038/sj.jp.7211290]

19 **Gilbert WM**, Brace RA. Amniotic fluid volume and normal flows to and from the amniotic cavity. *Semin Perinatol* 1993; **17**: 150-157 [PMID: 8378799]

20 **Gilbert WM**, Newman PS, Eby-Wilkens E, Brace RA. Technetium Tc 99m rapidly crosses the ovine placenta and intramembranous pathway. *Am J Obstet Gynecol* 1996; **175**: 1557-1562 [PMID: 8987941 DOI: 10.1016/S0002-9378(96)70106-0]

21 **Brace RA**. Physiology of amniotic fluid volume regulation. *Clin Obstet Gynecol* 1997; **40**: 280-289 [PMID: 9199840 DOI: 10.1097/00003081-199706000-00005]

22 **Chochinov RH**, Ketupanya A, Mariz IK, Underwood LE, Daughaday WH. Amniotic fluid reactivity detected by somatomedin C radioreceptor assay: correlation with growth hormone, prolactin and fetal renal maturation. *J Clin Endocrinol Metab* 1976; **42**: 983-986 [PMID: 1270588]

23 **Barka T**, van der Noen H, Gresik EW, Kerenyi T. Immunoreactive epidermal growth factor in human amniotic fluid. *Mt Sinai J Med* 1978; **45**: 679-684 [PMID: 310947]

24 **Maheshwari A**. Role of cytokines in human intestinal villous development. *Clin Perinatol* 2004; **31**: 143-155 [PMID: 15183663 DOI: 10.1016/j.clp.2004.03.003]

25 **Wagner CL**, Forsythe DW. Effect of human milk and recombinant EGF, TGFalpha, and IGF-1 on small intestinal cell proliferation. *Adv Exp Med Biol* 2000; **478**: 373-374 [PMID: 11065088 DOI: 10.1007/0-306-46830-1_33]

26 **Booth C**, Evans GS, Potten CS. Growth factor regulation of proliferation in primary cultures of small intestinal epithelium. *In Vitro Cell Dev Biol Anim* 1995; **31**: 234-243 [PMID: 7757306 DOI: 10.1007/BF02639439]

27 **Cohen S**. Isolation of a mouse submaxillary gland protein accelerating incisor eruption and eyelid opening in the new-born animal. *J Biol Chem* 1962; **237**: 1555-1562 [PMID: 13880319]

28 **Tarnawski A**, Stachura J, Durbin T, Sarfeh IJ, Gergely H. Increased expression of epidermal growth factor receptor during gastric ulcer healing in rats. *Gastroenterology* 1992; **102**: 695-698 [PMID: 1732139]

29 **Playford RJ**, Wright NA. Why is epidermal growth factor present in the gut lumen? *Gut* 1996; **38**: 303-305 [PMID: 8675078 DOI: 10.1136/gut.38.3.303]

30 **Lacy ER**. Epithelial restitution in the gastrointestinal tract. *J Clin Gastroenterol* 1988; **10** Suppl 1: S72-S77 [PMID: 3053884 DOI: 10.1097/00004836-198812001-00012]

31 **Playford RJ**, Marchbank T, Calnan DP, Calam J, Royston P, Batten JJ, Hansen HF. Epidermal growth factor is digested to smaller, less active forms in acidic gastric juice. *Gastroenterology* 1995; **108**: 92-101 [PMID: 7806067 DOI: 10.1016/0016-5085(95)90012-8]

32 **Varner MW**, Dildy GA, Hunter C, Dudley DJ, Clark SL, Mitchell MD. Amniotic fluid epidermal growth factor levels in normal and abnormal pregnancies. *J Soc Gynecol Investig* 1996; **3**: 17-19 [PMID: 8796801 DOI: 10.1016/1071-5576(95)00044-5]

33 **Tremblay E**, Monfils S, Ménard D. Epidermal growth factor influences cell proliferation, glycoproteins, and lipase activity in human fetal stomach. *Gastroenterology* 1997; **112**: 1188-1196 [PMID: 9098002 DOI: 10.1016/S0016-5085(97)70130-6]

34 **Claud EC**, Savidage T, Walker WA. Modulation of human intestinal epithelial cell IL-8 secretion by human milk factors. *Pediatr Res* 2003; **53**: 419-425 [PMID: 12595589 DOI: 10.1203/01.PDR.0000050141.73528.AD]

35 **Maheshwari A**, Kelly DR, Nicola T, Ambalavanan N, Jain SK, Murphy-Ullrich J, Athar M, Shimamura M, Bhandari V, Aprahamian C, Dimmitt RA, Serra R, Ohls RK. TGF-β2 suppresses macrophage cytokine production and mucosal inflammatory responses in the developing intestine. *Gastroenterology* 2011; **140**: 242-253 [PMID: 20875417 DOI: 10.1053/j.gastro.2010.09.043]

36 **Kimble RM**, Breier BH, Gluckman PD, Harding JE. Enteral IGF-I enhances fetal growth and gastrointestinal development in oesophageal ligated fetal sheep. *J Endocrinol* 1999; **162**: 227-235 [PMID: 10425460 DOI: 10.1677/joe.0.1620227]

37 **Merimee TJ**, Grant M, Tyson JE. Insulin-like growth factors in amniotic fluid. *J Clin Endocrinol Metab* 1984; **59**: 752-755 [PMID: 6384254 DOI: 10.1210/jcem-59-4-752]

38 **Lallemand AV**, Ruocco SM, Joly PM, Gaillard DA. In vivo localization of the insulin-like growth factors I and II (IGF I and IGF II) gene expression during human lung development. *Int J Dev Biol* 1995; **39**: 529-537 [PMID: 7577444]

39 **Constância** M, Hemberger M, Hughes J, Dean W, Ferguson-Smith A, Fundele R, Stewart F, Kelsey G, Fowden A, Sibley C, Reik W. Placental-specific IGF-II is a major modulator of placental and fetal growth. *Nature* 2002; **417**: 945-948 [PMID: 12087403 DOI: 10.1038/nature00819]

40 **Semba RD**, Juul SE. Erythropoietin in human milk: physiology and role in infant health. *J Hum Lact* 2002; **18**: 252-261 [PMID: 12192960 DOI: 10.1177/089033440201800307]

41 **Calhoun DA**, Lunoe M, Du Y, Christensen RD. Granulocyte colony-stimulating factor is present in human milk and its receptor is present in human fetal intestine. *Pediatrics* 2000; **105**: e7 [PMID: 10617744 DOI: 10.1542/peds.105.1.e7]

42 **Barmeyer C**, Horak I, Zeitz M, Fromm M, Schulzke JD. The interleukin-2-deficient mouse model. *Pathobiology* 2003; **70**: 139-142 [PMID: 12571417 DOI: 10.1159/000068145]

43 **Rollwagen FM**, Yu ZY, Li YY, Pacheco ND. IL-6 rescues enterocytes from hemorrhage induced apoptosis in vivo and in vitro by a bcl-2 mediated mechanism. *Clin Immunol Immunopathol* 1998; **89**: 205-213 [PMID: 9837690 DOI: 10.1006/clin.1998.4600]

44 **Lebenthal A**, Lebenthal E. The ontogeny of the small intestinal epithelium. *JPEN J Parenter Enteral Nutr* 1999; **23**: S3-S6 [PMID: 10483884 DOI: 10.1177/014860719902300502]

45 **Claud EC**, Walker WA. Hypothesis: inappropriate colonization of the premature intestine can cause neonatal necrotizing enterocolitis. *FASEB J* 2001; **15**: 1398-1403 [PMID: 11387237 DOI: 10.1096/fj.00-0833hyp]

46 **Altman DJ**, Schneider SL, Thompson DA, Cheng HL, Tomasi TB. A transforming growth factor beta 2 (TGF-beta 2)-like immunosuppressive factor in amniotic fluid and localization of TGF-beta 2 mRNA in the pregnant uterus. *J Exp Med* 1990; **172**: 1391-1401 [PMID: 1700055 DOI: 10.1084/jem.172.5.1391]

47 **Good M**, Siggers RH, Sodhi CP, Afrazi A, Alkhudari F, Egan CE, Neal MD, Yazji I, Jia H, Lin J, Branca MF, Ma C, Prindle T, Grant Z, Shah S, Slagle D, Paredes J, Ozolek J, Gittes GK, Hackam DJ. Amniotic fluid inhibits Toll-like receptor 4 signaling in the fetal and neonatal intestinal epithelium. *Proc Natl Acad Sci USA* 2012; **109**: 11330-11335 [PMID: 22733781 DOI: 10.1073/pnas.1200856109]

48 **Siggers J**, Ostergaard MV, Siggers RH, Skovgaard K, Mølbak L, Thymann T, Schmidt M, Møller HK, Purup S, Fink LN, Frøkiær H, Boye M, Sangild PT, Bering SB. Postnatal amniotic fluid intake reduces gut inflammatory responses and necrotizing enterocolitis in preterm neonates. *Am J Physiol Gastrointest Liver Physiol* 2013; **304**: G864-G875 [PMID: 23518680 DOI: 10.1152/ajpgi.00278.2012]

49 **Zani A**, Cananzi M, Fascetti-Leon F, Lauriti G, Smith VV, Bollini S, Ghionzoli M, D'Arrigo A, Pozzobon M, Piccoli M, Hicks A, Wells J, Siow B, Sebire NJ, Bishop C, Leon A, Atala A, Lythgoe MF, Pierro A, Eaton S, De Coppi P. Amniotic fluid stem cells improve survival and enhance repair of damaged intestine in necrotising enterocolitis via a COX-2 dependent mechanism. *Gut* 2014; **63**: 300-309 [PMID: 23525603]

50 **Jain SK**, Baggerman EW, Mohankumar K, Namachivayam K, Jagadeeswaran R, Reyes VE, Maheshwari A. Amniotic fluid-borne hepatocyte growth factor protects rat pups against experimental necrotizing enterocolitis. *Am J Physiol Gastrointest Liver Physiol* 2014; **306**: G361-G369 [PMID: 24407592 DOI: 10.1152/ajpgi.00272.2013]

51 **Golovin DI**, Nikonov AA. APUD cells and apudomas. *Arkh Patol* 1981; **43**: 3-11 [PMID: 6119068]

52 **Bosman FT**, Louwerens JW. APUD cells in teratomas. *Am J Pathol* 1981; **104**: 174-180 [PMID: 6114639]

53 **Andrew A**. An experimental investigation into the possible neural crest origin of pancreatic APUD (islet) cells. *J Embryol Exp Morphol* 1976; **35**: 577-593 [PMID: 781174]

54 **Petsche Connell J**, Camci-Unal G, Khademhosseini A, Jacot JG. Amniotic fluid-derived stem cells for cardiovascular tissue engineering applications. *Tissue Eng Part B Rev* 2013; **19**: 368-379 [PMID: 23350771 DOI: 10.1089/ten.teb.2012.0561]

55 **Playford RJ**. Peptides and gastrointestinal mucosal integrity. *Gut* 1995; **37**: 595-597 [PMID: 8549930 DOI: 10.1136/gut.37.5.595]

56 **Seare NJ**, Playford RJ. Growth factors and gut function. *Proc Nutr Soc* 1998; **57**: 403-408 [PMID: 9793997 DOI: 10.1079/PNS19980057]

57 **Balda MS**, Fallon MB, Van Itallie CM, Anderson JM. Structure, regulation, and pathophysiology of tight junctions in the gastrointestinal tract. *Yale J Biol Med* 1992; **65**: 725-735; discussion 737-740 [PMID: 1341075]

58 **Axelsson I**, Jakobsson I, Lindberg T, Polberger S, Benediktsson B, Räihä N. Macromolecular absorption in preterm and term infants. *Acta Paediatr Scand* 1989; **78**: 532-537 [PMID: 2782068 DOI: 10.1111/j.1651-2227.1989.tb17932.x]

59 **Dvorak B**, Halpern MD, Holubec H, Williams CS, McWilliam DL, Dominguez JA, Stepankova R, Payne CM, McCuskey RS. Epidermal growth factor reduces the development of necrotizing enterocolitis in a neonatal rat model. *Am J Physiol Gastrointest Liver Physiol* 2002; **282**: G156-G164 [PMID: 11751169 DOI: 10.1152/ajpgi.00196.2001]

60 **Sullivan SE**, Calhoun DA, Maheshwari A, Ashmeade TL, Auerbach DA, Hudak ML, Beltz SE, Christensen RD. Tolerance of simulated amniotic fluid in premature neonates. *Ann Pharmacother* 2002; **36**: 1518-1524 [PMID: 12243599 DOI: 10.1345/aph.1A439]

61 **Lima-Rogel V**, Calhoun DA, Maheshwari A, Torres-Montes A, Roque-Sanchez R, Garcia MG, Christensen RD. Tolerance of a sterile isotonic electrolyte solution containing select recombinant growth factors in neonates recovering from necrotizing enterocolitis. *J Perinatol* 2003; **23**: 200-204 [PMID: 12732856 DOI: 10.1038/sj.jp.7210894]

62 **Porter AE**, Auth J, Prince M, Ghidini A, Brenneman DE, Spong CY. Optimization of cytokine stability in stored amniotic fluid. *Am J Obstet Gynecol* 2001; **185**: 459-462 [PMID: 11518909 DOI: 10.1067/mob.2001.115106]

P- Reviewer: Caboclo JLF, Chow WK
S- Editor: Kong JX **L- Editor**: A **E- Editor**: Jiao XK

World Journal of Gastrointestinal Pathophysiology

Submit a Manuscript: http://www.wjgnet.com/esps/
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.4291/wjgp.v7.i1.48

World J Gastrointest Pathophysiol 2016 February 15; 7(1): 48-58
ISSN 2150-5330 (online)

REVIEW

Alcoholic pancreatitis: New insights into the pathogenesis and treatment

Dahn L Clemens, Katrina J Schneider, Christopher K Arkfeld, Jaclyn R Grode, Mark A Wells, Shailender Singh

Dahn L Clemens, Fred and Pamela Buffett Cancer Center, University of Nebraska Medical Center, Omaha, NE 68198-8098, United States

Dahn L Clemens, Nebraska-Western Iowa VA Heath Care System, University of Nebraska Medical Center, Omaha, NE 68198-8098, United States

Dahn L Clemens, Katrina J Schneider, Christopher K Arkfeld, Jaclyn R Grode, Mark A Wells, Shailender Singh, Department of Internal Medicine, Section of Gastroenterology and Hepatology, University of Nebraska Medical Center, Omaha, NE 68198-8098, United States

Author contributions: Clemens DL wrote the manuscript; all other authors contributed to the conceptual design of the manuscript, as well as the editing and final preparation.

Supported by Funds from the University of Nebraska Department of Internal Medicine, the Fred and Pamela Buffett Cancer Center, and the Nebraska Research Initiative (to Clemens DL); and by NIH, NIAAA grant AA020818 (to Arkfeld CK).

Conflict-of-interest statement: The authors declare no conflict of interests.

Correspondence to: Dahn L Clemens, PhD, Associate Professor, Department of Internal Medicine, Section of Gastroenterology and Hepatology, University of Nebraska Medical Center, Omaha, NE 68198-8098, United States. dclemens@unmc.edu
Telephone: +1-402-9953738
Fax: +1-402-9950604

Received: June 29, 2015
Peer-review started: July 2, 2015
First decision: August 4, 2015
Revised: October 15, 2015
Accepted: November 10, 2015
Article in press: November 11, 2015
Published online: February 15, 2016

Abstract

Acute pancreatitis is a necro-inflammatory disease of the exocrine pancreas that is characterized by inappropriate activation of zymogens, infiltration of the pancreas by inflammatory cells, and destruction of the pancreatic exocrine cells. Acute pancreatitis can progress to a severe life-threatening disease. Currently there is no pharmacotherapy to prevent or treat acute pancreatitis. One of the more common factors associated with acute pancreatitis is alcohol abuse. Although commonly associated with pancreatitis alcohol alone is unable to cause pancreatitis. Instead, it appears that alcohol and its metabolic by-products predispose the pancreas to damage from agents that normally do not cause pancreatitis, or to more severe disease from agents that normally cause mild pancreatic damage. Over the last 10 to 20 years, a tremendous amount of work has defined a number of alcohol-mediated biochemical changes in pancreatic cells. Among these changes are: Sustained levels of intracellular calcium, activation of the mitochondrial permeability transition pore, endoplasmic reticulum stress, impairment in autophagy, alteration in the activity of transcriptional activators, and colocalization of lysosomal and pancreatic digestive enzymes. Elucidation of these changes has led to a deeper understanding of the mechanisms by which ethanol predisposes acinar cells to damage. This greater understanding has revealed a number of promising targets for therapeutic intervention. It is hoped that further investigation of these targets will lead to the development of pharmacotherapy that is effective in treating and preventing the progression of acute pancreatitis.

Key words: Alcohol; Pancreatitis; Alcoholic pancreatitis; Ethanol metabolism; Acute pancreatitis; Fatty acid ethyl esters

Core tip: There is currently no specific pharmacotherapy for pancreatitis. Although ethanol abuse is commonly associated with both acute and chronic pancreatitis, ethanol does not itself cause pancreatitis. Instead it appears that ethanol and its metabolic by-products sensitize the pancreas to damage from other factors. Detailed understanding of the mechanisms by which ethanol sensitizes the pancreas to damage has identified a number of promising targets for therapy. It is hoped that further preclinical and clinical studies will lead to the development of successful treatment of both acute and chronic pancreatitis.

Clemens DL, Schneider KJ, Arkfeld CK, Grode JR, Wells MA, Singh S. Alcoholic pancreatitis: New insights into the pathogenesis and treatment. *World J Gastrointest Pathophysiol* 2016; 7(1): 48-58 Available from: URL: http://www.wjgnet.com/2150-5330/full/v7/i1/48.htm DOI: http://dx.doi.org/10.4291/wjgp.v7.i1.48

INTRODUCTION

The pancreas is a complex organ, containing both endocrine and exocrine components. The endocrine component of the pancreas is composed of the Islets of Langerhans, and comprises a relatively small portion of the pancreas, only about 1%-2% of the organ. The endocrine pancreas is responsible for the production of glucagon and insulin, hormones that regulate glucose homeostasis. The exocrine component comprises the vast majority of the pancreas and is comprised of acinar, ductal, and stellate cells. The acinar cells produce digestive enzymes that are synthesized as inactive zymogens and are secreted through ducts to the duodenum where they are activated. The ductal cells produce and secrete large quantities of bicarbonate (HCO_3^-) and form a network that serves as a conduit for the delivery of the digestive enzymes into the duodenum. The pancreatic stellate cells synthesize and degrade extracellular matrix proteins.

Pancreatitis is a serious gastrointestinal illness and an important health concern both in the United States and worldwide. Typically, pancreatitis is either classified as acute or chronic. Acute pancreatitis is a necro-inflammatory disease that is characterized by infiltration of the pancreas by inflammatory cells and destruction of the pancreatic exocrine cells. Based on clinical observations in human beings, it is believed that in cases of acute pancreatitis, which resolve, the pancreas regenerates to its full structural and functional capacity after an acute episode. This concept is supported by many studies in experimental animals, which have demonstrated structural and functional repair of the pancreas after experimentally induced pancreatitis[1-5]. In cases of severe acute pancreatitis systemic inflammation develops and can lead to multi-organ failure and death.

Of all gastrointestinal ailments, acute pancreatitis is the single most common cause of hospitalization in the United States. It has been estimated that acute pancreatitis accounts for approximately 2.6 billion dollars in annual inpatient costs[6]. Each year, approximately 220000 people are admitted to United States hospitals because of acute pancreatitis[7]. In up to 20% of these cases there are serious complications with a mortality rate ranging from 10% to 30%[8,9]. Currently, there is no specific pharmacotherapy for acute pancreatitis, underscoring the need for research that aids in the prevention and treatment of this disease.

Although the pathogenesis of acute pancreatitis is not entirely known, it appears that the disease originates in injured acinar cells. Over a hundred years ago, Chiari[10] suggested that acute pancreatitis is caused by inappropriate activation of digestive enzymes, ultimately leading to autodigestion of the pancreas. Although inappropriate activation of trypsinogen is important in causing pancreatic injury early in the disease, the induction and progression of local and systemic inflammation associated with acute pancreatitis does not require trypsinogen activation[11]. In fact, it has been demonstrated that intra-acinar cell activation of the transcriptional activator nuclear factor-κB (NF-κB) occurs simultaneous to, but independent of, trypsinogen activation[11]. NF-κB regulates a wide variety of genes involved in cell survival, cellular replication, immunity, and inflammation. The NF-κB-mediated inflammatory response appears to be responsible for up to half of the pancreatic tissue damage that is associated with acute pancreatitis, as well as the potentially fatal severe systemic inflammatory response[11].

Chronic pancreatitis, like acute pancreatitis, is thought to begin as a necro-inflammatory disease. The exact series of events that ultimately result in chronic pancreatitis are not known. Despite this fact, it is generally thought that chronic pancreatitis has an early stage that is characterized by recurrent attacks of acute pancreatitis, and a late stage associated with pancreatic insufficiency, steatorrhea, diabetes, pancreatic calcification, and fibrosis[12].

ALCOHOLIC PANCREATITIS

Alcohol abuse is commonly associated with pancreatitis. This association has been recognized for well over 100 years, yet to this day how alcohol abuse predisposes the pancreas to disease is not entirely understood[13]. In developing countries, approximately 35% of acute pancreatitis cases[9] and approximately 70% of chronic pancreatitis cases are associated with alcohol abuse[14]. Additionally, individuals diagnosed with

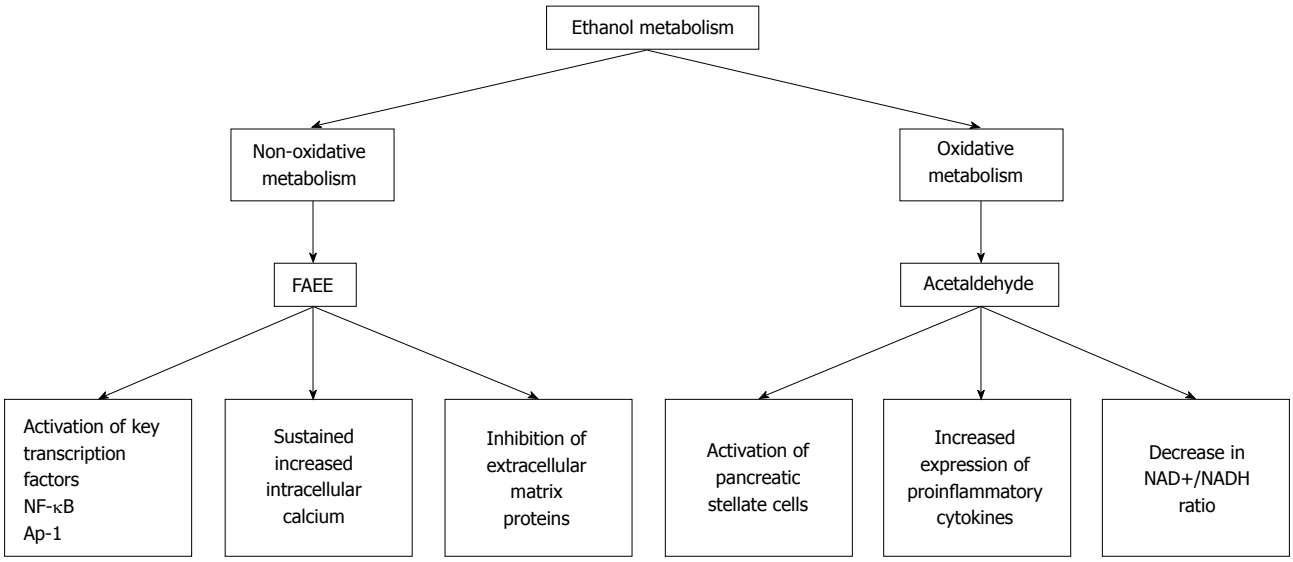


Figure 1 The by-products of ethanol metabolism cause a number of changes in the pancreas. In the pancreas, ethanol is metabolized by both nonoxidative and oxidative pathways. The major by-products of the nonoxidative metabolism of ethanol are FAEEs. The major metabolic by-product of the oxidative metabolism of ethanol is acetaldehyde. Metabolism of ethanol by both of these pathways has been shown to cause a number of changes that can predispose the pancreas to acute pancreatitis. FAEE: Fatty acid ethyl ester; NF-κB: Nuclear factor-κB.

chronic pancreatitis are 20-times more likely to develop pancreatic cancer[15], a disease with a dismal prognosis. It is thought that changes that occur in the pancreas during chronic injury are associated with, or predispose the organ to, the initiation of pancreatic neoplasia. This has led to the classification of chronic pancreatitis as a preneoplastic disease.

How alcohol abuse contributes to alcoholic pancreatitis is not fully understood. Although there is a tremendous association between alcohol abuse and pancreatitis, relatively few individuals who abuse alcohol develop alcoholic pancreatitis. This fact indicates that alcoholic pancreatitis is not caused by chronic alcohol abuse alone[16-18]. Instead, it appears that the pancreas is sensitized to injury by alcohol consumption, and external or environmental factors trigger initiation of this disease. A number of factors are believed to be triggers of alcoholic pancreatitis, among these are: Genetic predisposition, high lipid diet, cigarette smoking, and infectious agents[19].

Although alcoholic pancreatitis can remain an acute disease, in many cases this acute disease progresses to alcoholic chronic pancreatitis. Many times this progression from an acute disease to a chronic disease is associated with recurring bouts of acute pancreatitis. Interestingly, it was reported, that progression from acute to chronic pancreatitis is most common in habitual alcohol abusers[20]. This indicates that excessive alcohol consumption is involved in acute pancreatitis progressing to a chronic fibrotic disorder. Because ethanol alone is not capable of causing pancreatitis, the question is, how does ethanol alter the physiology of the pancreas and sensitize the organ to disease?

Developmentally, the liver and the pancreas are related[21]. Because of this, it is not terribly surprising that the pancreas can metabolize ethanol. In the pancreas, both nonoxidative and oxidative pathways of ethanol metabolism are functional and have been shown to have a number of deleterious effects on the pancreas (Figure 1).

Two enzymes, alcohol dehydrogenase (ADH) and cytochrome P450 2E1 (CYP 2E1) catalyze oxidative ethanol metabolism. Ethanol metabolized by both ADH and CYP 2E1 results in the production of reactive oxygen species (ROS) and acetaldehyde. Although the pancreas expresses both ADH and CYP 2E1, the expression of these enzymes is much lower than in the liver. Consequently, the oxidative metabolism of ethanol by the pancreas is also much lower than in the liver[22,23]. In spite of this fact, acetaldehyde, a reactive metabolite of ethanol oxidation, mediates some detrimental effects in pancreatic acinar cells[24].

Nonoxidative ethanol metabolism is accomplished by a diverse group of enzymes known as fatty acid ethyl ester (FAEE) synthases[25]. Ethanol metabolism by these enzymes combines free fatty acids (FA) with ethanol generating FAEEs. In the pancreas fatty acid ester synthase activity is relatively high. Therefore, ethanol metabolism by the nonoxidative pathway is also relatively high[26]. Because ADH and CYP 2E1 activity is relatively low in the pancreases, ethanol metabolism by FAEE synthases and the production of FAEEs likely has an important role in alcohol associated pancreatic dysfunctions and development of alcoholic pancreatitis.

Effects of ethanol on the cellular mobilization of calcium and the inappropriate activation of pancreatic enzymes

It is generally thought that one of the initiating events of acute pancreatitis is the intracellular activation of trypsinogen and other digestive enzymes produced by

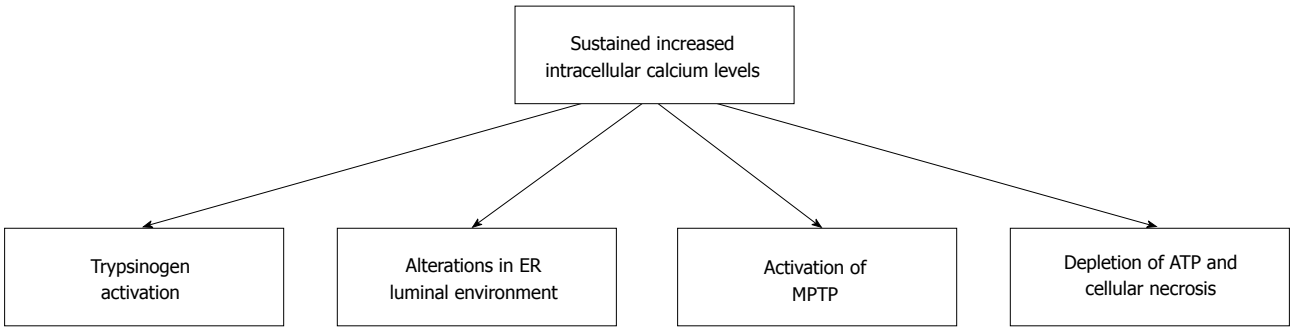


Figure 2 Consequences of sustained increased intracellular calcium in pancreatic acinar cells. Ethanol and its metabolic by-products can cause sustained increases in the level of intracellular calcium. Sustained increases in intracellular calcium results in cellular changes that can damage pancreatic acinar cells. Many of these changes can predispose individuals to the development of alcoholic acute pancreatitis. MPTP: Mitochondrial permeability transition pore; ER: Endoplasmic reticulum.

acinar cells. This inappropriate enzyme activation is mediated by sustained elevation in the concentration of cytoplasmic calcium[27,28].

Intracellular calcium has a critical role in both normal and pathologic actions of acinar cells. The majority of calcium in acinar cells is stored in the endoplasmic reticulum (ER), although there exists an important acidic granular reservoir located in the apical region of the cell. Zymogen granules contain substantial quantities of calcium and constitute a major portion of this acidic reservoir in acinar cells[29].

Secretion of zymogens from acinar cells is controlled by the local release of small quantities of calcium from the zymogen-containing granules. In contrast, global sustained release of calcium from intracellular stores sets in action a number of pathologic changes in acinar cells (Figure 2). Thus, intracellular calcium is involved both in the normal and the pathologic processes of acinar cells[30].

It has been demonstrated that stimulation of inositol trisphosphate (IP_3) type 2 and 3 receptors (IP_3Rs) and to a lesser extent ryanodine receptors, located on the ER and zymogen granules results in calcium release[30]. Importantly, in both whole cells and 2-photon permeabilized cells, ethanol and FAEEs induce the sustained release of calcium from intracellular stores by activation of IP_3Rs[31]. The critical role of IP_3Rs in the pathologic sustained intracellular release of calcium has been demonstrated by studies in which antibodies specific to IP_3R2 and 3, pharmacologic inhibition of IP_3Rs, and the use of genetically modified mice that lack IP_3R2 and 3, attenuate the intensity of calcium release, as well as the extent of trypsinogen activation and tissue necrosis[30-33].

Acinar cells do not contain infinite stores of calcium. In response to increases in cytosolic calcium concentrations, ATP-dependent calcium pumps located on the plasma membrane are activated and eliminate calcium. Therefore, to maintain sustained elevated levels of calcium there must be a mechanism by which acinar cells take up calcium from the extracellular environment.

Located on the basolateral portion of the plasma membrane of acinar cells are calcium-release activated calcium (CRAC) channels. When calcium concentrations in the ER are reduced, a calcium sensing protein (STIM1) located in the ER translocates to these CRAC channels where it interacts with Orai1. The channels are activated and extracellular calcium is taken up from the extracelluar environment. This uptake sustains the elevated levels of intracellular calcium[34]. Importantly, it has been shown that a CRAC channel inhibitor, GSK-7975A, inhibited the calcium entry into acinar cells. Inhibition of calcium entry was able to abrogate trypsin and protease activity, as well as necrosis induced by treatment of acinar cells with FAEEs[32].

Acinar cells are not without some protection from the deleterious effects of sustained elevated calcium levels. Using 2-photon permeabilized acinar cells Gerasimenko *et al*[33] showed that the actions of ethanol treatment were accentuated in permeabilized cells compared with intact cells. This increased severity could be overcome if physiologic concentrations of calmodulin were included in the extracellular media. The authors speculated that calmodulin was lost from the permeabilized cells and that inclusion of calmodulin in the extracellular media allowed calmodulin to reenter the cells and protect them from the actions of elevated calcium[33]. In support of this contention, the authors demonstrated that pharmacologic inhibition of calmodulin with calmodulin inhibitory peptide resulted in activation of trypsin in permeabilized cells. Conversely, pharmacologic activation of calmodulin with the cell permeable calmodulin activator, CALP-3, substantially abolished the detrimental actions of ethanol in both permeabilized and intact cells[33].

The findings that the addition of calmodulin and inhibition of CRAC channels attenuate the deleterious effects of sustained elevated levels of cytosolic calcium provide novel targets for therapeutic intervention and the treatment of acute pancreatitis[30].

Mitochondrial dysfunction in alcoholic pancreatitis

Mitochondria are intimately involved in the life and death of cells. Mitochondria are responsible for the production of ATP and thus, the energy required to perform all cellular functions. Conversely, mitochondrial

damage or dysfunction can lead to cell death by either apoptosis or necrosis. Mitochondria are involved in the activation of the classical apoptosis pathway of cell death in which there is little release of intracellular material and limited activation of the inflammatory response. If mitochondrial dysfunction becomes severe enough, ATP production is reduced or inhibited. In the absence of ATP cell death is necrotic. Cellular necrosis is characterized by disruption of the plasma membrane, release of cellular contents, including activated enzymes, and the activation of the inflammatory response. Of clinical importance, necrotic cell death is associated with more severe pancreatitis[35,36].

In pancreatic acinar cells mitochondria play an important role in maintaining calcium homeostasis. This may be, in part, because of their juxtaposition to sites of calcium release from the ER. It has been shown that peri-apical mitochondria take up cytosolic calcium released during local calcium spikes and respond by increasing ATP production. This ATP is used to drive the sarcoER Ca^{2+} ATPase pump (SERCA), which restores ER calcium and the plasma membrane Ca^{2+} pump (PMCA), which restores normal cytosolic calcium levels, thereby terminating the signal and preventing the spread of the signal throughout the cell[37,38]. Unfortunately, this normal physiologic response of mitochondria to increased cytosolic calcium levels can also lead to cell death. If the elevated cytoplasmic calcium concentration is global and sustained this normal cellular compensatory mechanism can be overwhelmed and result in cell death.

As mentioned above, the nonoxidative metabolites of ethanol, FAEEs, can bind IP_3Rs on the ER and zymogen granules causing the release of calcium. Excessive mitochondrial calcium can cause permeabilization of the mitochondrial membrane. Mitochondrial membrane permeabilization is a trigger that initiates both apoptotic and necrotic cell death pathways[39]. Permeabilization of the mitochondrial membrane leads to the loss of mitochondrial membrane potential ($\Delta\psi m$) by opening the mitochondrial permeability transition pore (MPTP). Activation of the MPTP allows nonspecific entry of substances with a nuclear mass of less than 15000 Daltons into the inner mitochondrial matrix. This can disrupt the ability of mitochondria to produce ATP and ultimately in necrosis.

FAEEs have also been shown to bind to the inner mitochondrial membrane where they undergo hydrolysis to FA by FAEE hydrolases[40]. This results in the production of locally high concentrations of FA, which can uncouple oxidative phosphorylation and deplete $\Delta\psi m$, thereby inhibiting ATP production[31]. The lack of ATP production exacerbates the effects of the cytosolic calcium because the lack of ATP to drive the ATP-dependent SERCA and PMCA pumps results in the inability of the cell to regain calcium homeostasis.

In cases where the oxidative metabolism of ethanol is diminished the levels of FAEE are increased and can result in tissue damage[41,42]. This is thought to be the explanation for the high level of FAEEs in the pancreas. In support of this, treatment of isolated pancreatic acinar cells with low levels of ethanol and the fatty acid palmitoleic acid caused transient rises in intracellular calcium. Inhibition of the oxidative metabolism of ethanol with 4-methylpyrazol in these cells resulted in the conversion of transient calcium rises to sustained increases in calcium. The sustained elevated levels of calcium resulted in mitochondrial membrane depolarization and cellular necrosis[43]. Inhibition of the FAEE synthase carboxylester lipase with 3-benzyl-6-chloro-2-pyrone (3-BCP) ameliorated the adverse actions of the combined treatment[43]. *In vivo* studies demonstrated that mice treated with ethanol and palmitoleic acid resulted in increased levels of palmitoleic acid ethyl ester, extensive edema, neutrophil infiltration and acinar cell necrosis. Furthermore, these pathologic changes were accentuated by the inclusion of 4-methylpyrazol treatment. Treatment of these mice with 3-BCP significantly reduced the pathologic effects[43]. Thus, inhibition of fatty acid ethyl synthases reduced the tissue injury associated with ethanol and fatty acid treatment and may be an effective strategy to attenuate the severity of alcohol acute pancreatitis.

Oxidative metabolism of ethanol has also been shown to have deleterious effects on mitochondria[24]. Using isolated mouse acinar cells, as well as, *in vivo* and *ex vivo* models of pancreatitis it has been shown that ethanol treatment reduces the $\Delta\psi m$ and converts the normal transient decrease in $\Delta\psi m$ caused by treatment with physiologic concentrations of cholecystokinin (CCK) to a sustained decrease in $\Delta\psi m$. The sustained decrease in $\Delta\psi m$ results in reduced cellular ATP concentrations and necrosis[24]. Using mice deficient in cyclophilin-D, a major component of the MPTP, it was demonstrated that the MPTP plays a major role in the ethanol-mediated sensitivity to mitochondrial depolarization. Further studies revealed that the ethanol-induced effects on $\Delta\psi m$ were dependent on the decreased NAD^+/NADH ratio associated with the oxidative metabolism of ethanol and its by-product acetaldehyde, and not dependent on calcium.

Metabolism of acetaldehyde is primarily carried out by aldehyde dehydrogenase-2 (ALDH2) a NAD^+ requiring enzyme residing on the inner mitochondrial membrane. Thus, metabolism of acetaldehyde by ALDH2 depletes NAD^+ and increases the concentration of NADH. Because of this, the authors speculated that the decreased mitochondrial NAD^+/NADH ratio and reduced $\Delta\psi m$ is a result of the metabolism of acetaldehyde to acetate. This contention is supported by the facts that pharmacologic depletion of NAD^+ with FK866 also results in mitochondrial depolarization, and the fact that supplementation with NAD^+ ameliorates the effects of ethanol[24].

Interestingly, in mitochondria isolated from the liver, ethanol metabolism induces activation of the MPTP, at least in part, through increased cyclophilin-D activity and the increased association of cyclophilin-D with adenine nucleotide translocator-1 (ANT-1)[44]. This

increased cyclophilin activity appears to be linked to sirtuin-3, a NAD^+-dependent deacetylase localized to the mitochondrial matrix that is involved in the regulation of cyclophilin-D acetylation[45]. Ethanol oxidation-mediated decrease in the NAD^+/NADH ratio leads to decreased sirtuin-3 activity and consequently, hyperacetylation of cyclophilin-D. Hyperacetylation of cyclophilin-D results in increased cyclophilin-D activity, increased binding to ANT-1, and MPTP induction[44]. The role of NAD^+ in MPTP in the pancreas makes it tempting to speculate that the ethanol oxidation-mediated induction of the MPTP in pancreatic mitochondria is mediated by a similar NAD^+-sirtuin-3-cyclophilin-D mechanism. Thus, NAD^+/NADH may be a novel target to ameliorate alcoholic acute pancreatitis.

ER-stress in alcoholic pancreatitis

The primary function of pancreatic acinar cells is to produce digestive enzymes[46]. Synthesis of these proteins requires an extensive ER network. In the ER, newly synthesized proteins undergo posttranslational modification, disulfide bond formation, and chaperone-facilitated protein folding before being transported to the Golgi apparatus. Once in the Golgi these proteins undergo further modification before being transported to zymogen granules or other cellular organelles. Proper protein folding and sorting are critical in preventing inappropriate activation of digestive proenzymes in acinar cells.

Proper protein modification and folding in the ER requires the appropriate levels of intraluminal calcium and ATP, as well as the proper oxidizing environment for disulfide bond formation. Perturbations in these environmental factors, results in the production of misfolded proteins and what is referred to as ER stress. Detection of these misfolded proteins initiates the unfolded protein response. The unfolded protein response is mediated by the activation of three pathways: (1) the inositol-requiring protein-1 pathway (IRE-1); (2) the activating transcription factor-6 pathway; and (3) the RNA-activated protein kinase-like ER kinase (PERK) pathway. In general, activation of the unfolded protein response decreases the production of cellular proteins and increases the expression of proteins involved in protein folding. It is thought that this adaptive response is protective and aids cells in riding themselves of misfolded proteins, the presence of which can be detrimental to cells[47,48].

It has been shown in mice that administration of ethanol by the intragastric feeding model causes ER stress in pancreatic acinar cells[49]. This is characterized by dilation of the ER, alteration of the redox state of the ER, and up regulation of the oxidoreductase, protein disulfide isomerase. It was suggested that the altered redox state was the result of ROS generated in the ER. Additionally, expression of IRE-1 and its downstream effector the spliced form of X-box binding protein-1 (sXBP1) were increased.

The authors speculated that the increased expression of IRE-1 and sXBP1 were critical for the adaptive protective response, and that induction of this pathway protected acinar cells from the detrimental effects of ROS-induced ER stress. To test this hypothesis, the authors examined the effects of ethanol in mice heterozygous for XBP1 ($XBP1^{+/-}$). Ethanol administration to $XBP1^{+/-}$ mice resulted in a number of ultrastructural changes. These changes included extensive dilation of the ER, a dramatic increase in the number of autophagic vesicles, a substantial decrease in the number of zymogen granules, and the inappropriate localization of zymogen granules throughout the cell. These changes in ultrastructure were also associated with decreased expression of the pancreatic digestive enzyme amylase[49]. Additionally, compared with wild type mice administered ethanol, $XBP1^{+/-}$ mice administered ethanol demonstrated marked increase in PERK, eIF2α phosphorylation, and expression of ATF4[49]; adaptations associated with severe ER stress[50]. Furthermore, approximately 20% of the pancreas in $XBP1^{+/-}$ mice administered ethanol contained pathologic lesions characterized by areas of necrosis, apoptosis, and inflammation[49]. Thus, it appears that ER stress and activation of the unfolded protein response if controlled can be a protective adaptive response. Alternatively, an uncontrolled unfolded protein response can result in cell death and tissue injury[51]. These findings indicate that modulation of ER stress and the unfolded protein response may provide some protection from alcoholic acute pancreatitis.

Pancreatitis and impaired autophagy

Autophagy is an important cellular process by which unneeded or damaged cellular components or organelles are sequestered in autophagic vacuoles and targeted to the lysosomes for degradation. Impairment of this process has been implicated in the pathogenesis of a number of diseases including pancreatitis[52-56]. Ethanol can affect autophagy in a number of organs, including the pancreas[53,57,58].

One histological characteristic of pancreatitis is the accumulation of large vacuoles within acinar cells[59]. It has been demonstrated in preclinical animal models of pancreatitis, as well as in tissue from human beings, that these vacuoles are autophagic vacuoles[55,56]. These vacuoles possess markers of both autophagosomes and lysosomes, and contain undegraded or partially degraded cellular material[56]. The finding that these vacuoles contain undegraded or partially degraded cellular material indicates that the degradation of the material in the autolysosomes, a late event in the autophagic process, is impaired during pancreatitis[56]. Thus, it appears that the inability to complete the autophagic process is responsible for the accumulation of the vacuoles characteristic of pancreatitis.

Cathepsin L and cathepsin B are two important lysosomal hydrolases. Cathepsin L degrades trypsinogen and trypsin, whereas cathepsin B cleaves trypsinogen forming active trypsin. Thus, the activity

of these two enzymes has a pivotal role in trypsin activity. Inappropriate trypsin activation is thought to be an early event in the initiation of pancreatitis. How trypsin is inappropriately activated in acinar cells is poorly understood. It has been proposed that cathepsin B is mis-sorted to the zymogen granules, where it cleaves trypsinogen, forming trypsin. How trypsinogen and cathepsin B come in contact has always been a mystery. Recent studies indicate that impairment in the completion of the autophagic process has a role in the co-localization of these two enzymes[56].

Pancreatitis leads to increased levels of cathepsin L and cathepsin B in the zymogen granule fraction. In alcoholic pancreatitis, as well as other forms of acute pancreatitis, the processing and activation of these two enzymes is impaired[56,60]. Importantly, the impairment in cathepsin L activity is more severe than the impairment in cathepsin B activity, especially in the zymogen granules[56]. Additionally, analysis of the autophagosome/autolysosome fraction revealed the presence of zymogen granules. Thus, in these zymogen granule-containing autophagosomes/autolysosomes trypsinogen and cathepsin B come in contact[56]. The imbalance between cathepsin B and cathepsin L activity in these vacuoles would favor the activation of trypsin, and the initiation of pancreatitis. Therefore, lysosomal dysfunction may not only contribute to the accumulation of vacuoles, but may also have an important role in the inappropriate intracellular activation of trypsin and the initiation of pancreatitis.

Ethanol impairs other aspects of autophagy. It has been demonstrated that lysosomal-associated membrane protein-2 (Lamp-2), a lysosomal membrane protein required for the fusion of autophagosomes with lysosomes, is depleted in the pancreata of rats suffering from alcoholic pancreatitis[53,61]. Importantly, analysis of pancreata from human beings revealed that in patients suffering from chronic alcoholic pancreatitis Lamp-2 expression is also decreased[53]. These results indicate that ethanol consumption can inhibit the expression of lysosomal proteins, particularly Lamp-2. Decreased expression of Lamp-2 impairs the fusion of autophagosomes with lysosomes, and autophagic flux. This impairment may be another contributing factor to alcoholic pancreatitis in human beings.

INVOLVEMENT OF PANCREATIC STELLATE CELLS IN ALCOHOLIC PANCREATITIS

The pancreas, like the liver, contains a population of periacinar vitamin A storing cells known as stellate cells[62,63]. These cells, like their hepatic counterparts, synthesize extracellular matrix proteins, as well as matrix metalloproteinases (enzymes that degrade extracellular matrix proteins) and in the healthy organ, function to maintain the architecture of the organ by regulating the deposition and degradation of extracellular matrix components[64]. In response to injury, pancreatic stellate cells transform into highly proliferative myofibroblast-like cells. These "activated" pancreatic stellate cells synthesize large amounts of extracellular matrix proteins, the accumulation of which results in fibrosis. Thus, pancreatic stellate cells are intimately involved in the regulation of both physiologic, as well as pathologic aspects of the pancreas[64,65].

Both rat and human pancreatic stellate cells express ADH[66,67]. Furthermore, ADH activity is up regulated in pancreatic stellate cells of individuals suffering from chronic pancreatitis and pancreatic cancer[67].

The fact that pancreatic stellate cells express ADH indicates that these cells can produce acetaldehyde when exposed to ethanol. Pancreatic stellate cells are activated when exposed to physiologic concentrations of either ethanol or acetaldehyde[66,68]. Ethanol and acetaldehyde not only activate pancreatic stellate cells, but also induce secretion of type-1 collagen and matrix metalloproteinases[66,68,69]. Thus, expression of ADH by pancreatic stellate cells may have an important role in the activation of these cells and development of alcoholic pancreatitis

Treatment of pancreatic stellate cells with ethanol or acetaldehyde also induces the synthesis of cytokines and growth factors involved in their activation[68,70]. These findings have lead to the suggestion that these cytokines and growth factors act on pancreatic stellate cells in an autocrine manner, thereby perpetuating their activation[16]. This autocrine loop may help to explain both the apparent inability of the pancreas to fully recover from injury in the continued presence of ethanol, and the extremely common association between alcohol abuse and chronic pancreatitis[3,14].

Although it is well established that pancreatic stellate cells are primarily responsible for the deposition and degradation of components of the extracellular matrix, acinar cells can also contribute to the deposition of extracellular matrix components. Using isolated rat acinar cells Lugea *et al*[71] demonstrated that treatment with FAEEs increase the levels of extracellular matrix proteins by inhibiting the acinar cell activity of plasmin and urokinase-type plasminogen activator, proteins that are involved in the degradation of the extracellular matrix components[71]. Thus, it is apparent that ethanol acts by a number of mechanisms to alter the extracellular environment of pancreatic cells.

EFFECTS OF ETHANOL ON PANCREATIC DUCTAL CELLS

In is now becoming evident that ductal cells are also affected by ethanol and thus may have a role in the development of alcoholic pancreatitis. Maléth *et al*[72] demonstrated that ethanol and FA inhibit the expression, localization, and activity of the cystic fibrosis transmembrane conductance regulator (CFTR) on pancreatic ductal epithelial cells[72]. This defect was

attributed to accelerated CFTR turnover and aberrant CFTR biosynthetic processing. Additionally, they demonstrated that high concentrations of ethanol inhibited the secretion of bicarbonate (HCO_3^-). Furthermore, they demonstrated that these dysfunctions were associated with a sustained increase in the levels of intracellular calcium as well as depletion of ATP. The authors suggest that methods to increase the levels and function of the CFTR may be an affective strategy to treat alcoholic pancreatitis[72].

EFFECTS OF ETHANOL ON PANCREATIC REPAIR

Repair of the exocrine pancreas requires the dedifferentiation of mature acinar cells and their redifferentiation back to a differentiated phenotype[73]. Thus, it appears that acinar cells can act as facultative progenitor cells. We have shown that chronic administration of ethanol delays the structural and functional regeneration of the pancreas in mice[1]. This delayed regeneration is associated with the decreased expression of a number of important pancreatic developmental factors including PDX-1 and Ptf-1α, as well as activation of the notch signaling pathway, a developmental pathway, which is required for pancreatic regeneration[3,4]. Ethanol-mediated alterations in the expression of these important developmental factors affect the dedifferentiation/redifferentiation of acinar cells and therefore, the repair of the damaged organ. These findings support the suggestion that repair of the damaged pancreas is never fully completed in the continued presence of ethanol[74]. Thus, ethanol consumption inhibits the repair mechanisms of the pancreas. These findings may help explain the extremely strong association between alcohol abuse and chronic pancreatitis.

ROLE OF THE INFLAMMATORY RESPONSE IN ALCOHOLIC PANCREATITIS

Inflammation mediated by cytokines, chemokines, and adhesion molecules is involved in the initiation and progression of pancreatitis[75-77]. In fact, the NF-κB-dependent inflammatory response is responsible for up to half of the pancreatic tissue damage that is associated with acute pancreatitis, as well as the potentially fatal severe systemic inflammatory response[11].

NF-κB and AP-1, important transcriptional activators involved in the inflammatory response have been shown to have prominent roles in pancreatitis[78,79]. Interestingly, it appears that oxidative and nonoxidative metabolites of ethanol have different effects on the expression of these two regulators of the inflammatory response.

Treatment of isolated acini with ethanol or acetaldehyde decreases the activity of these two factors, whereas; treatment with FAEEs increases their activity[78]. The activity of NF-κB is also reduced in the pancreata of animals chronically fed ethanol[17]. This finding led the authors to hypothesize that *in vivo* attenuation of NF-κB by ethanol reflects a mechanism to protect the pancreas from ethanol-induced damage[17]. Interestingly, administration of CCK, at concentrations that do not normally cause pancreatitis results in pancreatic damage in animals chronically fed ethanol. This damage is associated with increased NF-κB activity, as well as increases in the mRNA levels of a number of proinflammatory cytokines, including: Tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), macrophage inflammatory protein-1 (MIP-1), and monocyte chemotactic protein-1[17]. These findings demonstrate that although attenuation of NF-κB activity by ethanol may reflect a protective adaptation, ethanol also sensitizes the pancreas to damage that is at least partially mediated by the inflammatory response.

Ethanol abuse is one of the primary risk factors associated with chronic pancreatitis. Because of this, the inflammatory response in chronic alcoholic pancreatitis has also been investigated[80]. Using rats pair-fed the Lieber-Decarli diet, Deng *et al*[80] demonstrated that chronic ethanol administration reduced the number of resident mononuclear cells in the pancreas. They, like others, suggested that this reduction likely reflects a general immunologic suppression in the pancreas of animals chronically fed ethanol[17,78]. Furthermore, the authors suggested this suppression might explain why animals chronically provided ethanol do not develop chronic pancreatitis in the absence of acute pancreatic damage[80].

Similar to previous findings, induction of acute pancreatitis enhanced the inflammatory response in these animals[80]. Repeated episodes of caerulein-induced acute pancreatitis increased the expression of both pro-inflammatory cytokines such as TNF-α, MIP-1α, and RANTES, as well as anti-inflammatory cytokines such as tissue growth factor-β, and IL-10. The increases in cytokine expression were only detected in ethanol-fed rats in which repeated episodes of acute pancreatitis were induced. Additionally, increased activation of pancreatic stellate cells and fibrosis were observed in the animals. These findings led the authors to suggest that ethanol not only sensitizes the pancreas to acute pancreatitis, but also facilitates the progression of acute to chronic pancreatitis following repeated episodes of acute pancreatic injury[80].

CONCLUSION

Unfortunately, there is currently no pharmacotherapy to attenuate the severity of acute pancreatitis in general and alcoholic acute pancreatitis in particular. Despite the fact that ethanol is commonly associated with pancreatitis, it is apparent that ethanol itself is unable to cause pancreatitis and that an additional trigger is required for the initiation of alcoholic acute pancreatitis.

Although the actual triggers that initiate alcoholic acute pancreatitis may differ, the inappropriate activation of trypsin and other pancreatic enzymes, as well as the activation of the inflammatory response are key events in its development and progression.

A tremendous amount of work has revealed a number of mechanisms by which ethanol and both its oxidative and nonoxidative metabolites damage pancreatic cells. Greater understanding of the mechanisms by which ethanol alter the normal physiology of pancreatic cells has provided some promising therapeutic targets.

Among the potential targets are the transcriptional activators NF-κB and AP-1. Numerous studies have demonstrated the importance of these factors in the activation of the inflammatory response and alcoholic acute pancreatitis. Thus, attenuating or regulating the activation of these factors should decrease the severity of acute pancreatitis.

Ethanol and its metabolites cause sustained elevation of intracellular calcium, which results in a number of dysfunctions in acinar cells. Modulation of intracellular calcium attenuates many of these dysfunctions. Thus, regulation or modulation of intracellular calcium levels is an attractive strategy for the treatment of acute pancreatitis.

The nonoxidative metabolites of ethanol metabolism FAEEs have been shown to cause a number of pathologic changes in pancreatic cells. It has been demonstrated that inhibiting FAEE synthase attenuates experimental acute pancreatitis. This is also an extremely attractive area to pursue. Regulating or reestablishing the normal NAD^+/NADH ratio in acinar cells may also attenuate the severity of alcoholic acute pancreatitis. Lastly, numerous studies have demonstrated that the pancreas responds to ethanol by compensatory mechanisms such as the unfolded protein response and suppression of the inflammatory response. Thus, manipulation of these compensatory mechanisms may be a fruitful strategy for treatment of this disease.

It is clear that ethanol sensitizes the pancreas to injury. By understanding the mechanisms by which ethanol alter the normal physiology of the pancreas, we have uncovered potential targets for therapeutic intervention. Further experimental work and clinical studies are required to determine the utility of these targets in treating alcoholic pancreatitis.

REFERENCES

1 **Clemens DL**, Jerrells TR. Ethanol consumption potentiates viral pancreatitis and may inhibit pancreas regeneration: preliminary findings. *Alcohol* 2004; **33**: 183-189 [PMID: 15596086 DOI: 10.1016/j.alcohol.2004.07.001]

2 **Desai BM**, Oliver-Krasinski J, De Leon DD, Farzad C, Hong N, Leach SD, Stoffers DA. Preexisting pancreatic acinar cells contribute to acinar cell, but not islet beta cell, regeneration. *J Clin Invest* 2007; **117**: 971-977 [PMID: 17404620 DOI: 10.1172/JCI29988]

3 **Schneider KJ**, Scheer M, Suhr M, Clemens DL. Ethanol administration impairs pancreatic repair after injury. *Pancreas* 2012; **41**: 1272-1279 [PMID: 22617711 DOI: 10.1097/MPA.0b013e31824bde37]

4 **Siveke JT**, Lubeseder-Martellato C, Lee M, Mazur PK, Nakhai H, Radtke F, Schmid RM. Notch signaling is required for exocrine regeneration after acute pancreatitis. *Gastroenterology* 2008; **134**: 544-555 [PMID: 18242220 DOI: 10.1053/j.gastro.2007.11.003]

5 **Strobel O**, Dor Y, Alsina J, Stirman A, Lauwers G, Trainor A, Castillo CF, Warshaw AL, Thayer SP. In vivo lineage tracing defines the role of acinar-to-ductal transdifferentiation in inflammatory ductal metaplasia. *Gastroenterology* 2007; **133**: 1999-2009 [PMID: 18054571 DOI: 10.1053/j.gastro.2007.09.009]

6 **Peery AF**, Dellon ES, Lund J, Crockett SD, McGowan CE, Bulsiewicz WJ, Gangarosa LM, Thiny MT, Stizenberg K, Morgan DR, Ringel Y, Kim HP, Dibonaventura MD, Carroll CF, Allen JK, Cook SF, Sandler RS, Kappelman MD, Shaheen NJ. Burden of gastrointestinal disease in the United States: 2012 update. *Gastroenterology* 2012; **143**: 1179-1187.e1-3 [PMID: 22885331 DOI: 10.1053/j.gastro.2012.08.00]

7 **Whitcomb DC**. Clinical practice. Acute pancreatitis. *N Engl J Med* 2006; **354**: 2142-2150 [PMID: 16707751 DOI: 10.1056/NEJMcp054958]

8 **Pandol SJ**, Saluja AK, Imrie CW, Banks PA. Acute pancreatitis: bench to the bedside. *Gastroenterology* 2007; **132**: 1127-1151 [PMID: 17383433 DOI: 10.1053/j.gastro.2007.01.055]

9 **Wang GJ**, Gao CF, Wei D, Wang C, Ding SQ. Acute pancreatitis: etiology and common pathogenesis. *World J Gastroenterol* 2009; **15**: 1427-1430 [PMID: 19322914]

10 **Chiari H**. Uberdie Selbstverdauung des Menschlichen Pankreas. *Z Heilk* 1896; **(17)**: 69-96

11 **Dawra R**, Sah RP, Dudeja V, Rishi L, Talukdar R, Garg P, Saluja AK. Intra-acinar trypsinogen activation mediates early stages of pancreatic injury but not inflammation in mice with acute pancreatitis. *Gastroenterology* 2011; **141**: 2210-2217.e2 [PMID: 21875495 DOI: 10.1053/j.gastro.2011.08.033]

12 **Ammann RW**. The natural history of alcoholic chronic pancreatitis. *Intern Med* 2001; **40**: 368-375 [PMID: 11393404]

13 **Friederich N**. Disease of the Pancreas. Cycloaedia of the Practice of Medicine. Vol 18. New York: William Wood, 1878: 254-312

14 **Ammann RW**, Heitz PU, Klöppel G. Course of alcoholic chronic pancreatitis: a prospective clinicomorphological long-term study. *Gastroenterology* 1996; **111**: 224-231 [PMID: 8698203]

15 **Krejs GJ**. Pancreatic cancer: epidemiology and risk factors. *Dig Dis* 2010; **28**: 355-358 [PMID: 20814212 DOI: 10.1159/000319414]

16 **Apte MV**, Pirola RC, Wilson JS. Mechanisms of alcoholic pancreatitis. *J Gastroenterol Hepatol* 2010; **25**: 1816-1826 [PMID: 21091991]

17 **Pandol SJ**, Periskic S, Gukovsky I, Zaninovic V, Jung Y, Zong Y, Solomon TE, Gukovskaya AS, Tsukamoto H. Ethanol diet increases the sensitivity of rats to pancreatitis induced by cholecystokinin octapeptide. *Gastroenterology* 1999; **117**: 706-716 [PMID: 10464148]

18 **Yadav D**, Papachristou GI, Whitcomb DC. Alcohol-associated pancreatitis. *Gastroenterol Clin North Am* 2007; **36**: 219-238, vii [PMID: 17533076 DOI: 10.1016/j.gtc.2007.03.005]

19 **Sata N**, Koizumi M, Nagai H. Alcoholic pancreatopathy: a proposed new diagnostic category representing the preclinical stage of alcoholic pancreatic injury. *J Gastroenterol* 2007; **42** Suppl 17: 131-134 [PMID: 17238042 DOI: 10.1007/s00535-006-1936-5]

20 **Lankisch PG**, Breuer N, Bruns A, Weber-Dany B, Lowenfels AB, Maisonneuve P. Natural history of acute pancreatitis: a long-term population-based study. *Am J Gastroenterol* 2009; **104**: 2797-2805; quiz 2806 [PMID: 19603011 DOI: 10.1038/ajg.2009.405]

21 **Slack JM**. Developmental biology of the pancreas. *Development* 1995; **121**: 1569-1580 [PMID: 7600975]

22 **Haber PS**, Apte MV, Applegate TL, Norton ID, Korsten MA, Pirola RC, Wilson JS. Metabolism of ethanol by rat pancreatic acinar cells. *J Lab Clin Med* 1998; **132**: 294-302 [PMID: 9794700]

23 **Norton ID**, Apte MV, Haber PS, McCaughan GW, Pirola RC, Wilson JS. Cytochrome P4502E1 is present in rat pancreas and is

induced by chronic ethanol administration. *Gut* 1998; **42**: 426-430 [PMID: 9577353]
24 **Shalbueva N**, Mareninova OA, Gerloff A, Yuan J, Waldron RT, Pandol SJ, Gukovskaya AS. Effects of oxidative alcohol metabolism on the mitochondrial permeability transition pore and necrosis in a mouse model of alcoholic pancreatitis. *Gastroenterology* 2013; **144**: 437-446.e6 [PMID: 23103769 DOI: 10.1053/j.gastro.2012.10.037]
25 **Laposata M**. Fatty acid ethyl esters: ethanol metabolites which mediate ethanol-induced organ damage and serve as markers of ethanol intake. *Prog Lipid Res* 1998; **37**: 307-316 [PMID: 10209651]
26 **Laposata EA**, Lange LG. Presence of nonoxidative ethanol metabolism in human organs commonly damaged by ethanol abuse. *Science* 1986; **231**: 497-499 [PMID: 3941913]
27 **Krüger B**, Albrecht E, Lerch MM. The role of intracellular calcium signaling in premature protease activation and the onset of pancreatitis. *Am J Pathol* 2000; **157**: 43-50 [PMID: 10880374 DOI: 10.1016/S0002-9440(10)64515-4]
28 **Raraty M**, Ward J, Erdemli G, Vaillant C, Neoptolemos JP, Sutton R, Petersen OH. Calcium-dependent enzyme activation and vacuole formation in the apical granular region of pancreatic acinar cells. *Proc Natl Acad Sci USA* 2000; **97**: 13126-13131 [PMID: 11087863 DOI: 10.1073/pnas.97.24.13126]
29 **Gerasimenko JV**, Lur G, Sherwood MW, Ebisui E, Tepikin AV, Mikoshiba K, Gerasimenko OV, Petersen OH. Pancreatic protease activation by alcohol metabolite depends on Ca2+ release via acid store IP3 receptors. *Proc Natl Acad Sci USA* 2009; **106**: 10758-10763 [PMID: 19528657 DOI: 10.1073/pnas.0904818106]
30 **Gerasimenko JV**, Gerasimenko OV, Petersen OH. The role of Ca2+ in the pathophysiology of pancreatitis. *J Physiol* 2014; **592**: 269-280 [PMID: 23897234 DOI: 10.1113/jphysiol.2013.261784]
31 **Criddle DN**, Murphy J, Fistetto G, Barrow S, Tepikin AV, Neoptolemos JP, Sutton R, Petersen OH. Fatty acid ethyl esters cause pancreatic calcium toxicity via inositol trisphosphate receptors and loss of ATP synthesis. *Gastroenterology* 2006; **130**: 781-793 [PMID: 16530519 DOI: 10.1053/j.gastro.2005.12.031]
32 **Gerasimenko JV**, Gryshchenko O, Ferdek PE, Stapleton E, Hébert TO, Bychkova S, Peng S, Begg M, Gerasimenko OV, Petersen OH. Ca2+ release-activated Ca2+ channel blockade as a potential tool in antipancreatitis therapy. *Proc Natl Acad Sci USA* 2013; **110**: 13186-13191 [PMID: 23878235 DOI: 10.1073/pnas.1300910110]
33 **Gerasimenko JV**, Lur G, Ferdek P, Sherwood MW, Ebisui E, Tepikin AV, Mikoshiba K, Petersen OH, Gerasimenko OV. Calmodulin protects against alcohol-induced pancreatic trypsinogen activation elicited via Ca2+ release through IP3 receptors. *Proc Natl Acad Sci USA* 2011; **108**: 5873-5878 [PMID: 21436055 DOI: 10.1073/pnas.1016534108]
34 **Lur G**, Haynes LP, Prior IA, Gerasimenko OV, Feske S, Petersen OH, Burgoyne RD, Tepikin AV. Ribosome-free terminals of rough ER allow formation of STIM1 puncta and segregation of STIM1 from IP(3) receptors. *Curr Biol* 2009; **19**: 1648-1653 [PMID: 19765991 DOI: 10.1016/j.cub.2009.07.072]
35 **Kaiser AM**, Saluja AK, Sengupta A, Saluja M, Steer ML. Relationship between severity, necrosis, and apoptosis in five models of experimental acute pancreatitis. *Am J Physiol* 1995; **269**: C1295-C1304 [PMID: 7491921]
36 **Mareninova OA**, Sung KF, Hong P, Lugea A, Pandol SJ, Gukovsky I, Gukovskaya AS. Cell death in pancreatitis: caspases protect from necrotizing pancreatitis. *J Biol Chem* 2006; **281**: 3370-3381 [PMID: 16339139]
37 **Burdakov D**, Petersen OH, Verkhratsky A. Intraluminal calcium as a primary regulator of endoplasmic reticulum function. *Cell Calcium* 2005; **38**: 303-310 [PMID: 16076486 DOI: 10.1016/j.ceca.2005.06.010]
38 **Mukherjee R**, Criddle DN, Gukovskaya A, Pandol S, Petersen OH, Sutton R. Mitochondrial injury in pancreatitis. *Cell Calcium* 2008; **44**: 14-23 [PMID: 18207570 DOI: 10.1016/j.ceca.2007.11.013]
39 **Gukovskaya AS**, Gukovsky I. Which way to die: the regulation of acinar cell death in pancreatitis by mitochondria, calcium, and reactive oxygen species. *Gastroenterology* 2011; **140**: 1876-1880 [PMID: 21524653 DOI: 10.1053/j.gastro.2011.04.025]
40 **Lange LG**, Sobel BE. Mitochondrial dysfunction induced by fatty acid ethyl esters, myocardial metabolites of ethanol. *J Clin Invest* 1983; **72**: 724-731 [PMID: 6308061 DOI: 10.1172/JCI111022]
41 **Werner J**, Saghir M, Fernandez-del Castillo C, Warshaw AL, Laposata M. Linkage of oxidative and nonoxidative ethanol metabolism in the pancreas and toxicity of nonoxidative ethanol metabolites for pancreatic acinar cells. *Surgery* 2001; **129**: 736-744 [PMID: 11391373 DOI: 10.1067/msy.2001.113891]
42 **Wu H**, Cai P, Clemens DL, Jerrells TR, Ansari GA, Kaphalia BS. Metabolic basis of ethanol-induced cytotoxicity in recombinant HepG2 cells: role of nonoxidative metabolism. *Toxicol Appl Pharmacol* 2006; **216**: 238-247 [PMID: 16806343 DOI: 10.1016/j.taap.2006.05.003]
43 **Huang W**, Booth DM, Cane MC, Chvanov M, Javed MA, Elliott VL, Armstrong JA, Dingsdale H, Cash N, Li Y, Greenhalf W, Mukherjee R, Kaphalia BS, Jaffar M, Petersen OH, Tepikin AV, Sutton R, Criddle DN. Fatty acid ethyl ester synthase inhibition ameliorates ethanol-induced Ca2+-dependent mitochondrial dysfunction and acute pancreatitis. *Gut* 2014; **63**: 1313-1324 [PMID: 24162590 DOI: 10.1136/gutjnl-2012-304058]
44 **Shulga N**, Pastorino JG. Ethanol sensitizes mitochondria to the permeability transition by inhibiting deacetylation of cyclophilin-D mediated by sirtuin-3. *J Cell Sci* 2010; **123**: 4117-4127 [PMID: 21062897]
45 **Shulga N**, Wilson-Smith R, Pastorino JG. Sirtuin-3 deacetylation of cyclophilin D induces dissociation of hexokinase II from the mitochondria. *J Cell Sci* 2010; **123**: 894-902 [PMID: 20159966 DOI: 10.1242/jcs.061846]
46 **Williams JA**. Receptor-mediated signal transduction pathways and the regulation of pancreatic acinar cell function. *Curr Opin Gastroenterol* 2008; **24**: 573-579 [PMID: 19122497 DOI: 10.1097/MOG.0b013e32830b110c]
47 **Lugea A**, Waldron RT, Pandol SJ. Pancreatic adaptive responses in alcohol abuse: Role of the unfolded protein response. *Pancreatology* 2015; **15**: S1-S5 [PMID: 25736240 DOI: 10.1016/j.pan.2015.01.011]
48 **Pandol SJ**, Gorelick FS, Gerloff A, Lugea A. Alcohol abuse, endoplasmic reticulum stress and pancreatitis. *Dig Dis* 2010; **28**: 776-782 [PMID: 21525762 DOI: 10.1159/000327212]
49 **Lugea A**, Tischler D, Nguyen J, Gong J, Gukovsky I, French SW, Gorelick FS, Pandol SJ. Adaptive unfolded protein response attenuates alcohol-induced pancreatic damage. *Gastroenterology* 2011; **140**: 987-997 [PMID: 21111739 DOI: 10.1053/j.gastro.2010.11.038]
50 **Harding HP**, Zhang Y, Zeng H, Novoa I, Lu PD, Calfon M, Sadri N, Yun C, Popko B, Paules R, Stojdl DF, Bell JC, Hettmann T, Leiden JM, Ron D. An integrated stress response regulates amino acid metabolism and resistance to oxidative stress. *Mol Cell* 2003; **11**: 619-633 [PMID: 12667446]
51 **Lugea A**, Waldron RT, French SW, Pandol SJ. Drinking and driving pancreatitis: links between endoplasmic reticulum stress and autophagy. *Autophagy* 2011; **7**: 783-785 [PMID: 21460613]
52 **Levine B**, Kroemer G. Autophagy in the pathogenesis of disease. *Cell* 2008; **132**: 27-42 [PMID: 18191218 DOI: 10.1016/j.cell.2007.12.018]
53 **Fortunato F**, Bürgers H, Bergmann F, Rieger P, Büchler MW, Kroemer G, Werner J. Impaired autolysosome formation correlates with Lamp-2 depletion: role of apoptosis, autophagy, and necrosis in pancreatitis. *Gastroenterology* 2009; **137**: 350-360, 360.e1-5 [PMID: 19362087 DOI: 10.1053/j.gastro.2009.04.003]
54 **Gukovsky I**, Li N, Todoric J, Gukovskaya A, Karin M. Inflammation, autophagy, and obesity: common features in the pathogenesis of pancreatitis and pancreatic cancer. *Gastroenterology* 2013; **144**: 1199-1209.e4 [PMID: 23622129 DOI: 10.1053/j.gastro.20 13.02.007]
55 **Hashimoto D**, Ohmuraya M, Hirota M, Yamamoto A, Suyama K, Ida S, Okumura Y, Takahashi E, Kido H, Araki K, Baba H, Mizushima N, Yamamura K. Involvement of autophagy in trypsinogen activation within the pancreatic acinar cells. *J Cell Biol* 2008; **181**: 1065-1072 [PMID: 18591426 DOI: 10.1083/jcb.200712156]

56 **Mareninova OA**, Hermann K, French SW, O'Konski MS, Pandol SJ, Webster P, Erickson AH, Katunuma N, Gorelick FS, Gukovsky I, Gukovskaya AS. Impaired autophagic flux mediates acinar cell vacuole formation and trypsinogen activation in rodent models of acute pancreatitis. *J Clin Invest* 2009; **119**: 3340-3355 [PMID: 19805911 DOI: 10.1172/JCI38674]

57 **Ding WX**, Li M, Chen X, Ni HM, Lin CW, Gao W, Lu B, Stolz DB, Clemens DL, Yin XM. Autophagy reduces acute ethanol-induced hepatotoxicity and steatosis in mice. *Gastroenterology* 2010; **139**: 1740-1752 [PMID: 20659474]

58 **Thomes PG**, Ehlers RA, Trambly CS, Clemens DL, Fox HS, Tuma DJ, Donohue TM. Multilevel regulation of autophagosome content by ethanol oxidation in HepG2 cells. *Autophagy* 2013; **9**: 63-73 [PMID: 23090141 DOI: 10.4161/auto.22490]

59 **Helin H**, Mero M, Markkula H, Helin M. Pancreatic acinar ultrastructure in human acute pancreatitis. *Virchows Arch A Pathol Anat Histol* 1980; **387**: 259-270 [PMID: 7456314]

60 **Mareninova OA**, Yakubov I, French SW, Jia W, Lee MA, Pandol SJ, Gukovskaya AS, Gukovsky I. Ethanol feeding causes lysosomal dysfunction in exocrine pancreas similar to pancreatitis; but in contrast to pancreatitis, ethanol down-regulates autophagy. *Gastroenterol* 2010; **138**: S-148 [DOI: 10.1016/S0016-5085(10)60681-6]

61 **Huynh KK**, Eskelinen EL, Scott CC, Malevanets A, Saftig P, Grinstein S. LAMP proteins are required for fusion of lysosomes with phagosomes. *EMBO J* 2007; **26**: 313-324 [PMID: 17245426 DOI: 10.1038/sj.emboj.7601511]

62 **Apte MV**, Haber PS, Applegate TL, Norton ID, McCaughan GW, Korsten MA, Pirola RC, Wilson JS. Periacinar stellate shaped cells in rat pancreas: identification, isolation, and culture. *Gut* 1998; **43**: 128-133 [PMID: 9771417]

63 **Bachem MG**, Schneider E, Gross H, Weidenbach H, Schmid RM, Menke A, Siech M, Beger H, Grünert A, Adler G. Identification, culture, and characterization of pancreatic stellate cells in rats and humans. *Gastroenterology* 1998; **115**: 421-432 [PMID: 9679048 DOI: 10.1016/S0016-5085(98)70209-4]

64 **Apte M**, Pirola R, Wilson J. The fibrosis of chronic pancreatitis: new insights into the role of pancreatic stellate cells. *Antioxid Redox Signal* 2011; **15**: 2711-2722 [PMID: 21728885 DOI: 10.1089/ars.2011.4079]

65 **Apte MV**, Pirola RC, Wilson JS. Pancreatic stellate cells: a starring role in normal and diseased pancreas. *Front Physiol* 2012; **3**: 344 [PMID: 22973234 DOI: 10.3389/fphys.2012.00344]

66 **Apte MV**, Phillips PA, Fahmy RG, Darby SJ, Rodgers SC, McCaughan GW, Korsten MA, Pirola RC, Naidoo D, Wilson JS. Does alcohol directly stimulate pancreatic fibrogenesis? Studies with rat pancreatic stellate cells. *Gastroenterology* 2000; **118**: 780-794 [PMID: 10734030]

67 **Chiang CP**, Wu CW, Lee SP, Chung CC, Wang CW, Lee SL, Nieh S, Yin SJ. Expression pattern, ethanol-metabolizing activities, and cellular localization of alcohol and aldehyde dehydrogenases in human pancreas: implications for pathogenesis of alcohol-induced pancreatic injury. *Alcohol Clin Exp Res* 2009; **33**: 1059-1068 [PMID: 19382905 DOI: 10.1111/j.1530-0277.2009.00927.x]

68 **Masamune A**, Satoh A, Watanabe T, Kikuta K, Satoh M, Suzuki N, Satoh K, Shimosegawa T. Effects of ethanol and its metabolites on human pancreatic stellate cells. *Dig Dis Sci* 2010; **55**: 204-211 [PMID: 19165599 DOI: 10.1007/s10620-008-0695-y]

69 **Phillips PA**, McCarroll JA, Park S, Wu MJ, Pirola R, Korsten M, Wilson JS, Apte MV. Rat pancreatic stellate cells secrete matrix metalloproteinases: implications for extracellular matrix turnover. *Gut* 2003; **52**: 275-282 [PMID: 12524413 DOI: 10.1136/gut.52.2.275]

70 **Lawrencia C**, Charrier A, Huang G, Brigstock DR. Ethanol-mediated expression of connective tissue growth factor (CCN2) in mouse pancreatic stellate cells. *Growth Factors* 2009; **27**: 91-99 [PMID: 19280452 DOI: 10.1080/08977190902786319]

71 **Lugea A**, Gukovsky I, Gukovskaya AS, Pandol SJ. Nonoxidative ethanol metabolites alter extracellular matrix protein content in rat pancreas. *Gastroenterology* 2003; **125**: 1845-1859 [PMID: 14724836]

72 **Maléth J**, Balázs A, Pallagi P, Balla Z, Kui B, Katona M, Judák L, Németh I, Kemény LV, Rakonczay Z, Venglovecz V, Földesi I, Pető Z, Somorácz Á, Borka K, Perdomo D, Lukacs GL, Gray MA, Monterisi S, Zaccolo M, Sendler M, Mayerle J, Kühn JP, Lerch MM, Sahin-Tóth M, Hegyi P. Alcohol disrupts levels and function of the cystic fibrosis transmembrane conductance regulator to promote development of pancreatitis. *Gastroenterology* 2015; **148**: 427-439.e16 [PMID: 25447846 DOI: 10.1053/j.gastro.2014.11.002]

73 **Jensen JN**, Cameron E, Garay MV, Starkey TW, Gianani R, Jensen J. Recapitulation of elements of embryonic development in adult mouse pancreatic regeneration. *Gastroenterology* 2005; **128**: 728-741 [PMID: 15765408 DOI: 10.1053/j.gastro.2004.12.008]

74 **Perides G**, Tao X, West N, Sharma A, Steer ML. A mouse model of ethanol dependent pancreatic fibrosis. *Gut* 2005; **54**: 1461-1467 [PMID: 15870229 DOI: 10.1136/gut.2004.062919]

75 **Bhatia M**, Brady M, Shokuhi S, Christmas S, Neoptolemos JP, Slavin J. Inflammatory mediators in acute pancreatitis. *J Pathol* 2000; **190**: 117-125 [PMID: 10657008 DOI: 10.1002/(SICI)1096-9896(200002)190: 2<117: : AID-PATH494>3.0.CO; 2-K]

76 **Norman J**. The role of cytokines in the pathogenesis of acute pancreatitis. *Am J Surg* 1998; **175**: 76-83 [PMID: 9445247 DOI: 10.1016/S0002-9610(97)00240-7]

77 **Vonlaufen A**, Apte MV, Imhof BA, Frossard JL. The role of inflammatory and parenchymal cells in acute pancreatitis. *J Pathol* 2007; **213**: 239-248 [PMID: 17893879 DOI: 10.1002/path.2231]

78 **Gukovskaya AS**, Mouria M, Gukovsky I, Reyes CN, Kasho VN, Faller LD, Pandol SJ. Ethanol metabolism and transcription factor activation in pancreatic acinar cells in rats. *Gastroenterology* 2002; **122**: 106-118 [PMID: 11781286 DOI: 10.1053/gast.2002.30302]

79 **Gukovsky I**, Gukovskaya AS, Blinman TA, Zaninovic V, Pandol SJ. Early NF-kappaB activation is associated with hormone-induced pancreatitis. *Am J Physiol* 1998; **275**: G1402-G1414 [PMID: 9843778]

80 **Deng X**, Wang L, Elm MS, Gabazadeh D, Diorio GJ, Eagon PK, Whitcomb DC. Chronic alcohol consumption accelerates fibrosis in response to cerulein-induced pancreatitis in rats. *Am J Pathol* 2005; **166**: 93-106 [PMID: 15632003]

P- Reviewer: Bramhall S, Carrasco C, Demetter P, Phillip V, Rakonczay Z, Zhao JB
S- Editor: Ji FF **L- Editor**: A **E- Editor**: Jiao XK

World Journal of Gastrointestinal Pathophysiology

Submit a Manuscript: http://www.wjgnet.com/esps/
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.4291/wjgp.v7.i1.59

World J Gastrointest Pathophysiol 2016 February 15; 7(1): 59-71
ISSN 2150-5330 (online)

REVIEW

Faecal incontinence: Current knowledges and perspectives

Alban Benezech, Michel Bouvier, Véronique Vitton

Alban Benezech, Michel Bouvier, Véronique Vitton, Service de Gastroentérologie, Hôpital Nord, Assistance Publique-Hôpitaux de Marseille, 13915 Marseille, France

Michel Bouvier, Véronique Vitton, Plateforme d'interface clinique, CRN2M CNRS UMR 7286, Aix-Marseille Université, 13915 Marseille, France

Author contributions: All the authors equally contributed to the preparation of the manuscript.

Conflict-of-interest statement: The authors declare no conflicts of interest regarding this manuscript.

Correspondence to: Dr. Veronique Vitton, MD, PhD, Service de Gastroentérologie, Hôpital Nord, Assistance Publique-Hôpitaux de Marseille, Cedex 20, 13915 Marseille, France. vittonv@yahoo.com
Telephone: +33-04-91965598
Fax: +33-04-91965550

Received: June 16, 2015
Peer-review started: June 20, 2015
First decision: August 22, 2015
Revised: September 21, 2015
Accepted: December 9, 2015
Article in press: December 11, 2015
Published online: February 15, 2016

Abstract

Faecal incontinence (FI) is a disabling and frequent symptom since its prevalence can vary between 5% and 15% of the general population. It has a particular negative impact on quality of life. Many tools are currently available for the treatment of FI, from conservative measures to invasive surgical treatments. The conservative treatment may be dietetic measures, various pharmacological agents, anorectal rehabilitation, posterior tibial nerve stimulation, and transanal irrigation. If needed, patients may have miniinvasive approaches such as sacral nerve modulation or antegrade irrigation. In some cases, a surgical treatment is proposed, mainly external anal sphincter repair. Although these different therapeutic options are available, new techniques are arriving allowing new hopes for the patients. Moreover, most of them are non-invasive such as local application of an α1-adrenoceptor agonist, stem cell injections, rectal injection of botulinum toxin, acupuncture. New more invasive techniques with promising results are also coming such as anal magnetic sphincter and antropylorus transposition. This review reports the main current available treatments of FI and the developing therapeutics tools.

Key words: Faecal incontinence; Treatment

Core tip: Faecal incontinence (FI) is a disabling and frequent symptom. Many tools are available for its treatment from conservative measures to invasive surgical treatments. Although different therapeutic options are currently available, new techniques are arriving allowing new hopes for the patients. This review reports the main current available treatments of FI and the developing therapeutics tools.

Benezech A, Bouvier M, Vitton V. Faecal incontinence: Current knowledges and perspectives. *World J Gastrointest Pathophysiol* 2016; 7(1): 59-71 Available from: URL: http://www.wjgnet.com/2150-5330/full/v7/i1/59.htm DOI: http://dx.doi.org/10.4291/wjgp.v7.i1.59

INTRODUCTION

Faecal incontinence (FI) is defined as a complaint of involuntary loss of flatus and/or liquid or solid stool *via* the anus[1]. Its prevalence can vary between 5% and 15% of the general population, especially depending on the patient's age and gender[2]. Moreover, these rates are most probably underestimated since less than 25% of patients with FI report it to their physician[3]. As a debilitating condition it has considerable impact on patient quality of life (QOL), particularly from a sexual and social point of view[4].

Aetiologic factors for FI are mainly split between localized perineal pathologies and general pathologies (Table 1). Obstetric perineal lesions are the most frequent, including anal sphincter tears and stretch induced neuropathy[5]. Side effects of radiotherapy or chronic inflammatory bowel diseases can also lead to FI. General pathologies concerned include neurological diseases such as multiple sclerosis[6] or medullary lesions, metabolic disorders (diabetes)[7] and systemic diseases (systemic sclerosis). Aetiological diagnosis is essential for the management of FI. Indeed, any specific treatment available can be used to target the pathology, and thus improve FI.

Faecal continence relies on two systems: A resistive and a capacitive system. The rectum that is a reservoir for stool represents the capacitive system. The resistive system is made up of the anal sphincters and the pubococcygeus muscle that closes the anal canal and maintains optimal intra abdominal pressure. Continence is also tightly linked to a very elaborate sensory nervous system, capable of analysing the sense of urge as well as the exact contents of the rectum[8]. FI can result from the failure of one or more of these elements. Further useful examinations include anal endosonography to detect any damage to the anal sphincters, and anorectal manometry to measure compliance and rectal sensation as well as pelvic floor muscle strength. These examinations are sometimes completed with electromyography of the anal canal and measurement of pudendal nerve terminal motor latency to check them for damage. These examinations aim to identify defective mechanisms and set up appropriate healthcare.

Treatment has greatly progressed in recent years and the future holds interesting new therapies. This paper describes the current and future treatments for FI. The level of evidence of each current therapeutic modality, as summarized in Table 2, was given according to subdivisions of Level of Evidence as proposed by the Haute Autorité de Santé (French High Autority of Health) (Table 3).

CURRENT TREATMENTS

In order to offer targeted treatment, it is necessary to identify the pathophysiological mechanisms responsible for FI, but also the patient's expectations. The aim is to improve continence and to reduce the impact of FI on the patient's QOL. Currently, treatments revolve around three levels: Conservative treatment, minimally invasive treatment and surgical treatment. Figure 1 presents the current therapeutic strategy for the management of FI.

CONSERVATIVE TREATMENT

The first level in FI management may improve FI in over 60% of patients[9]. It is based on personal hygiene and diet control, the use of certain drugs, pelvic floor therapy, posterior tibial nerve stimulation (PTNS), transanal irrigation (TAI) and the use of anal plugs.

Hygiene and diet control

FI management is, above all, based on regularizing stool frequency and consistency. Use of a bowel diary combined with a food diary may help identify food that aggravates stool leakage, such as caffeine, fruit and vegetables, alcohol, or spicy food[10]. Cutting out these foods may alleviate FI for some patients even though definitive evidence is lacking[11]. A high fibre diet, or the consumption of mucilage can, in some cases, improves faecal continence by improving stool consistency, especially for patients with liquid stools or with associated constipation. In a randomized controlled study, Bliss *et al*[12] showed a 50% reduction in the number of FI episodes per week after daily consumption of Psyllium compared to placebo.

Pharmacological therapy

Several different medications can be used to regularize stool frequency, improve stool consistency or enhance anal canal tone. Anti-motility drugs (loperamide, codeine) have been scientifically proved to be efficient for patients suffering from FI[13]. These molecules seem to be the most useful in cases of loose of liquid stools by reducing stool frequency and thus the number of incontinence episodes. Indeed, in a double-blind cross-over trial comparing loperamide with placebo, about 60% of the patients had less incontinence episodes with loperamide than with placebo[14]. Their efficacy has not been proved in the cases of normal or hard stools.

Stool-bulking agents, such as mucilage, are also useful in management of FI with loose stool even if these practice is not supported by strong scientific studies. These synthetic fibres improve stool consistency by increasing water absorption by stools. Even though their efficacy has not been rigorously proved scientifically, mucilages are still recommended in clinical practice[15], particularly combined with other conservative treatments[16].

Ion-exchange resins may also be tried in FI management. Cholestyramine, in particular, has been shown to improve stool frequency and consistency in patients who have an FI in combination with diarrhea[17].

Several pharmaceutical substances have been studied in FI that aim to enhance anal canal tone and

Table 1 Main aetiologic factors for faecal incontinence

Localized perineal pathologies
Sphincter injury
Traumatic lesion (obstetric lesion, sexual abuse)
Surgical lesion (anal fistula surgery, hemorrhoidectomy, anal sphincterotomy)
Anoperineal lesion in Crohn's disease
Anal cancer
Pudendal neuropathy
Obstetric lesion
Dyschezia
Deficient rectal function
Chronic inflammatory bowel diseases
Radiation proctitis
Rectal cancer
Faecal impaction
Rectal surgery (anterior rectal resection, ileoanal pouch surgery)
Rectal prolapse
General pathologies
Acute or chronic diarrhea
Chronic inflammatory bowel diseases
Irritable bowel syndrome
Coeliac disease
Infectious diarrhea
Bile acid induced
Neurological diseases
Central (post stroke lesion, multiple sclerosis, medullary lesions)
Peripheral (diabetic or alcoholic neuropathy)
Systemic diseases (systemic sclerosis)

thus reduce the number of incontinence episodes. Topical agents such as phenylephrine[18-20], or oral treatments such as diazepam, amitriptyline or sodium valproate[21-23] have shown some efficacy in this indication, sometimes up to 100% of treated patients. However, given the small number of studies and the potentially disabling adverse effects, these treatments are not recommended in clinical practice.

Even though some data suggest a potential efficacy of hormone replacement therapy on FI in postmenopausal women, about 65% of treated patients[24], it is not currently recommended due to insufficient scientific proof[15].

Finally, in cases where FI is associated with rectal emptying disorders, help with rectal emptying is an essential point in incontinence management. Suppositories and rectal irrigation, combined with oral laxatives if necessary, all contribute towards eliminating faecal impaction, thus allowing a decrease of 35% in the number of FI episodes and of 42% in faecal soiling especially in geriatric patients[25].

Perineal rehabilitation

If the first line medical treatment fails, perineal rehabilitation strategies can be offered. These physiotherapy techniques are based on re-training faecal continence and target both anal sphincters and abdominal muscles. Anorectal manometry is used to determine the most adequate therapy.

These techniques mainly involve pelvic floor exercises, anal electrostimulation and biofeedback. Pelvic floor exercises are muscle strengthening exercises, especially of the pubococcygeus using Kegel exercises[26]. Anal electrostimulation uses surface electrodes on the perineum which either stimulate muscular contraction directly or indirectly *via* stimulation of peripheral nerves[27]. Biofeedback therapy helps to increase voluntary contraction of the external anal sphincter, but also to synchronize the different perineal muscles in response to a rectal stimulus in order to maintain continence[28]. This technique uses instruments capable of monitoring sphincter contractions and thus helps with training.

Perineal rehabilitation strategies have shown heterogeneous efficacy on FI depending on the study. Despite anal electrostimulation having been shown to be beneficial in this indication[27], its use alone is not recommended[15]. Biofeedback therapy seems to be the most widely used and the most efficient, with success rates between 50% and 90% that were maintained up to 24 mo[29,30]. Even though there are discordant results in scientific literature[31], these perineal rehabilitation techniques are still recommended in second line for FI management.

PTNS

PTNS appears to be a simple technique to use, it is non invasive and not costly. Two methods of stimulation exist: Percutaneous, using needle-electrodes, and transcutaneous using adhesive surface electrodes. Two electrodes are placed on the posterior tibial nerve pathway, and linked to a stimulator that can be controlled by the patient. The mechanism involved in FI treatment remains poorly understood but certainly involves afferences and somato-sympathetic reflexes. Even though there are only a few studies published with relevant different results for the two methods of stimulation[32,33], this technique reduced FI episodes for 63% to 82% of patients treated, with a follow-up of 1 to 30 mo[34]. To this day, there is no consensus concerning treatment duration, stimulation frequency/rythm or need for repeating treatment. However, this technique may be recommended for patients who do not respond to the other non-invasive techniques or suffering from FI without transit disorders[15].

TAI

TAI is currently recommended in second line management, after dietary measures and first-line medical treatment, in patients suffering from chronic neurological diseases[15]. The aim is to empty the colon of the maximum of faecal matter using regular irrigation, optimized using an inflatable rectal balloon catheter to make the system watertight. This method improves digestive symptoms, including FI, in between 40% and 75% of patients suffering from chronic neurological diseases[35-37]. It also helps to significantly enhance the patients' QOL, by significantly increasing their independence, and seems to decrease the risk of

Table 2 Level of scientific evidence for current treatments in faecal incontinence according to the Haute Autorité de Santé (French High Autority of Health)

Therapeutic modality	Levels of scientific evidence
Conservative treatment	
Hygiene and diet control	
Diet restriction	V
High fiber diet	II (Liquid stools)
	I (Constipation)
Pharmacological therapy	
Anti-motility drugs	I (Liquid stools)
Stool-bulking agents	IV
Cholestyramine	IV
Topical agents or oral treatment to enhance anal canal tone	V
Hormone replacement therapy	V
Suppositories, rectal irrigation, oral laxatives	I (Constipation)
Perineal rehabilitation	
Pelvic floor exercises	V
Anal electrostimulation	IV
Biofeedback therapy	II
Other conservative treatments	
Posterior tibial nerve stimulation	III
Transanal irrigation	I
Anal plugs	V
Minimally invasive treatment	
Sacral neuromodulation	IV
Antegrade irrigation	V
Anal radiofrequency	V
Intrasphincteric injections	V
Surgical treatment	
Sphincter repair	II
Graciloplasty	V
Artificial sphincter	V
Colostomy	V

Table 3 Level of scientific evidence (Haute Autorité de Santé, High Autority of Health)

I	Large randomized controlled trials with undeniable results
II	Small randomized controlled trials and uncertain outcomes
III	Non-randomized trials with control groups contemporaries
IV	Comparative non-randomized groups with historical controls and case-control studies
V	No control groups, patient series
	Case reports
	Expert recommandation

urinary infections[38]. However, this treatment can only work if the patient and their family are committed.

Moreover, this device may also be used in non-neurological patients as demonstrated by recent studies in patients suffering from anterior resection syndrome. For example, in the study by Rosen *et al*[39] after a mean follow-up of 29 mo, TAI allowed a significant improvement in the number of stools, the Cleveland Incontinence Score, and in QOL. A study by Koch *et al*[40] using a device from another manufacturer in thirty patients, showed a complete improvement in 57% of patients.

Anal plugs

Anal plugs come in different sizes and are made from different substances, they are easy to use and can be of great help on a daily basis. Tolerating anal plugs can be a problem but can be improved depending on the type of plug used[41,42]. Anal plugs have shown to be efficient on FI and can be used as a supplementary treatment to other therapies. Indeed, when patients tolerate them, up to 65% of patients reported the absence of soiling episodes[43].

Minimally invasive treatment: When conservative therapy has not been sufficient, some minimally invasive methods can be proposed to improve faecal continence. Currently, this type of therapy is mainly represented by sacral neuromodulation (SNM). Other techniques exist but they are more confidential, such as antegrade colon irrigation, anal radiofrequency treatment or intrasphincteric injections.

SNM

SNM is proposed to patients suffering from at least one

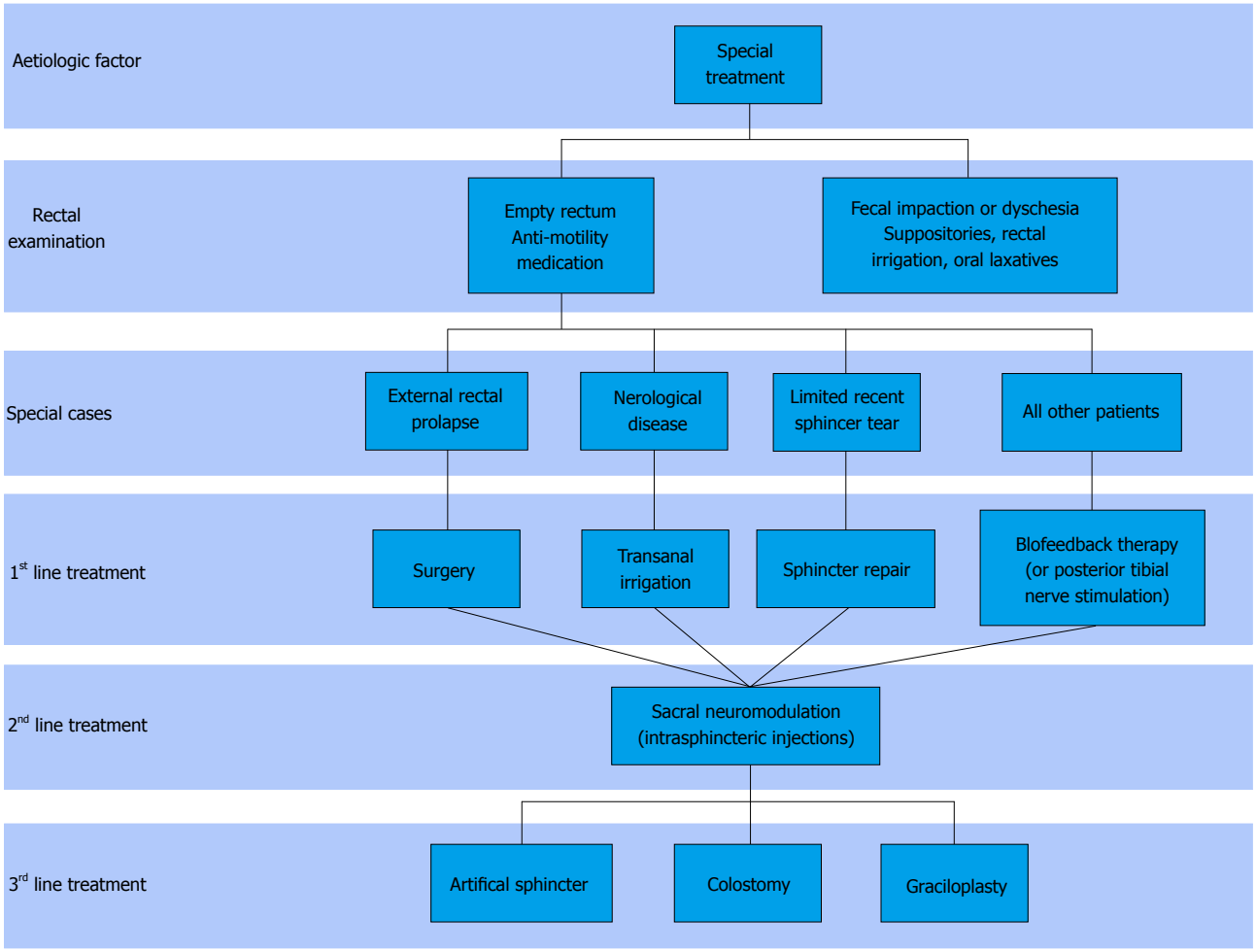


Figure 1 Algorithm for faecal incontinence current treatment.

FI episode a week and for whom conservative treatment, combining hygiene/dietary control and biofeedback, has failed[15]. The mechanism of action remains poorly understood to this day[44], although it seems to involve several mechanisms at different levels. Indeed, it could act on continence *via* somato-sympathetic medullary reflexes[45], but also *via* the activation or inhibition of areas of the brain responsible for continence[46]. This cerebral mechanism was also discussed in a recent study that showed a persistent efficacy of SNM even after stimulation had been stopped, suggesting that a long period of SNM could bring about a certain neuroplasticity[47].

This treatment stimulates the sacral nerves on a permanent basis *via* an electrode implanted in contact with the nerve where it exits the sacral foramen. The device is set up in two stages. The first stage, called peripheral nerve evaluation (PNE), is a test period. For 2 to 3 wk, the electrode is implanted, the most often in contact with the S3 root[48,49], and linked to an external stimulator. Stimulation parameters can therefore be modified to obtain sufficient efficacy on FI. The second stage, involving definitive implantation of the pulse generator under the skin, is only carried out if the patient has a 50% reduction of FI episodes with PNE[15].

On the whole, in a recent literary review, SNM seems to be efficient in approximately 60% of patients suffering from FI, for whom conservative therapy failed. The therapeutic effect persists over time, even though a 10% reduction in efficacy was noted during the first 5 years[50]. At the same time, 70% to 80% of patients who had this treatment declared that it had improved their QOL[51-53].

Indications for SNM are numerous. It can be recommended in cases of idiopathic FI, or FI linked to post-obstetric perineal injuries, such as anal sphincter tears or stretch-induced neuropathy[54-56]. It is also recommended in cases of FI of neurological origin, either central or peripheral[57-59]. Patients with systemic sclerosis suffering from FI can also benefit from SNM, although recent data have shown a lack of efficacy for this indication[60]. Finally, patients suffering from double incontinence, urinary and faecal, seem to respond to SNM[61,62]. Other aetiologies of FI are not currently considered to be indications for SNM[15], even though there is some data, particularly for Crohn's disease[63].

Several studies have tried to determine predictive factors for SNM efficacy on FI. Factors such as age, stool consistency, symptom duration, pre-therapeutic manometric data, or obtaining a motor or sensory

response at a low stimulation threshold have all been suggested to be predictive of a good or bad response to SNM. However, despite the fact that data is sometimes contradictory and that recent studies have not been able to identify any significant predictive factors[64], SNM should be considered to be a therapeutic option for all patients suffering from FI, except when contraindicated[65].

Antegrade irrigation (malone)

Based on the same principal as TAI, antegrade irrigation aims to restore continence by keeping the colon empty. The surgical procedure creates a caecostomy allowing patients to perform colon irrigation themselves.

With this technique 80% to 90% of patients reported pseudo continence, with, however, some complications that sometimes led to explantation of the device[66,67]. This method has also been shown to be efficient when combined with an artificial urinary sphincter in patients suffering from double incontinence[68].

More recently a technique of percutaneous endoscopic caecostomy has been proposed with interesting results but further studies are necessary to assess its success rate in the treatment of FI[69].

Anal radiofrequency (SECCA)

Temperature-controlled radiofrequency energy delivery to the anal canal (SECCA procedure) is an endoscopic technique mainly used for passive FI, with a success rate of close to 70%[70]. It is based on forming retractile fibrosis, with deposition of collagen and shrinkage in the internal anal sphincter. However, published results are sometimes contradictory[71] and in the long term its efficacy seems to rapidly decrease[72].

Intrasphincteric injections

In order to increase anal resting pressure, several different bulking agents, injected either into the submucosal or intersphincteric space, have been tested on FI[73]. Efficacy varied depending on the product tested[74-76], but only two studies compared bulking agent injections with sham injections. Dextranomer microspheres in stabilised hyaluronic acid (NASHA Dx) was the only product that showed a significant difference *vs* placebo. Indeed, a 50% or more reduction of the number of FI episodes was observed in 52% of patients who received NASHA Dx and 31% of patients who received placebo[77]. A large number of adverse effects were reported, but most of them were not serious. On the other hand, despite a significant improvement on continence, injections with silicone elastomers showed no significant difference *vs* sham injections, underlining the noticeable placebo effect of this procedure[78]. Finally, in a randomized clinical trial studying an injectable silicone biomaterial (PTP™), the success rate was 69% with endoanal ultrasound guidance and 40% without[79]. So, this technique seems to give better results when the injections are performed under endoanal ultrasound guidance.

Surgical treatment: Several different surgical solutions can be offered to patients with severe FI[80]. The most frequently used technique is sphincteroplasty. Given the good results obtained with conservative treatment and SNM, indications for surgical treatment have become relatively rare.

Sphincter repair

Sphincter repair is indicated in patients with symptomatic FI associated with external anal sphincter damage[15]. It aims to restore, at least partially, the anatomical barrier necessary for faecal continence and is recommended in priority in patients with recent sphincter injury that does not exceed half the circumference[81].

Several different surgical techniques have been described, notably direct repair and overlapping sphincter repair, but no significant difference was demonstrated[82,83]. Performing a temporary protective colostomy did not show any probing results either[84].

Short-term efficacy on FI is close to 70%[85]. This efficacy tends however, to decrease with time[86]. Despite this, patient satisfaction and QOL remained high even on a long-term basis[87]. Furthermore, in cases of persistent and debilitating FI, it is possible to repeat surgical repair on the external anal sphincter[88].

Graciloplasty

Techniques of muscle transposition aim to replace anal sphincters, especially in cases where sphincter damage is too severe. Several techniques have been described but graciloplasty, dynamic or not, is the most studied and used technique.

The success rate of graciloplasty is between 60% and 90%[89-93]. Originally data seemed to suggest that dynamic graciloplasty was the most efficient, however a recent study reported an identical success rate for both techniques[94].

Despite a high rate of complications, between 35% and 75%, (infections, wound healing, pain, stoma problems, constipation) and the common necessity for further surgical procedures[95-98], these methods of muscle transposition remain efficient on anal incontinence, with a success rate which persists over time[99,100]. Furthermore, patients who have had graciloplasty report an improved QOL[101].

However, in clinical practice, the indication of these techniques remains limited[15].

Artificial sphincter

An artificial anal sphincter, whatever the model, is made up of a band around the anal canal, a pump placed in the external genital organs, and a pressure-regulated balloon[102]. It is especially indicated in case of severe sphincter damage and/or severe local neurological lesions.

In a recent meta-analysis, the technique's success rate on FI was 75% in the first three years, followed by a progressive decrease since it was only 55% after 5 years of follow-up[103]. Other studies have shown more

disappointing long-term results, with only 3 out of 25 patients in the study by Altomare *et al*[104] and 4 out of 21 patients in the study by Darnis *et al*[105] showing a good functional result after 3 years of follow-up.

Along with these contrasting results, morbidity of the technique is far from being negligible with a rate of explantation of the device between 24% and 39% depending on the length of follow-up[103]. The majority of complications are infections, perianal pain, and, on a long-term basis, problems with rectal emptying. In a recent case study, all 21 patients treated had at least one complication during the 38-mo follow-up[105].

This high rate of complications combined with moderate efficacy limits the use of this technique in common practice.

Colostomy

Often reserved for patients for whom all the other treatments have failed, colostomy can be indicated to treat severe FI[106]. Comparison of QOL between patients with a colostomy and those suffering from FI, showed a higher social function and an improvement of the coping, embarrassment, lifestyle scales and depression scales in the colostomy group[107]. In the same way, after colostomy, patients who initially suffered from FI granted a high level of satisfaction, in spite of initial apprehension and some complications reported (bleeding, parastomal hernia, mucus leakage)[108]. Therefore, this technique, used in strategic FI management, should not be excluded as a failure since in the end it improves patient QOL and grants them more independence.

PERSPECTIVES

Numerous novel therapies are arriving in the field of the treatment of FI allowing patients to new hopes for this disabling symptom. These novel therapies may be local application of NRL001 (an α1-adrenoceptor agonist), stem cell injection, magnetic anal sphincter, antropylorus transposition, or other new options.

Local application of an α1 -adrenoceptor agonist

Alpha1-adrenoceptors have been tested for years since they are known to induce a contractile response of the human internal anal sphincter[109]. However because of their poor clinical tolerance (modifications of cardiovascular parameters), their use didn't initially spread in clinical practice. More recently, since NRL001 has a dose-dependent effect, the concentration was modified to improve tolerance. However, to date, only phase-Ⅰ studies are available[110-113]. We are currently waiting for the future results of the "Libertas" study that is a multicentre Phase Ⅱ, double-blind, randomised, placebo-controlled, parallel group. It will evaluate the efficacy, safety and tolerability of locally applied NRL001 in patients with FI[114].

Stem cell injection

The use of stem cell can be done in two main different ways: Direct injections in the sphincter or peri-anal implantation of a bioengineered sphincter. Stem cell involved may be mesenchymal stem cell or muscle-derived stem cell. For the moment, most of the studies have been done in animal models. The first study reporting that autologous muscle-derived stem cell grafts (in a rat model) may be a new tool for improving anal sphincter function was published in 2007[115]. Then, other studies have demonstrated the feasibility of the use of stem cells in a rat model, with myoblast[116] and with mesenchymal stem cells[117,118]. Another way to use stem cell is the perinanal implantation of a bioengineered sphincter. In an original study, isolated human internal anal sphincter circular smooth muscle cells and human enteric neuronal progenitor cells were use to construct a bioengineered internal anal sphincter. After maturation, it was implanted in the perianal region of athymic rats and retrieved from the animal after 4 wk. The implantation was well tolerated in all animals and there were no postoperative complications. Normal stooling was observed during the implantation period. After the 4 wk period, it was observed that implanted bioengineered sphincters were adherent to the perirectal rat tissue and appeared healthy and pink and immunohistochemical data showed neovascularization[119]. A particular interest of this model is that the bioengineered IAS may overcome the problems of foreign bodies in the perineum since it is purely made from autologous tissue. Further studies will be necessary to confirm this concept.

To date, only two works describe the use of stem cell in humans. The first one was published in 2010 and included 10 women suffering from FI due to obstetric anal sphincter injury. In this pilot study, autologous myoblasts were cultured from a pectoralis muscle biopsy, harvested, and then injected into the external anal sphincter defect under ultrasound guidance. At 12 mo a significant improvement in the Wexner score and in the QOL were observed. The anal squeeze pressures did rise significantly at 1 mo and 6 mo post-injection without persistence at 12 mo. The procedure was well tolerated and no adverse events were observed[120]. The second case, published in 2013, has reported one case of injection of myoblast also obtained from a sample of the quadriceps muscle. The patient was a 20-year old male with FI due to an old external anal sphincter rupture in a road accident[121]. Although this work also showed encouraging results, data are still scarce and these two studies essentially demonstrate the feasibility in humans. Further studies will be needed to confirm the feasibility of stem cell injection in FI and to assess the potential long term success of this method that is for the moment limited to experimented centres.

Magnetic anal sphincter

The Fenix® magnetic anal sphincter operates on the principle of a reverse stent system; it is composed of a magnetic bead that creates a negative pressure around the tube it encircles. The device is made of magnetic

balls tied together by titanium threads. Various magnetic sphincter lengths are available to accommodate the individual variations of anal circumference. This sphincter is designed to enhance the function of the anal sphincters without causing obstruction. It is functional immediately after implantation. During stool evacuation, the patient strains in a physiological way to create a sufficient force to separate the magnetic balls and to open the anal canal permitting thus the passage of stools.

The first feasibility study was published in 2010 and included results for 14 patients from[122]. The authors described a simple technique with a low morbidity (mainly represented by surgical site infections). Compared to the conventional artificial sphincter, the operative time and duration of hospitalization were much shorter. Since this first study, the results of the Fenix® magnetic anal sphincter device on FI have been reported in the literature, but few studies are currently available. In 2012, Wong *et al*[123] reported results from a single-center non-randomized study showing that the magnetic anal sphincter was as effective as sacral nerve stimulation in improving symptoms and QOL in patients suffering from faecal continence. Moreover, the morbidity was similar with the two techniques. Then, new studies have confirmed the success rate of this device, in 23 patients with a median follow-up of 17.6 mo in the study of Barussaud *et al*[124] and in 18 patients with a follow-up from 353 d to 738 d in the study of Pakravan *et al*[125]. In both studies, symptoms, FI severity scores and QOL were significantly improved. However, although it brings promising results, the efficacy of the magnetic anal sphincter needs to be confirmed in larger and randomized studies with longer follow-up.

Antropylorus transposition

Ger *et al*[126] published the first report of the transposition of the antropyloric valve as a living sphincter at the end of an ileostomy in 1982. After this first publication, successive other works demonstrated the feasibility and the interest of this technique in animals[127-129]. More recently, some studies have been published about this technique in adult patients. The first preliminary report in human was published in 2011[130]. Then, Goldsmith *et al*[130] published successive studies on this technique demonstrating the feasibility and the success rate of this technique on clinical and manometric parameters in patients requiring anal replacement. In particular, they demonstrated in a study in 17 patients, after a median follow-up of 18 mo, a definite tone of the transposed graft on digital examination, an improvement in the St Mark incontinence score and in the QOL score (SF-36) associated with a significant rise in the postoperative resting neosphincter pressure[131-133].

However, although this new technique is very interesting, it remains a surgical invasive approach and it is, for the moment, at a very early stage of its development. Further larger studies with long-term follow-up will be necessary to evaluate the validity and the place of this technique.

OTHER TREATMENTS

Toxine

Injection of Botulinum Toxin (BT) into the detrusor muscle is used for years by urologists to improve overactive bladder. In FI, especially in urge incontinence, a same mechanism may be hypothesized based on a rectal contractile disorder. A first study has been published to assess the efficacy of intrarectal injections of BT in the treatment of FI[134]. This prospective pilot study included 6 patients with high-amplitude contractions of the intact native rectum or of the reservoir (4 patients had a proctectomy for rectal cancer). Anal sphincters were intact in most of the patients. In this study, all the patients reported a clinical improvement based on the Cleveland Clinic Score at 3-6 mo that was sustained at 6 mo. Manometric data showed a decrease of the mean amplitude of contractions whereas the frequency of contractions remained unaffected by the BT injections. These results are interesting and encouraging, especially because it is a simple and non-invasive treatment. However, its efficacy needs to be demonstrated in larger studies with selected patients.

Vaginal bowel control system

A vaginal bowel-control system has been reported in the treatment of FI. This device (Eclipse System) is a non-invasive, non-surgical therapeutic option. It consists of a vaginal insert with a pressure-regulated pump. The insert is made of a silicone-coated stainless steel base balloon that is posteriorly directed. In the prospective study of Richter including 110 patients, the intention-to-treat success rate at 1 mo was 78.7% and the success rate at 3 mo was 86.4%[135]. A significant improvement was also observed and no serious adverse effects were reported (mainly pelvic cramping or discomfort). This device is simple to use and non invasive and self-managed by the patients. Further studies will be necessary to determine the place of this device in the therapeutic algorithm of FI.

Acupuncture

The effect of acupuncture on FI has been reported by an Italian study[136]. In this pilot study, 15 female patients were submitted to one acupuncture treatment per week for a 10-wk period, and a control session was repeated once per month up to 7 mo for six patients. After the 10-wk period, a significant improvement was observed with an overall mean continence score in the 15 patients from 10 (3-21) to zero (0-7). The continence index available in 14 patients at about 18 mo after start of treatment was 1 (0-8). Concerning manometric parameters, a significant increase was observed in the resting anal pressure and in the ability to sustain the squeeze pressure whereas the maximal sphincter squeeze pressure remained unchanged.

CONCLUSION

FI is a common and disabling symptom. It is often reported by the patients as embarrassing to report to their health care providers leading to an underestimated prevalence. Several tools are currently available for the treatment of FI. The therapeutics modalities are mainly conservative and mini-invasive, but may sometimes need a surgical invasive approach. However, different now therapeutic approach are currently developing, most of them being conservative, leading to optimistic perspectives for patients suffering from FI.

REFERENCES

1 **Bharucha AE**, Wald A, Enck P, Rao S. Functional anorectal disorders. *Gastroenterology* 2006; **130**: 1510-1518 [PMID: 16678564 DOI: 10.1053/j.gastro.2005.11.064]

2 **Macmillan AK**, Merrie AE, Marshall RJ, Parry BR. The prevalence of fecal incontinence in community-dwelling adults: a systematic review of the literature. *Dis Colon Rectum* 2004; **47**: 1341-1349 [PMID: 15484348]

3 **Faltin DL**, Sangalli MR, Curtin F, Morabia A, Weil A. Prevalence of anal incontinence and other anorectal symptoms in women. *Int Urogynecol J Pelvic Floor Dysfunct* 2001; **12**: 117-120; discussion 121 [PMID: 11374509]

4 **Damon H**, Guye O, Seigneurin A, Long F, Sonko A, Faucheron JL, Grandjean JP, Mellier G, Valancogne G, Fayard MO, Henry L, Guyot P, Barth X, Mion F. Prevalence of anal incontinence in adults and impact on quality-of-life. *Gastroenterol Clin Biol* 2006; **30**: 37-43 [PMID: 16514381]

5 **Kamm MA**. Obstetric damage and faecal incontinence. *Lancet* 1994; **344**: 730-733 [PMID: 7915781]

6 **Caruana BJ**, Wald A, Hinds JP, Eidelman BH. Anorectal sensory and motor function in neurogenic fecal incontinence. Comparison between multiple sclerosis and diabetes mellitus. *Gastroenterology* 1991; **100**: 465-470 [PMID: 1985043]

7 **Bytzer P**, Talley NJ, Leemon M, Young LJ, Jones MP, Horowitz M. Prevalence of gastrointestinal symptoms associated with diabetes mellitus: a population-based survey of 15,000 adults. *Arch Intern Med* 2001; **161**: 1989-1996 [PMID: 11525701]

8 **Bouvier M**. [Physiology of fecal continence and defecation]. *Arch Int Physiol Biochim Biophys* 1991; **99**: A53-A63 [PMID: 1720692]

9 **Demirci S**, Gallas S, Bertot-Sassigneux P, Michot F, Denis P, Leroi AM. Anal incontinence: the role of medical management. *Gastroenterol Clin Biol* 2006; **30**: 954-960 [PMID: 17075441]

10 **Croswell E**, Bliss DZ, Savik K. Diet and eating pattern modifications used by community-living adults to manage their fecal incontinence. *J Wound Ostomy Continence Nurs* 2010; **37**: 677-682 [PMID: 21076267 DOI: 10.1097/WON.0b013e3181feb017]

11 **Hansen JL**, Bliss DZ, Peden-McAlpine C. Diet strategies used by women to manage fecal incontinence. *J Wound Ostomy Continence Nurs* 2006; **33**: 52-61; discussion 61-62 [PMID: 16444104]

12 **Bliss DZ**, Savik K, Jung HJ, Whitebird R, Lowry A, Sheng X. Dietary fiber supplementation for fecal incontinence: a randomized clinical trial. *Res Nurs Health* 2014; **37**: 367-378 [PMID: 25155992 DOI: 10.1002/nur.21616]

13 **Palmer KR**, Corbett CL, Holdsworth CD. Double-blind cross-over study comparing loperamide, codeine and diphenoxylate in the treatment of chronic diarrhea. *Gastroenterology* 1980; **79**: 1272-1275 [PMID: 7002706]

14 **Read M**, Read NW, Barber DC, Duthie HL. Effects of loperamide on anal sphincter function in patients complaining of chronic diarrhea with fecal incontinence and urgency. *Dig Dis Sci* 1982; **27**: 807-814 [PMID: 7105952]

15 **Vitton V**, Soudan D, Siproudhis L, Abramowitz L, Bouvier M, Faucheron JL, Leroi AM, Meurette G, Pigot F, Damon H. Treatments of faecal incontinence: recommendations from the French national society of coloproctology. *Colorectal Dis* 2014; **16**: 159-166 [PMID: 24521273 DOI: 10.1111/codi.12410]

16 **Sjödahl J**, Walter SA, Johansson E, Ingemansson A, Ryn AK, Hallböök O. Combination therapy with biofeedback, loperamide, and stool-bulking agents is effective for the treatment of fecal incontinence in women - a randomized controlled trial. *Scand J Gastroenterol* 2015; **50**: 965-974 [PMID: 25892434 DOI: 10.3109/00365521.2014.999252]

17 **Remes-Troche JM**, Ozturk R, Philips C, Stessman M, Rao SS. Cholestyramine--a useful adjunct for the treatment of patients with fecal incontinence. *Int J Colorectal Dis* 2008; **23**: 189-194 [PMID: 17938939 DOI: 10.1007/s00384-007-0391-y]

18 **Park JS**, Kang SB, Kim DW, Namgung HW, Kim HL. The efficacy and adverse effects of topical phenylephrine for anal incontinence after low anterior resection in patients with rectal cancer. *Int J Colorectal Dis* 2007; **22**: 1319-1324 [PMID: 17569063 DOI: 10.1007/s00384-007-0335-6]

19 **Badvie S**, Andreyev HJ. Topical phenylephrine in the treatment of radiation-induced faecal incontinence. *Clin Oncol* (R Coll Radiol) 2005; **17**: 122-126 [PMID: 15830575]

20 **Carapeti EA**, Kamm MA, Nicholls RJ, Phillips RK. Randomized, controlled trial of topical phenylephrine for fecal incontinence in patients after ileoanal pouch construction. *Dis Colon Rectum* 2000; **43**: 1059-1063 [PMID: 10950003]

21 **Shoji Y**, Kusunoki M, Yanagi H, Sakanoue Y, Utsunomiya J. Effects of sodium valproate on various intestinal motor functions after ileal J pouch-anal anastomosis. *Surgery* 1993; **113**: 560-563 [PMID: 8488476]

22 **Maeda K**, Maruta M, Sato H, Masumori K, Matsumoto M. Effect of oral diazepam on anal continence after low anterior resection: a preliminary study. *Tech Coloproctol* 2002; **6**: 15-18 [PMID: 12077635 DOI: 10.1007/s101510200002]

23 **Santoro GA**, Eitan BZ, Pryde A, Bartolo DC. Open study of low-dose amitriptyline in the treatment of patients with idiopathic fecal incontinence. *Dis Colon Rectum* 2000; **43**: 1676-1681; discussion 1681-1682 [PMID: 11156450]

24 **Donnelly V**, O'Connell PR, O'Herlihy C. The influence of oestrogen replacement on faecal incontinence in postmenopausal women. *Br J Obstet Gynaecol* 1997; **104**: 311-315 [PMID: 9091007]

25 **Chassagne P**, Jego A, Gloc P, Capet C, Trivalle C, Doucet J, Denis P, Bercoff E. Does treatment of constipation improve faecal incontinence in institutionalized elderly patients? *Age Ageing* 2000; **29**: 159-164 [PMID: 10791451]

26 **Park SH**, Kang CB, Jang SY, Kim BY. [Effect of Kegel exercise to prevent urinary and fecal incontinence in antenatal and postnatal women: systematic review]. *J Korean Acad Nurs* 2013; **43**: 420-430 [PMID: 23893232 DOI: 10.4040/jkan.2013.43.3.420]

27 **Norton C**, Gibbs A, Kamm MA. Randomized, controlled trial of anal electrical stimulation for fecal incontinence. *Dis Colon Rectum* 2006; **49**: 190-196 [PMID: 16362803 DOI: 10.1007/s10350-005-0251-1]

28 **Norton C**, Cody JD. Biofeedback and/or sphincter exercises for the treatment of faecal incontinence in adults. *Cochrane Database Syst Rev* 2012; **7**: CD002111 [PMID: 22786479 DOI: 10.1002/14651858.CD002111.pub3]

29 **Heymen S**, Scarlett Y, Jones K, Ringel Y, Drossman D, Whitehead WE. Randomized controlled trial shows biofeedback to be superior to pelvic floor exercises for fecal incontinence. *Dis Colon Rectum* 2009; **52**: 1730-1737 [PMID: 19966605 DOI: 10.1007/DCR.0b013e3181b55455]

30 **Bartlett L**, Sloots K, Nowak M, Ho YH. Biofeedback for fecal incontinence: a randomized study comparing exercise regimens. *Dis Colon Rectum* 2011; **54**: 846-856 [PMID: 21654252 DOI: 10.1007/DCR.0b013e3182148fef]

31 **Norton C**, Chelvanayagam S, Wilson-Barnett J, Redfern S, Kamm MA. Randomized controlled trial of biofeedback for fecal incontinence. *Gastroenterology* 2003; **125**: 1320-1329 [PMID: 14598248]

32 **George AT**, Kalmar K, Sala S, Kopanakis K, Panarese A, Dudding TC, Hollingshead JR, Nicholls RJ, Vaizey CJ. Randomized

controlled trial of percutaneous versus transcutaneous posterior tibial nerve stimulation in faecal incontinence. *Br J Surg* 2013; **100**: 330-338 [PMID: 23300071 DOI: 10.1002/bjs.9000]
33 **Leroi AM**, Siproudhis L, Etienney I, Damon H, Zerbib F, Amarenco G, Vitton V, Faucheron JL, Thomas C, Mion F, Roumeguère P, Gourcerol G, Bouvier M, Lallouche K, Menard JF, Queralto M. Transcutaneous electrical tibial nerve stimulation in the treatment of fecal incontinence: a randomized trial (CONSORT 1a). *Am J Gastroenterol* 2012; **107**: 1888-1896 [PMID: 23032981 DOI: 10.1038/ajg.2012.330]
34 **Thomas GP**, Dudding TC, Rahbour G, Nicholls RJ, Vaizey CJ. A review of posterior tibial nerve stimulation for faecal incontinence. *Colorectal Dis* 2013; **15**: 519-526 [PMID: 23216902 DOI: 10.1111/codi.12093]
35 **Del Popolo G**, Mosiello G, Pilati C, Lamartina M, Battaglino F, Buffa P, Redaelli T, Lamberti G, Menarini M, Di Benedetto P, De Gennaro M. Treatment of neurogenic bowel dysfunction using transanal irrigation: a multicenter Italian study. *Spinal Cord* 2008; **46**: 517-522 [PMID: 18317488 DOI: 10.1038/sj.sc.3102167]
36 **Faaborg PM**, Christensen P, Kvitsau B, Buntzen S, Laurberg S, Krogh K. Long-term outcome and safety of transanal colonic irrigation for neurogenic bowel dysfunction. *Spinal Cord* 2009; **47**: 545-549 [PMID: 19104513]
37 **Ausili E**, Focarelli B, Tabacco F, Murolo D, Sigismondi M, Gasbarrini A, Rendeli C. Transanal irrigation in myelomeningocele children: an alternative, safe and valid approach for neurogenic constipation. *Spinal Cord* 2010; **48**: 560-565 [PMID: 20084075 DOI: 10.1038/sc.2009.186]
38 **Christensen P**, Bazzocchi G, Coggrave M, Abel R, Hultling C, Krogh K, Media S, Laurberg S. A randomized, controlled trial of transanal irrigation versus conservative bowel management in spinal cord-injured patients. *Gastroenterology* 2006; **131**: 738-747 [PMID: 16952543 DOI: 10.1053/j.gastro.2006.06.004]
39 **Rosen H**, Robert-Yap J, Tentschert G, Lechner M, Roche B. Transanal irrigation improves quality of life in patients with low anterior resection syndrome. *Colorectal Dis* 2011; **13**: e335-e338 [PMID: 21689359 DOI: 10.1111/j.1463-1318.2011.02692.x]
40 **Koch SM**, Rietveld MP, Govaert B, van Gemert WG, Baeten CG. Retrograde colonic irrigation for faecal incontinence after low anterior resection. *Int J Colorectal Dis* 2009; **24**: 1019-1022 [PMID: 19452159 DOI: 10.1007/s00384-009-0719-x]
41 **Pfrommer W**, Holschneider AM, Löffler N, Schauff B, Ure BM. A new polyurethane anal plug in the treatment of incontinence after anal atresia repair. *Eur J Pediatr Surg* 2000; **10**: 186-190 [PMID: 10982049 DOI: 10.1055/s-2008-1072354]
42 **Norton C**, Kamm MA. Anal plug for faecal incontinence. *Colorectal Dis* 2001; **3**: 323-327 [PMID: 12790954]
43 **Deutekom M**, Dobben AC. Plugs for containing faecal incontinence. *Cochrane Database Syst Rev* 2012; **4**: CD005086 [PMID: 22513927 DOI: 10.1002/14651858.CD005086.pub3]
44 **Gourcerol G**, Vitton V, Leroi AM, Michot F, Abysique A, Bouvier M. How sacral nerve stimulation works in patients with faecal incontinence. *Colorectal Dis* 2011; **13**: e203-e211 [PMID: 21689312 DOI: 10.1111/j.1463-1318.2011.02623.x]
45 **Vitton V**, Abysique A, Gaigé S, Leroi AM, Bouvier M. Colonosphincteric electromyographic responses to sacral root stimulation: evidence for a somatosympathetic reflex. *Neurogastroenterol Motil* 2008; **20**: 407-416 [PMID: 18034793 DOI: 10.1111/j.1365-2982.2007.01022.x]
46 **Sheldon R**, Kiff ES, Clarke A, Harris ML, Hamdy S. Sacral nerve stimulation reduces corticoanal excitability in patients with faecal incontinence. *Br J Surg* 2005; **92**: 1423-1431 [PMID: 16044426 DOI: 10.1002/bjs.5111]
47 **Altomare DF**, Giannini I, Giuratrabocchetta S, Digennaro R. The effects of sacral nerve stimulation on continence are temporarily maintained after turning the stimulator off. *Colorectal Dis* 2013; **15**: e741-e748 [PMID: 24102954 DOI: 10.1111/codi.12418]
48 **Dudding TC**, Parés D, Vaizey CJ, Kamm MA. Predictive factors for successful sacral nerve stimulation in the treatment of faecal incontinence: a 10-year cohort analysis. *Colorectal Dis* 2008; **10**: 249-256 [PMID: 17655722 DOI: 10.1111/j.1463-1318.2007.01319.x]
49 **Melenhorst J**, Koch SM, Uludag O, van Gemert WG, Baeten CG. Sacral neuromodulation in patients with faecal incontinence: results of the first 100 permanent implantations. *Colorectal Dis* 2007; **9**: 725-730 [PMID: 17509049 DOI: 10.1111/j.1463-1318.2007.01241.x]
50 **Thin NN**, Horrocks EJ, Hotouras A, Palit S, Thaha MA, Chan CL, Matzel KE, Knowles CH. Systematic review of the clinical effectiveness of neuromodulation in the treatment of faecal incontinence. *Br J Surg* 2013; **100**: 1430-1447 [PMID: 24037562 DOI: 10.1002/bjs.9226]
51 **Damon H**, Barth X, Roman S, Mion F. Sacral nerve stimulation for fecal incontinence improves symptoms, quality of life and patients' satisfaction: results of a monocentric series of 119 patients. *Int J Colorectal Dis* 2013; **28**: 227-233 [PMID: 22885883 DOI: 10.1007/s00384-012-1558-8]
52 **Devroede G**, Giese C, Wexner SD, Mellgren A, Coller JA, Madoff RD, Hull T, Stromberg K, Iyer S. Quality of life is markedly improved in patients with fecal incontinence after sacral nerve stimulation. *Female Pelvic Med Reconstr Surg* 2012; **18**: 103-112 [PMID: 22453321 DOI: 10.1097/SPV.0b013e3182486e60]
53 **Duelund-Jakobsen J**, van Wunnik B, Buntzen S, Lundby L, Baeten C, Laurberg S. Functional results and patient satisfaction with sacral nerve stimulation for idiopathic faecal incontinence. *Colorectal Dis* 2012; **14**: 753-759 [PMID: 21883814 DOI: 10.1111/j.1463-1318.2011.02800.x]
54 **Chan MK**, Tjandra JJ. Sacral nerve stimulation for fecal incontinence: external anal sphincter defect vs. intact anal sphincter. *Dis Colon Rectum* 2008; **51**: 1015-1024; discussion 1024-1025 [PMID: 18484136 DOI: 10.1007/s10350-008-9326-0]
55 **Boyle DJ**, Knowles CH, Lunniss PJ, Scott SM, Williams NS, Gill KA. Efficacy of sacral nerve stimulation for fecal incontinence in patients with anal sphincter defects. *Dis Colon Rectum* 2009; **52**: 1234-1239 [PMID: 19571698 DOI: 10.1007/DCR.0b013e31819f7400]
56 **Brouwer R**, Duthie G. Sacral nerve neuromodulation is effective treatment for fecal incontinence in the presence of a sphincter defect, pudendal neuropathy, or previous sphincter repair. *Dis Colon Rectum* 2010; **53**: 273-278 [PMID: 20173472 DOI: 10.1007/DCR.0b013e3181ceeb22]
57 **Kenefick NJ**, Vaizey CJ, Nicholls RJ, Cohen R, Kamm MA. Sacral nerve stimulation for faecal incontinence due to systemic sclerosis. *Gut* 2002; **51**: 881-883 [PMID: 12427794]
58 **Holzer B**, Rosen HR, Novi G, Ausch C, Hölbling N, Schiessel R. Sacral nerve stimulation for neurogenic faecal incontinence. *Br J Surg* 2007; **94**: 749-753 [PMID: 17410558 DOI: 10.1002/bjs.5499]
59 **Jarrett ME**, Matzel KE, Christiansen J, Baeten CG, Rosen H, Bittorf B, Stösser M, Madoff R, Kamm MA. Sacral nerve stimulation for faecal incontinence in patients with previous partial spinal injury including disc prolapse. *Br J Surg* 2005; **92**: 734-739 [PMID: 15838899 DOI: 10.1002/bjs.4859]
60 **Butt SK**, Alam A, Cohen R, Krogh K, Buntzen S, Emmanuel A. Lack of effect of sacral nerve stimulation for incontinence in patients with systemic sclerosis. *Colorectal Dis* 2015; **17**: 903-907 [PMID: 25850948 DOI: 10.1111/codi.12969]
61 **El-Gazzaz G**, Zutshi M, Salcedo L, Hammel J, Rackley R, Hull T. Sacral neuromodulation for the treatment of fecal incontinence and urinary incontinence in female patients: long-term follow-up. *Int J Colorectal Dis* 2009; **24**: 1377-1381 [PMID: 19488765 DOI: 10.1007/s00384-009-0745-8]
62 **Leroi AM**, Michot F, Grise P, Denis P. Effect of sacral nerve stimulation in patients with fecal and urinary incontinence. *Dis Colon Rectum* 2001; **44**: 779-789 [PMID: 11391135]
63 **Vitton V**, Gigout J, Grimaud JC, Bouvier M, Desjeux A, Orsoni P. Sacral nerve stimulation can improve continence in patients with Crohn's disease with internal and external anal sphincter disruption. *Dis Colon Rectum* 2008; **51**: 924-927 [PMID: 18259815 DOI: 10.1007/s10350-008-9209-4]
64 **Roy AL**, Gourcerol G, Menard JF, Michot F, Leroi AM, Bridoux

V. Predictive factors for successful sacral nerve stimulation in the treatment of fecal incontinence: lessons from a comprehensive treatment assessment. *Dis Colon Rectum* 2014; **57**: 772-780 [PMID: 24807603 DOI: 10.1097/DCR.0000000000000115]

65 **Maeda Y**, O'Connell PR, Lehur PA, Matzel KE, Laurberg S. Sacral nerve stimulation for faecal incontinence and constipation: a European consensus statement. *Colorectal Dis* 2015; **17**: O74-O87 [PMID: 25603960 DOI: 10.1111/codi.12905]

66 **Sinha CK**, Grewal A, Ward HC. Antegrade continence enema (ACE): current practice. *Pediatr Surg Int* 2008; **24**: 685-688 [PMID: 18408942 DOI: 10.1007/s00383-008-2130-z]

67 **Hoekstra LT**, Kuijper CF, Bakx R, Heij HA, Aronson DC, Benninga MA. The Malone antegrade continence enema procedure: the Amsterdam experience. *J Pediatr Surg* 2011; **46**: 1603-1608 [PMID: 21843730 DOI: 10.1016/j.jpedsurg.2011.04.050]

68 **Bar-Yosef Y**, Castellan M, Joshi D, Labbie A, Gosalbez R. Total continence reconstruction using the artificial urinary sphincter and the Malone antegrade continence enema. *J Urol* 2011; **185**: 1444-1447 [PMID: 21334669 DOI: 10.1016/j.juro.2010.11.049]

69 **Duchalais E**, Meurette G, Mantoo SK, Le Rhun M, Varannes SB, Lehur PA, Coron E. Percutaneous endoscopic caecostomy for severe constipation in adults: feasibility, durability, functional and quality of life results at 1 year follow-up. *Surg Endosc* 2015; **29**: 620-626 [PMID: 25030476 DOI: 10.1007/s00464-014-3709-1]

70 **Frascio M**, Mandolfino F, Imperatore M, Stabilini C, Fornaro R, Gianetta E, Wexner SD. The SECCA procedure for faecal incontinence: a review. *Colorectal Dis* 2014; **16**: 167-172 [PMID: 24034552 DOI: 10.1111/codi.12403]

71 **Kim DW**, Yoon HM, Park JS, Kim YH, Kang SB. Radiofrequency energy delivery to the anal canal: is it a promising new approach to the treatment of fecal incontinence? *Am J Surg* 2009; **197**: 14-18 [PMID: 18614149 DOI: 10.1016/j.amjsurg.2007.11.023]

72 **Abbas MA**, Tam MS, Chun LJ. Radiofrequency treatment for fecal incontinence: is it effective long-term? *Dis Colon Rectum* 2012; **55**: 605-610 [PMID: 22513440 DOI: 10.1097/DCR.0b013e3182415406]

73 **Maeda Y**, Laurberg S, Norton C. Perianal injectable bulking agents as treatment for faecal incontinence in adults. *Cochrane Database Syst Rev* 2010; **(5)**: CD007959 [PMID: 20464759 DOI: 10.1002/14651858.CD007959.pub2]

74 **Tjandra JJ**, Chan MK, Yeh HC. Injectable silicone biomaterial (PTQ) is more effective than carbon-coated beads (Durasphere) in treating passive faecal incontinence--a randomized trial. *Colorectal Dis* 2009; **11**: 382-389 [PMID: 18637935 DOI: 10.1111/j.1463-1318.2008.01634.x]

75 **Morris OJ**, Smith S, Draganic B. Comparison of bulking agents in the treatment of fecal incontinence: a prospective randomized clinical trial. *Tech Coloproctol* 2013; **17**: 517-523 [PMID: 23525964 DOI: 10.1007/s10151-013-1000-4]

76 **Maeda Y**, Vaizey CJ, Kamm MA. Pilot study of two new injectable bulking agents for the treatment of faecal incontinence. *Colorectal Dis* 2008; **10**: 268-272 [PMID: 17655723 DOI: 10.1111/j.1463-1318.2007.01318.x]

77 **Graf W**, Mellgren A, Matzel KE, Hull T, Johansson C, Bernstein M. Efficacy of dextranomer in stabilised hyaluronic acid for treatment of faecal incontinence: a randomised, sham-controlled trial. *Lancet* 2011; **377**: 997-1003 [PMID: 21420555 DOI: 10.1016/S0140-6736(10)62297-0]

78 **Siproudhis L**, Morcet J, Lainé F. Elastomer implants in faecal incontinence: a blind, randomized placebo-controlled study. *Aliment Pharmacol Ther* 2007; **25**: 1125-1132 [PMID: 17439514 DOI: 10.1111/j.1365-2036.2007.03293.x]

79 **Tjandra JJ**, Lim JF, Hiscock R, Rajendra P. Injectable silicone biomaterial for fecal incontinence caused by internal anal sphincter dysfunction is effective. *Dis Colon Rectum* 2004; **47**: 2138-2146 [PMID: 15657666 DOI: 10.1007/s10350-004-0760-3]

80 **Brown SR**, Wadhawan H, Nelson RL. Surgery for faecal incontinence in adults. *Cochrane Database Syst Rev* 2013; **7**: CD001757 [PMID: 23821339 DOI: 10.1002/14651858.CD001757.pub4]

81 **Rasmussen OO**, Puggaard L, Christiansen J. Anal sphincter repair in patients with obstetric trauma: age affects outcome. *Dis Colon Rectum* 1999; **42**: 193-195 [PMID: 10211495]

82 **Tjandra JJ**, Han WR, Goh J, Carey M, Dwyer P. Direct repair vs. overlapping sphincter repair: a randomized, controlled trial. *Dis Colon Rectum* 2003; **46**: 937-942; discussion 942-943 [PMID: 12847369 DOI: 10.1097/01.DCR.0000074687.97942.92]

83 **Garcia V**, Rogers RG, Kim SS, Hall RJ, Kammerer-Doak DN. Primary repair of obstetric anal sphincter laceration: a randomized trial of two surgical techniques. *Am J Obstet Gynecol* 2005; **192**: 1697-1701 [PMID: 15902180 DOI: 10.1016/j.ajog.2004.11.045]

84 **Young CJ**, Mathur MN, Eyers AA, Solomon MJ. Successful overlapping anal sphincter repair: relationship to patient age, neuropathy, and colostomy formation. *Dis Colon Rectum* 1998; **41**: 344-349 [PMID: 9514430]

85 **Engel AF**, Kamm MA, Sultan AH, Bartram CI, Nicholls RJ. Anterior anal sphincter repair in patients with obstetric trauma. *Br J Surg* 1994; **81**: 1231-1234 [PMID: 7953372]

86 **Lamblin G**, Bouvier P, Damon H, Chabert P, Moret S, Chene G, Mellier G. Long-term outcome after overlapping anterior anal sphincter repair for fecal incontinence. *Int J Colorectal Dis* 2014; **29**: 1377-1383 [PMID: 25185844 DOI: 10.1007/s00384-014-2005-9]

87 **Glasgow SC**, Lowry AC. Long-term outcomes of anal sphincter repair for fecal incontinence: a systematic review. *Dis Colon Rectum* 2012; **55**: 482-490 [PMID: 22426274 DOI: 10.1097/DCR.0b013e3182468c22]

88 **Giordano P**, Renzi A, Efron J, Gervaz P, Weiss EG, Nogueras JJ, Wexner SD. Previous sphincter repair does not affect the outcome of repeat repair. *Dis Colon Rectum* 2002; **45**: 635-640 [PMID: 12004213]

89 **Thornton MJ**, Kennedy ML, Lubowski DZ, King DW. Long-term follow-up of dynamic graciloplasty for faecal incontinence. *Colorectal Dis* 2004; **6**: 470-476 [PMID: 15521938 DOI: 10.1111/j.1463-1318.2004.00714.x]

90 **Christiansen J**, Sørensen M, Rasmussen OO. Gracilis muscle transposition for faecal incontinence. *Br J Surg* 1990; **77**: 1039-1040 [PMID: 2207570]

91 **Kumar D**, Hutchinson R, Grant E. Bilateral gracilis neosphincter construction for treatment of faecal incontinence. *Br J Surg* 1995; **82**: 1645-1647 [PMID: 8548229]

92 **Wexner SD**, Gonzalez-Padron A, Rius J, Teoh TA, Cheong DM, Nogueras JJ, Billotti VL, Weiss EG, Moon HK. Stimulated gracilis neosphincter operation. Initial experience, pitfalls, and complications. *Dis Colon Rectum* 1996; **39**: 957-964 [PMID: 8797641]

93 **Mavrantonis C**, Billotti VL, Wexner SD. Stimulated graciloplasty for treatment of intractable fecal incontinence: critical influence of the method of stimulation. *Dis Colon Rectum* 1999; **42**: 497-504 [PMID: 10215051]

94 **Walega P**, Romaniszyn M, Siarkiewicz B, Zelazny D. Dynamic versus Adynamic Graciloplasty in Treatment of End-Stage Fecal Incontinence: Is the Implantation of the Pacemaker Really Necessary? 12-Month Follow-Up in a Clinical, Physiological, and Functional Study. *Gastroenterol Res Pract* 2015; **2015**: 698516 [PMID: 25861261 DOI: 10.1155/2015/698516]

95 **Devesa JM**, Madrid JM, Gallego BR, Vicente E, Nuño J, Enríquez JM. Bilateral gluteoplasty for fecal incontinence. *Dis Colon Rectum* 1997; **40**: 883-888 [PMID: 9269802]

96 **Baeten CG**, Bailey HR, Bakka A, Belliveau P, Berg E, Buie WD, Burnstein MJ, Christiansen J, Coller JA, Galandiuk S, LaFontaine LJ, Lange J, Madoff RD, Matzel KE, Påhlman L, Parc R, Reilly JC, Seccia M, Thorson AG, Vernava AM, Wexner S. Safety and efficacy of dynamic graciloplasty for fecal incontinence: report of a prospective, multicenter trial. Dynamic Graciloplasty Therapy Study Group. *Dis Colon Rectum* 2000; **43**: 743-751 [PMID: 10859072]

97 **Matzel KE**, Madoff RD, LaFontaine LJ, Baeten CG, Buie WD, Christiansen J, Wexner S. Complications of dynamic graciloplasty: incidence, management, and impact on outcome. *Dis Colon Rectum*

2001; **44**: 1427-1435 [PMID: 11598470]
98 **Bresler L**, Reibel N, Brunaud L, Sielezneff I, Rouanet P, Rullier E, Slim K. [Dynamic graciloplasty in the treatment of severe fecal incontinence. French multicentric retrospective study]. *Ann Chir* 2002; **127**: 520-526 [PMID: 12404846]
99 **Rongen MJ**, Uludag O, El Naggar K, Geerdes BP, Konsten J, Baeten CG. Long-term follow-up of dynamic graciloplasty for fecal incontinence. *Dis Colon Rectum* 2003; **46**: 716-721 [PMID: 12794571 DOI: 10.1097/01.DCR.0000070035.45378.BD]
100 **Boyle DJ**, Murphy J, Hotouras A, Allison ME, Williams NS, Chan CL. Electrically stimulated gracilis neosphincter for end-stage fecal incontinence: the long-term outcome. *Dis Colon Rectum* 2014; **57**: 215-222 [PMID: 24401884 DOI: 10.1097/DCR.0b013e3182a4b55f]
101 **Wexner SD**, Baeten C, Bailey R, Bakka A, Belin B, Belliveau P, Berg E, Buie WD, Burnstein M, Christiansen J, Coller J, Galandiuk S, Lange J, Madoff R, Matzel KE, Påhlman L, Parc R, Reilly J, Seccia M, Thorson AG, Vernava AM. Long-term efficacy of dynamic graciloplasty for fecal incontinence. *Dis Colon Rectum* 2002; **45**: 809-818 [PMID: 12072635]
102 **Christiansen J**, Lorentzen M. Implantation of artificial sphincter for anal incontinence. *Lancet* 1987; **2**: 244-245 [PMID: 2886717]
103 **Hong KD**, Dasilva G, Kalaskar SN, Chong Y, Wexner SD. Long-term outcomes of artificial bowel sphincter for fecal incontinence: a systematic review and meta-analysis. *J Am Coll Surg* 2013; **217**: 718-725 [PMID: 23891075 DOI: 10.1016/j.jamcollsurg.2013.04.028]
104 **Altomare DF**, Binda GA, Dodi G, La Torre F, Romano G, Rinaldi M, Melega E. Disappointing long-term results of the artificial anal sphincter for faecal incontinence. *Br J Surg* 2004; **91**: 1352-1353 [PMID: 15376181 DOI: 10.1002/bjs.4600]
105 **Darnis B**, Faucheron JL, Damon H, Barth X. Technical and functional results of the artificial bowel sphincter for treatment of severe fecal incontinence: is there any benefit for the patient? *Dis Colon Rectum* 2013; **56**: 505-510 [PMID: 23478619 DOI: 10.1097/DCR.0b013e3182809490]
106 **Van Koughnett JA**, Wexner SD. Current management of fecal incontinence: choosing amongst treatment options to optimize outcomes. *World J Gastroenterol* 2013; **19**: 9216-9230 [PMID: 24409050 DOI: 10.3748/wjg.v19.i48.9216]
107 **Colquhoun P**, Kaiser R, Efron J, Weiss EG, Nogueras JJ, Vernava AM, Wexner SD. Is the quality of life better in patients with colostomy than patients with fecal incontience? *World J Surg* 2006; **30**: 1925-1928 [PMID: 16957817 DOI: 10.1007/s00268-006-0531-5]
108 **Norton C**, Burch J, Kamm MA. Patients' views of a colostomy for fecal incontinence. *Dis Colon Rectum* 2005; **48**: 1062-1069 [PMID: 15868244 DOI: 10.1007/s10350-004-0868-5]
109 **O'Kelly TJ**, Brading A, Mortensen NJ. In vitro response of the human anal canal longitudinal muscle layer to cholinergic and adrenergic stimulation: evidence of sphincter specialization. *Br J Surg* 1993; **80**: 1337-1341 [PMID: 8242318]
110 **Simpson JA**, Bush D, Gruss HJ, Jacobs A, Pediconi C, Scholefield JH. A randomised, controlled, crossover study to investigate the safety and response of 1R,2S-methoxamine hydrochloride (NRL001) on anal function in healthy volunteers. *Colorectal Dis* 2014; **16** Suppl 1: 5-15 [PMID: 24499492 DOI: 10.1111/codi.12541]
111 **Bell D**, Duffin A, Gruss HJ, Pediconi C, Jacobs A. A randomised, controlled, crossover study to investigate the pharmacodynamics, pharmacokinetics and safety of 1R,2S-methoxamine hydrochloride (NRL001) in healthy elderly subjects. *Colorectal Dis* 2014; **16** Suppl 1: 27-35 [PMID: 24499494 DOI: 10.1111/codi.12543]
112 **Bell D**, Duffin A, Jacobs A, Pediconi C, Gruss HJ. A double-blind, placebo-controlled, randomised, parallel-group, dose-escalating, repeat dose study in healthy volunteers to evaluate the safety, tolerability, pharmacodynamic effects and pharmacokinetics of the once daily rectal application of NRL001 suppositories for 14 days. *Colorectal Dis* 2014; **16** Suppl 1: 36-50 [PMID: 24499495 DOI: 10.1111/codi.12544]
113 **Gruss HJ**, Pediconi C, Jacobs A. Meta-analysis for cardiovascular effects of NRL001 after rectal application in healthy volunteers. *Colorectal Dis* 2014; **16** Suppl 1: 51-58 [PMID: 24499496 DOI: 10.1111/codi.12545]
114 **Siproudhis L**, Jones D, Shing RN, Walker D, Scholefield JH. Libertas: rationale and study design of a multicentre, Phase II, double-blind, randomised, placebo-controlled investigation to evaluate the efficacy, safety and tolerability of locally applied NRL001 in patients with faecal incontinence. *Colorectal Dis* 2014; **16** Suppl 1: 59-66 [PMID: 24499497 DOI: 10.1111/codi.12546]
115 **Kang SB**, Lee HN, Lee JY, Park JS, Lee HS, Lee JY. Sphincter contractility after muscle-derived stem cells autograft into the cryoinjured anal sphincters of rats. *Dis Colon Rectum* 2008; **51**: 1367-1373 [PMID: 18536965 DOI: 10.1007/s10350-008-9360-y]
116 **Saihara R**, Komuro H, Urita Y, Hagiwara K, Kaneko M. Myoblast transplantation to defecation muscles in a rat model: a possible treatment strategy for fecal incontinence after the repair of imperforate anus. *Pediatr Surg Int* 2009; **25**: 981-986 [PMID: 19690871 DOI: 10.1007/s00383-009-2454-3]
117 **Salcedo L**, Mayorga M, Damaser M, Balog B, Butler R, Penn M, Zutshi M. Mesenchymal stem cells can improve anal pressures after anal sphincter injury. *Stem Cell Res* 2013; **10**: 95-102 [PMID: 23147650 DOI: 10.1016/j.scr.2012.10.002]
118 **Salcedo L**, Penn M, Damaser M, Balog B, Zutshi M. Functional outcome after anal sphincter injury and treatment with mesenchymal stem cells. *Stem Cells Transl Med* 2014; **3**: 760-767 [PMID: 24797828 DOI: 10.5966/sctm.2013-0157]
119 **Raghavan S**, Miyasaka EA, Gilmont RR, Somara S, Teitelbaum DH, Bitar KN. Perianal implantation of bioengineered human internal anal sphincter constructs intrinsically innervated with human neural progenitor cells. *Surgery* 2014; **155**: 668-674 [PMID: 24582493 DOI: 10.1016/j.surg.2013.12.023]
120 **Frudinger A**, Kölle D, Schwaiger W, Pfeifer J, Paede J, Halligan S. Muscle-derived cell injection to treat anal incontinence due to obstetric trauma: pilot study with 1 year follow-up. *Gut* 2010; **59**: 55-61 [PMID: 19875391 DOI: 10.1136/gut.2009.181347]
121 **Romaniszyn M**, Rozwadowska N, Nowak M, Malcher A, Kolanowski T, Walega P, Richter P, Kurpisz M. Successful implantation of autologous muscle-derived stem cells in treatment of faecal incontinence due to external sphincter rupture. *Int J Colorectal Dis* 2013; **28**: 1035-1036 [PMID: 23549961 DOI: 10.1007/s00384-013-1692-y]
122 **Lehur PA**, McNevin S, Buntzen S, Mellgren AF, Laurberg S, Madoff RD. Magnetic anal sphincter augmentation for the treatment of fecal incontinence: a preliminary report from a feasibility study. *Dis Colon Rectum* 2010; **53**: 1604-1610 [PMID: 21178853 DOI: 10.1007/DCR.0b013e3181f5d5f7]
123 **Wong MT**, Meurette G, Wyart V, Lehur PA. Does the magnetic anal sphincter device compare favourably with sacral nerve stimulation in the management of faecal incontinence? *Colorectal Dis* 2012; **14**: e323-e329 [PMID: 22339789 DOI: 10.1111/j.1463-1318.2012.02995.x]
124 **Barussaud ML**, Mantoo S, Wyart V, Meurette G, Lehur PA. The magnetic anal sphincter in faecal incontinence: is initial success sustained over time? *Colorectal Dis* 2013; **15**: 1499-1503 [PMID: 24103055 DOI: 10.1111/codi.12423]
125 **Pakravan F**, Helmes C. Magnetic anal sphincter augmentation in patients with severe fecal incontinence. *Dis Colon Rectum* 2015; **58**: 109-114 [PMID: 25489702 DOI: 10.1097/DCR.0000000000000263]
126 **Ger R**, Condrea H, Raskin N, Addei K. Preliminary report. The transposition of a living sphincter. *J Surg Res* 1982; **33**: 69-73 [PMID: 7087449]
127 **Goldsmith HS**, Steward E. Fecal continence after abdominoperineal resection using the pedicled pyloric valve--an experimental study. *Clin Oncol* 1982; **8**: 313-317 [PMID: 7168924]
128 **Erdogan E**, Rode H, Hickman R, Cywes S. Transposition of the antropylorus for anal incontinence--an experimental model in the pig. *J Pediatr Surg* 1995; **30**: 795-800 [PMID: 7666309]
129 **Centeno Neto AA**, Veyrac M, Briand D, Spiliotis J, Saint-Aubert B, Joyeux H. Autotransplantation of the pylorus sphincter at the terminal abdominal colostomy. Experimental study in dogs. *Dis*

Colon Rectum 1991; **34**: 874-879 [PMID: 1914720]
130 **Goldsmith HS**, Chandra A. Pyloric valve transposition as substitute for a colostomy in humans: a preliminary report. *Am J Surg* 2011; **202**: 409-416 [PMID: 21545998 DOI: 10.1016/j.amjsurg.2010.11.012]
131 **Chandra A**, Kumar A, Noushif M, Gupta V, Singh D, Kumar M, Srivastava RN, Ghoshal UC. Perineal antropylorus transposition for end-stage fecal incontinence in humans: initial outcomes. *Dis Colon Rectum* 2013; **56**: 360-366 [PMID: 23392152 DOI: 10.1097/DCR.0b013e31827571ad]
132 **Chandra A**, Malhotra HS, M N, Gupta V, Singh SK, Kumar N, Lalla RS, Chandra A, Garg RK. Neuromodulation of perineally transposed antropylorus with pudendal nerve anastomosis following total anorectal reconstruction in humans. *Neurogastroenterol Motil* 2014; **26**: 1342-1348 [PMID: 25065404 DOI: 10.1111/nmo.12398]
133 **Chandra A**, Mishra B, Kumar S, Gupta V, Noushif M, Ghoshal UC, Misra A, Srivastava PK. Dynamic article: composite antropyloric valve and gracilis muscle transposition for total anorectal reconstruction: a preliminary report. *Dis Colon Rectum* 2015; **58**: 508-516 [PMID: 25850838 DOI: 10.1097/DCR.0000000000000319]
134 **Bridoux V**, Gourcerol G, Kianifard B, Touchais JY, Ducrotte P, Leroi AM, Michot F, Tuech JJ. Botulinum A toxin as a treatment for overactive rectum with associated faecal incontinence. *Colorectal Dis* 2012; **14**: 342-348 [PMID: 21689287 DOI: 10.1111/j.1463-1318.2011.02585.x]
135 **Richter HE**, Matthews CA, Muir T, Takase-Sanchez MM, Hale DS, Van Drie D, Varma MG. A vaginal bowel-control system for the treatment of fecal incontinence. *Obstet Gynecol* 2015; **125**: 540-547 [PMID: 25730213 DOI: 10.1097/AOG.0000000000000639]
136 **Scaglia M**, Delaini G, Destefano I, Hultén L. Fecal incontinence treated with acupuncture--a pilot study. *Auton Neurosci* 2009; **145**: 89-92 [PMID: 19059009 DOI: 10.1016/j.autneu.2008.10.014]

P- Reviewer: Miheller P
S- Editor: Qiu S **L- Editor**: A **E- Editor**: Jiao XK

World Journal of Gastrointestinal Pathophysiology

Submit a Manuscript: http://www.wjgnet.com/esps/
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.4291/wjgp.v7.i1.72

World J Gastrointest Pathophysiol 2016 February 15; 7(1): 72-85
ISSN 2150-5330 (online)

REVIEW

Esophageal testing: What we have so far

Nicola de Bortoli, Irene Martinucci, Lorenzo Bertani, Salvatore Russo, Riccardo Franchi, Manuele Furnari, Salvatore Tolone, Giorgia Bodini, Valeria Bolognesi, Massimo Bellini, Vincenzo Savarino, Santino Marchi, Edoardo Vincenzo Savarino

Nicola de Bortoli, Irene Martinucci, Lorenzo Bertani, Salvatore Russo, Riccardo Franchi, Valeria Bolognesi, Massimo Bellini, Santino Marchi, Gastroenterology Unit, Department of Translational Research and New Technologies in Medicine and Surgery, University of Pisa, 54124 Pisa, Italy

Manuele Furnari, Giorgia Bodini, Vincenzo Savarino, Gastroenterology Unit, Department of Internal Medicine, University of Genoa, 16146 Genoa, Italy

Salvatore Tolone, Surgery Unit, Department of Surgery, Second University of Naples, 81100 Naples, Italy

Edoardo Vincenzo Savarino, Gastroenterology Unit, Department Surgery, Oncology and Gastroenterology, University of Padua, 35128 Padua, Italy

Author contributions: de Bortoli N, Martinucci I, Bertani L, Russo S, Franchi R, Furnari M, Tolone S, Bodini G and Bolognesi V provided data collection and analysis, wrote the manuscript, approved the final version of the manuscript; Bellini M, Savarino V, Marchi S and Savarino EV reviewed the manuscript and approved the final version of the manuscript.

Conflict-of-interest statement: All the authors declare that there is no conflict of interest associated with any of the senior author or other co-authors contributed their efforts in this manuscript.

Correspondence to: Nicola de Bortoli, MD, Gastroenterology Unit, Department of Translational Research and New Technologies in Medicine and Surgery, University of Pisa, Lungarno Antonio Pacinotti, 43, 54124 Pisa, Italy. nick.debortoli@gmail.com
Telephone: +39-050-997448
Fax: +39-050-997395

Received: July 4, 2015
Peer-review started: July 12, 2015
First decision: September 22, 2015
Revised: December 2, 2015
Accepted: December 29, 2015
Article in press: January 4, 2016
Published online: February 15, 2016

Abstract

Gastroesophageal reflux disease (GERD) is a common disorder of the gastrointestinal tract. In the last few decades, new technologies have evolved and have been applied to the functional study of the esophagus, allowing for the improvement of our knowledge of the pathophysiology of GERD. High-resolution manometry (HRM) permits greater understanding of the function of the esophagogastric junction and the risks associated with hiatal hernia. Moreover, HRM has been found to be more reproducible and sensitive than conventional water-perfused manometry to detect the presence of transient lower esophageal sphincter relaxation. Esophageal 24-h pH-metry with or without combined impedance is usually performed in patients with negative endoscopy and reflux symptoms who have a poor response to anti-reflux medical therapy to assess esophageal acid exposure and symptom-reflux correlations. In particular, esophageal 24-h impedance and pH monitoring can detect acid and non-acid reflux events. EndoFLIP is a recent technique poorly applied in clinical practice, although it provides a large amount of information about the esophagogastric junction. In the coming years, laryngopharyngeal symptoms could be evaluated with up and coming non-invasive or minimally invasive techniques, such as pepsin detection in saliva or pharyngeal pH-metry. Future studies are required of these techniques to evaluate their diagnostic

accuracy and usefulness, although the available data are promising.

Key words: Gastroesophageal reflux disease; High resolution manometry; Multichannel impedance and pH; BRAVO; EndoFLIP; PEP-test; Restech

Core tip: In the last few decades, new technologies have evolved and have been applied to the functional study of the esophagus, allowing for the improvement of our knowledge of the pathophysiology of gastroesophageal reflux disease. High-resolution manometry permits a greater understanding of the function of the esophagogastric junction and the risks associated with hiatal hernia. The Chicago Classification V3.0 could define a hierarchic classification that accurately defines the major and minor disorders of esophageal motility. Esophageal 24-h pH-metry, especially when it is combined with impedance, is usually performed in patients with negative endoscopy and reflux symptoms who have a poor response to anti-reflux medical therapy to assess esophageal acid exposure and symptom-reflux correlations. In particular, esophageal 24-h impedance and pH monitoring are able to detect acid and non-acid reflux events. EndoFLIP is a recent technique poorly applied in clinical practice, although it provides a large amount of information about the esophagogastric junction. Recently, up and coming non-invasive or minimally invasive techniques, such as pepsin detection in saliva or pharyngeal pH-metry, have been suggested to detect laryngopharyngeal reflux disease. Future studies are required for these techniques to evaluate their accuracy and usefulness, although the available data are promising.

de Bortoli N, Martinucci I, Bertani L, Russo S, Franchi R, Furnari M, Tolone S, Bodini G, Bolognesi V, Bellini M, Savarino V, Marchi S, Savarino EV. Esophageal testing: What we have so far. *World J Gastrointest Pathophysiol* 2016; 7(1): 72-85 Available from: URL: http://www.wjgnet.com/2150-5330/full/v7/i1/72.htm DOI: http://dx.doi.org/10.4291/wjgp.v7.i1.72

INTRODUCTION

Gastroesophageal reflux disease (GERD) is a highly prevalent disease in Western countries, affecting up to 20% of the general population, with important impacts on health care costs and the quality of life of patients[1]. According to the Montreal Definition, GERD develops when the reflux of gastric contents causes troublesome symptoms and/or complications[2,3].

In the past decade, it was realized that, in addition to the presence of esophageal mucosal lesions (*i.e.,* erosions, intestinal metaplasia), the majority of GERD patients (approximately 70%) have typical reflux symptoms (*i.e.,* heartburn, regurgitation) without any esophageal mucosal breaks on upper endoscopy; thus, they are considered to have non-erosive reflux disease (NERD)[2,4]. In keeping with this definition, a GERD diagnosis can be based on the presence of typical symptoms only. In contrast, several recent studies have emphasized that NERD represents a heterogeneous group of patients with several pathophysiological and clinical differences, and it should be better classified using appropriate techniques able to characterize gastro-esophageal refluxate because the management and therapeutic response can change on the basis of the main mechanisms of symptom generation[5-7]. Conventional pH monitoring was first considered a useful tool to identify GERD patients, by evaluating distal esophageal acid exposure time (AET), number of acid reflux episodes and the association between symptoms and acid reflux[8,9]. However, the growing acknowledgment that factors/stimuli different from acid were involved in symptom generation in GERD has paved the way toward the search for innovative diagnostic and therapeutic approaches to GERD[10]. Moreover, the more frequent request to evaluate patients refractory to therapy with proton pump inhibitors (PPIs) has provided further impetus in this direction[11-13]. Finally, it is relevant to bear in mind the increasing referral to our outpatient clinics of subjects with extra-esophageal symptoms suggestive of GERD, such as laryngeal and pulmonary symptoms, representing a true challenge in our clinical practice due to the difficulties in evaluating the potential relationship of their symptoms and GERD with appropriate management[14,15]. In this context, the advent of novel esophageal function testing, such as impedance-pH monitoring (MII-pH) and high resolution manometry (HRM), has allowed for relevant progress in the understanding of the pathophysiological mechanisms contributing to the development of GERD and, thereafter, its diagnosis and management. Moreover, the role of new technology to detect laryngopharyngeal reflux (LPR)[16-18], as well as the presence of pepsin in clinical samples[19], deserves careful consideration. The aim of the present review article is to report on the current literature about recent advances in diagnosing GERD.

HIGH RESOLUTION MANOMETRY

GERD is primarily a motility disorder in which impairment of the esophago-gastric junction (EGJ) and ineffective esophageal motility (IEM) play an important roles[20-26].

Esophageal manometry, which assesses intraluminal esophageal pressures, peristalsis and bolus transit, is currently considered the gold standard to detect the esophageal motility abnormalities. Conventional manometry techniques record esophageal peristalsis using a catheter with 5 to 8 water-perfused channels, with or without a sleeve sensor to measure continuously the maximum lower esophageal sphincter (LES) pressure.

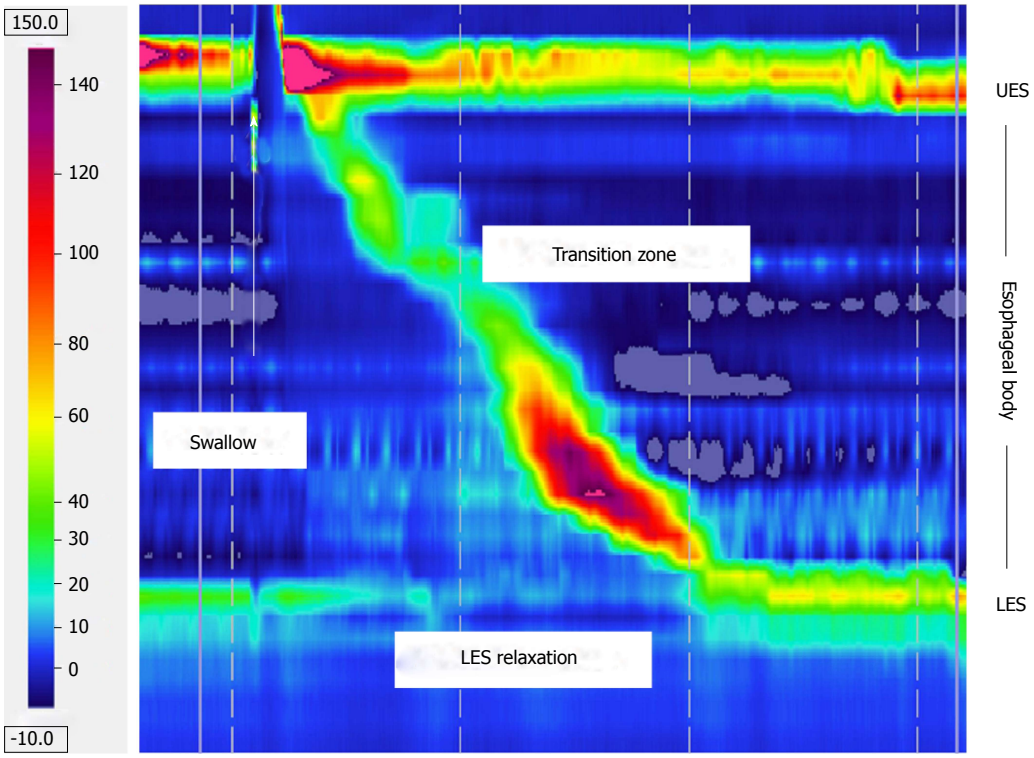


Figure 1 High resolution pressure topography of a peristaltic wave. In the picture, both the upper and lower esophageal sphincters and swallowing-induced relaxation of the lower esophageal sphincter are well represented. UES: Upper esophageal sphincter; LES: Lower esophageal sphincter.

HRM was described for the first time in 1991, introducing an increased number of pressure sensors along the catheter and the use of spatio-temporal plots[27,28], leading to the subsequent development of the Chicago Classification for primary esophageal motility disorders[29,30]. In HRM systems, multiple sensors (up to 36) are distributed longitudinally and radially, closely spaced along the length of the manometric catheter. Two main types of manometric catheters are currently available, solid state and water perfused, each with different physical and performance characteristics and specific advantages and disadvantages concerning costs, preparation, the location of transducers, autoclave possibility and the rate of pressure increase. Nevertheless, HRM allows for simultaneous pressure readings within both the sphincters and the esophageal body, providing detailed esophageal pressure topography (EPT) (Figure 1). With HRM, pull-through techniques became unnecessary and several problems, such as artifacts attributable to swallow-induced sphincter movement[31] or EGJ conformational changes that can spontaneously occur, were overcome[32-34]. RM provides a dynamic representation of the pressure within and across the EGJ, and it also creates opportunities to quantify more precise measurement of EGJ relaxation and morphology[25,26,31], providing the opportunity to detect expiratory LES pressure and crural diaphragm (CD) contraction[35-39]. On this basis, the EGJ was recently reviewed and classified into three types: Type Ⅰ (no LES-CD separation); type Ⅱ (the LES and CD are spatially separated such that there is a double-peaked pressure profile, but the nadir pressure between LES and CD does not decrease to gastric pressure); an type Ⅲ (the separation between peaks is > 2 cm, and the nadir pressure between LES-CD is equal to or less than the gastric pressure; in type Ⅲa, the pressure inversion point is located at the CD, while in type Ⅲb, it is placed at the LES level[30,36].

Pandolfino *et al*[36] compared the EPT attributes of the EGJ between 156 GERD patients and 75 asymptomatic controls. Although both lower LES pressure and greater LES-CD separation were associated with GERD, impaired CD function was most strongly associated factor and the only independent predictor of GERD. A study designed to analyze the relationship between obesity and the morphology of the EGJ pressure segment showed that obese subjects were more likely to have a spatial separation between the diaphragm and LES (Figure 2) and an augmented gastroesophageal pressure gradient[37]. These findings might partially explain why gastro-esophageal reflux is more frequent in obese subjects, as also evidenced in patients with NERD[40]. Bredenoord *et al*[32] showed that, in cases of small hiatal hernias in which intermittent reduction of the hernia frequently occurs, spatial separation of the CD and LES in the non-reduced state resulted in a 2-fold increase in acid and weakly acidic reflux.

Transient LES relaxations (TLESRs) are the most common mechanism of reflux. They occur independently from swallowing and are not accompanied by peristalsis,

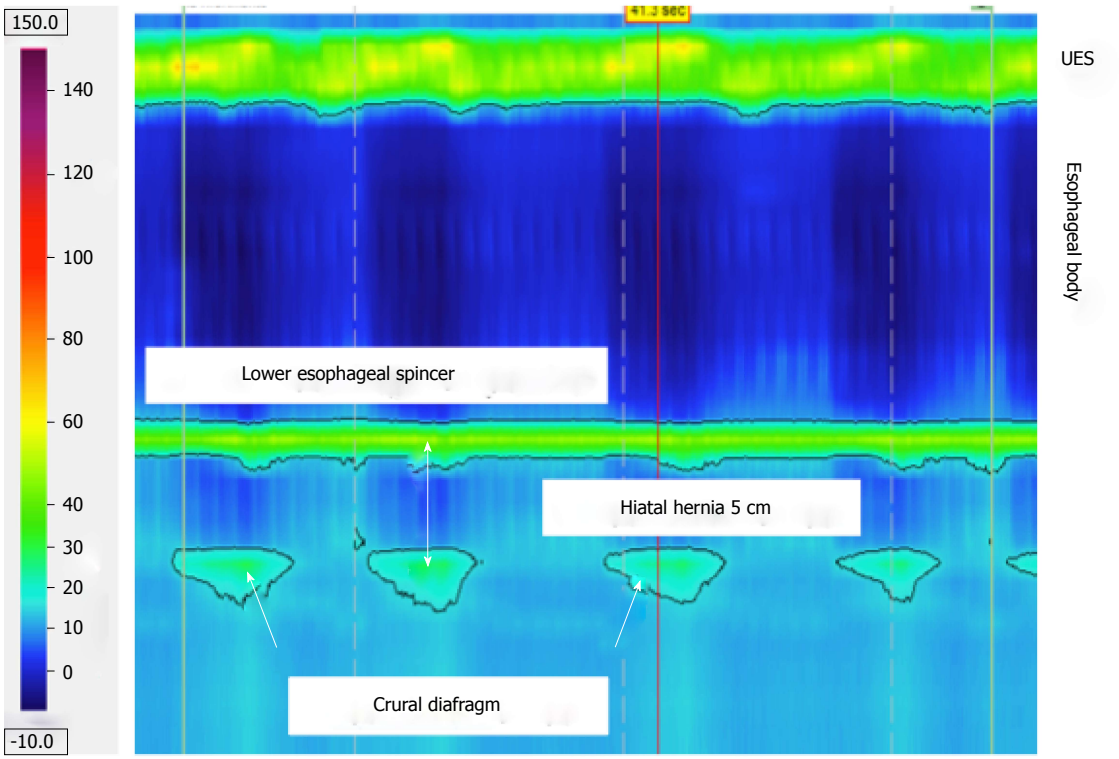


Figure 2 High resolution pressure topography at 30 s resting pressure calculation. In the picture, both the upper and lower esophageal sphincters and the crural diaphragm are well represented. This picture represents a large hiatal hernia (5 cm). UES: Upper esophageal sphincter.

but they are accompanied by diaphragmatic inhibition, and they persist for longer periods than swallow-induced LES relaxations (> 10 s)[41,42]. In a recent study, Roman *et al*[43] demonstrated that HRM is reproducible and more sensitive than perfused-sleeve manometry to detect TLESRs, providing better inter-observer agreement. Notably, in GERD patients, there is not an increased frequency of TLESR compared with controls but only a greater frequency of acid reflux during TLESRs[44]. Bredenoord *et al*[45] investigated the factors associated with reflux during TLESRs but no differences were observed in TLESR duration, trans-sphincteric pressure gradient, the prevalence, duration and amplitude of esophageal pre-contractions or sphincteric post-contractions. Pandolfino *et al*[35] studied the largest number of TLESRs in the postprandial period. They observed that the key events associated with EGJ opening were CD inhibition, LES relaxation, distal esophageal muscular contraction and a positive gradient between the stomach and the esophagus, but in only a few cases was manometric signature of EGJ opening associated with evidence of reflux on pH-metry.

It has been shown that 21%-38% of patients with GERD present with severely impaired esophageal peristalsis (Figure 3), resulting in more severe reflux, slower acid clearance, worse mucosal injury, and more frequent respiratory symptoms[46]. The Chicago Classification V3.0 approved the term "IEM", which is frequently used in conventional manometry[30]. In this recent Chicago Classification version, IEM was defined as 5 or more ineffective swallows of 10 with a DCI threshold of 450 mmHg-s-cm. No distinction need be made between failed swallows (DCI < 100 mmHg-s-cm) and weak swallows (450 mmHg-s-cm)[30].

Multiple rapid swallowing (MRS) as a provocative test has been suggested to diagnose IEM (in borderline conditions). MRS consists of administering 2 mL of water five times, for a total amount of 15 mL of water, in less than 10 s. MRS inhibits the esophageal body and LES during the first four swallows, and it is normally followed by an esophageal contraction of increased amplitude. It was suggested that many patients with suspected IEM had normalized esophageal contraction amplitude after MRS[47].

Manometric studies have described decreased pressure between striated and smooth esophageal muscle[48]. Pohl *et al*[49] correlated the size of the esophageal low-pressure zone and its possible relationship with esophageal symptoms (dysphagia, chest pain, and heartburn/regurgitation).

In conclusion, HRM is faster and easier to perform than conventional water-perfused manometry. Moreover, HRM does not require time-consuming pull-through maneuvers, and it allows for accurate evaluation of the intrinsic and extrinsic components of EGJ, thus improving the identification of TLESRs. However, the clinical application of this technique in GERD remains very limited; TLESRs cannot be used in the diagnosis of GERD because their prevalence is similar between GERD patients and normal subjects. Furthermore, minor

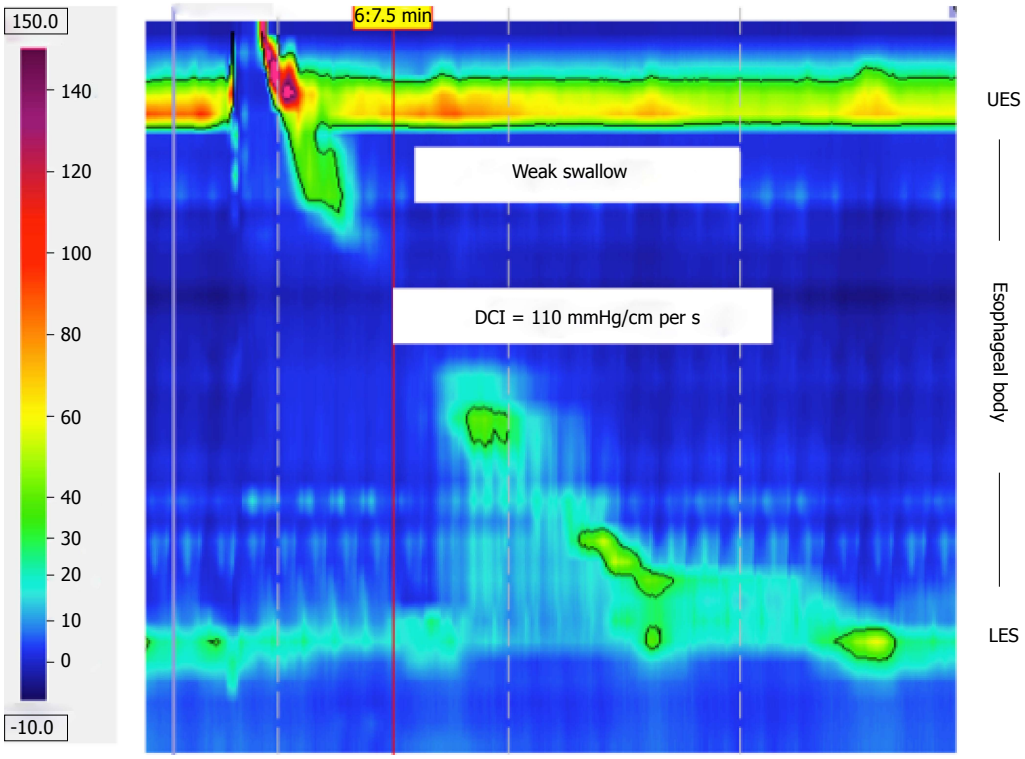


Figure 3 High resolution pressure topography of a weak peristaltic wave (distal contractile integral 110 mmHg-s-cm). UES: Upper esophageal sphincter; LES: Lower esophageal sphincter; DCI: Distal contractile integral.

esophageal motility abnormalities, observed in GERD patients, are not specific and can either be primarily or secondarily related to GERD[50]. However, HRM represents an important advance in the assessment of esophageal motor function and a promising technique for the evaluation of the mechanical abnormalities involved in GERD. Further studies are needed to evaluate additional possible benefits of this technique in clinical practice.

MULTICHANNEL INTRALUMINAL IMPEDANCE AND PH

Multichannel intraluminal impedance (MII) has been promoted to detect the movement of fluid, solid, and air in the esophagus regardless of its pH[51].

This new device, which combines MII-pH analysis, provides a sophisticated characterization of reflux episodes over a 24-h period. The most common catheter allows for 6 channels for intraluminal impedance (at 3, 5, 7, 9, 15, and 17 cm) and a pH sensor (at 5 cm above the upper border of the LES)[52].

MII-pH is an innovative technique that provides a detailed characterization of each reflux event including chemical (acid and non-acid reflux) and physical properties (liquid, mixed, gas) (Figure 4)[53]. To date, non-acid reflux represents the majority of reflux episodes in patients with GERD on PPI therapy[54,55]. Indeed, the total number of reflux episodes is not affected by acid suppressive therapy, and weakly acidic reflux accounts for approximately 90% of all reflux episodes in patients on PPIs, thus representing a potential mechanism underlying the failure of PPI treatment in patients with reflux-related symptoms[56,57]. Moreover, MII-pH monitoring, as well as pH-metry alone, provides the opportunity to assess the temporal relationship between the occurrence of refluxes and the onset of symptoms[58,59]. The relationship between symptoms and reflux events can be evaluated with the symptom index (SI) and symptom association probability (SAP), which are the most commonly used symptom indices[59].

Based on esophageal pH monitoring, NERD patients with physiological esophageal AET and a close temporal relationship between symptoms and reflux events have been defined as hypersensitive to acid stimuli. In contrast, in agreement with the Rome Ⅲ criteria, patients with heartburn, normal upper endoscopy, physiological AET, and negative correspondence between symptoms and reflux and who fail to respond to PPIs are defined as having functional heartburn (FH)[8,9,60]. In this regard, the advent of MII-pH monitoring has improved the diagnostic yield of GERD patients, mainly by identifying a positive SAP or SI with weakly acidic or non-acid reflux[61-68]. In particular, if pH-metry and the patient's response to PPI therapy are compared with MII-pH, we can observe an underestimation of GERD patients[69,70].

In contrast, all of the available diagnostic tests for GERD have some limitations. The drawbacks of MII-pH are mainly due to the day-to-day variability of the test[71-73]. Additionally, the reflux-symptom correlation in patients with GERD who do not respond to PPI therapy

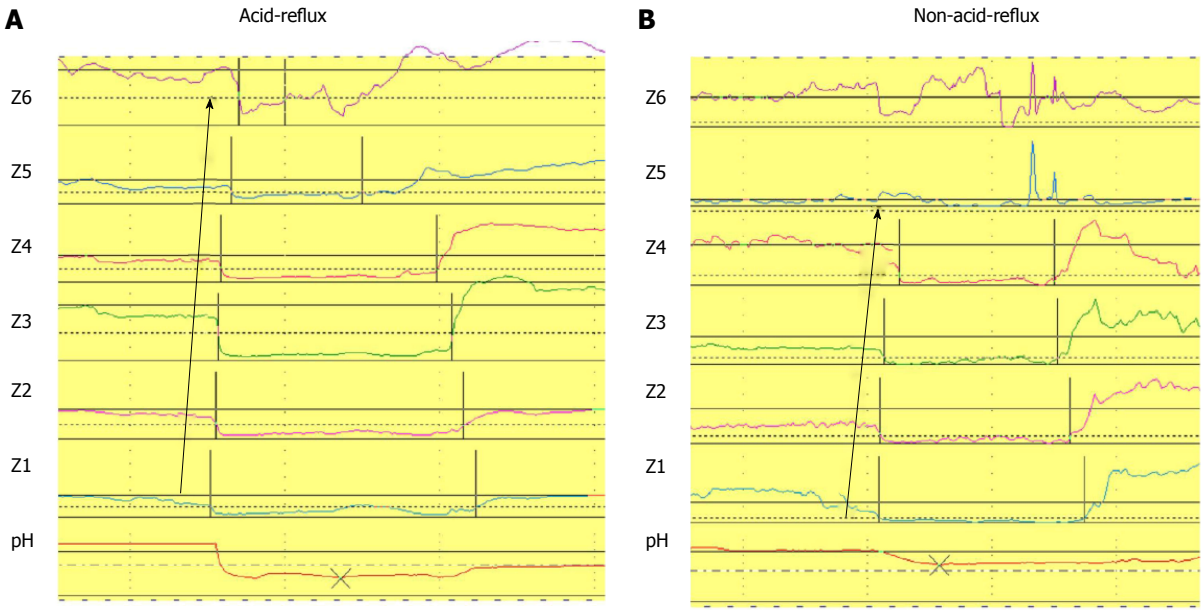


Figure 4 Graphic representation of MII-pH reflux events: (A) acid-proximal reflux; (B) non-acid distal reflux. Z1-Z6: Impedance channel; pH: Channel for pH recording.

is actually also calculated by the SI or SAP if its validity is still uncertain[74,75]. Recently Zerbib *et al*[76] reported that MII-pH findings were not always able to predict the response to PPIs in patients with typical reflux-related symptoms when the test is performed off PPI therapy.

Regarding the clinical utility of pathophysiological investigations in patients with heartburn, we described a group of patients (more than 19% of the whole population enrolled) with heartburn who completely responded to PPI, in whom GERD was not diagnosed with the MII-pH test[5]. Thus, our data suggested that PPI response alone should not be always considered a good predictor of a GERD diagnosis[5]. Overall, it is notable that NERD patients vary greatly from a pathophysiological point of view and should be accurately studied by means of MII-pH to undertake the best therapeutic approach[6]. Indeed, a meta-analysis found that the once defined low response rate in NERD was likely the result of the inclusion of patients with heartburn who did not have reflux disease[77].

Recently, the ability to perform MII-pH testing to understand GERD pathophysiology better has improved through the introduction of up and coming parameters, such as the post-reflux swallow-induced peristaltic wave (PSPW) index, which indicates the efficacy of esophageal clearance[78], and baseline impedance values, which indicate the presence of a lack of integrity in the esophageal mucosa[79]. Constant changes in esophageal chemical clearance could represent a specific mechanism involved in GERD pathophysiology. PSPW has been shown to be lower in patients with abnormal AET, compared to healthy volunteers (HVs) or FH patients. Moreover, this parameter was not altered after medical or surgical therapy[78]. Further, Kessing *et al*[80] described lower values of baseline impedance levels in the distal esophagus of patients with abnormal esophageal AET, compared to HVs. The authors described a negative correlation between baseline impedance levels and esophageal AET[80]. We recently described a large group of patients with typical GERD symptoms, negative endoscopy and any pathophysiological characteristics of GERD (normal AET, number of refluxes and negative SI and SAP). We observed that patients with good symptom relief after PPI therapy had lower baseline impedance values than FH patients (non-responders). FH patients showed similar baseline values to HVs. Moreover, we observed almost the same results when analyzing the PSPW index, which was lower in responders than in non-responders and HV groups. A direct linear correlation between PSPW and baseline impedance values has been described. Overall, these data suggest that baseline impedance values and PSPW could be considered up-and-coming parameters that could be helpful in better investigating patients with GERD-related symptoms, particularly when symptom-reflux association indexes fail to do so[81].

MII-pH testing showed that acid reflux events and their clearance were determinant factors that provoked esophageal mucosal breaks. Non-acid reflux does not appear to be directly related to the development of esophageal mucosa lesions; however, it is definitely involved in the genesis of symptoms in both NERD and erosive esophagitis (EE) patients. Ambulatory MII-pH studies have suggested that patients with moderate or severe esophagitis have rates of weakly acidic reflux similar to or slightly greater than healthy controls[69]. In this regard, it is important to emphasize that weakly acidic reflux is not synonymous with bile reflux. A simultaneous Bilitech and MII-pH study showed no relationship between the percentage of time for bilirubin

absorbance and weakly acidic or weakly alkaline reflux. Indeed, this study showed that the greatest number of bile refluxes occurred concomitantly with acid refluxes[82].

In patients with Barrett's esophagus (BE), MII-pH testing showed overall more severe reflux disease with a greater number of acid and weakly acidic reflux events and higher proximal extension[71]. By means of MII-pH, Savarino *et al*[71] showed that patients with BE and EE had greater numbers of acid and weakly acidic reflux episodes, higher percentages of proximal migration of the refluxate and higher total acid and volume clearance. Notably, it has been emphasized that a significantly increased amount of total reflux occurs in both the long and short segments of BE, compared with EE. In BE patients, the MII-pH tracks are not easy to analyze, especially in the long segment of BE. Inflammation and histologic modifications are supposed to reduce baseline impedance values thus impairing the ability to detect the real number of reflux episodes. This phenomenon has led some investigators to be more accurate during the manual analysis of MII-pH in patients with BE[62]. However, some authors have shown the viability of MII-pH even in BE patients[71,83]. In clinical practice, BE patients should be evaluated with MII-pH "on" PPI therapy to evaluate the effectiveness of acid suppression.

The presence of EE, which represents clear endoscopic evidence of esophageal mucosal injury (30% of all endoscopic series in patients with GERD-related symptoms), can obviously be responsible for symptoms provoked by the refluxate. In contrast, the absence of macroscopic damage makes it difficult to clarify the occurrence of the same symptoms in patients with NERD, and it is reasonable to hypothesize that microscopic damage is responsible for the GERD-related symptoms in them[84]. According to this assumption, recent investigations have focused their attention on the evaluation of the presence of dilated intercellular spaces, considered the most important microscopic alteration involved in symptom perception in patients with GERD[85]. Recently Savarino *et al*[86] showed that the frequency of microscopic esophagitis did not differ between patients with FH and control subjects and was significantly lower in FH patients than in the EE and well-defined NERD patients evaluated with MII-pH.

The overall MII-pH assessment "on" or "off" PPI therapy is actually a matter of discussion. In a recent seminar, Bredenoord *et al*[52] affirmed that combined MII-pH is better performed off PPIs when the diagnosis of GERD has not yet been established and on-PPIs when the diagnosis has been already made (*i.e.*, positive endoscopy for EE, MII-pH off-therapy already performed, BE surveillance). In particular, MII-pH off PPIs is useful to investigate the causes of ineffective PPI treatment. To confirm the presence of GERD, Hemmink *et al*[87] reported that PPI-resistant patients should preferably be evaluated with MII-pH monitoring after cessation of PPI treatment. This approach could increase the likelihood of observing a positive relationship between symptoms and reflux.

GERD is considered an important cause of laryngeal inflammation. Laryngoscopic examinations are very important to exclude abnormalities in the laryngeal mucosa or vocal cords (nodules or neoplastic lesions), but it is not sufficiently specific/sensitive to detect LPR[88]. We recently described that MII-pH analysis detected GERD at least in 40% of patients who were diagnosed with LPRD. In particular, MII-pH analysis was able to identify patients with NERD, those with HE and those without GERD, whereas laryngeal symptoms and laryngoscopic findings were not able to do so[89].

Finally, some interesting studies, performed by means of MII-pH analysis, evaluated the effectiveness of raft-forming gel formulation in "add-on" treatment for GERD symptoms. These studies have clearly demonstrated the efficacy of raft-forming gel preparation in reducing the total number of refluxes and their proximal migration[90-92].

To conclude, MII-pH is able to improve the ability to detect GERD compared with pH-metry alone in patients with GERD-related symptoms. MII-pH is able to provide more information and to exclude GERD diagnosis definitively in PPI non-responder patients (FH). The outcomes studies are unmet clinical needs for determining whether MII-pH truly leads to a change in management.

Combined multichannel intraluminal impedance and manometry has been considered a very helpful device because it provides information about esophageal contraction and bolus transit simultaneously. Tutuian *et al*[93] described results from a large cohort of patients (350), showing a very strong correlation between dysphagia and incomplete bolus transit. Moreover, the largest defects of bolus transit were correlated with diagnoses of achalasia, scleroderma, distal esophageal spasm and IEM. Outcome studies are needed to clarify better the role of this technique in clinical diagnosis.

WIRELESS PH CAPSULE (BRAVO)

The wireless pH capsule (BRAVO) is a novel technology able to assess esophageal exposure to acid. This device is placed transorally 6 cm above the EGJ during an upper endoscopy, usually under sedation, or 5 cm above the LES, previously detected by means of esophageal manometry[94]. The capsule transmits recording data via telemetry to an external receiver. This method is usually performed after the failure of a PPI test, alternatively at MII-pH or during an upper endoscopy before the PPI test. Capsule detachment occurs spontaneously in most cases between 2-3 d and 2 wk[95]. Adverse events or complications (severe or persistent chest pain) are very rare (1% to 2%)[94]. The technique is usually well tolerated, and the principal side effect reported is mild to moderate chest pain. The recording time of the wireless capsule is usually 48 h, showing overall good reproducibility between the 2 d of recording[96]. However, several studies have shown a relevant day-

to-day variability responsible for discrepancies in AET between the first and the second days of recording[97,98]. The reasons for these findings remain controversial and are the subject of debate.

A great day-to-day variability in AET might be frequent during 24-h pH-metry testing. pH monitoring prolonged to 48 h is routinely performed with the BRAVO technique, and it should provide a better overview of esophageal acid exposure and symptom-reflux correlation.

The prolonged pH-monitoring period increases the likelihood of detecting reflux events (12.5% increase), potentially improving symptom association (5.2% increase)[99]. These data were confirmed in patients on anti-reflux therapy[99], in patients with endoscopy negative heartburn[100] and in patients with non-cardiac chest pain[101].

Overall, BRAVO is a good method for reflux monitoring, especially in patients with less frequent symptoms (*i.e.*, non-cardiac chest pain) and in those who refuse catheter-based techniques, but it has some limitations, including higher cost than MII-pH and the possible risk of misplacement; more importantly, non-acid reflux events cannot be detected.

ENDOFLIP

An important property of the reflux barrier that is not assessed by manometry is distensibility (*i.e.*, the ease with which the EGJ is opened)[102]. If pressure and radius can be measured simultaneously in the LES, the circumferential tension in the wall can be estimated, as it has been firstly demonstrated by McMahon *et al*[103].

The EndoFLIP (Endo Functional Luminal Imaging Probe system; Crospon Ltd., Galway, Ireland) consists of a polyurethane balloon (maximum volume of 60 mL) mounted on the distal 14 cm of a probe (length 240 cm, diameter 25 mm) attached to the EndoFLIP unit. This balloon assumes a 10-cm long cylindrical shape with maximum diameter of 2.5 cm when filled. Along a 7.5-cm segment within the balloon, 17 ring electrodes are spaced 5 mm apart to obtain 16 CSA measurements using an impedance planimetry technique. The probe also contains a solid-state pressure transducer to measure intra-balloon pressure.

The EndoFLIP system provides real-time and dynamic information on EGJ distention that is visualized as cylinders of different diameters, based on instantaneous CSA measurements (included instant pressure measurements).

Using this technique, Kwiatek *et al*[104] compared GERD patients with healthy controls, with the following major findings: (1) the EGJ was 2 to 3 times more distensible in GERD patients than controls; (2) 20- to 30-mL distention volumes provided, in patients with GERD, a two- to three-fold increased EGJ distensibility, compared with controls; and (3) the endoscopic estimation of the flap valve grade seemed poorly correlated with EndoFLIP measurements.

Recently, Regan *et al*[105] used the EndoFLIP system to measure upper esophageal sphincter (UES) distensibility. EndoFLIP provided definitive information regarding UES compliance without the need for fluoroscopy. The results regarding the anatomic parameters and physiology of UES were directly matched with current knowledge. Few data are yet available, but EndoFLIP could be able to provide determinant information about UES in patients with dysphagia before and after surgery or rehabilitation.

DX-PH (RESTECH)

As mentioned above, the diagnosis and treatment of GERD are particularly difficult in cases of LPR[106].

Some authors have described LPR by means of dual-channel impedance and pH monitoring[107,108]. These studies have described the frequency, location, and direction of any gas or liquid refluxate along the esophagus, as well as in the hypopharynx[107]. Despite initial enthusiasm[108,109], outcome studies with impedance monitoring have been lacking, and their clinical significance with regard to medically recalcitrant LPR patients remains unclear[110].

The Dx-pH measurement system, called Restech (Respiratory Technology Corp., San Diego, CA, United States), was designed to detect aerosolized acid, thus identifying patients whose symptoms are due to acidic mist or liquid refluxing into the pharynx. Because the distal part containing the pH sensor does not traverse the UES, it is more comfortable than a conventional pH catheter, and it does not require esophageal manometry[17].

Restech has a higher frequency of measurement than older catheter-based pH probes and wireless pH probes, with a pH measurement obtained every 0.5 s (sampling rate, 2 Hz), compared with traditional catheter-based pH probes, which sample every 4 s to 5 s (at a rate of 0.2-0.25 Hz), and wireless pH probes, with a pH measurement every 6 s (0.17 Hz). This increased sampling rate theoretically allows Restech to detect more reflux events, which is a characteristic that could prove useful in patients with LPR[111].

Recently, Worrell *et al*[112] demonstrated, that in patients with extraesophageal reflux symptoms who underwent antireflux surgery, esophageal pH monitoring in the proximal esophagus failed to recognize 50% of the patients who recorded good outcomes post-antireflux surgery. Vailati *et al*[113] showed the high specificity and reasonable sensitivity of the Restech technique, which could be considered an interesting tool that can be used before therapy in patients with pharyngoesophageal reflux. In contrast, Ummarino *et al*[114] did not show any correlation, although chronic coughing was the only symptom reported by patients, and when they compared MII-pH and Restech, the superiority of the first technique seemed clear. Similarly, Becker *et al*[115] evaluated, in a prospective, single-center trial, the differences between MII-pH and Dx-pH. They demonstrated that acid pharyngeal pH levels detected with Dx-pH were not correlated with GERD, and acid

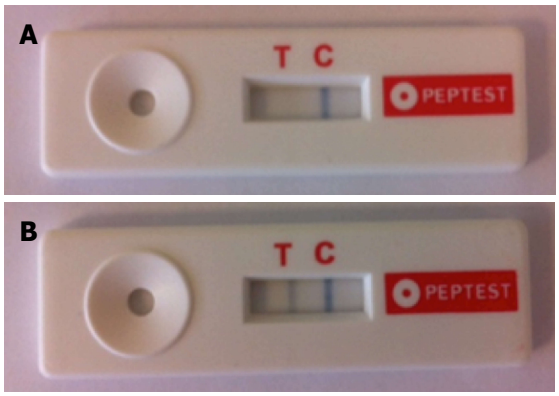


Figure 5 The picture shows the lateral flow device test to detect the presence of pepsin in saliva (PEP-test; RDBiomed, Hull, United Kingdom). A: A negative test indicates that no pepsin was detected in clinical sample (only C line is colored); B: A positive test, it indicates that pepsin is present in the sample (saliva/sputum). In the both T and C line are colored.

esophageal reflux episodes did not result in pharyngeal pH alterations. Mazzoleni *et al*[116], observed a very poor correlation between Dx-pH probe oropharyngeal monitoring and MII-pH in a group of 68 patients.

Until now, the use of Dx-pH recordings could not be recommended in clinical practice, given the discrepancies between traditional MII-pH monitoring and the current technologies used to measure oro-pharyngeal pH events.

PEP-TEST

Recent studies have shown that patients with LPR do not reap great benefit from PPI therapy because, at this level, the damage is not mediated by acid but rather by pepsin[117]. For this reason, it was recently decided to search for pepsin as a marker of reflux because the enzyme is secreted only by gastric main cells[118]; therefore, its presence above UES is a certain sign of GERD.

The pepsin lateral flow device (LFD), also called the PEP-Test (RDBiomed, Hull, United Kingdom), is a rapid, non-invasive test to detect salivary pepsin as a surrogate marker for GERD. The PEP-Test is an immunological *in-vitro* diagnostic medical device that contains two pepsin monoclonal antibodies; this test is able to detect pepsin in a clinical sample of saliva/sputum quickly and easily; the limit of detection is 16 ng/mL of pepsin. The patient must collect 30 mL of saliva, and this sample is centrifuged at 4000 rpm for 5 min; then, 80 μL from the surface layer are drawn up and mixed with a buffer solution using a vortex mixer. The sample obtained is applied in the circular well of the PEP-Test device, and after a few minutes, it is possible to check for the presence of pepsin: If it occurs on antigen-antibody binding, two blue lines appear on the display, while if pepsin is not present, it will be possible to see only the control line. The blue lines are visible on the display because the monoclonal antibody is directly labelled with blue latex beads, which detach at the time of Ag-Ab binding. Saritas Yuksel *et al*[19] evaluated the PEP-Test (defined as ELISA LFD) in 47 patients using pH-metry as a reference standard. The results were compared with a control population, which underwent only the PEP-Test, which has sensitivity and a specificity of 87% (Figure 5).

More recently Hayat *et al*[119] compared the PEP-test with MII-pH and showed a higher prevalence and concentration of salivary pepsin in patients with GERD or HE, compared with patients with FH and healthy controls. The authors suggested that salivary pepsin might complement questionnaires in the office-based diagnosis of patients with GERD-related symptoms.

The most important advantages of the PEP-Test are its low cost, its non-invasiveness, and, not to be underestimated, its feasibility while the patient is undergoing PPI therapy. However, given the very limited data available in the medical literature, further studies are needed to understand and assess the clinical utility of this test.

CONCLUSION

The reflux of gastric contents into the esophagus results in a broad and varied GERD spectrum. Thus, advances in the diagnosis of GERD represent the necessary progress in clinical investigation and therapeutic management as well as progress for more-in-depth assessment of the underlying pathophysiological mechanisms.

The pathogenesis of GERD is multifactorial, including esophageal motility abnormalities of which the most important are TLESRs and hypotensive LES and IEM. Recently, the role of esophageal dysmotility has gained relevant interest, showing an increased prevalence with increasing severity of GERD, from NERD to EE and BE[120]. To date, HRM is the gold standard for characterizing esophageal motility disorders. Indeed, HRM improves characterization of LES zones and esophageal body motility, increasing diagnostic yield and accuracy[121]. Moreover, HRM must be regarded as the new gold standard for detecting TLESRs[43]. However, the value of HRM in clinical practice has yet to be established fully Exclusion of severe esophageal motility disorders (*i.e.*, achalasia) is important because such diseases can present with heartburn and regurgitation, which could lead to an erroneous diagnosis of GERD[122].

Ambulatory MII-pH has become the gold standard for investigating GERD, owing mainly to its ability to detect both acid and non-acid reflux[68,123]. Of particular importance, MII-pH has enabled the subdivision of patients with NERD into different subsets, including patients with an excess of acid and those with symptomatic non-acid reflux, and MII-pH has the ability to identify patients with FH whose symptoms are not GERD related and who must be excluded from the realm of GERD[6,56,109].

Moreover, diagnosing patients with HE to non-acid reflux, MII-pH has contributed to narrowing down the population of patients with FH[70]. Recently, it has been

demonstrated that a more-in-depth pathophysiological evaluation of MII-pH tracings, including baseline impedance levels and PSPW index evaluation, could be helpful in better investigating patients with heartburn and appropriately identifying those with reflux disease and particularly those with HE, when symptom-reflux association analysis fails to do so[5,78,81].

According to the Montreal Classification, extra-esophageal symptoms of GERD can occur, such as hoarseness, coughing, and asthma[2]. However, establishing that extra-esophageal symptoms caused by GERD can be difficult, and in this context, the advent of new technologies deserves careful consideration. The Dx-pH measurement system is a sensitive and minimally invasive device for detecting acidic refluxate in the oropharynx. The PEP-Test is a sensitive and specific *in vitro* diagnostic test that allows for the rapid detection of pepsin, a marker of reflux, in a clinical sample. Overall, further studies are warranted to substantiate the clinical roles of these new technologies in diagnosing GERD.

REFERENCES

1 **Dent J**, El-Serag HB, Wallander MA, Johansson S. Epidemiology of gastro-oesophageal reflux disease: a systematic review. *Gut* 2005; **54**: 710-717 [PMID: 15831922 DOI: 10.1136/gut.2004.051821]

2 **Vakil N**, van Zanten SV, Kahrilas P, Dent J, Jones R. The Montreal definition and classification of gastroesophageal reflux disease: a global evidence-based consensus. *Am J Gastroenterol* 2006; **101**: 1900-1920; quiz 1943 [PMID: 16928254]

3 **Fuchs KH**, Babic B, Breithaupt W, Dallemagne B, Fingerhut A, Furnee E, Granderath F, Horvath P, Kardos P, Pointner R, Savarino E, Van Herwaarden-Lindeboom M, Zaninotto G. EAES recommendations for the management of gastroesophageal reflux disease. *Surg Endosc* 2014; **28**: 1753-1773 [PMID: 24789125 DOI: 10.1007/s00464-014-3431-z]

4 **Modlin IM**, Hunt RH, Malfertheiner P, Moayyedi P, Quigley EM, Tytgat GN, Tack J, Heading RC, Holtman G, Moss SF. Diagnosis and management of non-erosive reflux disease--the Vevey NERD Consensus Group. *Digestion* 2009; **80**: 74-88 [PMID: 19546560]

5 **de Bortoli N**, Martinucci I, Savarino E, Bellini M, Bredenoord AJ, Franchi R, Bertani L, Furnari M, Savarino V, Blandizzi C, Marchi S. Proton pump inhibitor responders who are not confirmed as GERD patients with impedance and pH monitoring: who are they? *Neurogastroenterol Motil* 2014; **26**: 28-35 [PMID: 23992024 DOI: 10.1111/nmo.12221]

6 **Savarino E**, Zentilin P, Savarino V. NERD: an umbrella term including heterogeneous subpopulations. *Nat Rev Gastroenterol Hepatol* 2013; **10**: 371-380 [PMID: 23528345 DOI: 10.1038/nrgastro.2013.50]

7 **Giacchino M**, Savarino V, Savarino E. Distinction between patients with non-erosive reflux disease and functional heartburn. *Ann Gastroenterol* 2013; **26**: 283-289 [PMID: 24714313]

8 **Martinez SD**, Malagon IB, Garewal HS, Cui H, Fass R. Non-erosive reflux disease (NERD)--acid reflux and symptom patterns. *Aliment Pharmacol Ther* 2003; **17**: 537-545 [PMID: 12622762]

9 **Fass R**, Fennerty MB, Vakil N. Nonerosive reflux disease--current concepts and dilemmas. *Am J Gastroenterol* 2001; **96**: 303-314 [PMID: 11232668 DOI: 10.1111/j.1572-0241.2001.03511.x]

10 **Woodland P**, Sifrim D. The refluxate: The impact of its magnitude, composition and distribution. *Best Pract Res Clin Gastroenterol* 2010; **24**: 861-871 [PMID: 21126699 DOI: 10.1016/j.bpg.2010.09.002]

11 **Sifrim D**, Zerbib F. Diagnosis and management of patients with reflux symptoms refractory to proton pump inhibitors. *Gut* 2012; **61**: 1340-1354 [PMID: 22684483 DOI: 10.1136/gutjnl-2011-301897]

12 **Smout AJ**. The patient with GORD and chronically recurrent problems. *Best Pract Res Clin Gastroenterol* 2007; **21**: 365-378 [PMID: 17544105 DOI: 10.1016/j.bpg.2007.01.007]

13 **Furnari M**, de Bortoli N, Savarino V, Marchi S, Savarino E. Not all anti-reflux treatment failures are due to persistence of abnormal esophageal acid exposure. *Surg Endosc* 2014; **28**: 1382-1383 [PMID: 24162141 DOI: 10.1007/s00464-013-3291-y]

14 **Savarino E**, Carbone R, Marabotto E, Furnari M, Sconfienza L, Ghio M, Zentilin P, Savarino V. Gastro-oesophageal reflux and gastric aspiration in idiopathic pulmonary fibrosis patients. *Eur Respir J* 2013; **42**: 1322-1331 [PMID: 23471347 DOI: 10.1183/09031936.00101212]

15 **Savarino E**, Bazzica M, Zentilin P, Pohl D, Parodi A, Cittadini G, Negrini S, Indiveri F, Tutuian R, Savarino V, Ghio M. Gastroesophageal reflux and pulmonary fibrosis in scleroderma: a study using pH-impedance monitoring. *Am J Respir Crit Care Med* 2009; **179**: 408-413 [PMID: 19096004 DOI: 10.1164/rccm.200808-1359OC]

16 **Martinucci I**, de Bortoli N, Savarino E, Nacci A, Romeo SO, Bellini M, Savarino V, Fattori B, Marchi S. Optimal treatment of laryngopharyngeal reflux disease. *Ther Adv Chronic Dis* 2013; **4**: 287-301 [PMID: 24179671 DOI: 10.1177/2040622313503485]

17 **Wiener GJ**, Tsukashima R, Kelly C, Wolf E, Schmeltzer M, Bankert C, Fisk L, Vaezi M. Oropharyngeal pH monitoring for the detection of liquid and aerosolized supraesophageal gastric reflux. *J Voice* 2009; **23**: 498-504 [PMID: 18468849 DOI: 10.1016/j.jvoice.2007.12.005]

18 **Savarino E**, Zentilin P, Savarino V, Tenca A, Penagini R, Clarke JO, Bravi I, Zerbib F, Yüksel ES. Functional testing: pharyngeal pH monitoring and high-resolution manometry. *Ann N Y Acad Sci* 2013; **1300**: 226-235 [PMID: 24117645 DOI: 10.1111/nyas.12255]

19 **Saritas Yuksel E**, Hong SK, Strugala V, Slaughter JC, Goutte M, Garrett CG, Dettmar PW, Vaezi MF. Rapid salivary pepsin test: blinded assessment of test performance in gastroesophageal reflux disease. *Laryngoscope* 2012; **122**: 1312-1316 [PMID: 22447277 DOI: 10.1002/lary.23252]

20 **Savarino E**, Giacchino M, Savarino V. Dysmotility and reflux disease. *Curr Opin Otolaryngol Head Neck Surg* 2013; **21**: 548-556 [PMID: 24240131 DOI: 10.1097/MOO.0b013e3283658edf]

21 **Martinucci I**, de Bortoli N, Giacchino M, Bodini G, Marabotto E, Marchi S, Savarino V, Savarino E. Esophageal motility abnormalities in gastroesophageal reflux disease. *World J Gastrointest Pharmacol Ther* 2014; **5**: 86-96 [PMID: 24868489 DOI: 10.4292/wjgpt.v5.i2.86]

22 **Dodds WJ**, Dent J, Hogan WJ, Helm JF, Hauser R, Patel GK, Egide MS. Mechanisms of gastroesophageal reflux in patients with reflux esophagitis. *N Engl J Med* 1982; **307**: 1547-1552 [PMID: 7144836 DOI: 10.1056/NEJM198212163072503]

23 **Orlando RC**. Overview of the mechanisms of gastroesophageal reflux. *Am J Med* 2001; **111** Suppl 8A: 174S-177S [PMID: 11749946]

24 **Dent J**, Holloway RH, Toouli J, Dodds WJ. Mechanisms of lower oesophageal sphincter incompetence in patients with symptomatic gastrooesophageal reflux. *Gut* 1988; **29**: 1020-1028 [PMID: 3410327]

25 **Tolone S**, de Cassan C, de Bortoli N, Roman S, Galeazzi F, Salvador R, Marabotto E, Furnari M, Zentilin P, Marchi S, Bardini R, Sturniolo GC, Savarino V, Savarino E. Esophagogastric junction morphology is associated with a positive impedance-pH monitoring in patients with GERD. *Neurogastroenterol Motil* 2015; **27**: 1175-1182 [PMID: 26010058 DOI: 10.1111/nmo.12606]

26 **Tolone S**, De Bortoli N, Marabotto E, de Cassan C, Bodini G, Roman S, Furnari M, Savarino V, Docimo L, Savarino E. Esophagogastric junction contractility for clinical assessment in patients with GERD: a real added value? *Neurogastroenterol Motil* 2015; **27**: 1423-1431 [PMID: 26227513 DOI: 10.1111/nmo.12638]

27 **Clouse RE**, Staiano A. Topography of the esophageal peristaltic pressure wave. *Am J Physiol* 1991; **261**: G677-G684 [PMID: 1928353]

28 **Clouse RE**, Staiano A, Alrakawi A. Development of a topographic analysis system for manometric studies in the gastrointestinal tract. *Gastrointest Endosc* 1998; **48**: 395-401 [PMID: 9786113]

29 **Kahrilas PJ**, Ghosh SK, Pandolfino JE. Esophageal motility disorders in terms of pressure topography: the Chicago Classification. *J Clin Gastroenterol* 2008; **42**: 627-635 [PMID: 18364587 DOI: 10.1097/MCG.0b013e31815ea291]

30 **Kahrilas PJ**, Bredenoord AJ, Fox M, Gyawali CP, Roman S, Smout AJ, Pandolfino JE. The Chicago Classification of esophageal motility disorders, v3.0. *Neurogastroenterol Motil* 2015; **27**: 160-174 [PMID: 25469569 DOI: 10.1111/nmo.12477]

31 **Pandolfino JE**, Ghosh SK, Zhang Q, Jarosz A, Shah N, Kahrilas PJ. Quantifying EGJ morphology and relaxation with high-resolution manometry: a study of 75 asymptomatic volunteers. *Am J Physiol Gastrointest Liver Physiol* 2006; **290**: G1033-G1040 [PMID: 16455788 DOI: 10.1152/ajpgi.00444.2005]

32 **Bredenoord AJ**, Weusten BL, Timmer R, Smout AJ. Intermittent spatial separation of diaphragm and lower esophageal sphincter favors acidic and weakly acidic reflux. *Gastroenterology* 2006; **130**: 334-340 [PMID: 16472589 DOI: 10.1053/j.gastro.2005.10.053]

33 **Edmundowicz SA**, Clouse RE. Shortening of the esophagus in response to swallowing. *Am J Physiol* 1991; **260**: G512-G516 [PMID: 2003613]

34 **Staiano A**, Clouse RE. Detection of incomplete lower esophageal sphincter relaxation with conventional point-pressure sensors. *Am J Gastroenterol* 2001; **96**: 3258-3267 [PMID: 11774934 DOI: 10.1111/j.1572-0241.2001.05323.x]

35 **Pandolfino JE**, Zhang QG, Ghosh SK, Han A, Boniquit C, Kahrilas PJ. Transient lower esophageal sphincter relaxations and reflux: mechanistic analysis using concurrent fluoroscopy and high-resolution manometry. *Gastroenterology* 2006; **131**: 1725-1733 [PMID: 17087957 DOI: 10.1053/j.gastro.2006.09.009]

36 **Pandolfino JE**, Kim H, Ghosh SK, Clarke JO, Zhang Q, Kahrilas PJ. High-resolution manometry of the EGJ: an analysis of crural diaphragm function in GERD. *Am J Gastroenterol* 2007; **102**: 1056-1063 [PMID: 17319930 DOI: 10.1111/j.1572-0241.2007.01138.x]

37 **Pandolfino JE**, El-Serag HB, Zhang Q, Shah N, Ghosh SK, Kahrilas PJ. Obesity: a challenge to esophagogastric junction integrity. *Gastroenterology* 2006; **130**: 639-649 [PMID: 16530504 DOI: 10.1053/j.gastro.2005.12.016]

38 **Bredenoord AJ**, Weusten BL, Roelofs JM, Smout AJ. The gastro-oesophageal pressure inversion point revisited. *Scand J Gastroenterol* 2003; **38**: 812-818 [PMID: 12940432]

39 **Bredenoord AJ**, Weusten BL, Carmagnola S, Smout AJ. Double-peaked high-pressure zone at the esophagogastric junction in controls and in patients with a hiatal hernia: a study using high-resolution manometry. *Dig Dis Sci* 2004; **49**: 1128-1135 [PMID: 15387333]

40 **Savarino E**, Zentilin P, Marabotto E, Bonfanti D, Inferrera S, Assandri L, Sammito G, Gemignani L, Furnari M, Dulbecco P, Savarino V. Overweight is a risk factor for both erosive and non-erosive reflux disease. *Dig Liver Dis* 2011; **43**: 940-945 [PMID: 21944835 DOI: 10.1016/j.dld.2011.07.014]

41 **Mittal RK**, Holloway RH, Penagini R, Blackshaw LA, Dent J. Transient lower esophageal sphincter relaxation. *Gastroenterology* 1995; **109**: 601-610 [PMID: 7615211]

42 **Holloway RH**, Penagini R, Ireland AC. Criteria for objective definition of transient lower esophageal sphincter relaxation. *Am J Physiol* 1995; **268**: G128-G133 [PMID: 7840195]

43 **Roman S**, Zerbib F, Belhocine K, des Varannes SB, Mion F. High resolution manometry to detect transient lower oesophageal sphincter relaxations: diagnostic accuracy compared with perfused-sleeve manometry, and the definition of new detection criteria. *Aliment Pharmacol Ther* 2011; **34**: 384-393 [PMID: 21651594 DOI: 10.1111/j.1365-2036.2011.04728.x]

44 **Sifrim D**, Holloway R. Transient lower esophageal sphincter relaxations: how many or how harmful? *Am J Gastroenterol* 2001; **96**: 2529-2532 [PMID: 11569671 DOI: 10.1111/j.1572-0241.2001.04095.x]

45 **Bredenoord AJ**, Weusten BL, Timmer R, Smout AJ. Gastro-oesophageal reflux of liquids and gas during transient lower oesophageal sphincter relaxations. *Neurogastroenterol Motil* 2006; **18**: 888-893 [PMID: 16961691 DOI: 10.1111/j.1365-2982.2006.00817.x]

46 **Diener U**, Patti MG, Molena D, Fisichella PM, Way LW. Esophageal dysmotility and gastroesophageal reflux disease. *J Gastrointest Surg* 2001; **5**: 260-265 [PMID: 11360049]

47 **Fornari F**, Bravi I, Penagini R, Tack J, Sifrim D. Multiple rapid swallowing: a complementary test during standard oesophageal manometry. *Neurogastroenterol Motil* 2009; **21**: 718-e41 [PMID: 19222762 DOI: 10.1111/j.1365-2982.2009.01273.x]

48 **Ghosh SK**, Janiak P, Schwizer W, Hebbard GS, Brasseur JG. Physiology of the esophageal pressure transition zone: separate contraction waves above and below. *Am J Physiol Gastrointest Liver Physiol* 2006; **290**: G568-G576 [PMID: 16282364 DOI: 10.1152/ajpgi.00280.2005]

49 **Pohl D**, Ribolsi M, Savarino E, Frühauf H, Fried M, Castell DO, Tutuian R. Characteristics of the esophageal low-pressure zone in healthy volunteers and patients with esophageal symptoms: assessment by high-resolution manometry. *Am J Gastroenterol* 2008; **103**: 2544-2549 [PMID: 18684179 DOI: 10.1111/j.1572-0241.2008.02062.x]

50 **Vinjirayer E**, Gonzalez B, Brensinger C, Bracy N, Obelmejias R, Katzka DA, Metz DC. Ineffective motility is not a marker for gastroesophageal reflux disease. *Am J Gastroenterol* 2003; **98**: 771-776 [PMID: 12738454 DOI: 10.1111/j.1572-0241.2003.07391.x]

51 **Dolder M**, Tutuian R. Laboratory based investigations for diagnosing gastroesophageal reflux disease. *Best Pract Res Clin Gastroenterol* 2010; **24**: 787-798 [PMID: 21126694 DOI: 10.1016/j.bpg.2010.10.005]

52 **Bredenoord AJ**, Pandolfino JE, Smout AJ. Gastro-oesophageal reflux disease. *Lancet* 2013; **381**: 1933-1942 [PMID: 23477993 DOI: 10.1016/S0140-6736(12)62171-0]

53 **Zentilin P**, Iiritano E, Dulbecco P, Bilardi C, Savarino E, De Conca S, Parodi A, Reglioni S, Vigneri S, Savarino V. Normal values of 24-h ambulatory intraluminal impedance combined with pH-metry in subjects eating a Mediterranean diet. *Dig Liver Dis* 2006; **38**: 226-232 [PMID: 16480938]

54 **Frazzoni M**, Savarino E, Manno M, Melotti G, Mirante VG, Mussetto A, Bertani H, Manta R, Conigliaro R. Reflux patterns in patients with short-segment Barrett's oesophagus: a study using impedance-pH monitoring off and on proton pump inhibitor therapy. *Aliment Pharmacol Ther* 2009; **30**: 508-515 [PMID: 19519732]

55 **Boeckxstaens GE**, Smout A. Systematic review: role of acid, weakly acidic and weakly alkaline reflux in gastro-oesophageal reflux disease. *Aliment Pharmacol Ther* 2010; **32**: 334-343 [PMID: 20491749 DOI: 10.1111/j.1365-2036.2010.04358.x]

56 **Mainie I**, Tutuian R, Shay S, Vela M, Zhang X, Sifrim D, Castell DO. Acid and non-acid reflux in patients with persistent symptoms despite acid suppressive therapy: a multicentre study using combined ambulatory impedance-pH monitoring. *Gut* 2006; **55**: 1398-1402 [PMID: 16556669]

57 **Zerbib F**, Roman S, Ropert A, des Varannes SB, Pouderoux P, Chaput U, Mion F, Vérin E, Galmiche JP, Sifrim D. Esophageal pH-impedance monitoring and symptom analysis in GERD: a study in patients off and on therapy. *Am J Gastroenterol* 2006; **101**: 1956-1963 [PMID: 16848801]

58 **Savarino E**, Zentilin P, Tutuian R, Pohl D, Gemignani L, Malesci A, Savarino V. Impedance-pH reflux patterns can differentiate non-erosive reflux disease from functional heartburn patients. *J Gastroenterol* 2012; **47**: 159-168 [PMID: 22038553 DOI: 10.1007/s00535-011-0480-0]

59 **Bredenoord AJ**, Weusten BL, Smout AJ. Symptom association analysis in ambulatory gastro-oesophageal reflux monitoring. *Gut* 2005; **54**: 1810-1817 [PMID: 16284291]

60 **de Bortoli N**, Martinucci I, Bellini M, Savarino E, Savarino V, Blandizzi C, Marchi S. Overlap of functional heartburn and gastroesophageal reflux disease with irritable bowel syndrome.

World J Gastroenterol 2013; **19**: 5787-5797 [PMID: 24124323 DOI: 10.3748/wjg.v19.i35.5787]

61 **Savarino E**, Zentilin P, Tutuian R, Pohl D, Casa DD, Frazzoni M, Cestari R, Savarino V. The role of nonacid reflux in NERD: lessons learned from impedance-pH monitoring in 150 patients off therapy. *Am J Gastroenterol* 2008; **103**: 2685-2693 [PMID: 18775017 DOI: 10.1111/j.1572-0241.2008.02119.x]

62 **Sifrim D**, Castell D, Dent J, Kahrilas PJ. Gastro-oesophageal reflux monitoring: review and consensus report on detection and definitions of acid, non-acid, and gas reflux. *Gut* 2004; **53**: 1024-1031 [PMID: 15194656]

63 **Shay S**, Tutuian R, Sifrim D, Vela M, Wise J, Balaji N, Zhang X, Adhami T, Murray J, Peters J, Castell D. Twenty-four hour ambulatory simultaneous impedance and pH monitoring: a multicenter report of normal values from 60 healthy volunteers. *Am J Gastroenterol* 2004; **99**: 1037-1043 [PMID: 15180722 DOI: 10.1111/j.1572-0241.2004.04172.x]

64 **Blondeau K**, Tack J. Pro: Impedance testing is useful in the management of GERD. *Am J Gastroenterol* 2009; **104**: 2664-2666 [PMID: 19888230 DOI: 10.1038/ajg.2009.568]

65 **Chen CL**, Hsu PI. Current advances in the diagnosis and treatment of nonerosive reflux disease. *Gastroenterol Res Pract* 2013; **2013**: 653989 [PMID: 23935610 DOI: 10.1155/2013/653989]

66 **Bredenoord AJ**, Weusten BL, Timmer R, Conchillo JM, Smout AJ. Addition of esophageal impedance monitoring to pH monitoring increases the yield of symptom association analysis in patients off PPI therapy. *Am J Gastroenterol* 2006; **101**: 453-459 [PMID: 16464226 DOI: 10.1111/j.1572-0241.2006.00427.x]

67 **Savarino E**, Pohl D, Zentilin P, Dulbecco P, Sammito G, Sconfienza L, Vigneri S, Camerini G, Tutuian R, Savarino V. Functional heartburn has more in common with functional dyspepsia than with non-erosive reflux disease. *Gut* 2009; **58**: 1185-1191 [PMID: 19460766]

68 **de Bortoli N**, Martinucci I, Savarino E, Franchi R, Bertani L, Russo S, Ceccarelli L, Costa F, Bellini M, Blandizzi C, Savarino V, Marchi S. Lower pH values of weakly acidic refluxes as determinants of heartburn perception in gastroesophageal reflux disease patients with normal esophageal acid exposure. *Dis Esophagus* 2014; Epub ahead of print [PMID: 25212408 DOI: 10.1111/dote.12284]

69 **Savarino E**, Tutuian R, Zentilin P, Dulbecco P, Pohl D, Marabotto E, Parodi A, Sammito G, Gemignani L, Bodini G, Savarino V. Characteristics of reflux episodes and symptom association in patients with erosive esophagitis and nonerosive reflux disease: study using combined impedance-pH off therapy. *Am J Gastroenterol* 2010; **105**: 1053-1061 [PMID: 19997095 DOI: 10.1038/ajg.2009.670]

70 **Savarino E**, Marabotto E, Zentilin P, Frazzoni M, Sammito G, Bonfanti D, Sconfienza L, Assandri L, Gemignani L, Malesci A, Savarino V. The added value of impedance-pH monitoring to Rome III criteria in distinguishing functional heartburn from non-erosive reflux disease. *Dig Liver Dis* 2011; **43**: 542-547 [PMID: 21376679 DOI: 10.1016/j.dld.2011.01.016]

71 **Savarino E**, Zentilin P, Frazzoni M, Cuoco DL, Pohl D, Dulbecco P, Marabotto E, Sammito G, Gemignani L, Tutuian R, Savarino V. Characteristics of gastro-esophageal reflux episodes in Barrett's esophagus, erosive esophagitis and healthy volunteers. *Neurogastroenterol Motil* 2010; **22**: 1061-e1280 [PMID: 20557468 DOI: 10.1111/j.1365-2982.2010.01536.x]

72 **Zentilin P**, Dulbecco P, Savarino E, Giannini E, Savarino V. Combined multichannel intraluminal impedance and pH-metry: a novel technique to improve detection of gastro-oesophageal reflux literature review. *Dig Liver Dis* 2004; **36**: 565-569 [PMID: 15460839 DOI: 10.1016/j.dld.2004.03.019]

73 **Pandolfino JE**, Vela MF. Esophageal-reflux monitoring. *Gastrointest Endosc* 2009; **69**: 917-30, 930.e1 [PMID: 19249037]

74 **Hershcovici T**, Wendel CS, Fass R. Symptom indexes in refractory gastroesophageal reflux disease: overrated or misunderstood? *Clin Gastroenterol Hepatol* 2011; **9**: 816-817 [PMID: 21791195 DOI: 10.1016/j.cgh.2011.07.010]

75 **Slaughter JC**, Goutte M, Rymer JA, Oranu AC, Schneider JA, Garrett CG, Hagaman D, Vaezi MF. Caution about overinterpretation of symptom indexes in reflux monitoring for refractory gastroesophageal reflux disease. *Clin Gastroenterol Hepatol* 2011; **9**: 868-874 [PMID: 21782769 DOI: 10.1016/j.cgh.2011.07.009]

76 **Zerbib F**, Belhocine K, Simon M, Capdepont M, Mion F, Bruley des Varannes S, Galmiche JP. Clinical, but not oesophageal pH-impedance, profiles predict response to proton pump inhibitors in gastro-oesophageal reflux disease. *Gut* 2012; **61**: 501-506 [PMID: 21997546 DOI: 10.1136/gutjnl-2011-300798]

77 **Weijenborg PW**, Cremonini F, Smout AJ, Bredenoord AJ. PPI therapy is equally effective in well-defined non-erosive reflux disease and in reflux esophagitis: a meta-analysis. *Neurogastroenterol Motil* 2012; **24**: 747-757, e350 [PMID: 22309489 DOI: 10.1111/j.1365-2982.2012.01888.x]

78 **Frazzoni M**, Manta R, Mirante VG, Conigliaro R, Frazzoni L, Melotti G. Esophageal chemical clearance is impaired in gastro-esophageal reflux disease--a 24-h impedance-pH monitoring assessment. *Neurogastroenterol Motil* 2013; **25**: 399-406, e295 [PMID: 23360178 DOI: 10.1111/nmo.12080]

79 **Farré R**, Blondeau K, Clement D, Vicario M, Cardozo L, Vieth M, Mertens V, Pauwels A, Silny J, Jimenez M, Tack J, Sifrim D. Evaluation of oesophageal mucosa integrity by the intraluminal impedance technique. *Gut* 2011; **60**: 885-892 [PMID: 21303918 DOI: 10.1136/gut.2010.233049]

80 **Kessing BF**, Bredenoord AJ, Weijenborg PW, Hemmink GJ, Loots CM, Smout AJ. Esophageal acid exposure decreases intraluminal baseline impedance levels. *Am J Gastroenterol* 2011; **106**: 2093-2097 [PMID: 21844921 DOI: 10.1038/ajg.2011.276]

81 **Martinucci I**, de Bortoli N, Savarino E, Piaggi P, Bellini M, Antonelli A, Savarino V, Frazzoni M, Marchi S. Esophageal baseline impedance levels in patients with pathophysiological characteristics of functional heartburn. *Neurogastroenterol Motil* 2014; **26**: 546-555 [PMID: 24433456 DOI: 10.1111/nmo.12299]

82 **Pace F**, Sangaletti O, Pallotta S, Molteni P, Porro GB. Biliary reflux and non-acid reflux are two distinct phenomena: a comparison between 24-hour multichannel intraesophageal impedance and bilirubin monitoring. *Scand J Gastroenterol* 2007; **42**: 1031-1039 [PMID: 17710667 DOI: 10.1080/00365520701245645]

83 **Eisen GM**, Sandler RS, Murray S, Gottfried M. The relationship between gastroesophageal reflux disease and its complications with Barrett's esophagus. *Am J Gastroenterol* 1997; **92**: 27-31 [PMID: 8995932]

84 **Dent J**. Microscopic esophageal mucosal injury in nonerosive reflux disease. *Clin Gastroenterol Hepatol* 2007; **5**: 4-16 [PMID: 17157563 DOI: 10.1016/j.cgh.2006.08.006]

85 **Zentilin P**, Savarino V, Mastracci L, Spaggiari P, Dulbecco P, Ceppa P, Savarino E, Parodi A, Mansi C, Fiocca R. Reassessment of the diagnostic value of histology in patients with GERD, using multiple biopsy sites and an appropriate control group. *Am J Gastroenterol* 2005; **100**: 2299-2306 [PMID: 16181384 DOI: 10.1111/j.1572-0241.2005.50209.x]

86 **Savarino E**, Zentilin P, Mastracci L, Dulbecco P, Marabotto E, Gemignani L, Bruzzone L, de Bortoli N, Frigo AC, Fiocca R, Savarino V. Microscopic esophagitis distinguishes patients with non-erosive reflux disease from those with functional heartburn. *J Gastroenterol* 2013; **48**: 473-482 [PMID: 23001252 DOI: 10.1007/s00535-012-0672-2]

87 **Hemmink GJ**, Bredenoord AJ, Weusten BL, Monkelbaan JF, Timmer R, Smout AJ. Esophageal pH-impedance monitoring in patients with therapy-resistant reflux symptoms: 'on' or 'off' proton pump inhibitor? *Am J Gastroenterol* 2008; **103**: 2446-2453 [PMID: 18684197 DOI: 10.1111/j.1572-0241.2008.02033.x]

88 **Vaezi MF**. Gastroesophageal reflux-related chronic laryngitis: con. *Arch Otolaryngol Head Neck Surg* 2010; **136**: 908-909 [PMID: 20855684 DOI: 10.1001/archoto.2010.149]

89 **de Bortoli N**, Nacci A, Savarino E, Martinucci I, Bellini M, Fattori B, Ceccarelli L, Costa F, Mumolo MG, Ricchiuti A, Savarino V, Berrettini S, Marchi S. How many cases of laryngopharyngeal reflux suspected by laryngoscopy are gastroesophageal reflux disease-related? *World J Gastroenterol* 2012; **18**: 4363-4370 [PMID:

22969200 DOI: 10.3748/wjg.v18.i32.4363]
90 **Zentilin P**, Dulbecco P, Savarino E, Parodi A, Iiritano E, Bilardi C, Reglioni S, Vigneri S, Savarino V. An evaluation of the antireflux properties of sodium alginate by means of combined multichannel intraluminal impedance and pH-metry. *Aliment Pharmacol Ther* 2005; **21**: 29-34 [PMID: 15644042 DOI: 10.1111/j.1365-2036.2004.02298.x]
91 **Giannini EG**, Zentilin P, Dulbecco P, Iiritano E, Bilardi C, Savarino E, Mansi C, Savarino V. A comparison between sodium alginate and magaldrate anhydrous in the treatment of patients with gastroesophageal reflux symptoms. *Dig Dis Sci* 2006; **51**: 1904-1909 [PMID: 16977507 DOI: 10.1007/s10620-006-9284-0]
92 **Savarino E**, de Bortoli N, Zentilin P, Martinucci I, Bruzzone L, Furnari M, Marchi S, Savarino V. Alginate controls heartburn in patients with erosive and nonerosive reflux disease. *World J Gastroenterol* 2012; **18**: 4371-4378 [PMID: 22969201 DOI: 10.3748/wjg.v18.i32.4371]
93 **Tutuian R**, Castell DO. Combined multichannel intraluminal impedance and manometry clarifies esophageal function abnormalities: study in 350 patients. *Am J Gastroenterol* 2004; **99**: 1011-1019 [PMID: 15180718 DOI: 10.1111/j.1572-0241.2004.30035.x]
94 **Pandolfino JE**, Richter JE, Ours T, Guardino JM, Chapman J, Kahrilas PJ. Ambulatory esophageal pH monitoring using a wireless system. *Am J Gastroenterol* 2003; **98**: 740-749 [PMID: 12738450 DOI: 10.1111/j.1572-0241.2003.07398.x]
95 **Marchese M**, Spada C, Iacopini F, Familiari P, Shah SG, Tringali A, Costamagna G. Nonendoscopic transnasal placement of a wireless capsule for esophageal pH monitoring: feasibility, safety, and efficacy of a manometry-guided procedure. *Endoscopy* 2006; **38**: 813-818 [PMID: 17001571 DOI: 10.1055/s-2006-944526]
96 **Bhat YM**, McGrath KM, Bielefeldt K. Wireless esophageal pH monitoring: new technique means new questions. *J Clin Gastroenterol* 2006; **40**: 116-121 [PMID: 16394871]
97 **Lacy BE**, O'Shana T, Hynes M, Kelley ML, Weiss JE, Paquette L, Rothstein RI. Safety and tolerability of transoral Bravo capsule placement after transnasal manometry using a validated conversion factor. *Am J Gastroenterol* 2007; **102**: 24-32 [PMID: 17100980 DOI: 10.1111/j.1572-0241.2006.00889.x]
98 **Ayazi S**, Lipham JC, Portale G, Peyre CG, Streets CG, Leers JM, Demeester SR, Banki F, Chan LS, Hagen JA, Demeester TR. Bravo catheter-free pH monitoring: normal values, concordance, optimal diagnostic thresholds, and accuracy. *Clin Gastroenterol Hepatol* 2009; **7**: 60-67 [PMID: 18976965 DOI: 10.1016/j.cgh.2008.08.020]
99 **Prakash C**, Clouse RE. Value of extended recording time with wireless pH monitoring in evaluating gastroesophageal reflux disease. *Clin Gastroenterol Hepatol* 2005; **3**: 329-334 [PMID: 15822037]
100 **Ang D**, Teo EK, Ang TL, Ong J, Poh CH, Tan J, Fock KM. To Bravo or not? A comparison of wireless esophageal pH monitoring and conventional pH catheter to evaluate non-erosive gastroesophageal reflux disease in a multiracial Asian cohort. *J Dig Dis* 2010; **11**: 19-27 [PMID: 20132427 DOI: 10.1111/j.1751-2980.2009.00409.x]
101 **Prakash C**, Clouse RE. Wireless pH monitoring in patients with non-cardiac chest pain. *Am J Gastroenterol* 2006; **101**: 446-452 [PMID: 16542279 DOI: 10.1111/j.1572-0241.2006.00425.x]
102 **Fox M**. Review article: identifying the causes of reflux events and symptoms - new approaches. *Aliment Pharmacol Ther* 2011; **33** (Supp 1): 36-42
103 **McMahon BP**, Frøkjaer JB, Drewes AM, Gregersen H. A new measurement of oesophago-gastric junction competence. *Neurogastroenterol Motil* 2004; **16**: 543-546 [PMID: 15500510 DOI: 10.1111/j.1365-2982.2004.00540.x]
104 **Kwiatek MA**, Pandolfino JE, Hirano I, Kahrilas PJ. Esophagogastric junction distensibility assessed with an endoscopic functional luminal imaging probe (EndoFLIP). *Gastrointest Endosc* 2010; **72**: 272-278 [PMID: 20541755 DOI: 10.1016/j.gie.2010.01.069]
105 **Regan J**, Walshe M, Rommel N, Tack J, McMahon BP. New measures of upper esophageal sphincter distensibility and opening patterns during swallowing in healthy subjects using EndoFLIP ®. *Neurogastroenterol Motil* 2013; **25**: e25-e34 [PMID: 23240693 DOI: 10.1111/nmo.12041]
106 **Vaezi MF**, Hicks DM, Abelson TI, Richter JE. Laryngeal signs and symptoms and gastroesophageal reflux disease (GERD): a critical assessment of cause and effect association. *Clin Gastroenterol Hepatol* 2003; **1**: 333-344 [PMID: 15017651]
107 **Carroll TL**, Fedore LW, Aldahlawi MM. pH Impedance and high-resolution manometry in laryngopharyngeal reflux disease high-dose proton pump inhibitor failures. *Laryngoscope* 2012; **122**: 2473-2481 [PMID: 22965767 DOI: 10.1002/lary.23518]
108 **Richter JE**. Typical and atypical presentations of gastroesophageal reflux disease. The role of esophageal testing in diagnosis and management. *Gastroenterol Clin North Am* 1996; **25**: 75-102 [PMID: 8682579]
109 **Mainie I**, Tutuian R, Agrawal A, Adams D, Castell DO. Combined multichannel intraluminal impedance-pH monitoring to select patients with persistent gastro-oesophageal reflux for laparoscopic Nissen fundoplication. *Br J Surg* 2006; **93**: 1483-1487 [PMID: 17051602 DOI: 10.1002/bjs.5493]
110 **Vaezi MF**. Laryngitis and gastroesophageal reflux disease: increasing prevalence or poor diagnostic tests? *Am J Gastroenterol* 2004; **99**: 786-788 [PMID: 15128337 DOI: 10.1111/j.1572-0241.2004.40290.x]
111 **Sun G**, Muddana S, Slaughter JC, Casey S, Hill E, Farrokhi F, Garrett CG, Vaezi MF. A new pH catheter for laryngopharyngeal reflux: Normal values. *Laryngoscope* 2009; **119**: 1639-1643 [PMID: 19504553 DOI: 10.1002/lary.20282]
112 **Worrell SG**, DeMeester SR, Greene CL, Oh DS, Hagen JA. Pharyngeal pH monitoring better predicts a successful outcome for extraesophageal reflux symptoms after antireflux surgery. *Surg Endosc* 2013; **27**: 4113-4118 [PMID: 23836124 DOI: 10.1007/s00464-013-3076-3]
113 **Vailati C**, Mazzoleni G, Bondi S, Bussi M, Testoni PA, Passaretti S. Oropharyngeal pH monitoring for laryngopharyngeal reflux: is it a reliable test before therapy? *J Voice* 2013; **27**: 84-89 [PMID: 23159026 DOI: 10.1016/j.jvoice.2012.08.006]
114 **Ummarino D**, Vandermeulen L, Roosens B, Urbain D, Hauser B, Vandenplas Y. Gastroesophageal reflux evaluation in patients affected by chronic cough: Restech versus multichannel intraluminal impedance/pH metry. *Laryngoscope* 2013; **123**: 980-984 [PMID: 23023943 DOI: 10.1002/lary.23738]
115 **Becker V**, Graf S, Schlag C, Schuster T, Feussner H, Schmid RM, Bajbouj M. First agreement analysis and day-to-day comparison of pharyngeal pH monitoring with pH/impedance monitoring in patients with suspected laryngopharyngeal reflux. *J Gastrointest Surg* 2012; **16**: 1096-1101 [PMID: 22450948 DOI: 10.1007/s11605-012-1866-x]
116 **Mazzoleni G**, Vailati C, Lisma DG, Testoni PA, Passaretti S. Correlation between oropharyngeal pH-monitoring and esophageal pH-impedance monitoring in patients with suspected GERD-related extra-esophageal symptoms. *Neurogastroenterol Motil* 2014; **26**: 1557-1564 [PMID: 25208949 DOI: 10.1111/nmo.12422]
117 **Bardhan KD**, Strugala V, Dettmar PW. Reflux revisited: advancing the role of pepsin. *Int J Otolaryngol* 2012; **2012**: 646901 [PMID: 22242022 DOI: 10.1155/2012/646901]
118 **Knight J**, Lively MO, Johnston N, Dettmar PW, Koufman JA. Sensitive pepsin immunoassay for detection of laryngopharyngeal reflux. *Laryngoscope* 2005; **115**: 1473-1478 [PMID: 16094128 DOI: 10.1097/01.mlg.0000172043.51871.d9]
119 **Hayat JO**, Gabieta-Somnez S, Yazaki E, Kang JY, Woodcock A, Dettmar P, Mabary J, Knowles CH, Sifrim D. Pepsin in saliva for the diagnosis of gastro-oesophageal reflux disease. *Gut* 2015; **64**: 373-380 [PMID: 24812000 DOI: 10.1136/gutjnl-2014-307049]
120 **Savarino E**, Gemignani L, Pohl D, Zentilin P, Dulbecco P, Assandri L, Marabotto E, Bonfanti D, Inferrera S, Fazio V, Malesci A, Tutuian R, Savarino V. Oesophageal motility and bolus transit abnormalities increase in parallel with the severity of gastro-oesophageal reflux disease. *Aliment Pharmacol Ther* 2011; **34**: 476-486 [PMID: 21671968 DOI: 10.1111/j.1365-2036.2011.0474

2.x]
121 **Fox MR**, Bredenoord AJ. Oesophageal high-resolution manometry: moving from research into clinical practice. *Gut* 2008; **57**: 405-423 [PMID: 17895358 DOI: 10.1136/gut.2007.127993]
122 **Kessing BF**, Bredenoord AJ, Smout AJ. Erroneous diagnosis of gastroesophageal reflux disease in achalasia. *Clin Gastroenterol Hepatol* 2011; **9**: 1020-1024 [PMID: 21683804 DOI: 10.1016/j.cgh.2011.04.022]
123 **Shay SS**, Bomeli S, Richter J. Multichannel intraluminal impedance accurately detects fasting, recumbent reflux events and their clearing. *Am J Physiol Gastrointest Liver Physiol* 2002; **283**: G376-G383 [PMID: 12121885 DOI: 10.1152/ajpgi.00470.2001]

P- Reviewer: Dumitrascu DL, Hillman LC, Pehl C, Solazzo A
S- Editor: Ji FF **L- Editor**: A **E- Editor**: Jiao XK

World Journal of Gastrointestinal Pathophysiology

Submit a Manuscript: http://www.wjgnet.com/esps/
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.4291/wjgp.v7.i1.86

World J Gastrointest Pathophysiol 2016 February 15; 7(1): 86-96
ISSN 2150-5330 (online)

REVIEW

Upper gastrointestinal bleeding risk scores: Who, when and why?

Sara Monteiro, Tiago Cúrdia Gonçalves, Joana Magalhães, José Cotter

Sara Monteiro, Tiago Cúrdia Gonçalves, Joana Magalhães, José Cotter, Department of Gastroenterology, Hospital Senhora da Oliveira-Guimarães, Creixomil, 4835-044 Guimarães, Portugal

José Cotter, Life and Health Sciences Research Institute (ICVS), School of Health Sciences, ICVS/3B's Associate Laboratory, University of Minho, Campus de Gualtar, 4835-044 Braga/Guimarães, Portugal

Author contributions: Monteiro S performed the literature search; Monteiro S, Gonçalves TC and Magalhães J wrote the paper; Cotter J designed and conducted the manuscript, critically revised the paper and finally approved the version to be submitted. All authors read and approved the final paper.

Conflict-of-interest statement: The authors have no conflict of interest to disclose.

Correspondence to: Sara Monteiro, MD, Department of Gastroenterology, Hospital Senhora da Oliveira-Guimarães, Rua dos Cutileiros, Creixomil, 4835-044 Guimarães, Portugal. sara.s.o.monteiro@gmail.com
Telephone: +351-25-3540330
Fax: +351-25-3513592

Received: June 26, 2015
Peer-review started: June 26, 2015
First decision: August 3, 2015
Revised: October 14, 2015
Accepted: December 9, 2015
Article in press: December 11, 2015
Published online: February 15, 2016

Abstract

Upper gastrointestinal bleeding (UGIB) remains a significant cause of hospital admission. In order to stratify patients according to the risk of the complications, such as rebleeding or death, and to predict the need of clinical intervention, several risk scores have been proposed and their use consistently recommended by international guidelines. The use of risk scoring systems in early assessment of patients suffering from UGIB may be useful to distinguish high-risks patients, who may need clinical intervention and hospitalization, from low risk patients with a lower chance of developing complications, in which management as outpatients can be considered. Although several scores have been published and validated for predicting different outcomes, the most frequently cited ones are the Rockall score and the Glasgow Blatchford score (GBS). While Rockall score, which incorporates clinical and endoscopic variables, has been validated to predict mortality, the GBS, which is based on clinical and laboratorial parameters, has been studied to predict the need of clinical intervention. Despite the advantages previously reported, their use in clinical decisions is still limited. This review describes the different risk scores used in the UGIB setting, highlights the most important research, explains why and when their use may be helpful, reflects on the problems that remain unresolved and guides future research with practical impact.

Key words: Upper gastrointestinal bleeding; Risk scores; Risk assessment; Rockall score; Glasgow blatchford score

Core tip: Upper gastrointestinal bleeding (UGIB) remains a significant cause of hospital admission. In order to stratify patients according to the risk of complications, such as rebleeding or death, and to predict the need of clinical intervention, several risk scores have been proposed and their use consistently recommended by international guidelines. This review describes the different risk scores used in the UGIB setting, highlights the most important research, explains why and when

their use may be helpful, reflects on the problems that remain unresolved and guides future research with practical impact.

Monteiro S, Gonçalves TC, Magalhães J, Cotter J. Upper gastrointestinal bleeding risk scores: Who, when and why? *World J Gastrointest Pathophysiol* 2016; 7(1): 86-96 Available from: URL: http://www.wjgnet.com/2150-5330/full/v7/i1/86.htm DOI: http://dx.doi.org/10.4291/wjgp.v7.i1.86

INTRODUCTION

Acute upper gastrointestinal bleeding (UGIB) remains a common cause of visits to the Emergency Department, with an estimated incidence of about 100 per 100000 hospitalizations[1], and being associated to significant morbidity, 30 d mortality and health-care costs[2,3].

Many risk factors are known to influence the outcome in UGIB setting. Age, comorbidities, presence of shock, endoscopic diagnosis, haemoglobin values at the time, ulcers' size, stigmata of recent haemorrhage and need for a blood transfusion have all been described as significant risk factors for rebleeding and death[4-8].

Patients suffering from UGIB are generally admitted for observation with or without upper endoscopy. However, emergency endoscopy is not continuously available in many hospitals. An United Kingdom audit of 6750 patients suffering from UGIB has revealed that only 52% of the hospitals had an out-of-hours endoscopy service and only 50% patients received endoscopy within 24 h[9].

In order to stratify the risk of complications, rebleeding, need of clinical intervention or death, several clinical scores are in use. Although recommended in the prevailing guidelines[10,11] they are erratically applied in the clinical practice. To encourage the use of a risk score in the context of UGIB, it should be easy to calculate, contain easy access variables, have high accuracy in predicting relevant outcomes and distinguish low-risk from high-risk patients.

The purpose of this paper is to describe the different risk scores already in use, explain why and when they may be useful, reflect on their limitations in clinical practice and direct future investigations. Since the majority of the literature is limited to non-variceal UGIB (NVUGIB), this review article will focus on such condition.

UPPER GI BLEEDING RISK SCORES - WHO?

From the different UGIB risk scores described, three main groups can be established: The scores only require endoscopic parameters, those that incorporate clinical and endoscopic parameters and those based solely on clinical parameters.

Score with endoscopic variables only

Forrest classification is based on endoscopic findings of an ulcer and is still useful to stratify patients into high- and low-risk categories in terms of rebleeding[12].

The Forrest classification divides ulcers in six different categories, depending on the endoscopic findings. High risk lesions include those characterized by spurting haemorrhages (Forrest Ⅰa), oozing haemorrhages (Forrest Ⅰb), nonbleeding visible vessels (Forrest Ⅱa), adherent clots (Forrest Ⅱb). Low risk lesions include haematin on the ulcer base (Forrest Ⅱc), and clean ulcer base (Forrest Ⅲ).

Forrest Ⅰa, Ⅰb and Ⅱa lesions require endoscopic treatment. For the ulcers with adherent clots (Forrest Ⅱb) clot removal should be attempted by vigorous irrigation and should be treated according to the underlying lesion[13,14].

Clinical and endoscopic scores

The most cited score incorporating clinical and endoscopic elements is the Rockall score. However, other scores such as the Baylor Bleeding Score (BBS), the Cedars-Sinai Medical Centre Predictive Index (CSMCPI), and more recently the progetto nazionale emorragia digestiva (PNED) score have also been reported.

The Rockall score, which ranges from 0 to 11, was developed in 1996 to predict mortality due to UGIB[15]. This score incorporates five variables: Age, haemodynamic status, patient's comorbidities, endoscopic diagnosis and presence of major stigmata of recent haemorrhage (Table 1). Patients' stigmata of recent haemorrhage (blood in upper gastrointestinal tract, adherent clot, visible or spurting vessel) are recognised risk factors for rebleeding, surgery and death and are indications for endoscopic therapy[16-18].

The BBS was originally developed for predicting rebleeding in patients with non-variceal haemorrhage[19]. This scoring system, which ranges from 0 to 24, is divided into three parts: (1) a pre-endoscopy score based on age and number and severity of concurrent diseases; (2) an endoscopic score based on site and stigmata of bleeding; and (3) a post-endoscopy score, which includes both the pre-endoscopy and the endoscopy score (Table 2).

The CSMCPI was developed as a guideline for determining the appropriate length of stay for patients admitted suffering from UGIB. Developed in 1996, by Hay *et al*[20], CSMCPI is based on four variables previously identified as independent predictors of outcome in patients suffering from UGIB : Endoscopic findings, symptoms at the time, haemodynamic instability, and number of comorbidities. The CSMCPI ranges from 0 to 11 (Table 3).

An Italian score of 10, the PNED score[21], was developed and validated to predict 30 d mortality after non-variceal bleeding. The PNED score is based on ten variables, (8 clinical and 2 laboratorial), and ranges from 0 to 24 (Table 4).

Table 1 Rockall score

Score Variable	0	1	2	3
Age	< 60 yr	60-79 yr	⩾ 80 yr	
Shock	No shock, systolic BP ⩾ 100, pulse < 100	Tachycardia, systolic BP ⩾ 100, pulse ⩾ 100	Hypotension, systolic BP < 100	
Comorbidity	No major comorbidity		Cardiac failure, ischaemic heart disease, any major comorbidity	Renal failure, liver failure, disseminated malignancy
Diagnosis	Mallory-Weiss tear, no lesion identified and no SRH	All other diagnoses	Malignancy of UGI tract	
Major SRH	None or dark spot only		Blood in UGI tract, adherent clot, visible or spurting vessel	

Admission score: Sum of age, shock and comorbidity; full score: Sum of age, shock, comorbidity, diagnosis and major SRH; BP: Blood pressure (measured in mmHg); UGI: Upper gastrointestinal; SRH: Stigmata of recent haemorrhage.

Table 2 Baylor bleeding score

Pre-endoscopy score	1	2	3	4	5
Age (yr)	30-49	50-59	60-69		⩾ 70
No. of illnesses	1-2			3-4	> 5
Severity of illnesses				Chronic[1]	Acute[2]
Endoscopy Score					
Site of bleeding				Posterior wall bulb	
Stigmata of bleeding	Clot		Visible vessel		Active bleeding

Pre-endoscopy score: Sum of the scores for age and the number and severity of concurrent illnesses; Endoscopy score: Sum of the scores for site and stigmata of haemorrhage; Post-endoscopy score: Sum of the pre-endoscopy and endoscopy score; [1]Chronic: Presence of a concurrent chronic life-threatening illness; [2]Acute: Presence of a concurrent acute life-threatening illness.

Scores with clinical variables only

Several studies have tried to develop clinical scoring systems to stratify the patients' risks based on the data immediately available at the time of the visit to the Emergency Department. These risk scores can be used to help physicians decide on the need for hospital admission, inpatient monitoring level and time for endoscopic evaluation.

Clinical risk scores may be useful to identify high-risk patients requiring immediate intervention and low-risk patients that can be safely discharged[11]. The main clinical scores reported in the literature are the clinical Rockall score and the Glasgow-Blatchford Score (GBS). Other scores such as the AIMS65 and the T-score were only recently described.

The clinical Rockall score is calculated without the endoscopic findings[15,22] (Table 1), and only includes 3 clinical variables: The patient's age, the haemodynamic status, and the occurrence of a comorbid disease. A maximum score of 7 is possible.

The GBS incorporates 8 clinical or laboratorial variables (heart rate, haemoglobin value, blood urea nitrogen, systolic blood pressure, melena occurrence, syncope, hepatic disease, or heart failure) (Table 5). The GBS ranges from 0 to 23, with higher scores indicating higher likelihood of a need for an endoscopic intervention.

Saltzman *et al*[23] developed an acronymic risk score named AIMS65 which incorporates albumin level < 3.0 g/dL (A), international normalized ratio (INR) > 1.5 (I), altered mental status (M), systolic blood pressure ⩽ 90 mmHg (S), and age > 65 years (65) (Table 6).

An Italian study developed the T-score which included 4 clinical parameters commonly assessed in the UGIB setting: (1) general conditions (poor, intermediate, good); (2) pulse (< 90 beats/min, 90-110 beats/min, > 110 beats/min); (3) systolic blood pressure (< 90 mmHg, 90-110 mmHg, > 110 mmHg); and (4) haemoglobin level (⩽ 8 g/dL, 9-10 g/dL, > 10 g/dL)[24] (Table 7).

Although some other non-endoscopic scores have been developed, they still present some limitations which preclude their use in the clinical practice. For example, the Cambridge score[25], described by Cameron and colleagues, incorporates 14 clinical and laboratorial variables, but has not been externally validated. An American score based on artificial neural networks (ANN) has been assessed, not only as a means to predict endoscopic findings, but also for the need for endoscopic treatment in patients suffering from UGIB[26,27]. However, it requires the inclusion of 27 of the patients' variables and a specialized computer software for analysis.

THE IMPORTANCE OF OUTCOMES

When should we use a risk score?

Despite methodological and demographic differences,

Table 3 Cedars-sinai medical centre predictive index

Score	EGD findings[1]	Time[2]	Haemodynamics	Comorbidities
0	Ulcer without SHR, non-bleeding MW tear Erosive disease, normal EGD	> 48 h	Stable	⩽ 1
1	Ulcer with flat spot or clot, erosive disease with SHR, angiodysplasia	< 48 h	Intermediate	2
2	Ulcer with non- bleeding visible vessel or SHR	In hospital	Unstable	3
3				⩾ 4
4	Persistent haemorrhage, varices UGI cancer			

[1]Score for endoscopic findings was reduced by 1 point if effective endoscopic therapy was applied (not applicable to varices or cancer); [2]Time from onset of symptoms to hospitalization; SHR: Stigmata of recent haemorrhage; MW: Mallory-Weiss tear; EGD: Esophagogastroduodenoscopy; UGI: Upper gastrointestinal.

Table 4 Progetto nazionale emorragia digestiva score

Score	1	2	3	4
Variables	ASA 3 Time to admission < 8 h	Hb level ⩽ 7 g/dL Age ⩾ 80 Renal failure	Rebleeding ASA 4 Neoplasia Liver cirrhosis	Failure of endoscopic treatment

ASA: American Society of Anaesthesiology; GI: Gastrointestinal.

the outcomes evaluated in the different studies are relatively similar. However, when attempting to implement different scoring systems in clinical practice it is important to know the primary outcome variable that was measured in each developed study. A summary of the main outcomes of each score is listed in Table 8.

There is evidence that most of the mortality in patients suffering from NVUGIB is not directly related to bleeding[3]. So, adding clinical variables to a risk score may increase its ability to predict a specific outcome.

The Rockall score should be used as a tool for identifying patients with low risk of rebleeding and death according to the clinical and endoscopic risk factors. Patients with Rockall scores of less than or equal to 2 should be considered for management in the community. This score was prospectively derived from 4185 cases of UGIB over a 4 mo period in 1993 and was afterwards validated by the same investigators on the following year in 1625 cases from the same hospitals[15]. A higher Rockall score indicates a higher risk of a poor outcome. In a prospective validation of this score, Rockall *et al*[28] showed that the patients with a score of 2 or less (29.4% of the cohort) had a rebleeding rate of 4.3% and a mortality rate of 0.1%, suggesting that such patients could have been safely managed in the outpatient setting.

The Rockall score has been prospectively and externally validated in different populations[29-32]. Church and Palmer from Edinburgh proposed that the Rockall score could be used to predict rebleeding and death by doing a retrospective analysis of cases of peptic ulceration enrolled into two trials of endoscopic haemostasis. The authors showed a correlation between the Rockall score and mortality or rebleeding. Patients with scores of 8 or greater had a significantly poorer outcome[33].

Several studies have shown that the Rockall score was closely correlated with the probability of death, but not so close to the chance of rebleeding[30-32,34,35]. This observation may be partly explained by the fact that the Rockall score was originally developed for the prediction of mortality rather than for the prediction of rebleeding and also because not all patients received endoscopic therapy[15].

The clinical Rockall score, without endoscopy, can be used to improve the quality of patients' care by identifying those patients less likely to require intensive health care services and selecting them for endoscopic evaluation as outpatients, allowing substantial resource savings.

Tham *et al*[22] reported that patients classified as low risk, *i.e.,* clinical Rockall score of 0, can be managed in the outpatient setting because these patients had no adverse outcomes and did not require transfusion.

Phang *et al*[36] demonstrated that clinical Rockall may be helpful to determine the appropriate environment into which UGIB patients should be admitted. In this study 60.5% of the patients with a clinical Rockall score of < 4 (low risk) had a mortality rate of 3.2%, meaning that they could have been managed in a general ward. On the other hand, from the 39.5% of patients with a clinical Rockall score of ⩾ 4 (high risk), the mortality had a rate of 22.4%, indicating that those patients should be admitted to an intensive care unit. However, Gralnek *et al*[37] found that the complete rockall Score identified more low-risk patients than the clinical Rockall score.

Regarding peptic ulcer disease, BBS was initially proposed to predict rebleeding after endoscopic therapy, but was rarely used in the clinical practice. The original BBS study revealed that the cut-off of ⩾ 11 had a sensitivity of 100% and a specificity of 79% for the prediction of rebleeding[19]. The same authors who

Table 5 Glasgow blatchford score

Variable	Score
Blood urea (mmol/L)	
6.5-8	2
8-10	3
10-25	4
> 25	6
Hb (g/L) for men	
120-130	1
100-120	3
< 100	6
Hb (g/L) for women	
100-120	1
< 100	6
Systolic blood pressure (mmHg)	
100-109	1
90-99	2
< 90	3
Pulse ⩾ 100/min	1
History and comorbidities	
Melaena	1
Syncope	2
Hepatic disease[1]	2
Cardiac failure[2]	2

[1]Known history or clinical and laboratory evidence, of chronic or acute liver disease; [2]Known history or clinical and echocardiographic evidence, of cardiac failure.

Table 6 AIMS65 Score

Variable	Score
Albumin < 3 g/dL	1
International normalized ratio > 1.5	1
Systolic blood pressure < 90 mmHg	1
Altered mental status	1
Age > 65 yr	1

Table 7 T-score

Score Variable	1	2	3
General conditions	Poor	Intermediate	Good
Pulse (beats/min)	> 110	90-110	< 90
Systolic blood pressure (mmHg)	< 90	90-110	> 110
Haemoglobin levels (g/dL)	⩽ 8	9-10	> 10

Table 8 Outcomes

Scores	Primary outcome	Original studies
Endoscopy based score		
Forrest classification	Rebleeding	[12]
BBS	Rebleeding	[19]
CSMCPI	Mean length of stay	[20]
Rockall	Mortality	[15]
PNED	Mortality	[21]
Clinical scores		
Clinical rockall	Mortality	[15]
GBS	Need intervention	[41]
AIMS65	Mean length of stay/mortality	[23]
T-score	Time to endoscopy	[24,65]

BBS: Baylor bleeding score; CSMCPI: Cedars-sinai medical centre predictive index; PNED: Progetto nazionale emorragia digestiva; GBS: Glasgow blatchford score.

created this score had validated it prospectively in a cohort of 47 patients with bleeding peptic ulcer who had undergone endoscopic therapy[38]. Twenty-six patients were categorized as high-risk and 19 as low-risk. The rebleeding rate for high-risk patients was 31% and 0% for low-risk patients.

The CSMCPI may help to select low-risk patients suitable for early discharge[20,39]. In the original study of Hay *et al*[20] patients with a CSMCPI of 3 were considered suitable for discharge within 24 h. Seventy percent of UGIB patients (349 of 500) were considered as low-risk according to this cut-off. Complications occurred in 2 patients (0.6%) classified as low-risk. The routine use of the CSMCPI was associated with a reduced time of admission in 79% of all low-risk cases with a potential reduction of 2.1 bed-days per patient.

An italian study compared the three main endoscopy-based scores (CSMCPI, BBS and Rockall) and found out that the full Rockall score was superior in predicting mortality and rebleeding, particularly in low risk patients. In this study all scores were better at predicting mortality than rebleeding[29].

Marmo *et al*[21] developed the PNED score to predict 30 d mortality in patients suffering from acute NVUGB and validated it in a large cohort of patients. The PNED score is simple, reliable and accurate in identifying high-risk patients (score > 4 points) most likely to benefit from high levels of care and prevent death[21]. In a previous study the PNED score was significantly more accurate than the Rockall score in predicting death in non-variceal bleeders. This score introduces the failure to perform endoscopic haemostasis as a variable. As a matter of fact, in a former study by the same authors, the impossibility to perform endoscopic therapy was the strongest predictor of a negative outcome, being thus associated with an 11-fold risk of death[40].

The GBS should be used to predict the need for treatment (blood transfusion, endoscopic therapy or surgical intervention). This clinical score was developed from a prospective cohort involving 1748 patients admitted for UGIH in 19th centres in Scotland[41]. The greatest feature of the GBS is its ability to identify low-risk patients (GBS = 0) who do not need to be admitted into a hospital. With a high sensitivity and a high negative predictive value, the GBS indicates that almost all patients with a score equal to 0 can be safely discharged. In return, its positive predictive value remains low due to the low specificity (32%), significantly overestimating the risk of poor outcomes. A GBS of 0 was validated in the United Kingdom to safely discharge patients from Emergency Departments with suspected UGIB: Eighty-four patients with a GBS equal to 0 were managed as outpatients without adverse events during the follow-up[42]. In another United Kingdom study, 142 low-risk patients were managed

without admission, and none of them required endoscopic intervention, blood transfusion or surgery. In this cohort of patients the 28 d mortality rate was 0[43].

The GBS has been shown to be as good as the Rockall score in predicting the need for any intervention, namely the need for therapeutic endoscopy[41,42,44-47]. The GBS has also been shown to be superior to the clinical Rockall score in identifying patients with suspected UGIB who have a low likelihood of an adverse clinical outcome (blood transfusion, endoscopic therapy, interventional radiology, surgery or 30 d mortality) and can be considered for early discharge[48].

A multicentre study by Stanley *et al*[47] found that the GBS was equivalent to both the full and clinical Rockall scores in predicting death. It was however superior to the clinical Rockall score, and similar to the full Rockall score in predicting the need for endoscopic therapy or surgical intervention and superior to both in predicting the need for blood transfusion. This late finding is similar in both scores, because the GBS includes the measurement of haemoglobin levels on admission.

A study by Ahn *et al*[49] including patients with cancer who visited the Emergency Department, reported a greater accuracy of the GBS when compared to the Rockall score in predicting intervention in those patients.

Clinical variables such as liver disease, cardiovascular disease, presence of syncope, altered mental status and melena are susceptible to a subjective interpretation in GBS. Thus, a modified GBS could be used to eliminate subjective variables. A multicentre North American study reported that a modified GBS (without urea or syncope variables) was superior to the clinical Rockall score in predicting high risk of endoscopic stigmata in bleeding or rebleeding[50]. Another North American study has only incorporated the four quantitative variables, eliminating the subjective variables such as history of syncope, presence of melena, liver disease, cardiovascular disease, as well as altered mental status. The reported conclusion is that the modified GBS performed as well as the full GBS while outperforming both clinical and endoscopic Rockall Scores for prediction of clinical outcomes[51].

Some studies have suggested that the rate of identified low-risk patients could be increased by using a higher GBS cut-off value[45,52-57] or by incorporating age as a variable[52,58]. Stephens *et al*[52] showed that there was a relationship between age and significant endoscopic findings in patients categorized as low-risk by GBS. In this study, patients with a GBS of $\leqslant$ 2, for each additional year of age, had the chance of a significant endoscopic finding increased by 8%. The authors concluded that using GBS $\leqslant$ 2 and age of less than 70 years to define low-risk patients allows 10.5% of patients suffering from UGIB to be safely managed in the community. Over a 5-year period of managing such patients without hospital admission, McLaughlin *et al*[43] showed that any of them required endoscopic intervention, blood transfusion or surgery, and that the 28 d mortality was nil. A recent multicentre Danish study reported that a GBS cut-off value of $\leqslant$ 1 and an age modified low-risk version can be safely and effectively used to reduce unnecessary admissions for suspected UGIB[55].

AIMS65 can potentially be used to predict in-hospital mortality, length of stay, and cost in patients with acute UGIB[23]. When more than two components of AIMS65 are present, the mortality risk is considered to be high.

Hyett *et al*[59] has reported the superiority of the AIMS65 score when compared to the GBS in predicting inpatient mortality, but inferiority in predicting the need for blood transfusion. In a north American retrospective study, the AIMS65 showed no ability to predict the need for blood transfusion[60]. In a retrospective Japanese study, the AIMS65, but not the GBS, was considered an independent prognostic factor for poor overall survival[61]. On the other hand, in a recent study by Jung *et al*[62], the AIMS65 was insufficient in predicting outcomes in peptic ulcer bleeding.

In a recent Turkish study, the GBS was found to have superior sensitivity when compared to the AIMS65 score in identifying patients who were not likely to require interventions, including emergency endoscopy[63]. Such finding can be linked to the lower number of variables considered in this score. This score may not be able to detect low-risk patients with UGIB, but further studies are required.

Masaoka *et al*[64] recently proposed an algorithm to assess the mortality risk, which consisted in applying the AIMS65 score after detecting low-risk patients with GBS $\leqslant$ 2.

The T-score can be used to triage patients who are likely to have high-risk endoscopic stigmata and therefore need intervention[24]. It has the ability to predict high-risk endoscopic stigmata, rebleeding and mortality with an accuracy similar to the GBS[65]. According to Tammaro and collaborators, a T-score of $\leqslant$ 6 was able to predict the presence of high risk endoscopic stigmata and the need for an early endoscopy with a specificity of 96% and a positive predictive value of 74.5%[65].

Das *et al*[26] showed that ANN was superior to the admission Rockall score and similar to the full Rockall score to predict the need for endoscopic intervention, but its applicability in clinical practice is complex and time consuming.

Therapeutic decisions - why or why not should we use a risk score?

The consensus opinion recommends the early use of risk stratification scores in patients suffering from UGIB[10,11,13]. Many of these differ in the outcomes they were suggested for (risk of mortality, rebleeding and need for therapeutic intervention). However, in the era of increased outpatient management of UGIB, predicting the need for therapeutic intervention may be as useful as predicting rebleeding and death. Stratification risk systems could reduce the resources and costs without adversely influencing the patients' outcomes[66].

The greatest interest of clinical scores lies in their

ability to identify patients at low risk of complications who are suitable for early discharge without endoscopy. There is considerable evidence from several geographical regions (both in the United Kingdom and around the world)[41,42,44,46,48,56] that the GBS is an excellent risk assessment tool and accurately identifies patients with a low risk of requiring intervention or death. However, the best GBS cut-off for these situations is not clearly defined. Moreover, using a cut-off of 0 to predict low risk for adverse events has a practical limitation, since most of the patients who visit the Emergency Department with UGIB will score at least 1 point[41,42,46,65,67]. Possible explanations for the reported cut-off variation are differences in the demographics characteristics, aetiology of UGIB, routine use of proton pump inhibitors before endoscopy and adherence to guidelines regarding the need for endoscopic therapy.

Although with high sensitivity to identify patients at high risk for developing the need for blood transfusion, endoscopic therapy, or surgical intervention, the GBS has a low specificity[44,45,48,56,68,69].

Advanced age is a risk factor for death[70] and low-risk patients in general are younger than high-risk patients with UGIB. Despite not including age and being developed for predicting clinical intervention after UGIB detection, the GBS has proven to be equivalent to the Rockall score in predicting death[47]. Nevertheless, the ability of the GBS to identify low-risk patients may be enhanced by incorporating age as a variable[52,58].

Although there is greater consensus that certain endoscopic findings are associated with a high risk for adverse outcomes (*e.g.,* active bleeding or non-bleeding visible vessel), and other findings indicate a low risk for such outcomes (*e.g.,* clean-base ulcer, Mallory-Weiss tear), some controversy remains in what concerns the need of endoscopy as a component for early risk stratification at the initial patients' triage[71].

Almost all patients in the low risk group of the Rockall score had no stigmata of recent haemorrhage[29,35] and in clinical practice decisions regarding patient length of stay, admission place (intensive care unit versus regular ward) and therapeutic decisions are usually made on the basis of endoscopic appearance rather than the Rockall score[13]. The Forrest classification has shown a higher specificity and positive predictive value for the prediction of rebleeding and death when compared to other four scoring systems that were evaluated (the Rockall, the CSMCPI, the GBS and the BBS scores)[34].

The responsibility for initial patient assessment lies on the Emergency Department staff who invariably are general physicians or surgeons and may be uncomfortable about discharging patients without an endoscopy. Thus, although the GBS has shown a great ability to detect patients with low risk of complications in the Emergency Department setting, an endoscopy continues to support the patient management. On the other hand, by adopting a policy of urgent endoscopies in all patients with acute UGIB, several patients will undergo an unnecessary urgent procedure.

Another important key question in the management of patients with UGIB is the timing of the endoscopy, even though the overall consensus suggests that it should be performed within 24 h from admission[10]. Earlier endoscopy was not associated with a reduction in mortality or need for surgery. However, it was associated with an increased efficiency of care, a potential improvement in the control of haemorrhage in high-risk patients, and a reduction in the length of stay. All these factors support the routine use of early endoscopies, unless specific contraindications occur[72,73].

The Rockall Score is unable to address this question, since it requires endoscopic findings. A retrospective study by Lim *et al*[74] revealed that performing an endoscopy within 13 h for high-risk patients with a GBS of > 12 is associated with a reduced mortality. The timing of urgent endoscopy following an episode of UGIB may be also differentiated according to the simplified clinical T-score of $\leqslant 6$[65].

The need for a therapeutic endoscopy may also be a subjective decision[75] and a score that would equally help endoscopists in the decision to perform an urgent intervention is still warranted. Ideally, simple clinical scores could facilitate the identification of high-risk patients who could benefit from an early endoscopy with therapeutic intervention. Farooq *et al*[76] reported that the use of clinical Rockall and GBS was less accurate than a clinical triage decision in predicting the need for endoscopic therapy. In the study by Attar *et al*[67], the GBS showed an equivalent sensitivity when compared to endoscopists (both 98%) in the detection of urgent upper endoscopy necessity. However, both GBS and endoscopists showed a very poor specificity, being unable to detect non urgent patients to endoscopy.

In a recent multicentre study, although clinical knowledge of the endoscopists (described as "gut feeling") was an independent predictor for an adverse outcome, it had a lower sensitivity and a worst predictive power compared to prediction scores[77].

The reasons for not using clinical scores may be that they are difficult to calculate and time consuming and do not add much information to the physician's knowledge. Furthermore, no clinicians will feel comfortable in managing an elderly patient as an outpatient even if he has a GBS of 0. When there is clinical concern on avoiding admission in elderly patients, the use of an age modified GBS should be considered[55].

In real life, patients may also take antiplatelet and anticoagulant medications that may further increase the rebleeding rate and mortality, an issue not addressed in most reported studies. However, the AIMS65 includes the INR as a risk factor and an INR > 1.5 has been shown to be independently associated with in-hospital mortality in acute NVUGIB in a recent multicentre United Kingdom national audit[78].

The variables of AIMS65, with the exception of blood pressure, are different from those in the GBS. Albumin level and age may also contribute to the superiority of this score for the prediction of mortality[59]. Furthermore,

it has advantages over the existing risk scores, including the fact of being easy to remember and lacking the subjectivity in its calculation.

All scores seem to have lower performance in high-risk patients. Most clinical scores have poor specificity, possibly leading to unjustified upper endoscopies in the emergency context. However, the sensitivity of these scores may be likely more important than specificity, since they may help in physician's decisions, ensuring that any patient who may have a poor prognosis is discharged.

Extending the definition of low-risk patient may lead to outpatient management of patients who may actually need clinical intervention. Indeed, the cut-off value for considering patients to be at low or high risk may depend on local healthcare assistance and outpatient support and therefore needs to be carefully assessed in different populations.

Personalized medicine can help in stratification of patients according to biomarkers and guide optimal treatment and prevention. The molecular pathological epidemiology (MPE) is a recently established interdisciplinary and transdisciplinary field, which emerged from the complex relationship between etiological factors, molecular alterations, and disease evolution[79,80]. MPE may stratify UGIB into different subtypes according to the pathogenic mechanisms, enabling a more efficient and individualized approach.

To date, most of MPE research is applied to cancer[81,82], but this approach may also be important to UGIB and further investigation is needed to evaluate its contribution.

CONCLUSION

We believe that the value of risk scores in predicting the outcomes in acute UGIB has been proven far beyond any scepticism. Routine use of scoring systems by unspecialized medical staff could save lives, alert to the severity of a patient's condition and lead to an immediate referral. Furthermore, it could be an auxiliary tool for endoscopists that are often asked to perform an urgent endoscopy and have to decide whether the procedure should be done immediately or delayed up to 24 h. Endoscopic based scores can determine intensive care strategies, endoscopic therapy and length of hospitalization. As a means to predict low risk patients amenable to an early discharge and outpatient management, the Rockall and GBS are the two most commonly used and recommended risk stratification systems[13].

T-score, recently described, can potentially be useful to predict high-risk endoscopic stigmata and the need of early intervention[65]. We recommend the use of non-endoscopic scores as the pre-endoscopic Rockall score or the GBS, as a decision tools for patients with acute UGIB. The scores may be useful when endoscopy are not available in the emergency department. A patient with Rockall score or the GBS equal to 0 can be safely discharged.

Moreover, we also advocate early endoscopy (within 12 to 24 h of admission) and early discharge of patients with low risk lesions or low post-endoscopic risk scores (*e.g.,* post-endoscopic Rockall score $\leqslant$ 2).

Theoretically, the perfect score would be applicable in the two different stages of the patient's assessment, pre- and post-endoscopy, with excellent accuracy for the main outcomes in the context of UGIB: Rebleeding, death, and need of clinical intervention. However, since both scores have reached sufficient levels of efficiency that enable their safe employment in clinical practice, and until further research proves this premise, endoscopists should continue to rely on their "gut feeling" and on all the endoscopic findings as the key factors to guide their therapeutic decisions in patients with UGIB.

REFERENCES

1 **Longstreth GF**. Epidemiology of hospitalization for acute upper gastrointestinal hemorrhage: a population-based study. *Am J Gastroenterol* 1995; **90**: 206-210 [PMID: 7847286]

2 **Lanas A**, García-Rodríguez LA, Polo-Tomás M, Ponce M, Alonso-Abreu I, Perez-Aisa MA, Perez-Gisbert J, Bujanda L, Castro M, Muñoz M, Rodrigo L, Calvet X, Del-Pino D, Garcia S. Time trends and impact of upper and lower gastrointestinal bleeding and perforation in clinical practice. *Am J Gastroenterol* 2009; **104**: 1633-1641 [PMID: 19574968 DOI: 10.1038/ajg.2009.164]

3 **Sung JJ**, Tsoi KK, Ma TK, Yung MY, Lau JY, Chiu PW. Causes of mortality in patients with peptic ulcer bleeding: a prospective cohort study of 10,428 cases. *Am J Gastroenterol* 2010; **105**: 84-89 [PMID: 19755976 DOI: 10.1038/ajg.2009.507]

4 **Chiu PW**, Ng EK. Predicting poor outcome from acute upper gastrointestinal hemorrhage. *Gastroenterol Clin North Am* 2009; **38**: 215-230 [PMID: 19446255 DOI: 10.1016/j.gtc.2009.03.009]

5 **Klebl F**, Bregenzer N, Schöfer L, Tamme W, Langgartner J, Schölmerich J, Messmann H. Risk factors for mortality in severe upper gastrointestinal bleeding. *Int J Colorectal Dis* 2005; **20**: 49-56 [PMID: 15322836 DOI: 10.1007/s00384-004-0624-2]

6 **Zimmerman J**, Siguencia J, Tsvang E, Beeri R, Arnon R. Predictors of mortality in patients admitted to hospital for acute upper gastrointestinal hemorrhage. *Scand J Gastroenterol* 1995; **30**: 327-331 [PMID: 7610347]

7 **Imperiale TF**, Dominitz JA, Provenzale DT, Boes LP, Rose CM, Bowers JC, Musick BS, Azzouz F, Perkins SM. Predicting poor outcome from acute upper gastrointestinal hemorrhage. *Arch Intern Med* 2007; **167**: 1291-1296 [PMID: 17592103 DOI: 10.1001/archinte.167.12.1291]

8 **Nahon S**, Hagège H, Latrive JP, Rosa I, Nalet B, Bour B, Faroux R, Gower P, Arpurt JP, Denis J, Henrion J, Rémy AJ, Pariente A. Epidemiological and prognostic factors involved in upper gastrointestinal bleeding: results of a French prospective multicenter study. *Endoscopy* 2012; **44**: 998-1008 [PMID: 23108771 DOI: 10.1055/s-0032-1310006]

9 **Hearnshaw SA**, Logan RF, Lowe D, Travis SP, Murphy MF, Palmer KR. Use of endoscopy for management of acute upper gastrointestinal bleeding in the UK: results of a nationwide audit. *Gut* 2010; **59**: 1022-1029 [PMID: 20357318 DOI: 10.1136/gut.2008.174599]

10 **Barkun AN**, Bardou M, Kuipers EJ, Sung J, Hunt RH, Martel M, Sinclair P. International consensus recommendations on the management of patients with nonvariceal upper gastrointestinal bleeding. *Ann Intern Med* 2010; **152**: 101-113 [PMID: 20083829 DOI: 10.7326/0003-4819-152-2-201001190-00009]

11 **Dworzynski K**, Pollit V, Kelsey A, Higgins B, Palmer K. Management of acute upper gastrointestinal bleeding: summary of

NICE guidance. *BMJ* 2012; **344**: e3412 [PMID: 22695897 DOI: 10.1136/bmj.e3412]
12 **Forrest JA**, Finlayson ND, Shearman DJ. Endoscopy in gastrointestinal bleeding. *Lancet* 1974; **2**: 394-397 [PMID: 4136718]
13 **Laine L**, Jensen DM. Management of patients with ulcer bleeding. *Am J Gastroenterol* 2012; **107**: 345-360; quiz 361 [PMID: 22310222 DOI: 10.1038/ajg.2011.480]
14 **Holster IL**, Kuipers EJ. Update on the endoscopic management of peptic ulcer bleeding. *Curr Gastroenterol Rep* 2011; **13**: 525-531 [PMID: 21918857 DOI: 10.1007/s11894-011-0223-7]
15 **Rockall TA**, Logan RF, Devlin HB, Northfield TC. Risk assessment after acute upper gastrointestinal haemorrhage. *Gut* 1996; **38**: 316-321 [PMID: 8675081]
16 **Lin HJ**, Perng CL, Lee FY, Lee CH, Lee SD. Clinical courses and predictors for rebleeding in patients with peptic ulcers and non-bleeding visible vessels: a prospective study. *Gut* 1994; **35**: 1389-1393 [PMID: 7959193]
17 **Laine L**, Peterson WL. Bleeding peptic ulcer. *N Engl J Med* 1994; **331**: 717-727 [PMID: 8058080 DOI: 10.1056/nejm199409153311107]
18 **Laine L**, Cohen H, Brodhead J, Cantor D, Garcia F, Mosquera M. Prospective evaluation of immediate versus delayed refeeding and prognostic value of endoscopy in patients with upper gastrointestinal hemorrhage. *Gastroenterology* 1992; **102**: 314-316 [PMID: 1727765]
19 **Saeed ZA**, Winchester CB, Michaletz PA, Woods KL, Graham DY. A scoring system to predict rebleeding after endoscopic therapy of nonvariceal upper gastrointestinal hemorrhage, with a comparison of heat probe and ethanol injection. *Am J Gastroenterol* 1993; **88**: 1842-1849 [PMID: 8237930]
20 **Hay JA**, Lyubashevsky E, Elashoff J, Maldonado L, Weingarten SR, Ellrodt AG. Upper gastrointestinal hemorrhage clinical--guideline determining the optimal hospital length of stay. *Am J Med* 1996; **100**: 313-322 [PMID: 8629677]
21 **Marmo R**, Koch M, Cipolletta L, Capurso L, Grossi E, Cestari R, Bianco MA, Pandolfo N, Dezi A, Casetti T, Lorenzini I, Germani U, Imperiali G, Stroppa I, Barberani F, Boschetto S, Gigliozzi A, Gatto G, Peri V, Buzzi A, Della Casa D, Di Cicco M, Proietti M, Aragona G, Giangregorio F, Allegretta L, Tronci S, Michetti P, Romagnoli P, Piubello W, Ferri B, Fornari F, Del Piano M, Pagliarulo M, Di Mitri R, Trallori G, Bagnoli S, Frosini G, Macchiarelli R, Sorrentini I, Pietrini L, De Stefano S, Ceglia T, Chiozzini G, Salvagnini M, Di Muzio D, Rotondano G. Predicting mortality in non-variceal upper gastrointestinal bleeders: validation of the Italian PNED Score and Prospective Comparison with the Rockall Score. *Am J Gastroenterol* 2010; **105**: 1284-1291 [PMID: 20051943 DOI: 10.1038/ajg.2009.687]
22 **Tham TC**, James C, Kelly M. Predicting outcome of acute non-variceal upper gastrointestinal haemorrhage without endoscopy using the clinical Rockall Score. *Postgrad Med J* 2006; **82**: 757-759 [PMID: 17099097 DOI: 10.1136/pmj.2006.048462]
23 **Saltzman JR**, Tabak YP, Hyett BH, Sun X, Travis AC, Johannes RS. A simple risk score accurately predicts in-hospital mortality, length of stay, and cost in acute upper GI bleeding. *Gastrointest Endosc* 2011; **74**: 1215-1224 [PMID: 21907980 DOI: 10.1016/j.gie.2011.06.024]
24 **Tammaro L**, Di Paolo MC, Zullo A, Hassan C, Morini S, Caliendo S, Pallotta L. Endoscopic findings in patients with upper gastrointestinal bleeding clinically classified into three risk groups prior to endoscopy. *World J Gastroenterol* 2008; **14**: 5046-5050 [PMID: 18763288 DOI: 10.3748/wjg.14.5046]
25 **Cameron EA**, Pratap JN, Sims TJ, Inman S, Boyd D, Ward M, Middleton SJ. Three-year prospective validation of a pre-endoscopic risk stratification in patients with acute upper-gastrointestinal haemorrhage. *Eur J Gastroenterol Hepatol* 2002; **14**: 497-501 [PMID: 11984147]
26 **Das A**, Ben-Menachem T, Farooq FT, Cooper GS, Chak A, Sivak MV, Wong RC. Artificial neural network as a predictive instrument in patients with acute nonvariceal upper gastrointestinal hemorrhage. *Gastroenterology* 2008; **134**: 65-74 [PMID: 18061180 DOI: 10.1053/j.gastro.2007.10.037]
27 **Rotondano G**, Cipolletta L, Grossi E, Koch M, Intraligi M, Buscema M, Marmo R. Artificial neural networks accurately predict mortality in patients with nonvariceal upper GI bleeding. *Gastrointest Endosc* 2011; **73**: 218-226, 226.e1-2 [PMID: 21295635 DOI: 10.1016/j.gie.2010.10.006]
28 **Rockall TA**, Logan RF, Devlin HB, Northfield TC. Selection of patients for early discharge or outpatient care after acute upper gastrointestinal haemorrhage. National Audit of Acute Upper Gastrointestinal Haemorrhage. *Lancet* 1996; **347**: 1138-1140 [PMID: 8609747]
29 **Camellini L**, Merighi A, Pagnini C, Azzolini F, Guazzetti S, Scarcelli A, Manenti F, Rigo GP. Comparison of three different risk scoring systems in non-variceal upper gastrointestinal bleeding. *Dig Liver Dis* 2004; **36**: 271-277 [PMID: 15115340 DOI: 10.1016/j.dld.2003.10.017]
30 **Vreeburg EM**, Terwee CB, Snel P, Rauws EA, Bartelsman JF, Meulen JH, Tytgat GN. Validation of the Rockall risk scoring system in upper gastrointestinal bleeding. *Gut* 1999; **44**: 331-335 [PMID: 10026316]
31 **Enns RA**, Gagnon YM, Barkun AN, Armstrong D, Gregor JC, Fedorak RN. Validation of the Rockall scoring system for outcomes from non-variceal upper gastrointestinal bleeding in a Canadian setting. *World J Gastroenterol* 2006; **12**: 7779-7785 [PMID: 17203520 DOI: 10.3748/wjg.v12.i48.7779]
32 **Church NI**, Dallal HJ, Masson J, Mowat NA, Johnston DA, Radin E, Turner M, Fullarton G, Prescott RJ, Palmer KR. Validity of the Rockall scoring system after endoscopic therapy for bleeding peptic ulcer: a prospective cohort study. *Gastrointest Endosc* 2006; **63**: 606-612 [PMID: 16564860 DOI: 10.1016/j.gie.2005.06.042]
33 **Church NI**, Palmer KR. Relevance of the Rockall score in patients undergoing endoscopic therapy for peptic ulcer haemorrhage. *Eur J Gastroenterol Hepatol* 2001; **13**: 1149-1152 [PMID: 11711769]
34 **Kim BJ**, Park MK, Kim SJ, Kim ER, Min BH, Son HJ, Rhee PL, Kim JJ, Rhee JC, Lee JH. Comparison of scoring systems for the prediction of outcomes in patients with nonvariceal upper gastrointestinal bleeding: a prospective study. *Dig Dis Sci* 2009; **54**: 2523-2529 [PMID: 19104934 DOI: 10.1007/s10620-008-0654-7]
35 **Bessa X**, O'Callaghan E, Ballesté B, Nieto M, Seoane A, Panadès A, Vazquez DJ, Andreu M, Bory F. Applicability of the Rockall score in patients undergoing endoscopic therapy for upper gastrointestinal bleeding. *Dig Liver Dis* 2006; **38**: 12-17 [PMID: 16314150 DOI: 10.1016/j.dld.2005.05.012]
36 **Phang TS**, Vornik V, Stubbs R. Risk assessment in upper gastrointestinal haemorrhage: implications for resource utilisation. *N Z Med J* 2000; **113**: 331-333 [PMID: 11008609]
37 **Gralnek IM**, Dulai GS. Incremental value of upper endoscopy for triage of patients with acute non-variceal upper-GI hemorrhage. *Gastrointest Endosc* 2004; **60**: 9-14 [PMID: 15229418]
38 **Saeed ZA**, Ramirez FC, Hepps KS, Cole RA, Graham DY. Prospective validation of the Baylor bleeding score for predicting the likelihood of rebleeding after endoscopic hemostasis of peptic ulcers. *Gastrointest Endosc* 1995; **41**: 561-565 [PMID: 7672549]
39 **Garripoli A**, Mondardini A, Turco D, Martinoglio P, Secreto P, Ferrari A. Hospitalization for peptic ulcer bleeding: evaluation of a risk scoring system in clinical practice. *Dig Liver Dis* 2000; **32**: 577-582 [PMID: 11142555]
40 **Marmo R**, Koch M, Cipolletta L, Capurso L, Pera A, Bianco MA, Rocca R, Dezi A, Fasoli R, Brunati S, Lorenzini I, Germani U, Di Matteo G, Giorgio P, Imperiali G, Minoli G, Barberani F, Boschetto S, Martorano M, Gatto G, Amuso M, Pastorelli A, Torre ES, Triossi O, Buzzi A, Cestari R, Della Casa D, Proietti M, Tanzilli A, Aragona G, Giangregorio F, Allegretta L, Tronci S, Michetti P, Romagnoli P, Nucci A, Rogai F, Piubello W, Tebaldi M, Bonfante F, Casadei A, Cortini C, Chiozzini G, Girardi L, Leoci C, Bagnalasta G, Segato S, Chianese G, Salvagnini M, Rotondano G. Predictive factors of mortality from nonvariceal upper gastrointestinal hemorrhage: a multicenter study. *Am J Gastroenterol* 2008; **103**: 1639-1647; quiz 1648 [PMID: 18564127 DOI: 10.1111/j.1572-0241.2008.01865.x]
41 **Blatchford O**, Murray WR, Blatchford M. A risk score to predict

need for treatment for upper-gastrointestinal haemorrhage. *Lancet* 2000; **356**: 1318-1321 [PMID: 11073021 DOI: 10.1016/s0140-6736(00)02816-6]

42 **Stanley AJ**, Ashley D, Dalton HR, Mowat C, Gaya DR, Thompson E, Warshow U, Groome M, Cahill A, Benson G, Blatchford O, Murray W. Outpatient management of patients with low-risk upper-gastrointestinal haemorrhage: multicentre validation and prospective evaluation. *Lancet* 2009; **373**: 42-47 [PMID: 19091393 DOI: 10.1016/s0140-6736(08)61769-9]

43 **McLaughlin C**, Vine L, Chapman L, Deering P, Whittaker S, Beckly J, Fortun P, Murray IA, Hussaini SH, Michell NP, Stableforth B, Thatcher P, Hare NC, Palmer J, Dalton HR. The management of low-risk primary upper gastrointestinal haemorrhage in the community: a 5-year observational study. *Eur J Gastroenterol Hepatol* 2012; **24**: 288-293 [PMID: 22189690 DOI: 10.1097/MEG.0b013e32834febef]

44 **Chen IC**, Hung MS, Chiu TF, Chen JC, Hsiao CT. Risk scoring systems to predict need for clinical intervention for patients with nonvariceal upper gastrointestinal tract bleeding. *Am J Emerg Med* 2007; **25**: 774-779 [PMID: 17870480 DOI: 10.1016/j.ajem.2006.12.024]

45 **Srirajaskanthan R**, Conn R, Bulwer C, Irving P. The Glasgow Blatchford scoring system enables accurate risk stratification of patients with upper gastrointestinal haemorrhage. *Int J Clin Pract* 2010; **64**: 868-874 [PMID: 20337750 DOI: 10.1111/j.1742-1241.2009.02267.x]

46 **Pang SH**, Ching JY, Lau JY, Sung JJ, Graham DY, Chan FK. Comparing the Blatchford and pre-endoscopic Rockall score in predicting the need for endoscopic therapy in patients with upper GI hemorrhage. *Gastrointest Endosc* 2010; **71**: 1134-1140 [PMID: 20598244 DOI: 10.1016/j.gie.2010.01.028]

47 **Stanley AJ**, Dalton HR, Blatchford O, Ashley D, Mowat C, Cahill A, Gaya DR, Thompson E, Warshow U, Hare N, Groome M, Benson G, Murray W. Multicentre comparison of the Glasgow Blatchford and Rockall Scores in the prediction of clinical end-points after upper gastrointestinal haemorrhage. *Aliment Pharmacol Ther* 2011; **34**: 470-475 [PMID: 21707681 DOI: 10.1111/j.1365-2036.2011.04747.x]

48 **Le Jeune IR**, Gordon AL, Farrugia D, Manwani R, Guha IN, James MW. Safe discharge of patients with low-risk upper gastrointestinal bleeding (UGIB): can the use of Glasgow-Blatchford Bleeding Score be extended? *Acute Med* 2011; **10**: 176-181 [PMID: 22111089]

49 **Ahn S**, Lim KS, Lee YS, Lee JL. Blatchford score is a useful tool for predicting the need for intervention in cancer patients with upper gastrointestinal bleeding. *J Gastroenterol Hepatol* 2013; **28**: 1288-1294 [PMID: 23432611 DOI: 10.1111/jgh.12179]

50 **Romagnuolo J**, Barkun AN, Enns R, Armstrong D, Gregor J. Simple clinical predictors may obviate urgent endoscopy in selected patients with nonvariceal upper gastrointestinal tract bleeding. *Arch Intern Med* 2007; **167**: 265-270 [PMID: 17296882 DOI: 10.1001/archinte.167.3.265]

51 **Cheng DW**, Lu YW, Teller T, Sekhon HK, Wu BU. A modified Glasgow Blatchford Score improves risk stratification in upper gastrointestinal bleed: a prospective comparison of scoring systems. *Aliment Pharmacol Ther* 2012; **36**: 782-789 [PMID: 22928529 DOI: 10.1111/apt.12029]

52 **Stephens JR**, Hare NC, Warshow U, Hamad N, Fellows HJ, Pritchard C, Thatcher P, Jackson L, Michell N, Murray IA, Hyder Hussaini S, Dalton HR. Management of minor upper gastrointestinal haemorrhage in the community using the Glasgow Blatchford Score. *Eur J Gastroenterol Hepatol* 2009; **21**: 1340-1346 [PMID: 19738479 DOI: 10.1097/MEG.0b013e32831bc3ec]

53 **Mustafa Z**, Cameron A, Clark E, Stanley AJ. Outpatient management of low-risk patients with upper gastrointestinal bleeding: can we safely extend the Glasgow Blatchford Score in clinical practice? *Eur J Gastroenterol Hepatol* 2015; **27**: 512-515 [PMID: 25822859 DOI: 10.1097/meg.0000000000000333]

54 **Bryant RV**, Kuo P, Williamson K, Yam C, Schoeman MN, Holloway RH, Nguyen NQ. Performance of the Glasgow-Blatchford score in predicting clinical outcomes and intervention in hospitalized patients with upper GI bleeding. *Gastrointest Endosc* 2013; **78**: 576-583 [PMID: 23790755 DOI: 10.1016/j.gie.2013.05.003]

55 **Laursen SB**, Dalton HR, Murray IA, Michell N, Johnston MR, Schultz M, Hansen JM, Schaffalitzky de Muckadell OB, Blatchford O, Stanley AJ. Performance of new thresholds of the Glasgow Blatchford score in managing patients with upper gastrointestinal bleeding. *Clin Gastroenterol Hepatol* 2015; **13**: 115-21.e2 [PMID: 25058843 DOI: 10.1016/j.cgh.2014.07.023]

56 **Masaoka T**, Suzuki H, Hori S, Aikawa N, Hibi T. Blatchford scoring system is a useful scoring system for detecting patients with upper gastrointestinal bleeding who do not need endoscopic intervention. *J Gastroenterol Hepatol* 2007; **22**: 1404-1408 [PMID: 17716345 DOI: 10.1111/j.1440-1746.2006.04762.x]

57 **Schiefer M**, Aquarius M, Leffers P, Stassen P, van Deursen C, Oostenbrug L, Jansen L, Masclee A, Keulemans YC. Predictive validity of the Glasgow Blatchford Bleeding Score in an unselected emergency department population in continental Europe. *Eur J Gastroenterol Hepatol* 2012; **24**: 382-387 [PMID: 22228368 DOI: 10.1097/MEG.0b013e3283505965]

58 **Laursen SB**, Hansen JM, Schaffalitzky de Muckadell OB. The Glasgow Blatchford score is the most accurate assessment of patients with upper gastrointestinal hemorrhage. *Clin Gastroenterol Hepatol* 2012; **10**: 1130-1135.e1 [PMID: 22801061 DOI: 10.1016/j.cgh.2012.06.022]

59 **Hyett BH**, Abougergi MS, Charpentier JP, Kumar NL, Brozovic S, Claggett BL, Travis AC, Saltzman JR. The AIMS65 score compared with the Glasgow-Blatchford score in predicting outcomes in upper GI bleeding. *Gastrointest Endosc* 2013; **77**: 551-557 [PMID: 23357496 DOI: 10.1016/j.gie.2012.11.022]

60 **Chandra S**. AIMS65 score predicts short-term mortality but not the need for intervention in acute upper GI bleeding. *Gastrointest Endosc* 2013; **78**: 381-382 [PMID: 23867377 DOI: 10.1016/j.gie.2013.02.034]

61 **Nakamura S**, Matsumoto T, Sugimori H, Esaki M, Kitazono T, Hashizume M. Emergency endoscopy for acute gastrointestinal bleeding: prognostic value of endoscopic hemostasis and the AIMS65 score in Japanese patients. *Dig Endosc* 2014; **26**: 369-376 [PMID: 24168099 DOI: 10.1111/den.12187]

62 **Jung SH**, Oh JH, Lee HY, Jeong JW, Go SE, You CR, Jeon EJ, Choi SW. Is the AIMS65 score useful in predicting outcomes in peptic ulcer bleeding? *World J Gastroenterol* 2014; **20**: 1846-1851 [PMID: 24587662 DOI: 10.3748/wjg.v20.i7.1846]

63 **Yaka E**, Yılmaz S, Doğan NÖ, Pekdemir M. Comparison of the Glasgow-Blatchford and AIMS65 scoring systems for risk stratification in upper gastrointestinal bleeding in the emergency department. *Acad Emerg Med* 2015; **22**: 22-30 [PMID: 25556538 DOI: 10.1111/acem.12554]

64 **Masaoka T**, Suzuki H. Does the AIMS65, a new risk score for upper gastrointestinal bleeding, work in Japan? *Dig Endosc* 2014; **26**: 331-332 [PMID: 24754239 DOI: 10.1111/den.12224]

65 **Tammaro L**, Buda A, Di Paolo MC, Zullo A, Hassan C, Riccio E, Vassallo R, Caserta L, Anderloni A, Natali A. A simplified clinical risk score predicts the need for early endoscopy in non-variceal upper gastrointestinal bleeding. *Dig Liver Dis* 2014; **46**: 783-787 [PMID: 24953205 DOI: 10.1016/j.dld.2014.05.006]

66 **Cipolletta L**, Bianco MA, Rotondano G, Marmo R, Piscopo R. Outpatient management for low-risk nonvariceal upper GI bleeding: a randomized controlled trial. *Gastrointest Endosc* 2002; **55**: 1-5 [PMID: 11756905 DOI: 10.1067/mge.2002.119219]

67 **Attar A**, Sebbagh V, Vicaut E, Le Toumelin P, Bouhnik Y. Urgent endoscopy in severe non-variceal upper gastrointestinal hemorrhage: does the Glasgow-Blatchford score help endoscopists? *Scand J Gastroenterol* 2012; **47**: 1086-1093 [PMID: 22775006 DOI: 10.3109/00365521.2012.703237]

68 **Chandra S**, Hess EP, Agarwal D, Nestler DM, Montori VM, Song LM, Wells GA, Stiell IG. External validation of the Glasgow-Blatchford Bleeding Score and the Rockall Score in the US setting. *Am J Emerg Med* 2012; **30**: 673-679 [PMID: 21641145 DOI: 10.1016/j.ajem.2011.03.010]

69 **Wang CH**, Chen YW, Young YR, Yang CJ, Chen IC. A prospective comparison of 3 scoring systems in upper gastrointestinal bleeding. *Am J Emerg Med* 2013; **31**: 775-778 [PMID: 23465874 DOI: 10.1016/j.ajem.2013.01.007]

70 **Rockall TA**, Logan RF, Devlin HB, Northfield TC. Incidence of and mortality from acute upper gastrointestinal haemorrhage in the United Kingdom. Steering Committee and members of the National Audit of Acute Upper Gastrointestinal Haemorrhage. *BMJ* 1995; **311**: 222-226 [PMID: 7627034]

71 **Das A**, Wong RC. Prediction of outcome of acute GI hemorrhage: a review of risk scores and predictive models. *Gastrointest Endosc* 2004; **60**: 85-93 [PMID: 15229431]

72 **Jairath V**, Kahan BC, Logan RF, Hearnshaw SA, Doré CJ, Travis SP, Murphy MF, Palmer KR. Outcomes following acute nonvariceal upper gastrointestinal bleeding in relation to time to endoscopy: results from a nationwide study. *Endoscopy* 2012; **44**: 723-730 [PMID: 22752889 DOI: 10.1055/s-0032-1309736]

73 **Cooper GS**, Chak A, Way LE, Hammar PJ, Harper DL, Rosenthal GE. Early endoscopy in upper gastrointestinal hemorrhage: associations with recurrent bleeding, surgery, and length of hospital stay. *Gastrointest Endosc* 1999; **49**: 145-152 [PMID: 9925690]

74 **Lim LG**, Ho KY, Chan YH, Teoh PL, Khor CJ, Lim LL, Rajnakova A, Ong TZ, Yeoh KG. Urgent endoscopy is associated with lower mortality in high-risk but not low-risk nonvariceal upper gastrointestinal bleeding. *Endoscopy* 2011; **43**: 300-306 [PMID: 21360421 DOI: 10.1055/s-0030-1256110]

75 **Lau JY**, Sung JJ, Chan AC, Lai GW, Lau JT, Ng EK, Chung SC, Li AK. Stigmata of hemorrhage in bleeding peptic ulcers: an interobserver agreement study among international experts. *Gastrointest Endosc* 1997; **46**: 33-36 [PMID: 9260702]

76 **Farooq FT**, Lee MH, Das A, Dixit R, Wong RC. Clinical triage decision vs risk scores in predicting the need for endotherapy in upper gastrointestinal bleeding. *Am J Emerg Med* 2012; **30**: 129-134 [PMID: 21185674 DOI: 10.1016/j.ajem.2010.11.007]

77 **de Groot N**, van Oijen M, Kessels K, Hemmink M, Weusten B, Timmer R, Hazen W, van Lelyveld N, Vermeijden W, Baak L, Verburg R, Bosman J, de Wijkerslooth L, de Rooij J, Venneman N, Pennings M, van Hee K, Scheffer R, van Eijk R, Meiland R, Siersema P, Bredenoord A. Prediction scores or gastroenterologists' Gut Feeling for triaging patients that present with acute upper gastrointestinal bleeding. *United European Gastroenterol J* 2014; **2**: 197-205 [PMID: 25360303 DOI: 10.1177/2050640614531574]

78 **Jairath V**, Kahan BC, Stanworth SJ, Logan RF, Hearnshaw SA, Travis SP, Palmer KR, Murphy MF. Prevalence, management, and outcomes of patients with coagulopathy after acute nonvariceal upper gastrointestinal bleeding in the United Kingdom. *Transfusion* 2013; **53**: 1069-1076 [PMID: 22897615 DOI: 10.1111/j.1537-2995.2012.03849.x]

79 **Ogino S**, King EE, Beck AH, Sherman ME, Milner DA, Giovannucci E. Interdisciplinary education to integrate pathology and epidemiology: towards molecular and population-level health science. *Am J Epidemiol* 2012; **176**: 659-667 [PMID: 22935517 DOI: 10.1093/aje/kws226]

80 **Ogino S**, Lochhead P, Chan AT, Nishihara R, Cho E, Wolpin BM, Meyerhardt JA, Meissner A, Schernhammer ES, Fuchs CS, Giovannucci E. Molecular pathological epidemiology of epigenetics: emerging integrative science to analyze environment, host, and disease. *Mod Pathol* 2013; **26**: 465-484 [PMID: 23307060 DOI: 10.1038/modpathol.2012.214]

81 **Lochhead P**, Chan AT, Giovannucci E, Fuchs CS, Wu K, Nishihara R, O'Brien M, Ogino S. Progress and opportunities in molecular pathological epidemiology of colorectal premalignant lesions. *Am J Gastroenterol* 2014; **109**: 1205-1214 [PMID: 24935274 DOI: 10.1038/ajg.2014.153]

82 **Ogino S**, Chan AT, Fuchs CS, Giovannucci E. Molecular pathological epidemiology of colorectal neoplasia: an emerging transdisciplinary and interdisciplinary field. *Gut* 2011; **60**: 397-411 [PMID: 21036793 DOI: 10.1136/gut.2010.217182]

P- Reviewer: Bustamante-Balen M, Ogino S
S- Editor: Qiu S **L- Editor**: A **E- Editor**: Jiao XK

World Journal of Gastrointestinal Pathophysiology

Submit a Manuscript: http://www.wjgnet.com/esps/
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.4291/wjgp.v7.i1.97

World J Gastrointest Pathophysiol 2016 February 15; 7(1): 97-107
ISSN 2150-5330 (online)

REVIEW

Role of *Helicobacter pylori* infection in pathogenesis of gastric carcinoma

Rong-Guang Zhang, Guang-Cai Duan, Qing-Tang Fan, Shuai-Yin Chen

Rong-Guang Zhang, Guang-Cai Duan, Shuai-Yin Chen, Department of Epidemiology and Biostatistics, College of Public Health, Zhengzhou University, Zhengzhou 450001, Henan Province, China

Qing-Tang Fan, Henan Key Laboratory of Molecular Medicine, Zhengzhou University, Zhengzhou 450001, Henan Province, China

Author contributions: Zhang RG performed the review and wrote the paper; Duan GC conducted the review and inspected the manuscript; Fan QT and Chen SY took part in literature retrieval and analysis of the reported research findings.

Supported by The China Postdoctoral Science Foundation, No. 200801273.

Conflict-of-interest statement: No conflict-of-interest exists.

Correspondence to: Guang-Cai Duan, PhD, Department of Epidemiology and Biostatistics, College of Public Health, Zhengzhou University, 100 Kexue Avenue, Zhengzhou 450001, Henan Province, China. gcduan@zzu.edu.cn
Telephone: +86-371-67781472
Fax: +86-371-66997182

Received: April 28, 2015
Peer-review started: May 5, 2015
First decision: July 27, 2015
Revised: September 18, 2015
Accepted: November 3, 2015
Article in press: November 4, 2015
Published online: February 15, 2016

Abstract

Gastric cancer (GC) is one of the most common carcinoma and the second leading cause of cancer-related deaths worldwide. *Helicobacter pylori* (*H. pylori*) infection causes a series of precancerous lesions like gastritis, atrophy, intestinal metaplasia and dysplasia, and is the strongest known risk factor for GC, as supported by epidemiological, preclinical and clinical studies. However, the mechanism of *H. pylori* developing gastric carcinoma has not been well defined. Among infected individuals, approximately 10% develop severe gastric lesions such as peptic ulcer disease, 1%-3% progresses to GC. The outcomes of *H. pylori* infection are determined by bacterial virulence, genetic polymorphism of hosts as well as environmental factors. It is important to gain further understanding of the pathogenesis of *H. pylori* infection for developing more effective treatments for this common but deadly malignancy. The recent findings on the bacterial virulence factors, effects of *H. pylori* on epithelial cells, genetic polymorphism of both the bacterium and its host, and the environmental factors for GC are discussed with focus on the role of *H. pylori* in gastric carcinogenesis in this review.

Key words: *Helicobacter pylori*; Virulence factors; Gastric cancer; Genetic polymorphism; Environmental factors

Core tip: It is important to gain further understanding of the pathogenesis of *Helicobacter pylori* (*H. pylori*) infection for developing more effective treatments for this common but deadly malignancy. The recent findings on the bacterial virulence factors, effects of *H. pylori* on epithelial cells, genetic polymorphism of both the bacterium and its host, and the environmental factors for gastric cancer are discussed with focus on the role of *H. pylori* in gastric carcinogenesis in this review.

Zhang RG, Duan GC, Fan QT, Chen SY. Role of *Helicobacter pylori* infection in pathogenesis of gastric carcinoma. *World J Gastrointest Pathophysiol* 2016; 7(1): 97-107 Available from: URL: http://www.wjgnet.com/2150-5330/full/v7/i1/97.htm DOI: http://dx.doi.org/10.4291/wjgp.v7.i1.97

INTRODUCTION

Gastric cancer (GC) is one of the most common malignancies globally[1]. The risk factors for GC consist of *Helicobacter pylori* (*H. pylori*) infection, genetic and environmental factors[1]. *H. pylori* mainly colonized in human stomach, has coexisted with humans for nearly sixty thousand years[2]. The outcome of infection is affected by the environmental and genetic factors, the infection in most individuals does not develop distinct disease or even become beneficial, leading to the hypothesis that *H. pylori* might be commensal[3]. However, accumulating evidences support that *H. pylori* infection cause a list of diseases, ranging from gastric to extra-gastric diseases, from chronic gastritis to gastric carcinoma, and thus this bacterium is recognized as the Class Ⅰ carcinogenic pathogen in human with less than 3% of the infected eventually suffering GC[3].

Mechanism of *H. pylori*-associated gastric carcinogenesis has not been well defined. *H. pylori* infection commonly lasts for decades, provoking a series of histological changes including destruction of intercellular junctions, apoptosis and proliferation of epithelial cells and malignant transformation[4,5]. The genotypes of *H. pylori* strains, host genetic polymorphisms, environmental factors like high salt diet, smoking habit and certain gastric commensal organisms have been determined to be associated with occurrence of GC[6]. *H. pylori* genetic polymorphisms, effects of specific *H. pylori* products on gastric epithelium and cellular signaling process have been intensively investigated in recent decades[6]. This review is performed to discuss the role of *H. pylori* in gastric carcinogenesis.

H. PYLORI VIRULENCE FACTORS

Studies on *H. pylori* heterogeneity have proved that the strongest virulence factors were amongst the genes within the *cag* pathogenicity island (PAI).

CagA

CagA, a highly immunogenic protein, is encoded at one end of the *cag* PAI, which encode the components to form the type Ⅳ secretion system (T4SS)[7]. As a component of T4SS, CagL protein binds to and activates the integrin $\alpha5\beta1$ receptor on gastric epithelial cells and triggers CagA delivery into the target cells[8], CagM, along with CagX and CagT, forms an outer membrane-associated T4SS subcomplex[9], CagX and CagT interact directly[10]. As reported, CagA also facilitates its translocation into host epithelial cells by T4SS-induced externalization of phosphatidylserine from inner leaflet of the cellular membrane[8,11]. Recent studies demonstrated that fibronectinand peptidoglycan was also transported into epithelial cells by T4SS, suggesting that T4SS might have more CagA-independent functions than its ability to inject CagA[10]. CagA and CagM are important for assessing virulence of *H. pylori* strains.

H. pylori strains harboring the *cag* PAI or producing CagA are related to enhanced inflammation and risk of ulcers and carcinoma[12]. CagA contributes to myriad signaling alterations, which profoundly affects physiology of host epithelial cells. The Glu-Pro-Ile-Tyr-Ala (EPIYA) motifs in CagA are phosphorylation sites and play crucial roles in pathogenesis of *H. pylori* infection[13,14]. Once inside host cells, CagA is tyrosine phosphorylated by Src and Abl kinases at EPIYA motifs, and binds the SH2 domain of the SHP-2 phosphatase involved in transduction of signaling[15,16]. Phosphorylated CagA triggers the cellular signaling pathways leading to expression of proinflammatory cytokines and chemokines, and deregulates the signaling pathways that control host cell shape, adhesion and transformation[17,18]. Unphosphorylated CagA interacts with certain intracellular proteins, up-regulate production of proinflammatory cytokines, provoke mitogenic responses and disrupt intercellular junctions and epithelial cell polarity[17,19]. Additionally, CagA intoxicates dendritic cells leading to impaired activation, decreased inflammatory cytokine production and Th1 immune response[20]. Recently, it was confirmed that *H. pylori* infection resulted in rapid association of the virulence factor CagA with the c-Met receptor, activation of signaling and epithelial proliferation[21].

Vacuolating cytotoxin A

Vacuolating cytotoxin A (VacA) contributes to multiple structural and functional alterations of epithelial cells. After secretion by the bacterium through the type Ⅴ secretion system, VacA binds to host cells interfering with endosomal maturation and leading to vacuolation, enhances leakage of nutrients by destruction of barrier function at tight junctions of epithelial cells, provokes mitochondrial damage and cell apoptosis, which improves *H. pylori* growth[22-24]. Recent studies proved that VacA could disrupt phagocytosis, interfere with antigen presentation, restrain T cell activation *in vitro* and inhibit T cell proliferation independing of NFAT (nuclear factor of activated T cells) activation or IL-2 expression[25-27]. These effects of VacA on the immune system may explain how *H. pylori* evades adaptive immune responses to establish persistent infection.

BabA and SabA

Adherence to epithelial cells is important for *H. pylori* colonization and delivery of virulence factors to host cells[6]. BabA and SabA are two sialic acid-binding adhesins variably expressed by *H. pylori*. Among *babA* and *babB* genes, only the *babA2* allele possesses active

function[6,28]. BabA can bind to sialyl-Lewis x/a antigens and the Lewis b ABO blood group antigen (Leb), which are mainly distributed in red blood cells and certain epithelial cells[29], and this binding activity is commonly present in CagA positive strains[30]. Adherence mediated by BabA enhances the ability of T4SS to contact host cells, thus strengthen inflammatory response[31]. BabA binding to Leb contributes to gene mutations through formation of double stranded DNA breaks in host cell lines[32]. SabA can facilitate colonization in patients lacking Leb by binding to the sialyl-Lewis antigens[33], and mediate binding of *H. pylori* to sialylated structures of neutrophils[34]. The data suggest that BabA and SabA might be involved in carcinogenesis as abundance of sialyl-Lewis antigens is commonly enhanced in inflamed or cancerous gastric tissues[35].

OipA

OipA is an inflammation-related outer membrane protein, and the functional *oipA* gene is associated with more severe clinic outcomes[36,37]. *OipA* is commonly expressed together with CagA in *cagA* positive strains, which make it rather difficult to identify the effects of *oipA* alone in *H. pylori* infected human body or animal modules[38]. OipA expression is linked to increased production of proinflammatory cytokines like IL-8, IL-1, IL-17 and TNFα[39] as well as other host effector proteins including those associated with GC[40]. OipA can activate β-catenin, and mutant *H. pylori* strains lacking OipA decrease nuclear translocation of β-catenin, while tumorigenesis can be depressed by inactivating *oipA* of *H. pylori* strain in experimental animals[33,37]. These data suggest that OipA might take part in gastric carcinogenesis[33].

Gamma-glutamyl transpeptidase

Gamma-glutamyl transpeptidase (GGT) is mainly found in outer membrane vesicles of *H. pylori*, and has been proved to be related to enhanced levels of hydrogen peroxide and IL-8 production in epithelial cells and *H. pylori*-associated diseases[41-43]. GGT accelerates glutathione degradation, pro-oxidant compounds and reactive oxygen production[41]. GGT adjusts IL-8 expression by depletion of glutamine[41]. These findings indicate that GGT plays a significant role in *H. pylori*-related chronic inflammation and tissue damage.

It should be noted that interaction of the factors commonly exists *in vivo*, actions that a virulence factor take under the conditions with presence of other virulence factors might be different from those observed *in vitro*[44]. The influence of interactions between virulence factors in multifactorial pathogenesis is still uncertain. The main virulence factors in pathogenesis of *H. pylori* infection are shown in Figure 1[6].

EFFECTS OF *H. PYLORI* ON GASTRIC EPITHELIAL CELLS

Beside responsibility for digestive processes, the gastric epithelium has the function to protect underlying tissues from infection by pathogens[45]. *H. pylori* take specialized mechanisms to avoid host defense and adaptive immune for persistent colonization in human body, such as disruption of epithelial junctions, stimulation of cytokine production, overproliferation, DNA damage, apoptosis and cell transformation.

H. pylori disrupts junctions and polarity of epithelial cells

Intercellular apical junctions of epithelial cells are critical in keeping integrity of gastric epithelial barrier and essential cellular functions[25]. *H. pylori* disrupts epithelial tight junctions through binding to specific cellular receptors and stimulating the signaling pathways. As transported into epithelial cells through T4SS, CagA interacts with junction proteins like E-cadherin and ZO-1, and alters the tight or adherence junctions[25,46]. It has been confirmed that E-cadherin, a transmembrane protein, localizes at cell-to-cell junctions and interacts with β-catenin to form the E-cadherin/β-catenin complex, which play a key role in interaction of epithelial cells and stabilization of cellular architecture[46]. However, the complex is destabilized by translocated CagA in a phosphorylation independent manner during *H. pylori* infection[46]. As reported, CagA translocation is relevant to mislocalization of ZO-1 in epithelial cells[47,48]. Studies revealed that *H. pylori* altered expression and localisation of claudin-7, a cancer-associated tight junction protein, in gastroids and human epithelial cells, which was mediated by β-catenin and snail activation[49]. A recent study demonstrated that *H. pylori* diminished acid-induced tightening of cell junctions, affected the response of epithelial cells to acid, which took effects in inflammatory response and alteration of the barrier function[50].

H. pylori cause defect of epithelial cell polarity by targeting the epithelial adhesion receptors like E-cadherin and β1-integrin to modulate formation of cytoskeleton[51]. CagA disrupts polarity of epithelial cells through interaction with PAR1/MARK kinase[52]. As proved, an atypical protein kinase C (aPKC) contributes to disaggregation of PAR1 from tight junctions by phosphorylation of PAR1 at the junctions[52], and PAR1b binding to CagA restrains PAR1b activity and phosphorylation by aPKC to promote disruption of cellular polarity[48,52].

Induction of gastric epithelial cell autophagy or apoptosis

H. pylori not only colonize the mucus layer covering gastric mucosa, but also invade gastric epithelial cells, and even immunocytes[53]. Recent studies demonstrated that *H. pylori* induced autophagy of epithelial cells and phagocytes[53]. The autophagy of epithelial cells is modulated by *H. pylori*, and can be inducted by acute VacA exposure, and prolonged exposure to the toxin disrupts autophagy by preventing maturation of the autolysosome. The evidences support that *H. pylori*-

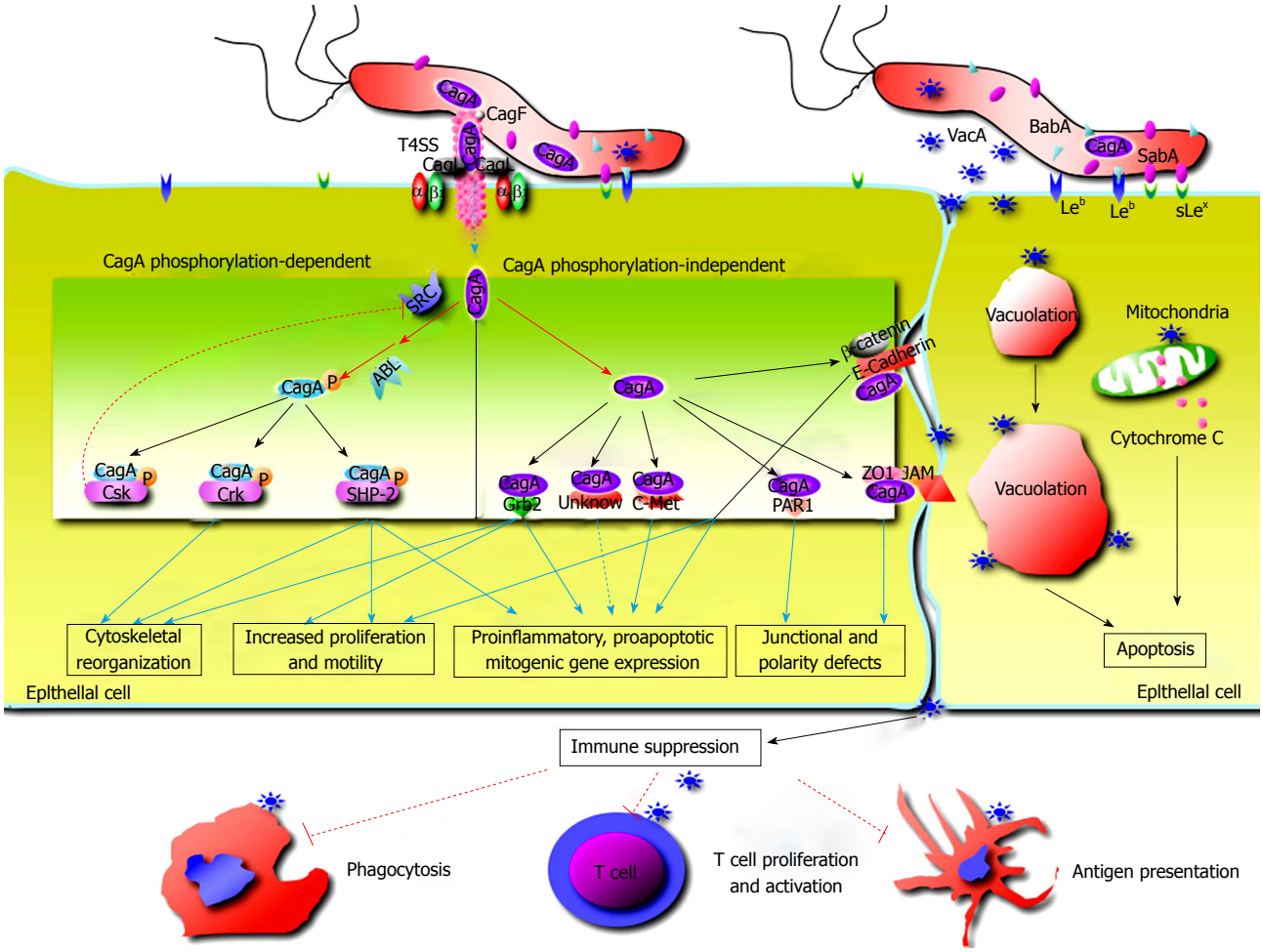


Figure 1 The roles of the main virulence factors in pathogenesis of *Helicobacter pylori* infection[6]. Adherence of *Helicobacter pylori* to gastric epithelial cells is mediated by BabA and SabA binding Leb and Lewis x/a respectively. CagA is translocated into epithelial cells through T4SS, and then tyrosine phosphorylated at EPIYA sites by Src and Abl kinases. CagA contributes to alteration of myriad signaling transduction, which affects host cell physiology with disruption of intercellular junctions, loss of cell polarity, promotion of inflammation, dysregulation of cellular apoptosis and proliferation. VacA inducts cytoplasmic vacuolation, apoptosis and immune suppression[6].

suppressed autophagy facilitates intracellular survival of this bacterium and generates an environment favoring carcinogenesis[54].

Rapid turnover of epithelial cells contributes to protect the epithelium from infection. *H. pylori* disrupts the balance of the proliferation and turnover of gastric epithelium to facilitate its survival[55]. Apoptosis is a regulated and conserved process in tissue, and takes the key role in tissue homeostasis[56]. *H. pylori* regulates the balance of epithelial cell apoptosis and proliferation for its reproduction[57,58]. The mechanism for this phenomenon remains to be well defined. The damage of gastric mucosa, stimulation of inflammatory immune responses by the enzymes like urease and VacA contribute to cellular apoptosis. The elevated level of free radicals produced by neutrophils and TH1 cytokines like IFN-γ in inflammatory response can damage DNA and induce apoptosis of epithelial cells[5,59]. *H. pylori* adhering to the epithelial surface also stimulate cellular apoptosis[60]. Studies demonstrated that human gastric epithelial cells sensitized to *H. pylori* confer susceptibility to TRAIL-mediated apoptosis through regulation of cellular FLICE-inhibitory protein activity and assembly of death-inducing signaling complex[61].

Cytokines secretion

The secretion of proinflammatory cytokines by gastric epithelial cells plays significant roles in pathogenesis of *H. pylori*-related gastric diseases. The cytokines involved in *H. pylori* infection include IL-8, IL-6, MCP-1, TNF-α, MIF, IL-1α, TGF-β, IL-1β and GMCSF[17]. The production of IL-8, a chemokine mediating accumulation of neutrophils, is related to the expression of CagA[17]. Further study confirmed that IL-8 and NF-κB expression was activated by urease B subunit[62], and the urease stimulates gastric epithelial cells to produce TNF-α and IL-6[63]. As reported, both *cag* PAI and OipA up-regulate IL-6 production in gastric epithelial cells[64]. Additionally, Th17 subsets are enriched in *H. pylori* infected mucosa[65]. The expression level of interleukin-17 (IL-17) has been observed to be up-regulated in gastric tissues of both human and animal during *H. pylori* infection, while

IL-17 can enhance expression of IL-8 in epithelial cells[66]. On the other hand, elevated levels of IL-21 and IL-23 expression in gastric mucosa induce and sustain IL-17 production[66]. Recently, a new clue for the pathogenesis of *H. pylori*-related gastric inflammation and GC is impairment of ghrelin synthesis in *H. pylori*-colonized stomach. Ghrelin, the ligand of growth hormone secretagogue receptor 1a, has immunoregulatory properties and function of certain inflammatory pathways inhibitor[67]. The defective ghrelin synthesis may contribute to sustain the ongoing inflammatory response in the gastric diseases[67].

Pro-carcinogenic responses

The mechanism underlying *H. pylor*-related gastric carcinogenesis remains unclear. CagA interacts with E-cadherin, deregulates β-catenin signal transduction and promotes gastric-to-intestinal transdifferentiation[47]. As observed, CagA translocated intracellularly binds to PAR1, destroys cellular junctions and polarity, and fosters carcinogenesis[48]. Development of gastric and hematological carcinoma has been observed in the mice that were genetically modified to express CagA[68]. Studies revealed that *cagA+/vacAs1+/vacAm1+H. pylori* strains promoted pathogenesis of intestinal metaplasia and gastric carcinoma[69].

Aditionally, *H. pylori* regulates expression of such toll-like receptors as toll-like receptor (TLR) 4 and TLR9 in epithelial cells during gastric carcinogenesis[70]. Caveolin-1 plays a protective role in immunologic injury caused by *H. pylori*[71]. Rapid association of the virulence factor CagA with the c-Met receptor, activation of signaling and induction of epithelial proliferation have been observed by using pluripotent stem-cell-derived gastric organoids[21].

GENETIC POLYMORPHISMS AFFECTING *H. PYLORI*-ASSOCIATED CARCINOGENESIS

H. pylori genetic variability

Genetic diversity of *H. pylori* has major contribution in the pathogenesis[72]. Studies have been conducted focusing on polymorphism of the main virulence factors, such as *cagA*, *vacA*, *oipA*, *iceA* and *hopQ*.

The highly polymorphic EPIYA motifs at the C-terminal of CagA are involved in pathogenesis of *H. pylori*-related gastroduodenal diseases[73]. CagA containing EPIYA motifs can activate the STAT3 pathway and promote cell migration[74]. The EPIYA motifs are distinguished by different amino acid sequences surrounding the EPIYA motif, and an increased number of CagA EPIYA-C sites confers a heightened risk for GC developing[75,76]. The sequences from Western and East Asian strains contain EPIYA-C and -D, respectively, and the strains with two segment C have more chances to develop GC than those with one[73]. The significantly higher prevalence of East Asian CagA in patients from Japan with *H. pylori* infection may be involved in the pathogenesis of GC[77].

Polymorphisms among the *vacA* alleles of *H. pylori* strains contribute to various levels of cytotoxicity, while variations in various regions can influence activity of VacA, including vacuolating activity[78,79]. It has been confirmed that vacuolating activity is highest in s1/m1 strains, *vacA* s1/m1 strains are closely relevant with GC in western countries[80,81]. Nevertheless, this situation is not universal worldwide, for example, s1/m1 strains in districts of Asia is irrelevant to clinical outcome[82,83].

Additionally, investigation of the prevalence of *oipA* and *iceA1/iceA2* positive strains among patients suffering from GC or gastritis results in that the frequency of *iceA1* allele in patients with GC is significantly higher than those with gastritis[72]. However, there is no significant difference in prevalence of *oipA* and *iceA2* genes among the two groups of patients ($P > 0.05$), suggesting the *iceA1* gene might take a role in pathogenesis of *H. pylori*-induced GC[72]. Studies also indicated that certain genetic types of *H. pylori hopQ* were closely related to GC[84].

Genetic polymorphism of H. pylori hosts

Polymorphisms in the genes encoding innate immune factors are involved in pathogenesis of *H. pylori*-related diseases, and the polymorphisms of cytokine genes cause inter-individual variation in cytokine responses which contributes to diversity of clinical outcome[85].

As reported, the risk of GC in many populations was affected by the polymorphism of the genes encoding IL-1β, TNFα, IL-8, IL-17 and IL-10 or their receptor antagonist[25]. An elevated risk of GC was observed in IL-8-251 AA or IL-10-1082 G genotype carriers with *H. pylori* infection[86]. IL-17 A/F plays critical function in inflammation and probably in cancer. Studies concluded that polymorphism of IL-17F was involved in susceptibility to GC[87].

Current evidences support that TLRs are play roles in both recognition of *H. pylori* and gastric carcinogenesis, and polymorphisms in genes involved in the TLR signaling pathways modulate the risk of GC[88].

Additionally, peroxisome proliferator-activated receptors may play roles in *H. pylori*-related gastric carcinogenesis[89]. The G/G variant rs2076167 is relevant to increased risk of GC in an animal model. The association between G/G variants of rs2016167 and GC is close among those consuming higher salt diet[89]. The insertion/deletion polymorphism of the angiotensin I-converting enzyme gene was recently proved to be linked to the pathogenesis and progression of human cancers[90]. As demonstrated, both bacterial and host gene polymorphisms affect oxidative stress and DNA damage, as thought to be a key mechanism in gastric carcinogenesis. The interaction of bacterial and host gene polymorphisms may become an explanation for why GC only occurs in a small proportion of *H. pylori*-infected individuals[91].

Table 1 Association between cagA status and tobacco smoking[98]

	N cagA neg	*N* cagA pos	OR	95%CI	OR[1]	95%CI
Smoking status at endoscope						
Non active smokers	34	31	1.00		1.00	
Active smokers	8	23	3.15	1.23-8.07	4.52	1.28-15.98
Smoking status						
Never	22	23	1.00		1.00	
Former smoker	12	8	0.64	0.22-1.86	0.36	0.09-1.53
Current smoker	8	23	2.75	1.02-7.43	3.24	0.84-12.47
P value for linear trend				0.067		0.123

[1]Adjusted for sex, age (≤ 50 years, > 50 years)[98].

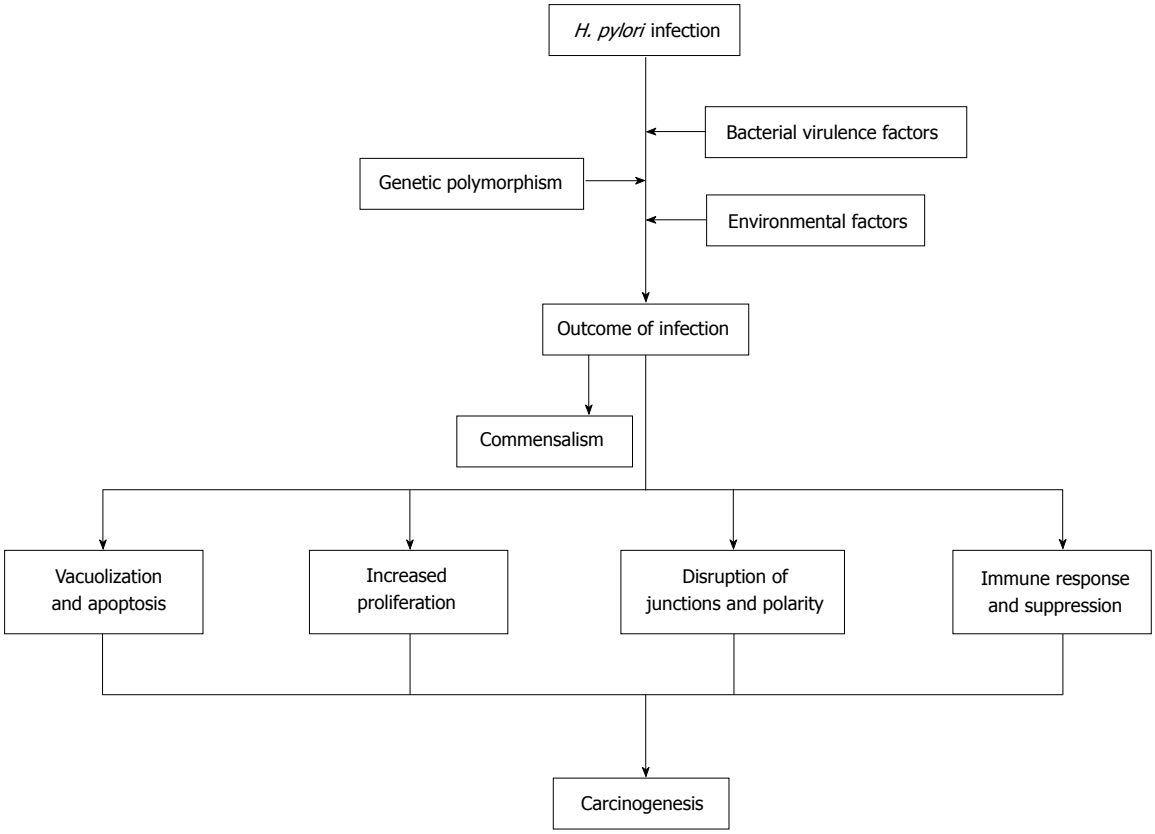


Figure 2 The pathogenesis of *Helicobacter pylori*-associated gastric cancer. The pathogenesis of *H. pylori*-associated GC is a multi-factorial process, its development depends on a combination of host, bacterial and environmental factors, and the pathological changes might progress in steps. *H. pylori*: *Helicobacter pylori*; GC: Gastric cancer.

ENVIRONMENTAL FACTORS IN *H. PYLORI*-RELATED GC

There are multiple ways by which *H. pylori* manipulates the host to lower the threshold for carcinogenesis, gastric microbiota, high-salt diet, smoking habit, low iron levels and use of proton pump inhibitors (PPIs) may enhance risk of *H. pylori*-associated carcinogenesis[92].

Gastric microbiota

Alterations of microbiota inhabiting human digestive tract can favor carcinogenesis[93]. Conventional wisdom espoused the dogma that pH values < 4 were able to sterilize the stomach, but since the discovery of *H. pylori*[94], a complex community of noncultivatable inhabitants have been uncovered in the stomach[95]. The interaction of gastric microbiota with *H. pylori* likely affects gastric immunobiology and the outcome of infection[95]. Data indicate that the microbial density in the normal stomach is low (10^1-10^3 CFU/g)[94], and the low bacterial densities within this portion of gastrointestinal tract is attributed to rapid peristalsis, low pH and/or high bile concentration[96]. The parietal cell loss caused by *H. pylori* infection leads to hypochlorhydria or even achlorhydria, thereby increase the risk of bacterial overgrowth and detrimental infection[97]. Alteration of gastric microbiota may promote the development of GC by up-regulating production of N-nitroso

compounds[93].

Smoking

Studies demonstrated that virulence factors like *cagA* and smoking might have synergistic effect in carcinogenesis of GC, and *cagA* genotype of *H. pylori* strains was closely related to active-smoking in population with *H. pylori* infection as shown in Table 1[98].

High salt diet

As evidenced, CagA expression is significantly upregulated when certain *H. pylori* strains are cultured in a medium of high salt concentrations. Through sequence analysis and site-directed mutagenesis, it was determined that salt-responsive *H. pylori* strains were more likely to contain two copies of TAATGA motif within the *cagA* gene promoter, while the strains containing only a single copy of this motif were less likely to possess properties of salt-responsive CagA expression[92,99]. However, another study showed that the severity of gastritis in *H. pylori* infected population might be unassociated with high-salt diet[100].

Iron levels

The iron level in the host has also been proved to manipulate the virulence potential of *H. pylori*. The bacteria harvested from gerbils with low iron levels were found to assemble more T4SS pili per bacterium, translocate increased amounts of CagA, and augment more IL-8 secretion compared to those isolated from gerbils with normal iron levels[101,102]. Furthermore, the *H. pylori* strains isolated from patients with low ferritin levels induce significantly higher levels of IL-8 compared to the strains from patients with the highest ferritin levels, suggesting that iron deficiency in the host might enhance the bacterial virulence and the risk for carcinogenesis of gastric tissues[101,102].

PPIs

It has been evidenced that long-term use of proton PPIs might aggravate corpus atrophic gastritis in *H. pylori*-infected patients[97]. The worsening atrophic gastritis contributes to development of gastric carcinoma, particularly owing increasing production of potentially carcinogenic N-nitroso compounds by the bacteria overgrowing under conditions of hypochlorhydria[97]. Hypergastrinemia induced by PPI administration might also promote the development of GC[97].

Di (2-ethylhexyl) phthalate

Di (2-ethylhexyl) phthalate (DEHP), as an essential additive in plastic manufacturing, has been used as plasticizer for many products including plastic food packaging[103]. Recent studies confirmed that DEHP was a teratogenic compound closely related to carcinogenesis[103]. DEHP may enhance *H. pylori* cytotoxicity, induce gastric epithelial cell apoptosis, disrupt the gastric mucosa integrity and promote pathogenesis of gastric carcinogenesis[103].

CONCLUSION

The genomes of *H. pylori* are highly diverse, multiple virulence factors take effects on host epithelium in various manners, including direct action and indirect action like eliciting immune response. Genetic polymorphism of host, dietary factors, smoking, gastric microbiota and long-term consuming of PPIs influence the progression of *H. pylori*-relate gastric lesion. The pathogenesis of *H. pylori*-associated GC is a multifactorial and multi-step process, and its development depends on a combination of host, bacterial and environmental factors as shown in Figure 2. It is important to further reveal the carcinogenesis of *H. pylori*-related GC in order to develop more effective treatments for this common but deadly malignancy.

REFERENCES

1 **Goh LY**, Leow AH, Goh KL. Observations on the epidemiology of gastrointestinal and liver cancers in the Asia-Pacific region. *J Dig Dis* 2014; **15**: 463-468 [PMID: 24894597 DOI: 10.1111/1751-2980.12164]

2 **Linz B**, Balloux F, Moodley Y, Manica A, Liu H, Roumagnac P, Falush D, Stamer C, Prugnolle F, van der Merwe SW, Yamaoka Y, Graham DY, Perez-Trallero E, Wadstrom T, Suerbaum S, Achtman M. An African origin for the intimate association between humans and Helicobacter pylori. *Nature* 2007; **445**: 915-918 [PMID: 17287725 DOI: 10.1038/nature05562]

3 **Mishra S**. Is Helicobacter pylori good or bad? *Eur J Clin Microbiol Infect Dis* 2013; **32**: 301-304 [PMID: 23132690 DOI: 10.1007/s10096-012-1773-9]

4 **Watari J**, Chen N, Amenta PS, Fukui H, Oshima T, Tomita T, Miwa H, Lim KJ, Das KM. Helicobacter pylori associated chronic gastritis, clinical syndromes, precancerous lesions, and pathogenesis of gastric cancer development. *World J Gastroenterol* 2014; **20**: 5461-5473 [PMID: 24833876 DOI: 10.3748/wjg.v20.i18.5461]

5 **Xia HH**, Talley NJ. Apoptosis in gastric epithelium induced by Helicobacter pylori infection: implications in gastric carcinogenesis. *Am J Gastroenterol* 2001; **96**: 16-26 [PMID: 11197247 DOI: 10.1016/S0002-9270(00)02240-1]

6 **Wen S**, Moss SF. Helicobacter pylori virulence factors in gastric carcinogenesis. *Cancer Lett* 2009; **282**: 1-8 [PMID: 19111390 DOI: 10.1016/j.canlet.2008.11.016]

7 **Tegtmeyer N**, Wessler S, Backert S. Role of the cag-pathogenicity island encoded type IV secretion system in Helicobacter pylori pathogenesis. *FEBS J* 2011; **278**: 1190-1202 [PMID: 21352489 DOI: 10.1111/j.1742-4658.2011.08035.x]

8 **Murata-Kamiya N**, Kikuchi K, Hayashi T, Higashi H, Hatakeyama M. Helicobacter pylori exploits host membrane phosphatidylserine for delivery, localization, and pathophysiological action of the CagA oncoprotein. *Cell Host Microbe* 2010; **7**: 399-411 [PMID: 20478541 DOI: 10.1016/j.chom.2010.04.005]

9 **Fischer W**. Assembly and molecular mode of action of the Helicobacter pylori Cag type IV secretion apparatus. *FEBS J* 2011; **278**: 1203-1212 [PMID: 21352490 DOI: 10.1111/j.1742-4658.2011.08036.x]

10 **Gopal GJ**, Pal J, Kumar A, Mukhopadhyay G. C-terminal domain of CagX is responsible for its interaction with CagT protein of Helicobacter pylori type IV secretion system. *Biochem Biophys Res Commun* 2015; **456**: 98-103 [PMID: 25446105 DOI: 10.1016/j.bbrc.2014.11.041]

11 **Hayashi T**, Senda M, Morohashi H, Higashi H, Horio M, Kashiba Y, Nagase L, Sasaya D, Shimizu T, Venugopalan N, Kumeta H,

Noda NN, Inagaki F, Senda T, Hatakeyama M. Tertiary structure-function analysis reveals the pathogenic signaling potentiation mechanism of Helicobacter pylori oncogenic effector CagA. *Cell Host Microbe* 2012; **12**: 20-33 [PMID: 22817985 DOI: 10.1016/j.chom.2012.05.010]

12 **Kim SS**, Ruiz VE, Carroll JD, Moss SF. Helicobacter pylori in the pathogenesis of gastric cancer and gastric lymphoma. *Cancer Lett* 2011; **305**: 228-238 [PMID: 20692762 DOI: 10.1016/j.canlet.2010.07.014]

13 **Kalaf EA**, Al-Khafaji ZM, Yassen NY, Al-Abbudi FA, Sadwen SN. Study of the cytoxin-associated gene a (CagA gene) in Helicobacter pylori using gastric biopsies of Iraqi patients. *Saudi J Gastroenterol* 2013; **19**: 69-74 [PMID: 23481132 DOI: 10.4103/1319-3767.108474]

14 **Stein M**, Bagnoli F, Halenbeck R, Rappuoli R, Fantl WJ, Covacci A. c-Src/Lyn kinases activate Helicobacter pylori CagA through tyrosine phosphorylation of the EPIYA motifs. *Mol Microbiol* 2002; **43**: 971-980 [PMID: 11929545 DOI: 10.1046/j.1365-2958.2002.02781.x]

15 **Higashi H**, Tsutsumi R, Muto S, Sugiyama T, Azuma T, Asaka M, Hatakeyama M. SHP-2 tyrosine phosphatase as an intracellular target of Helicobacter pylori CagA protein. *Science* 2002; **295**: 683-686 [PMID: 11743164 DOI: 10.1126/science.1067147]

16 **Bourzac KM**, Guillemin K. Helicobacter pylori-host cell interactions mediated by type IV secretion. *Cell Microbiol* 2005; **7**: 911-919 [PMID: 15953024 DOI: 10.1111/j.1462-5822.2005.00541.x]

17 **Alzahrani S**, Lina TT, Gonzalez J, Pinchuk IV, Beswick EJ, Reyes VE. Effect of Helicobacter pylori on gastric epithelial cells. *World J Gastroenterol* 2014; **20**: 12767-12780 [PMID: 25278677 DOI: 10.3748/wjg.v20.i36.12767]

18 **Backert S**, Clyne M. Pathogenesis of Helicobacter pylori infection. *Helicobacter* 2011; **16** Suppl 1: 19-25 [PMID: 21896081 DOI: 10.1111/j.1523-5378.2011.00876.x]

19 **Amieva MR**, Vogelmann R, Covacci A, Tompkins LS, Nelson WJ, Falkow S. Disruption of the epithelial apical-junctional complex by Helicobacter pylori CagA. *Science* 2003; **300**: 1430-1434 [PMID: 12775840 DOI: 10.1126/science.1081919]

20 **Tanaka H**, Yoshida M, Nishiumi S, Ohnishi N, Kobayashi K, Yamamoto K, Fujita T, Hatakeyama M, Azuma T. The CagA protein of Helicobacter pylori suppresses the functions of dendritic cell in mice. *Arch Biochem Biophys* 2010; **498**: 35-42 [PMID: 20363211 DOI: 10.1016/j.abb.2010.03.021]

21 **McCracken KW**, Catá EM, Crawford CM, Sinagoga KL, Schumacher M, Rockich BE, Tsai YH, Mayhew CN, Spence JR, Zavros Y, Wells JM. Modelling human development and disease in pluripotent stem-cell-derived gastric organoids. *Nature* 2014; **516**: 400-404 [PMID: 25363776 DOI: 10.1038/nature13863]

22 **Palframan SL**, Kwok T, Gabriel K. Vacuolating cytotoxin A (VacA), a key toxin for Helicobacter pylori pathogenesis. *Front Cell Infect Microbiol* 2012; **2**: 92 [PMID: 22919683 DOI: 10.3389/fcimb.2012.00092]

23 **Manente L**, Perna A, Buommino E, Altucci L, Lucariello A, Citro G, Baldi A, Iaquinto G, Tufano MA, De Luca A. The Helicobacter pylori's protein VacA has direct effects on the regulation of cell cycle and apoptosis in gastric epithelial cells. *J Cell Physiol* 2008; **214**: 582-587 [PMID: 17786942 DOI: 10.1002/jcp.21242]

24 **Papini E**, Satin B, Norais N, de Bernard M, Telford JL, Rappuoli R, Montecucco C. Selective increase of the permeability of polarized epithelial cell monolayers by Helicobacter pylori vacuolating toxin. *J Clin Invest* 1998; **102**: 813-820 [PMID: 9710450 DOI: 10.1172/JCI2764]

25 **Amieva MR**, El-Omar EM. Host-bacterial interactions in Helicobacter pylori infection. *Gastroenterology* 2008; **134**: 306-323 [PMID: 18166359 DOI: 10.1053/j.gastro.2007.11.009]

26 **Allen LA**, Schlesinger LS, Kang B. Virulent strains of Helicobacter pylori demonstrate delayed phagocytosis and stimulate homotypic phagosome fusion in macrophages. *J Exp Med* 2000; **191**: 115-128 [PMID: 10620610 DOI: 10.1084/jem.191.1.115]

27 **Torres VJ**, VanCompernolle SE, Sundrud MS, Unutmaz D, Cover TL. Helicobacter pylori vacuolating cytotoxin inhibits activation-induced proliferation of human T and B lymphocyte subsets. *J Immunol* 2007; **179**: 5433-5440 [PMID: 17911630 DOI: 10.4049/jimmunol.179.8.5433]

28 **Ilver D**, Arnqvist A, Ogren J, Frick IM, Kersulyte D, Incecik ET, Berg DE, Covacci A, Engstrand L, Borén T. Helicobacter pylori adhesin binding fucosylated histo-blood group antigens revealed by retagging. *Science* 1998; **279**: 373-377 [PMID: 9430586 DOI: 10.1126/science.279.5349.373]

29 **Mahdavi J**, Sondén B, Hurtig M, Olfat FO, Forsberg L, Roche N, Angstrom J, Larsson T, Teneberg S, Karlsson KA, Altraja S, Wadström T, Kersulyte D, Berg DE, Dubois A, Petersson C, Magnusson KE, Norberg T, Lindh F, Lundskog BB, Arnqvist A, Hammarström L, Borén T. Helicobacter pylori SabA adhesin in persistent infection and chronic inflammation. *Science* 2002; **297**: 573-578 [PMID: 12142529 DOI: 10.1126/science.1069076]

30 **Lu H**, Yamaoka Y, Graham DY. Helicobacter pylori virulence factors: facts and fantasies. *Curr Opin Gastroenterol* 2005; **21**: 653-659 [PMID: 16220040 DOI: 10.1097/01.mog.0000181711.04529.d5]

31 **Ishijima N**, Suzuki M, Ashida H, Ichikawa Y, Kanegae Y, Saito I, Borén T, Haas R, Sasakawa C, Mimuro H. BabA-mediated adherence is a potentiator of the Helicobacter pylori type IV secretion system activity. *J Biol Chem* 2011; **286**: 25256-25264 [PMID: 21596743 DOI: 10.1074/jbc.M111.233601]

32 **Toller IM**, Neelsen KJ, Steger M, Hartung ML, Hottiger MO, Stucki M, Kalali B, Gerhard M, Sartori AA, Lopes M, Müller A. Carcinogenic bacterial pathogen Helicobacter pylori triggers DNA double-strand breaks and a DNA damage response in its host cells. *Proc Natl Acad Sci USA* 2011; **108**: 14944-14949 [PMID: 21896770 DOI: 10.1073/pnas.1100959108]

33 **Testerman TL**, Morris J. Beyond the stomach: an updated view of Helicobacter pylori pathogenesis, diagnosis, and treatment. *World J Gastroenterol* 2014; **20**: 12781-12808 [PMID: 25278678 DOI: 10.3748/wjg.v20.i36.12781]

34 **Unemo M**, Aspholm-Hurtig M, Ilver D, Bergström J, Borén T, Danielsson D, Teneberg S. The sialic acid binding SabA adhesin of Helicobacter pylori is essential for nonopsonic activation of human neutrophils. *J Biol Chem* 2005; **280**: 15390-15397 [PMID: 15689619 DOI: 10.1074/jbc.M412725200]

35 **Yamaoka Y**. Increasing evidence of the role of Helicobacter pylori SabA in the pathogenesis of gastroduodenal disease. *J Infect Dev Ctries* 2008; **2**: 174-181 [PMID: 19738347 DOI: 10.3855/jidc.259]

36 **Yamaoka Y**, Kwon DH, Graham DY. A M(r) 34,000 proinflammatory outer membrane protein (oipA) of Helicobacter pylori. *Proc Natl Acad Sci USA* 2000; **97**: 7533-7538 [PMID: 10852959 DOI: 10.1073/pnas.130079797]

37 **Franco AT**, Johnston E, Krishna U, Yamaoka Y, Israel DA, Nagy TA, Wroblewski LE, Piazuelo MB, Correa P, Peek RM. Regulation of gastric carcinogenesis by Helicobacter pylori virulence factors. *Cancer Res* 2008; **68**: 379-387 [PMID: 18199531 DOI: 10.1158/0008-5472.CAN-07-0824]

38 **Yamaoka Y**. Mechanisms of disease: Helicobacter pylori virulence factors. *Nat Rev Gastroenterol Hepatol* 2010; **7**: 629-641 [PMID: 20938460 DOI: 10.1038/nrgastro.2010.154]

39 **Sugimoto M**, Ohno T, Graham DY, Yamaoka Y. Gastric mucosal interleukin-17 and -18 mRNA expression in Helicobacter pylori-induced Mongolian gerbils. *Cancer Sci* 2009; **100**: 2152-2159 [PMID: 19694753 DOI: 10.1111/j.1349-7006.2009.01291.x]

40 **Backert S**, Clyne M, Tegtmeyer N. Molecular mechanisms of gastric epithelial cell adhesion and injection of CagA by Helicobacter pylori. *Cell Commun Signal* 2011; **9**: 28 [PMID: 22044679 DOI: 10.1186/1478-811X-9-28]

41 **Rimbara E**, Mori S, Kim H, Shibayama K. Role of γ-glutamyltranspeptidase in the pathogenesis of Helicobacter pylori infection. *Microbiol Immunol* 2013; **57**: 665-673 [PMID: 23937242 DOI: 10.1111/1348-0421.12089]

42 **Olofsson A**, Vallström A, Petzold K, Tegtmeyer N, Schleucher J, Carlsson S, Haas R, Backert S, Wai SN, Gröbner G, Arnqvist A.

Biochemical and functional characterization of Helicobacter pylori vesicles. *Mol Microbiol* 2010; **77**: 1539-1555 [PMID: 20659286 DOI: 10.1111/j.1365-2958.2010.07307.x]

43 **Gong M**, Ling SS, Lui SY, Yeoh KG, Ho B. Helicobacter pylori gamma-glutamyl transpeptidase is a pathogenic factor in the development of peptic ulcer disease. *Gastroenterology* 2010; **139**: 564-573 [PMID: 20347814 DOI: 10.1053/j.gastro.2010.03.050]

44 **Kim IJ**, Blanke SR. Remodeling the host environment: modulation of the gastric epithelium by the Helicobacter pylori vacuolating toxin (VacA). *Front Cell Infect Microbiol* 2012; **2**: 37 [PMID: 22919629 DOI: 10.3389/fcimb.2012.00037]

45 **Wroblewski LE**, Peek RM. "Targeted disruption of the epithelial-barrier by Helicobacter pylori". *Cell Commun Signal* 2011; **9**: 29 [PMID: 22044698 DOI: 10.1186/1478-811X-9-29]

46 **Fischer W**, Prassl S, Haas R. Virulence mechanisms and persistence strategies of the human gastric pathogen Helicobacter pylori. *Curr Top Microbiol Immunol* 2009; **337**: 129-171 [PMID: 19812982 DOI: 10.1007/978-3-642-01846-6_5]

47 **Murata-Kamiya N**, Kurashima Y, Teishikata Y, Yamahashi Y, Saito Y, Higashi H, Aburatani H, Akiyama T, Peek RM, Azuma T, Hatakeyama M. Helicobacter pylori CagA interacts with E-cadherin and deregulates the beta-catenin signal that promotes intestinal transdifferentiation in gastric epithelial cells. *Oncogene* 2007; **26**: 4617-4626 [PMID: 17237808 DOI: 10.1038/sj.onc.1210251]

48 **Saadat I**, Higashi H, Obuse C, Umeda M, Murata-Kamiya N, Saito Y, Lu H, Ohnishi N, Azuma T, Suzuki A, Ohno S, Hatakeyama M. Helicobacter pylori CagA targets PAR1/MARK kinase to disrupt epithelial cell polarity. *Nature* 2007; **447**: 330-333 [PMID: 17507984 DOI: 10.1038/nature05765]

49 **Wroblewski LE**, Piazuelo MB, Chaturvedi R, Schumacher M, Aihara E, Feng R, Noto JM, Delgado A, Israel DA, Zavros Y, Montrose MH, Shroyer N, Correa P, Wilson KT, Peek RM. Helicobacter pylori targets cancer-associated apical-junctional constituents in gastroids and gastric epithelial cells. *Gut* 2015; **64**: 720-730 [PMID: 25123931 DOI: 10.1136/gutjnl-2014-307650]

50 **Marcus EA**, Vagin O, Tokhtaeva E, Sachs G, Scott DR. Helicobacter pylori impedes acid-induced tightening of gastric epithelial junctions. *Am J Physiol Gastrointest Liver Physiol* 2013; **305**: G731-G739 [PMID: 23989011 DOI: 10.1152/ajpgi.00209.2013]

51 **Osman MA**, Bloom GS, Tagoe EA. Helicobacter pylori-induced alteration of epithelial cell signaling and polarity: a possible mechanism of gastric carcinoma etiology and disparity. *Cytoskeleton* (Hoboken) 2013; **70**: 349-359 [PMID: 23629919 DOI: 10.1002/cm.21114]

52 **Lu H**, Murata-Kamiya N, Saito Y, Hatakeyama M. Role of partitioning-defective 1/microtubule affinity-regulating kinases in the morphogenetic activity of Helicobacter pylori CagA. *J Biol Chem* 2009; **284**: 23024-23036 [PMID: 19553659 DOI: 10.1074/jbc.M109.001008]

53 **Deen NS**, Huang SJ, Gong L, Kwok T, Devenish RJ. The impact of autophagic processes on the intracellular fate of Helicobacter pylori: more tricks from an enigmatic pathogen? *Autophagy* 2013; **9**: 639-652 [PMID: 23396129 DOI: 10.4161/auto.23782]

54 **Greenfield LK**, Jones NL. Modulation of autophagy by Helicobacter pylori and its role in gastric carcinogenesis. *Trends Microbiol* 2013; **21**: 602-612 [PMID: 24156875 DOI: 10.1016/j.tim.2013.09.004]

55 **Saberi S**, Douraghi M, Azadmanesh K, Shokrgozar MA, Zeraati H, Hosseini ME, Mohagheghi MA, Parsaeian M, Mohammadi M. A potential association between Helicobacter pylori CagA EPIYA and multimerization motifs with cytokeratin 18 cleavage rate during early apoptosis. *Helicobacter* 2012; **17**: 350-357 [PMID: 22967118 DOI: 10.1111/j.1523-5378.2012.00954.x]

56 **Jiang X**, Wang X. Cytochrome C-mediated apoptosis. *Annu Rev Biochem* 2004; **73**: 87-106 [PMID: 15189137 DOI: 10.1146/annurev.biochem.73.011303]

57 **Ashktorab H**, Dashwood RH, Dashwood MM, Zaidi SI, Hewitt SM, Green WR, Lee EL, Daremipouran M, Nouraie M, Malekzadeh R, Smoot DT. H. pylori-induced apoptosis in human gastric cancer cells mediated via the release of apoptosis-inducing factor from mitochondria. *Helicobacter* 2008; **13**: 506-517 [PMID: 19166416 DOI: 10.1111/j.1523-5378.2008.00646.x]

58 **Iwai H**, Kim M, Yoshikawa Y, Ashida H, Ogawa M, Fujita Y, Muller D, Kirikae T, Jackson PK, Kotani S, Sasakawa C. A bacterial effector targets Mad2L2, an APC inhibitor, to modulate host cell cycling. *Cell* 2007; **130**: 611-623 [PMID: 17719540 DOI: 10.1016/j.cell.2007.06.043]

59 **Fan X**, Crowe SE, Behar S, Gunasena H, Ye G, Haeberle H, Van Houten N, Gourley WK, Ernst PB, Reyes VE. The effect of class II major histocompatibility complex expression on adherence of Helicobacter pylori and induction of apoptosis in gastric epithelial cells: a mechanism for T helper cell type 1-mediated damage. *J Exp Med* 1998; **187**: 1659-1669 [PMID: 9584144 DOI: 10.1084/jem.187.10.1659]

60 **Fan X**, Gunasena H, Cheng Z, Espejo R, Crowe SE, Ernst PB, Reyes VE. Helicobacter pylori urease binds to class II MHC on gastric epithelial cells and induces their apoptosis. *J Immunol* 2000; **165**: 1918-1924 [PMID: 10925273 DOI: 10.4049/jimmunol.165.4.1918]

61 **Lin WC**, Tsai HF, Liao HJ, Tang CH, Wu YY, Hsu PI, Cheng AL, Hsu PN. Helicobacter pylori sensitizes TNF-related apoptosis-inducing ligand (TRAIL)-mediated apoptosis in human gastric epithelial cells through regulation of FLIP. *Cell Death Dis* 2014; **5**: e1109 [PMID: 24603337 DOI: 10.1038/cddis.2014.81]

62 **Beswick EJ**, Pinchuk IV, Minch K, Suarez G, Sierra JC, Yamaoka Y, Reyes VE. The Helicobacter pylori urease B subunit binds to CD74 on gastric epithelial cells and induces NF-kappaB activation and interleukin-8 production. *Infect Immun* 2006; **74**: 1148-1155 [PMID: 16428763 DOI: 10.1128/IAI.74.2.1148-1155.2006]

63 **Tanahashi T**, Kita M, Kodama T, Yamaoka Y, Sawai N, Ohno T, Mitsufuji S, Wei YP, Kashima K, Imanishi J. Cytokine expression and production by purified Helicobacter pylori urease in human gastric epithelial cells. *Infect Immun* 2000; **68**: 664-671 [PMID: 10639431 DOI: 10.1128/IAI.68.2.664-671.2000]

64 **Lu H**, Wu JY, Kudo T, Ohno T, Graham DY, Yamaoka Y. Regulation of interleukin-6 promoter activation in gastric epithelial cells infected with Helicobacter pylori. *Mol Biol Cell* 2005; **16**: 4954-4966 [PMID: 16030249 DOI: 10.1091/mbc.E05-05-0426]

65 **Pinchuk IV**, Morris KT, Nofchissey RA, Earley RB, Wu JY, Ma TY, Beswick EJ. Stromal cells induce Th17 during Helicobacter pylori infection and in the gastric tumor microenvironment. *PLoS One* 2013; **8**: e53798 [PMID: 23365642 DOI: 10.1371/journal.pone.0053798]

66 **Kabir S**. The role of interleukin-17 in the Helicobacter pylori induced infection and immunity. *Helicobacter* 2011; **16**: 1-8 [PMID: 21241406 DOI: 10.1111/j.1523-5378.2010.00812.x]

67 **Paoluzi OA**, Blanco del VG, Caruso R, Monteleone I, Monteleone G, Pallone F. Impairment of ghrelin synthesis in Helicobacter pylori-colonized stomach: new clues for the pathogenesis of H. pylori-related gastric inflammation. *World J Gastroenterol* 2014; **20**: 639-646 [PMID: 24574737 DOI: 10.3748/wjg.v20.i3.639]

68 **Ohnishi N**, Yuasa H, Tanaka S, Sawa H, Miura M, Matsui A, Higashi H, Musashi M, Iwabuchi K, Suzuki M, Yamada G, Azuma T, Hatakeyama M. Transgenic expression of Helicobacter pylori CagA induces gastrointestinal and hematopoietic neoplasms in mouse. *Proc Natl Acad Sci USA* 2008; **105**: 1003-1008 [PMID: 18192401 DOI: 10.1073/pnas.0711183105]

69 **Wang F**, Wu X, Liu Z, Bu G, Li X, Qu N, Peng J, Xu C, Shen S, Yuan Y. Association between Virulence Factors and TRAF1/4-1BB/Bcl-xL Expression in Gastric Mucosa Infected with Helicobacter pylori. *Gastroenterol Res Pract* 2015; **2015**: 648479 [PMID: 25737718 DOI: 10.1155/2015/648479]

70 **Wang TR**, Peng JC, Qiao YQ, Zhu MM, Zhao D, Shen J, Ran ZH. Helicobacter pylori regulates TLR4 and TLR9 during gastric carcinogenesis. *Int J Clin Exp Pathol* 2014; **7**: 6950-6955 [PMID: 25400780]

71 **Hitkova I**, Yuan G, Anderl F, Gerhard M, Kirchner T, Reu S,

Röcken C, Schäfer C, Schmid RM, Vogelmann R, Ebert MP, Burgermeister E. Caveolin-1 protects B6129 mice against Helicobacter pylori gastritis. *PLoS Pathog* 2013; **9**: e1003251 [PMID: 23592983 DOI: 10.1371/journal.ppat.1003251]

72 **Aghdam SM**, Sardari Z, Safaralizadeh R, Bonyadi M, Abdolmohammadi R, Moghadam MS, Khalilnezhad A. Investigation of association between oipA and iceA1/iceA2 genotypes of Helicobacter pylori and gastric cancer in Iran. *Asian Pac J Cancer Prev* 2014; **15**: 8295-8299 [PMID: 25339020 DOI: 10.7314/APJCP.2014.15.19.8295]

73 **Xia Y**, Yamaoka Y, Zhu Q, Matha I, Gao X. A comprehensive sequence and disease correlation analyses for the C-terminal region of CagA protein of Helicobacter pylori. *PLoS One* 2009; **4**: e7736 [PMID: 19893742 DOI: 10.1371/journal.pone.0007736]

74 **Lee IO**, Kim JH, Choi YJ, Pillinger MH, Kim SY, Blaser MJ, Lee YC. Helicobacter pylori CagA phosphorylation status determines the gp130-activated SHP2/ERK and JAK/STAT signal transduction pathways in gastric epithelial cells. *J Biol Chem* 2010; **285**: 16042-16050 [PMID: 20348091 DOI: 10.1074/jbc.M110.111054]

75 **Naito M**, Yamazaki T, Tsutsumi R, Higashi H, Onoe K, Yamazaki S, Azuma T, Hatakeyama M. Influence of EPIYA-repeat polymorphism on the phosphorylation-dependent biological activity of Helicobacter pylori CagA. *Gastroenterology* 2006; **130**: 1181-1190 [PMID: 16618412 DOI: 10.1053/j.gastro.2005.12.038]

76 **Ferreira RM**, Machado JC, Leite M, Carneiro F, Figueiredo C. The number of Helicobacter pylori CagA EPIYA C tyrosine phosphorylation motifs influences the pattern of gastritis and the development of gastric carcinoma. *Histopathology* 2012; **60**: 992-998 [PMID: 22348604 DOI: 10.1111/j.1365-2559]

77 **Fujiya K**, Nagata N, Uchida T, Kobayakawa M, Asayama N, Akiyama J, Shimbo T, Igari T, Banerjee R, Nageshwar Reddy D, Mizokami M, Uemura N. Different gastric mucosa and CagA status of patients in India and Japan infected with Helicobacter pylori. *Dig Dis Sci* 2014; **59**: 631-637 [PMID: 24282059 DOI: 10.1007/s10620-013-2961-x]

78 **Rhead JL**, Letley DP, Mohammadi M, Hussein N, Mohagheghi MA, Eshagh Hosseini M, Atherton JC. A new Helicobacter pylori vacuolating cytotoxin determinant, the intermediate region, is associated with gastric cancer. *Gastroenterology* 2007; **133**: 926-936 [PMID: 17854597 DOI: 10.1053/j.gastro.2007.06.056]

79 **Ji X**, Fernandez T, Burroni D, Pagliaccia C, Atherton JC, Reyrat JM, Rappuoli R, Telford JL. Cell specificity of Helicobacter pylori cytotoxin is determined by a short region in the polymorphic midregion. *Infect Immun* 2000; **68**: 3754-3757 [PMID: 10816542 DOI: 10.1128/IAI.68.6.3754-3757.2000]

80 **Atherton JC**, Cao P, Peek RM, Tummuru MK, Blaser MJ, Cover TL. Mosaicism in vacuolating cytotoxin alleles of Helicobacter pylori. Association of specific vacA types with cytotoxin production and peptic ulceration. *J Biol Chem* 1995; **270**: 17771-17777 [PMID: 7629077 DOI: 10.1074/jbc.270.30.17771]

81 **Miehlke S**, Kirsch C, Agha-Amiri K, Günther T, Lehn N, Malfertheiner P, Stolte M, Ehninger G, Bayerdörffer E. The Helicobacter pylori vacA s1, m1 genotype and cagA is associated with gastric carcinoma in Germany. *Int J Cancer* 2000; **87**: 322-327 [PMID: 10897035 DOI: 10.1002/1097-0215(20000801)87: 3<322: : AID-IJC3>3.3.CO; 2-D]

82 **Basso D**, Zambon CF, Letley DP, Stranges A, Marchet A, Rhead JL, Schiavon S, Guariso G, Ceroti M, Nitti D, Rugge M, Plebani M, Atherton JC. Clinical relevance of Helicobacter pylori cagA and vacA gene polymorphisms. *Gastroenterology* 2008; **135**: 91-99 [PMID: 18474244 DOI: 10.1053/j.gastro.2008.03.041]

83 **Ogiwara H**, Graham DY, Yamaoka Y. vacA i-region subtyping. *Gastroenterology* 2008; **134**: 1267; author reply 1268 [PMID: 18395110 DOI: 10.1053/j.gastro.2007.11.062]

84 **Talebi Bezmin Abadi A**, Mohabbati Mobarez A. High Prevalence of Helicobacter pylori hopQ II Genotype Isolated from Iranian Patients with Gastroduodenal Disorders. *J Pathog* 2014; **2014**: 842469 [PMID: 24672729 DOI: 10.1155/2014/842469]

85 **Oluwasola AO**. Genetic determinants and clinico-pathological outcomes of helicobacter pylori infection. *Ann Ib Postgrad Med* 2014; **12**: 22-30 [PMID: 25332697]

86 **Lu W**, Pan K, Zhang L, Lin D, Miao X, You W. Genetic polymorphisms of interleukin (IL)-1B, IL-1RN, IL-8, IL-10 and tumor necrosis factor {alpha} and risk of gastric cancer in a Chinese population. *Carcinogenesis* 2005; **26**: 631-636 [PMID: 15579481 DOI: 10.1093/carcin/bgh349]

87 **Wu X**, Zeng Z, Chen B, Yu J, Xue L, Hao Y, Chen M, Sung JJ, Hu P. Association between polymorphisms in interleukin-17A and interleukin-17F genes and risks of gastric cancer. *Int J Cancer* 2010; **127**: 86-92 [PMID: 19904747 DOI: 10.1002/ijc.25027]

88 **Castaño-Rodríguez N**, Kaakoush NO, Mitchell HM. Pattern-recognition receptors and gastric cancer. *Front Immunol* 2014; **5**: 336 [PMID: 25101079 DOI: 10.3389/fimmu.2014.00336]

89 **Jeon C**, Chang SC, Mu L, Zhao J, Rao JY, Lu QY, Zhang ZF. Genetic variants of peroxisome proliferator-activated receptor δ are associated with gastric cancer. *Dig Dis Sci* 2013; **58**: 2881-2886 [PMID: 23907334 DOI: 10.1007/s10620-013-2770-2]

90 **Ebert MP**, Lendeckel U, Westphal S, Dierkes J, Glas J, Folwaczny C, Roessner A, Stolte M, Malfertheiner P, Röcken C. The angiotensin I-converting enzyme gene insertion/deletion polymorphism is linked to early gastric cancer. *Cancer Epidemiol Biomarkers Prev* 2005; **14**: 2987-2989 [PMID: 16365022 DOI: 10.1158/1055-9965.EPI-05-0411]

91 **Izzotti A**, De Flora S, Cartiglia C, Are BM, Longobardi M, Camoirano A, Mura I, Dore MP, Scanu AM, Rocca PC, Maida A, Piana A. Interplay between Helicobacter pylori and host gene polymorphisms in inducing oxidative DNA damage in the gastric mucosa. *Carcinogenesis* 2007; **28**: 892-898 [PMID: 17127715 DOI: 10.1093/carcin/bgl208]

92 **Wroblewski LE**, Peek RM. Helicobacter pylori in gastric carcinogenesis: mechanisms. *Gastroenterol Clin North Am* 2013; **42**: 285-298 [PMID: 23639641 DOI: 10.1016/j.gtc.2013.01.006]

93 **Wang LL**, Yu XJ, Zhan SH, Jia SJ, Tian ZB, Dong QJ. Participation of microbiota in the development of gastric cancer. *World J Gastroenterol* 2014; **20**: 4948-4952 [PMID: 24803806 DOI: 10.3748/wjg.v20.i17.4948]

94 **Sheh A**, Fox JG. The role of the gastrointestinal microbiome in Helicobacter pylori pathogenesis. *Gut Microbes* 2013; **4**: 505-531 [PMID: 23962822 DOI: 10.4161/gmic.26205]

95 **Brawner KM**, Morrow CD, Smith PD. Gastric microbiome and gastric cancer. *Cancer J* 2014; **20**: 211-216 [PMID: 24855010 DOI: 10.1097/PPO.0000000000000043]

96 **Manson JM**, Rauch M, Gilmore MS. The commensal microbiology of the gastrointestinal tract. *Adv Exp Med Biol* 2008; **635**: 15-28 [PMID: 18841700 DOI: 10.1007/978-0-387-09550-9_2]

97 **Hagiwara T**, Mukaisho K, Nakayama T, Hattori T, Sugihara H. Proton pump inhibitors and helicobacter pylori-associated pathogenesis. *Asian Pac J Cancer Prev* 2015; **16**: 1315-1319 [PMID: 25743791 DOI: 10.7314/APJCP.2015.16.4.1315]

98 **Santibáñez M**, Aguirre E, Belda S, Aragones N, Saez J, Rodríguez JC, Galiana A, Sola-Vera J, Ruiz-García M, Paz-Zulueta M, Sarabia-Lavín R, Brotons A, López-Girona E, Pérez E, Sillero C, Royo G. Relationship between tobacco, cagA and vacA i1 virulence factors and bacterial load in patients infected by Helicobacter pylori. *PLoS One* 2015; **10**: e0120444 [PMID: 25794002 DOI: 10.1371/journal.pone.0120444]

99 **Loh JT**, Torres VJ, Cover TL. Regulation of Helicobacter pylori cagA expression in response to salt. *Cancer Res* 2007; **67**: 4709-4715 [PMID: 17510398 DOI: 10.1158/0008-5472.CAN-06-4746]

100 **Lee JY**, Kim N, Nam RH, Choi YJ, Seo JH, Lee HS, Oh JC, Lee DH. No Correlation of Inflammation With Colonization of Helicobacter pylori in the Stomach of Mice Fed High-salt Diet. *J Cancer Prev* 2014; **19**: 144-151 [PMID: 25337583 DOI: 10.15430/JCP.2014.19.2.144]

101 **Noto JM**, Gaddy JA, Lee JY, Piazuelo MB, Friedman DB, Colvin DC, Romero-Gallo J, Suarez G, Loh J, Slaughter JC, Tan S, Morgan DR, Wilson KT, Bravo LE, Correa P, Cover TL, Amieva MR, Peek RM. Iron deficiency accelerates Helicobacter pylori-induced

carcinogenesis in rodents and humans. *J Clin Invest* 2013; **123**: 479-492 [PMID: 23257361 DOI: 10.1172/JCI64373]

102 **Loh JT**, Friedman DB, Piazuelo MB, Bravo LE, Wilson KT, Peek RM, Correa P, Cover TL. Analysis of Helicobacter pylori cagA promoter elements required for salt-induced upregulation of CagA expression. *Infect Immun* 2012; **80**: 3094-3106 [PMID: 22710874 DOI: 10.1128/IAI.00232-12]

103 **Lin CH**, Wu CY, Kou HS, Chen CY, Huang MC, Hu HM, Wu MC, Lu CY, Wu DC, Wu MT, Kuo FC. Effect of Di(2-ethylhexyl)phthalate on Helicobacter pylori-Induced Apoptosis in AGS Cells. *Gastroenterol Res Pract* 2013; **2013**: 924769 [PMID: 24454344 DOI: 10.1155/2013/924769]

P- Reviewer: Adachi Y
S- Editor: Tian YL **L- Editor**: A **E- Editor**: Jiao XK

World Journal of Gastrointestinal Pathophysiology

Submit a Manuscript: http://www.wjgnet.com/esps/
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.4291/wjgp.v7.i1.108

World J Gastrointest Pathophysiol 2016 February 15; 7(1): 108-116
ISSN 2150-5330 (online)

MINIREVIEWS

Mechanisms of interleukin-22's beneficial effects in acute pancreatitis

Chongmin Huan, Daniel Kim, Peiqi Ou, Antonio Alfonso, Albert Stanek

Chongmin Huan, Daniel Kim, Antonio Alfonso, Albert Stanek, Department of Surgery, Downstate Medical Center, State University of New York, Brooklyn, NY 11203, United States

Peiqi Ou, Program of Molecular and Cellular Biology, School of Graduate Studies, Downstate Medical Center, State University of New York, Brooklyn, NY 11203, United States

Albert Stanek, Department of Pathology, Downstate Medical Center, State University of New York, Brooklyn, NY 11203, United States

Author contributions: Huan C and Kim D contributed equally to this work; Huan C wrote the manuscript and directed the acute pancreatitis study; Kim D generated and analyzed the major sources of data; Ou P measured IL-22 mRNA levels; Alfonso A contributed to the writing of the manuscript; Stanek A assisted the direction of the study, performed histology study, and made important revisions of the manuscript.

Conflict-of-interest statement: The authors declare no conflicts of interest.

Correspondence to: Chongmin Huan, MD, PhD, Assistant Professor, Department of Surgery, Downstate Medical Center, State University of New York, 450 Clarkson Avenue, Brooklyn, NY 11203, United States. chongmin.huan@downstate.edu
Telephone: +1-718-2706772
Fax: +1-718-2216132

Received: August 11, 2015
Peer-review started: August 11, 2015
First decision: September 28, 2015
Revised: November 11, 2015
Accepted: November 17, 2015
Article in press: November 25, 2015
Published online: February 15, 2016

Abstract

Acute pancreatitis (AP) is a disorder characterized by parenchymal injury of the pancreas controlled by immune cell-mediated inflammation. AP remains a significant challenge in the clinic due to a lack of specific and effective treatment. Knowledge of the complex mechanisms that regulate the inflammatory response in AP is needed for the development of new approaches to treatment, since immune cell-derived inflammatory cytokines have been recognized to play critical roles in the pathogenesis of the disease. Recent studies have shown that interleukin (IL)-22, a cytokine secreted by leukocytes, when applied in the severe animal models of AP, protects against the inflammation-mediated acinar injury. In contrast, in a mild AP model, endogenous IL-22 has been found to be a predominantly anti-inflammatory mediator that inhibits inflammatory cell infiltration *via* the induction of Reg3 proteins in acinar cells, but does not protect against acinar injury in the early stage of AP. However, constitutively over-expressed IL-22 can prevent the initial acinar injury caused by excessive autophagy through the induction of the anti-autophagic proteins Bcl-2 and Bcl-X_L. Thus IL-22 plays different roles in AP depending on the severity of the AP model. This review focuses on these recently reported findings for the purpose of better understanding IL-22's regulatory roles in AP which could help to develop a novel therapeutic strategy.

Key words: Interleukin-22; Acute pancreatitis; Cytokine; Inflammatory response; Acinar cell

Core tip: Interleukin (IL)-22 has been recognized as a potential therapeutic agent for acute pancreatitis (AP) treatment due to its discovered beneficial effects in inflammatory diseases. However, according to recent publications and our results, IL-22 appears to have differential effects in AP depending on the severity of the disorder. In this review we discuss the different regulatory mechanisms of IL-22 in mild and severe AP models in order to promote development of an effective and efficient therapeutic approach.

Huan C, Kim D, Ou P, Alfonso A, Stanek A. Mechanisms of interleukin-22's beneficial effects in acute pancreatitis. *World J Gastrointest Pathophysiol* 2016; 7(1): 108-116 Available from: URL: http://www.wjgnet.com/2150-5330/full/v7/i1/108.htm DOI: http://dx.doi.org/10.4291/wjgp.v7.i1.108

INTRODUCTION

Despite recent improvements in the management of acute pancreatitis (AP), it remains a persistent challenge in clinical medicine[1]. In the United States, AP is the leading cause of hospitalizations among gastrointestinal diseases with a mortality rate which ranges from 3% to 17%. This is largely because of the unpredictable outcome early in AP, and the lack of a specific and effective treatment to block the inflammatory injury in AP[2-4]. Knowledge of the complex inflammatory regulation in AP is required to provide a basis for developing new strategies in the management of AP.

The inflammation in AP is initiated by local acinar cell injury caused by ductal obstruction, bile salts, ethanol or hyperlipidemia. The injured acinar cells release inflammatory mediators which activate immune cells in the pancreas and induce inflammatory cell infiltration to promote the tissue repair[5]. However, if the local injury cannot be resolved, escalated cytokine production by activated immune cells may bring about more severe inflammatory damage in the pancreas[6]. When the pro-inflammatory response is not sufficiently countered by an anti-inflammatory mechanism, a cascade of inflammatory reactions can lead to a systemic response and result in multiple organ dysfunction syndrome (MODS)[6,7]. Thus cytokines control the evolution of AP by mediating the progression of the pathogenesis in AP. It is known that tumor necrosis factor (TNF), interleukin (IL)-1, IL-6, IL-8, platelet activating factor and chemokines are major pro-inflammatory cytokines in AP, whereas IL-10 is an anti-inflammatory mediator[4,6]. Recently, IL-22, a member of the IL-10 family, has been described as a key protector in rodent AP models, suggesting a potential therapeutic approach for AP patients by targeting IL-22[8-11].

IL-22 is produced by T helper 17 cells, γδ T cells, NKT cells and innate lymphoid cells (ILCs). Unlike other cytokines that act on both immune and non-immune cells, IL-22 has no effect on immune cells but primarily targets cells of epithelial origin due to the restrictive expression of its receptor[12,13]. The IL-22 receptor is a heterodimer composed of IL-22R1 and IL-10R2. While IL-10R2 is ubiquitously expressed, IL-22R1 is predominantly limited to epithelial cells, with its highest expression found in pancreatic acinar cells[8,14,15]. The main function of IL-22 is to regulate the host defense at barrier surfaces. However, in diseases of different organs and tissues, it can be either protective or pathogenic[12,13,16,17]. IL-22 has been reported to be tissue protective in pancreas[8-11], liver[18-28], intestine[29-36] and lung[9,37-40]. However, in psoriasis[41-45] and arthritis[46-49], dysregulated expression of IL-22 is pro-inflammatory and involved in the pathogenesis of the diseases. In addition, IL-22 has been found to have tumorigenic potential due to its ability to activate STAT3 signaling[23,27,35,42,50]. In AP, IL-22 appears to be involved in the pathogenesis in different animal models, but has disparate effects depending on the severity of the inflammation. As studies of the potential role of IL22 in AP prognosis are still currently lacking, in this review we focus on the different regulatory mechanisms of IL-22 in mild and severe AP models in order to help develop an effective and efficient therapeutic approach.

EXPRESSION OF IL-22 AND THE IL-22 RECEPTOR IN AP

It has been shown that IL-22 is produced by the resident $CD4^+$ T cells in normal pancreas. However, in the late stages of AP, the production of IL-22 was found to be reduced when the numbers of IL-22 producing $CD4^+$ T cells were significantly decreased in the pancreas. On the contrary, ILCs, another type of IL-22 producing cell, were highly increased in inflamed pancreas and partially restored the IL-22 production[8]. ILCs normally reside in the gut-associated lymphoid tissue to mediate mucosal immunity in the intestine[51,52]. Increased numbers of ILCs in inflamed pancreas suggest a possible migration of ILCs from the intestine after the onset of AP[8]. In support of the activation of intestinal ILCs in AP, we found that shortly after the AP induction by cerulein overstimulation in the mouse, increased IL-22 transcription was detected in intestinal tissues but not in the pancreas or spleen (Figure 1). Cerulein-induced secreted bile in the intestine is likely associated with IL-22 expression in ILCs, as administration of biliverdin, a major pigment in bile in the intestine[53], significantly increased pancreatic IL-22 levels and ILCs *via* activation of the aryl hydrocarbon receptor, a ligand-dependent transcription factor for IL-22 expression in leukocytes[8,54]. Thus intestinal ILCs could provide an important source of pancreatic IL-22 in AP. Interestingly, IL-22 produced by intestinal ILCs has also been found to regulate the selective containment of lymphoid-resident bacteria to prevent systemic inflammation[55]. However, it is unknown whether the failed containment

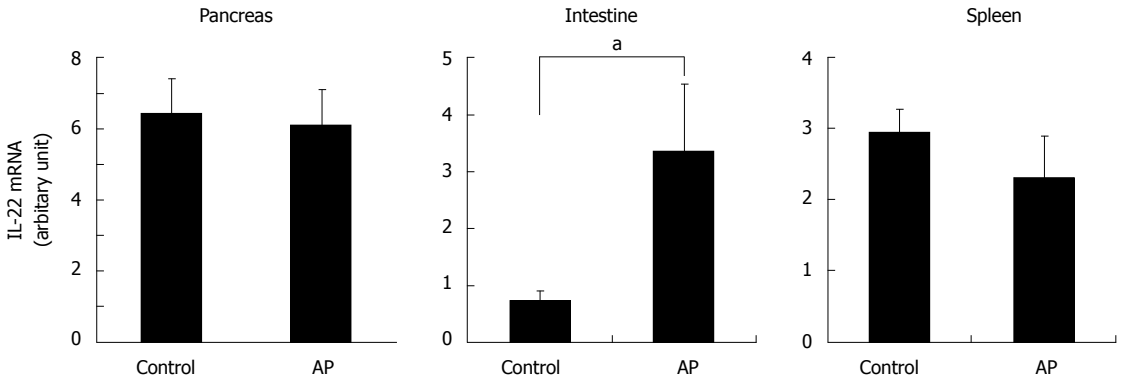


Figure 1 Increased interleukin-22 mRNA expression in small intestine in the early stage of acute pancreatitis. IL-22 mRNA levels in the tissues of pancreas, small intestine and spleen at 2 h after the induction of cerulein-AP were analyzed by real-time RT-PCR. In contrast to the pancreas and spleen, only the small intestine has increased IL-22 mRNA expression in the AP mice compared to the control mice. [a]*t*-test, $P < 0.05$. IL: Interleukin; AP: Acute pancreatitis; RT-PCR: Reverse transcription polymerase chain reaction.

of such lymphoid-resident bacteria is associated with the development of pancreatitis.

In contrast to IL-22, the expression of IL-22R1 on acinar cells is significantly increased in the acute phase of AP[8]. While the mechanism that regulates IL-22R1 expression in acinar cells is unknown, it has been shown that intra-peritoneal administration of LPS in mice increased IL-22R1 mRNA levels in liver, kidney and lung[56]. Thus it is possible that IL-22R1 behaves like an acute-phase protein whose expression is induced in response to the stress or injury in acinar cells, and then decreased in the late stage of AP[8]. Another study, however, showed that the IL-22R1 level was down-regulated by IL-22 in keratinocytes. This was because miR-197, a transcriptional inhibitor of IL-22R1, is actually up-regulated by IL-22[57]. Given the opposite changes in expression of IL-22 and IL-22R1 in the inflamed pancreas[8], the altered expression of IL-22R1 by IL-22 cannot be excluded.

DIFFERENT BENEFICIAL ROLES OF IL-22 IN AP

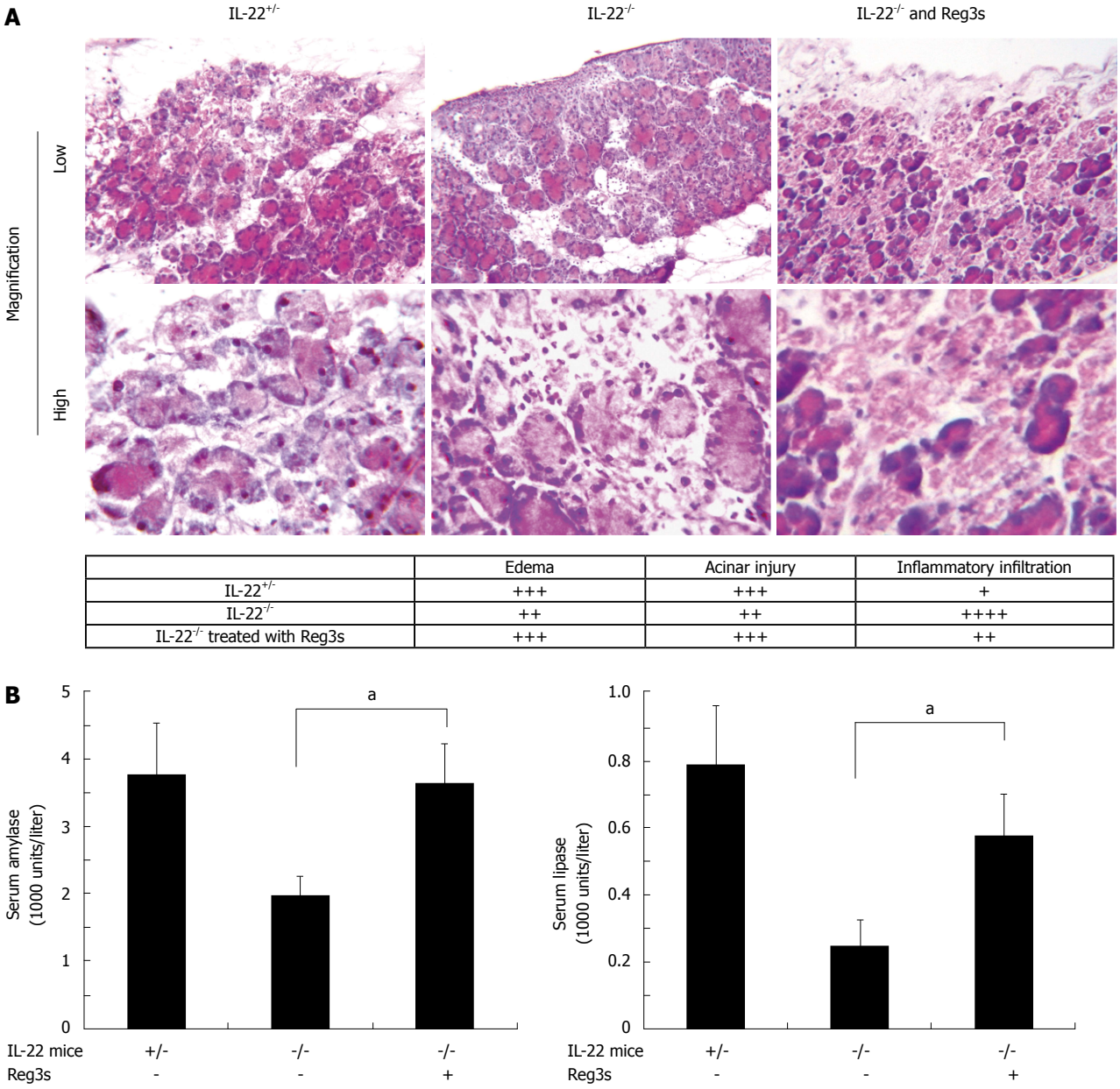
The beneficial effects of IL-22 appear to be dependent on the severity of AP as well as on the timing of IL-22's activity in AP. In a model of mild AP induced by cerulein, administration of IL-22 or constitutively over-expressing IL-22 before AP onset prevented the AP induction. However, in the same study, IL-22 deficiency did not worsen the acinar damage[11]. Similarly, using the same AP model, we observed that IL-22 knockout mice had less acinar cell injury but significantly increased inflammatory cell infiltration in the pancreas compared to the control mice (Figure 2). In contrast to mild AP, in models of severe AP, IL-22 administration following AP onset significantly ameliorated the injury in the pancreas[8,10]. Consistently, blocking endogenous IL-22 with an anti-IL-22 antibody worsened pancreatic injury in the severe AP model[8]. These seemingly conflicting effects of IL-22 in mild and severe AP actually suggest the existence of different regulatory mechanisms by IL-22 in AP of different levels of severity.

INCREASED IL-22 INHIBITS EXCESSIVE AUTOPHAGY IN ACINAR CELLS AND RESULTS IN RESISTANCE TO AP INDUCTION

Recent evidence has suggested that excessive autophagy leads to premature trypsinogen activation which initiates the acinar damage in AP[58,59]. Interestingly, it has been shown that genetically over-expressed IL-22 inhibited autophagosome formation in acinar cells and makes mice resistant to cerulein-induced AP[11]. This is associated with the increased levels of the anti-autophagic proteins Bcl-2 and Bcl-X_L detected in the pancreas of IL-22 transgenic mice[11]. Expressions of Bcl-2 and Bcl-X_L are likely regulated by IL-22 in these mice since the transcription of Bcl-2 and Bcl-X_L is regulated by STAT3[60], a signaling molecule known to be activated by IL-22[23,27,35,42,50]. Similar resistance to AP induction has also been observed in mice to which IL-22 was administrated before AP onset[11]. Thus it appears that when the level of IL-22 is higher than normal it blocks cerulein-induced excessive autophagy in acinar cells, and thereby prevents the initiation of acinar injury in AP. However, this protective mechanism of IL-22 may not affect acinar survival after the initial acute phase in severe AP, when the acinar injury is mainly associated with cytokine-mediated inflammation rather than excessive autophagy[61].

PHSYOLOGICAL IL-22 BLOCKS INFLAMMATORY INFILTRATION *VIA* THE INDUCTION OF REG3S IN AP

The Reg3 family includes Reg3α, Reg3β and Reg3γ. These are also known as pancreatitis associated proteins (PAP2, PAP1 and PAP3 respectively). Reg3s are evolutionarily conserved C-type lectin-like proteins



	Edema	Acinar injury	Inflammatory infiltration
IL-22$^{+/-}$	+++	+++	+
IL-22$^{-/-}$	++	++	++++
IL-22$^{-/-}$ treated with Reg3s	+++	+++	++

Figure 2 Interleukin-22 deficiency results in reduced acinar injury and increased inflammatory infiltration that are reversed by the administration of Reg3 proteins. In the upper panel of Figure 2A, H and E staining of pancreatic tissues shows that in mild AP, IL-22$^{-/-}$ mice have reduced tissue damage but increased inflammatory cell infiltration compared to IL-22$^{+/-}$ mice. These changes in IL-22$^{-/-}$ mice can be reversed by intravenous administration of Regα and Regβ. The lower panel in Figure 2A represents comparative severity of edema, acinar injury and inflammatory cell infiltration among the pancreata in the IL-22$^{+/-}$, IL-22$^{-/-}$ and Reg3s treated IL-22$^{-/-}$ mice. Consistent with the histology study, Figure 2B shows that IL-22$^{-/-}$ mice have reduced serum amylase and lipase levels in AP that could be restored by the administration of Reg3s. ^{a}t-test, $P < 0.05$. IL: Interleukin; AP: Acute pancreatitis.

that are most abundantly produced by pancreatic acinar cells in AP, but are almost undetectable in the normal pancreas[62-65]. In addition to pancreatic acinar cells, Reg3 proteins are also found in α-cells of Langerhans islets[66], intestine[36,66-69], brain[70,71], hepatocellular carcinomas[66,72] and pancreatic cancers[73,74]. Our laboratory has reported that Reg3 proteins protect against pancreatic acinar cell injury, since inhibition of their expression by either antisense DNA oligos or RNAi exacerbated experimental necrotizing AP induced by sodium taurocholate[65,75].

IL-22 signaling is required for up-regulating Reg3 expression in cerulein-induced AP as we have determined that pancreatic Reg3 levels were significantly reduced in IL-22 deficient mice (Figure 3). In line with this, according to our unpublished observation, the expressions of Reg3 proteins were induced coincidentally with the peak expression of IL-22R1 in cerulein AP[8]. IL-22 likely regulates Reg3 expression *via* STAT3 in acinar cells. This is because that STAT3 is known to be activated by IL-22 in acinar cells[8,14,15], and that STAT3 has been found to be required for the induction of Reg3β in AP[76]. Interestingly the study of Reg3β knockout mice showed reduced acinar injury but more severe inflammatory infiltration in cerulein-induced mild AP[77]. This phenotype is almost identical to that

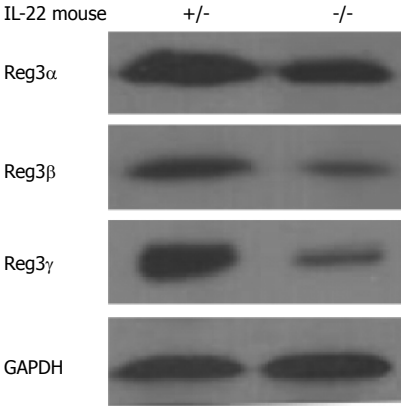


Figure 3 Reduced levels of Reg3 proteins in the pancreas of interleukin-22 deficient mice in acute pancreatitis. Western blot analysis of pancreatic tissues shows reduced levels of Reg3α, Reg3β and Reg3γ in IL-22$^{-/-}$ mice compared to IL-22$^{+/-}$ mice in cerulein-induced acute pancreatitis. IL: Interleukin; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

observed in IL-22 deficient mice. Importantly, we found that the reduced acinar injury in IL-22 knockout mice could be made worse by intravenous administration of recombinant Reg3α and Reg3β (Figure 2). Additionally, similar to IL-22[8], endogenous Reg3 proteins have been found to be required to inhibit inflammatory cell infiltration in severe AP as well[65,75]. Thus we conclude that IL-22 inhibits inflammation in AP *via* induction of Reg3 proteins. It is noteworthy that in addition to pancreatic acinar cells, Reg3s serve as downstream effectors of IL-22 in intestinal epithelial cells. It has been shown that in the intestine, IL-22-induced expression of Reg3β and Reg3γ contributes to IL-22's role in the innate immunity against C. rodentium[36].

The anti-inflammatory activities of IL-22 and Reg3 in severe AP are consistent with their roles in tissue protection. On the other hand, despite their inhibition of inflammatory cell infiltration, IL-22 and Reg3 appear to make tissue injury worse in mild AP[77] (Figure 2). One possible explanation of these adverse effects in mild AP could be that their restriction of inflammatory cell infiltration leads to a reduction in the number of protective inflammatory cells such as M2 and hemin-activated macrophages in the pancreas[78,79]. If this is so, it suggests that the inflammatory infiltrates in mild AP are composed of some cells with distinctly different functions than those in severe AP, in agreement with the finding of different types of peritoneal inflammatory cells in AP of different degrees of severity[80]. An alternative explanation is that the detrimental effects of IL-22 and Reg3 in mild AP may be related to their anti-apoptotic activity which leads to a "destructive" necrosis rather than a "clean" apoptosis of injured acinar cells in mild AP[77].

ADMINISTRATION OF IL-22 PROTECTS AGAINST INFLAMMATORY INJURY IN SEVERE AP

In models of severe AP, IL-22 administration has been found to be tissue protective[8,10]. This is at least partially contributed by its anti-inflammatory activity, since reduced IL-22 serum levels were found to be associated with lung injury in a necrotizing AP model induced by sodium taurocholate[9]. In addition, IL-22 administration not only alleviated the tissue damage in the pancreas but also rescued lung injury mediated by the systemic inflammatory response in the CDE (choline-deficient diet supplemented with DL-ethionine) AP model[8]. This is also in keeping with the lack of protection against acinar injury by endogenous IL-22 in mild AP where the acinar injury is largely the result of pathogenic factors other than inflammation[58,59,61].

In addition to its anti-inflammatory activity, the potential protective role of the interplay of IL-22 with other cytokines that are highly induced in severe but not mild AP may also help to explain IL-22's beneficial effects in severe AP. TNF-α is a pro-inflammatory cytokine that was found to be involved in AP pathogenesis and highly induced in severe AP patients[81-83]. Recent evidence, however, showed that the TNF-α expression profiles between the severe AP patients with and without MODS are different[84]. Another study claims that less TNF-α induction predicts the development of MODS and a fatal outcome in severe AP patients[85], suggesting an essential protective role of TNF-α in severe AP but with an undetermined mechanism. Interestingly, it has been shown that IL-22 and TNF-α synergistically conserve the integrity of the epidermal barrier in a skin infection model[86]. It is thus possible that the interplay between IL-22 and TNF-α could have a significant protective role in severe AP as well. In addition, IL-17, a pro-inflammatory cytokine involved in the development of necrotizing AP in rats as well as in severe AP in patients[87,88], may also coordinate IL-22 function in AP, since it has been shown that the pathological *vs* protective functions of IL-22 in airway inflammation were regulated by IL-17A[89]. Furthermore, IL-22 and IL-17 cooperatively enhance antimicrobial activity in keratinocytes[90]. Thus the protective function of IL-22 could be linked to other cytokines which are highly expressed in severe AP.

IL-22 IS A POTENTIAL THERAPEUTIC TARGET FOR AP TREATMENT

Immune modulation presents promising possibilities for AP treatment, since the balance between pro- and anti-inflammatory responses determines the progression and outcome of AP[2-7]. Many immune modulating strategies have been developed by either inhibiting pro-inflammatory cytokines or strengthening the effects of anti-inflammatory mediators. Although many of these strategies have been proven to be beneficial in AP animal models, their therapeutic effects in AP patients have been far from satisfactory. This could be due to the difficulties in clinical translation of animal experimental data, poor understanding of the regulation of inflammation, or the need for multimodal approaches[4]. Thus

in order to achieve convincing therapeutic effects in patients, a more thorough and practical strategy that targets multiple inflammatory mediators is needed to be able to control the network of inflammation in AP.

The restrictive expression of IL-22R1 on cells of epithelial origin limits the effects of IL-22 in the body. This makes IL-22 an ideal target for drug development. Given that IL-22 has either pathogenic or beneficial effects depending on the inflammatory diseases, both anti-IL-22 antibody and recombinant IL-22 have been developed for inhibiting or strengthening IL-22 signaling respectively[17,91,92]. For example, ILV-094, an anti-IL-22 antibody that blocks IL-22 activity, is being tested in a phase Ⅱ clinical trial for the treatment of atopic dermatitis; while, IL-22 IgG2-Fc, a human recombinant IL-22 developed for grade Ⅱ-Ⅳ lower GI acute graft-*vs*-host disease, is also in a phase Ⅱ clinical trial[93]. Recombinant IL-22 is a potential therapeutic agent for severe AP patients, since animal studies have proven that IL-22 administration after the onset of AP protects against inflammatory injury in different severe AP models[8,10]. Interestingly, even in a mouse model of chronic pancreatitis, IL-22 derived from an adenovirus vector ameliorates the pancreatic tissue injury[11]. It is noteworthy, however, that long-term application of IL-22 that maintains a higher than normal IL-22 level in the body is limited by its potential role in tumorigenesis[23,27,35,42,50].

CONCLUSION

Treatment of severe AP by temporally strengthening IL-22 signaling in acinar cells is an attractive concept supported by several independent studies of animal AP. The highest expression level of the IL-22 receptor on acinar cells strengthens IL-22 signaling in AP. This enables the acinar cells to abundantly produce Reg3s to combat inflammatory cell infiltration, and other pro-survival mediators to protect against tissue injury. However, in order to prepare IL-22 as a therapeutic agent for AP treatment, more thorough research is needed to comprehend the detailed mechanisms of IL-22-mediated anti-inflammatory and tissue protective mechanisms in severe AP, particularly the interplay of IL-22 with other pro-inflammatory cytokines. This will enable the development of a complex immune-modulating strategy to effectively control the cytokine-orchestrated inflammation network in severe AP.

ACKNOWLEDGMENTS

We thank Dr. Wenjun Ouyang at Genentech for providing us with the IL-22 knockout mice and other experimental reagents. We appreciate his comments to our manuscript as well. We also thank Cathy Mueller at SUNY Downstate for the technical support.

REFERENCES

1 **Working Group IAP/APA Acute Pancreatitis Guidelines**. IAP/APA evidence-based guidelines for the management of acute pancreatitis. *Pancreatology* 2013; **13**: e1-15 [PMID: 24054878 DOI: 10.1016/j.pan.2013.07.063]
2 **Singh VK**, Bollen TL, Wu BU, Repas K, Maurer R, Yu S, Mortele KJ, Conwell DL, Banks PA. An assessment of the severity of interstitial pancreatitis. *Clin Gastroenterol Hepatol* 2011; **9**: 1098-1103 [PMID: 21893128 DOI: 10.1016/j.cgh.2011.08.026]
3 **Lankisch PG**, Apte M, Banks PA. Acute pancreatitis. *Lancet* 2015; **386**: 85-96 [PMID: 25616312 DOI: 10.1016/S0140-6736(14)60649-8]
4 **Akinosoglou K**, Gogos C. Immune-modulating therapy in acute pancreatitis: fact or fiction. *World J Gastroenterol* 2014; **20**: 15200-15215 [PMID: 25386069 DOI: 10.3748/wjg.v20.i41.15200]
5 **Raraty MG**, Murphy JA, Mcloughlin E, Smith D, Criddle D, Sutton R. Mechanisms of acinar cell injury in acute pancreatitis. *Scand J Surg* 2005; **94**: 89-96 [PMID: 16111088]
6 **Makhija R**, Kingsnorth AN. Cytokine storm in acute pancreatitis. *J Hepatobiliary Pancreat Surg* 2002; **9**: 401-410 [PMID: 12483260 DOI: 10.1007/s005340200049]
7 **Bhatia M**. Inflammatory response on the pancreatic acinar cell injury. *Scand J Surg* 2005; **94**: 97-102 [PMID: 16111089]
8 **Xue J**, Nguyen DT, Habtezion A. Aryl hydrocarbon receptor regulates pancreatic IL-22 production and protects mice from acute pancreatitis. *Gastroenterology* 2012; **143**: 1670-1680 [PMID: 23022954 DOI: 10.1053/j.gastro.2012.08.051]
9 **Huai JP**, Sun XC, Chen MJ, Jin Y, Ye XH, Wu JS, Huang ZM. Melatonin attenuates acute pancreatitis-associated lung injury in rats by modulating interleukin 22. *World J Gastroenterol* 2012; **18**: 5122-5128 [PMID: 23049224 DOI: 10.3748/wjg.v18.i36.5122]
10 **El-Shemi AG**, Basalamah MA, Kensara OA, Ashshi AM. Interleukin-22 therapy attenuates the development of acute pancreatitis in rats. *J Clin Med Res* 2011; **3**: 82-88
11 **Feng D**, Park O, Radaeva S, Wang H, Yin S, Kong X, Zheng M, Zakhari S, Kolls JK, Gao B. Interleukin-22 ameliorates cerulein-induced pancreatitis in mice by inhibiting the autophagic pathway. *Int J Biol Sci* 2012; **8**: 249-257 [PMID: 22253568 DOI: 10.7150/ijbs.3967]
12 **Wolk K**, Witte E, Witte K, Warszawska K, Sabat R. Biology of interleukin-22. *Semin Immunopathol* 2010; **32**: 17-31 [PMID: 20127093 DOI: 10.1007/s00281-009-0188-x]
13 **Dudakov JA**, Hanash AM, van den Brink MR. Interleukin-22: immunobiology and pathology. *Annu Rev Immunol* 2015; **33**: 747-785 [PMID: 25706098 DOI: 10.1146/annurev-immunol-032414-112123]
14 **Aggarwal S**, Xie MH, Maruoka M, Foster J, Gurney AL. Acinar cells of the pancreas are a target of interleukin-22. *J Interferon Cytokine Res* 2001; **21**: 1047-1053 [PMID: 11798462]
15 **Gurney AL**. IL-22, a Th1 cytokine that targets the pancreas and select other peripheral tissues. *Int Immunopharmacol* 2004; **4**: 669-677 [PMID: 15120651]
16 **Mühl H**, Scheiermann P, Bachmann M, Härdle L, Heinrichs A, Pfeilschifter J. IL-22 in tissue-protective therapy. *Br J Pharmacol* 2013; **169**: 761-771 [PMID: 23530726 DOI: 10.1111/bph.12196]
17 **Sabat R**, Ouyang W, Wolk K. Therapeutic opportunities of the IL-22-IL-22R1 system. *Nat Rev Drug Discov* 2014; **13**: 21-38 [PMID: 24378801 DOI: 10.1038/nrd4176]
18 **Scheiermann P**, Bachmann M, Goren I, Zwissler B, Pfeilschifter J, Mühl H. Application of interleukin-22 mediates protection in experimental acetaminophen-induced acute liver injury. *Am J Pathol* 2013; **182**: 1107-1113 [PMID: 23375450 DOI: 10.1016/j.ajpath.2012.12.010]
19 **Kong X**, Feng D, Wang H, Hong F, Bertola A, Wang FS, Gao B. Interleukin-22 induces hepatic stellate cell senescence and restricts liver fibrosis in mice. *Hepatology* 2012; **56**: 1150-1159 [PMID: 22473749 DOI: 10.1002/hep.25744]
20 **Chestovich PJ**, Uchida Y, Chang W, Ajalat M, Lassman C, Sabat R, Busuttil RW, Kupiec-Weglinski JW. Interleukin-22: implications for liver ischemia-reperfusion injury. *Transplantation* 2012; **93**: 485-492 [PMID: 22262131 DOI: 10.1097/TP.0b013e3182449136]
21 **Meng F**, Wang K, Aoyama T, Grivennikov SI, Paik Y, Scholten D, Cong M, Iwaisako K, Liu X, Zhang M, Osterreicher CH, Stickel

F, Ley K, Brenner DA, Kisseleva T. Interleukin-17 signaling in inflammatory, Kupffer cells, and hepatic stellate cells exacerbates liver fibrosis in mice. *Gastroenterology* 2012; **143**: 765-766.e1-3 [PMID: 22687286 DOI: 10.1053/j.gastro.2012.05.049]

22 **Xing WW**, Zou MJ, Liu S, Xu T, Gao J, Wang JX, Xu DG. Hepatoprotective effects of IL-22 on fulminant hepatic failure induced by d-galactosamine and lipopolysaccharide in mice. *Cytokine* 2011; **56**: 174-179 [PMID: 21843953 DOI: 10.1016/j.cyto.2011.07.022]

23 **Xing WW**, Zou MJ, Liu S, Xu T, Wang JX, Xu DG. Interleukin-22 protects against acute alcohol-induced hepatotoxicity in mice. *Biosci Biotechnol Biochem* 2011; **75**: 1290-1294 [PMID: 21737938]

24 **Park O**, Wang H, Weng H, Feigenbaum L, Li H, Yin S, Ki SH, Yoo SH, Dooley S, Wang FS, Young HA, Gao B. In vivo consequences of liver-specific interleukin-22 expression in mice: Implications for human liver disease progression. *Hepatology* 2011; **54**: 252-261 [PMID: 21465510 DOI: 10.1002/hep.24339]

25 **Ki SH**, Park O, Zheng M, Morales-Ibanez O, Kolls JK, Bataller R, Gao B. Interleukin-22 treatment ameliorates alcoholic liver injury in a murine model of chronic-binge ethanol feeding: role of signal transducer and activator of transcription 3. *Hepatology* 2010; **52**: 1291-1300 [PMID: 20842630 DOI: 10.1002/hep.23837]

26 **Yang L**, Zhang Y, Wang L, Fan F, Zhu L, Li Z, Ruan X, Huang H, Wang Z, Huang Z, Huang Y, Yan X, Chen Y. Amelioration of high fat diet induced liver lipogenesis and hepatic steatosis by interleukin-22. *J Hepatol* 2010; **53**: 339-347 [PMID: 20452699 DOI: 10.1016/j.jhep.2010.03.004]

27 **Pan H**, Hong F, Radaeva S, Gao B. Hydrodynamic gene delivery of interleukin-22 protects the mouse liver from concanavalin A-, carbon tetrachloride-, and Fas ligand-induced injury *via* activation of STAT3. *Cell Mol Immunol* 2004; **1**: 43-49 [PMID: 16212920]

28 **Radaeva S**, Sun R, Pan HN, Hong F, Gao B. Interleukin 22 (IL-22) plays a protective role in T cell-mediated murine hepatitis: IL-22 is a survival factor for hepatocytes *via* STAT3 activation. *Hepatology* 2004; **39**: 1332-1342 [PMID: 15122762 DOI: 10.1002/hep.20184]

29 **Sovran B**, Loonen LM, Lu P, Hugenholtz F, Belzer C, Stolte EH, Boekschoten MV, van Baarlen P, Kleerebezem M, de Vos P, Dekker J, Renes IB, Wells JM. IL-22-STAT3 pathway plays a key role in the maintenance of ileal homeostasis in mice lacking secreted mucus barrier. *Inflamm Bowel Dis* 2015; **21**: 531-542 [PMID: 25636123 DOI: 10.1097/MIB.0000000000000319]

30 **Qiu J**, Heller JJ, Guo X, Chen ZM, Fish K, Fu YX, Zhou L. The aryl hydrocarbon receptor regulates gut immunity through modulation of innate lymphoid cells. *Immunity* 2012; **36**: 92-104 [PMID: 22177117 DOI: 10.1016/j.immuni.2011.11.011]

31 **Tumanov AV**, Koroleva EP, Guo X, Wang Y, Kruglov A, Nedospasov S, Fu YX. Lymphotoxin controls the IL-22 protection pathway in gut innate lymphoid cells during mucosal pathogen challenge. *Cell Host Microbe* 2011; **10**: 44-53 [PMID: 21767811 DOI: 10.1016/j.chom.2011.06.002]

32 **Sugimoto K**, Ogawa A, Mizoguchi E, Shimomura Y, Andoh A, Bhan AK, Blumberg RS, Xavier RJ, Mizoguchi A. IL-22 ameliorates intestinal inflammation in a mouse model of ulcerative colitis. *J Clin Invest* 2008; **118**: 534-544 [PMID: 18172556 DOI: 10.1172/JCI33194]

33 **Leung JM**, Davenport M, Wolff MJ, Wiens KE, Abidi WM, Poles MA, Cho I, Ullman T, Mayer L, Loke P. IL-22-producing CD4+ cells are depleted in actively inflamed colitis tissue. *Mucosal Immunol* 2014; **7**: 124-133 [PMID: 23695510 DOI: 10.1038/mi.2013.31]

34 **Rendon JL**, Li X, Akhtar S, Choudhry MA. Interleukin-22 modulates gut epithelial and immune barrier functions following acute alcohol exposure and burn injury. *Shock* 2013; **39**: 11-18 [PMID: 23143063 DOI: 10.1097/SHK.0b013e3182749f96]

35 **Pickert G**, Neufert C, Leppkes M, Zheng Y, Wittkopf N, Warntjen M, Lehr HA, Hirth S, Weigmann B, Wirtz S, Ouyang W, Neurath MF, Becker C. STAT3 links IL-22 signaling in intestinal epithelial cells to mucosal wound healing. *J Exp Med* 2009; **206**: 1465-1472 [PMID: 19564350 DOI: 10.1084/jem.20082683]

36 **Zheng Y**, Valdez PA, Danilenko DM, Hu Y, Sa SM, Gong Q, Abbas AR, Modrusan Z, Ghilardi N, de Sauvage FJ, Ouyang W. Interleukin-22 mediates early host defense against attaching and effacing bacterial pathogens. *Nat Med* 2008; **14**: 282-289 [PMID: 18264109 DOI: 10.1038/nm1720]

37 **Pociask DA**, Scheller EV, Mandalapu S, McHugh KJ, Enelow RI, Fattman CL, Kolls JK, Alcorn JF. IL-22 is essential for lung epithelial repair following influenza infection. *Am J Pathol* 2013; **182**: 1286-1296 [PMID: 23490254 DOI: 10.1016/j.ajpath.2012.12.007]

38 **Takahashi K**, Hirose K, Kawashima S, Niwa Y, Wakashin H, Iwata A, Tokoyoda K, Renauld JC, Iwamoto I, Nakayama T, Nakajima H. IL-22 attenuates IL-25 production by lung epithelial cells and inhibits antigen-induced eosinophilic airway inflammation. *J Allergy Clin Immunol* 2011; **128**: 1067-1076.e1-6 [PMID: 21794904 DOI: 10.1016/j.jaci.2011.06.018]

39 **Taube C**, Tertilt C, Gyülveszi G, Dehzad N, Kreymborg K, Schneeweiss K, Michel E, Reuter S, Renauld JC, Arnold-Schild D, Schild H, Buhl R, Becher B. IL-22 is produced by innate lymphoid cells and limits inflammation in allergic airway disease. *PLoS One* 2011; **6**: e21799 [PMID: 21789181 DOI: 10.1371/journal.pone.0021799]

40 **Simonian PL**, Wehrmann F, Roark CL, Born WK, O'Brien RL, Fontenot AP. γδ T cells protect against lung fibrosis *via* IL-22. *J Exp Med* 2010; **207**: 2239-2253 [PMID: 20855496 DOI: 10.1084/jem.20100061]

41 **Ma HL**, Liang S, Li J, Napierata L, Brown T, Benoit S, Senices M, Gill D, Dunussi-Joannopoulos K, Collins M, Nickerson-Nutter C, Fouser LA, Young DA. IL-22 is required for Th17 cell-mediated pathology in a mouse model of psoriasis-like skin inflammation. *J Clin Invest* 2008; **118**: 597-607 [PMID: 18202747 DOI: 10.1172/JCI33263]

42 **Wolk K**, Witte E, Wallace E, Döcke WD, Kunz S, Asadullah K, Volk HD, Sterry W, Sabat R. IL-22 regulates the expression of genes responsible for antimicrobial defense, cellular differentiation, and mobility in keratinocytes: a potential role in psoriasis. *Eur J Immunol* 2006; **36**: 1309-1323 [PMID: 16619290]

43 **Boniface K**, Guignouard E, Pedretti N, Garcia M, Delwail A, Bernard FX, Nau F, Guillet G, Dagregorio G, Yssel H, Lecron JC, Morel F. A role for T cell-derived interleukin 22 in psoriatic skin inflammation. *Clin Exp Immunol* 2007; **150**: 407-415 [PMID: 17900301]

44 **Nakajima H**, Nakajima K, Tarutani M, Morishige R, Sano S. Kinetics of circulating Th17 cytokines and adipokines in psoriasis patients. *Arch Dermatol Res* 2011; **303**: 451-455 [PMID: 21681565 DOI: 10.1007/s00403-011-1159-3]

45 **Cheuk S**, Wikén M, Blomqvist L, Nylén S, Talme T, Ståhle M, Eidsmo L. Epidermal Th22 and Tc17 cells form a localized disease memory in clinically healed psoriasis. *J Immunol* 2014; **192**: 3111-3120 [PMID: 24610014 DOI: 10.4049/jimmunol.1302313]

46 **Ikeuchi H**, Kuroiwa T, Hiramatsu N, Kaneko Y, Hiromura K, Ueki K, Nojima Y. Expression of interleukin-22 in rheumatoid arthritis: potential role as a proinflammatory cytokine. *Arthritis Rheum* 2005; **52**: 1037-1046 [PMID: 15818686]

47 **Leipe J**, Schramm MA, Grunke M, Baeuerle M, Dechant C, Nigg AP, Witt MN, Vielhauer V, Reindl CS, Schulze-Koops H, Skapenko A. Interleukin 22 serum levels are associated with radiographic progression in rheumatoid arthritis. *Ann Rheum Dis* 2011; **70**: 1453-1457 [PMID: 21593004 DOI: 10.1136/ard.2011.152074]

48 **Carrión M**, Juarranz Y, Martínez C, González-Álvaro I, Pablos JL, Gutiérrez-Cañas I, Gomariz RP. IL-22/IL-22R1 axis and S100A8/A9 alarmins in human osteoarthritic and rheumatoid arthritis synovial fibroblasts. *Rheumatology* (Oxford) 2013; **52**: 2177-2186 [PMID: 24056519 DOI: 10.1093/rheumatology/ket315]

49 **Carrión M**, Juarranz Y, Seoane IV, Martínez C, González-Álvaro I, Pablos JL, Gutiérrez-Cañas I, Gomariz RP. VIP modulates IL-22R1 expression and prevents the contribution of rheumatoid synovial fibroblasts to IL-22-mediated joint destruction. *J Mol Neurosci* 2014; **52**: 10-17 [PMID: 24254222 DOI: 10.1007/s12031-013-0177-3]

50 **Lejeune D**, Dumoutier L, Constantinescu S, Kruijer W, Schuringa JJ, Renauld JC. Interleukin-22 (IL-22) activates the JAK/STAT, ERK, JNK, and p38 MAP kinase pathways in a rat hepatoma cell line. Pathways that are shared with and distinct from IL-10. *J Biol Chem* 2002; **277**: 33676-33682 [PMID: 12087100]

51 **Xu H**, Wang X, Liu DX, Moroney-Rasmussen T, Lackner AA, Veazey RS. IL-17-producing innate lymphoid cells are restricted to mucosal tissues and are depleted in SIV-infected macaques. *Mucosal Immunol* 2012; **5**: 658-669 [PMID: 22669579 DOI: 10.1038/mi.2012.39]

52 **Colonna M**. Interleukin-22-producing natural killer cells and lymphoid tissue inducer-like cells in mucosal immunity. *Immunity* 2009; **31**: 15-23 [PMID: 19604490 DOI: 10.1016/j.immuni.2009.06.008]

53 **Bulmer AC**, Coombes JS, Blanchfield JT, Toth I, Fassett RG, Taylor SM. Bile pigment pharmacokinetics and absorption in the rat: therapeutic potential for enteral administration. *Br J Pharmacol* 2011; **164**: 1857-1870 [PMID: 21486273 DOI: 10.1111/j.1476-5381.2011.01413.x]

54 **Lee JS**, Cella M, McDonald KG, Garlanda C, Kennedy GD, Nukaya M, Mantovani A, Kopan R, Bradfield CA, Newberry RD, Colonna M. AHR drives the development of gut ILC22 cells and postnatal lymphoid tissues *via* pathways dependent on and independent of Notch. *Nat Immunol* 2012; **13**: 144-151 [PMID: 22101730 DOI: 10.1038/ni.2187]

55 **Sonnenberg GF**, Monticelli LA, Alenghat T, Fung TC, Hutnick NA, Kunisawa J, Shibata N, Grunberg S, Sinha R, Zahm AM, Tardif MR, Sathaliyawala T, Kubota M, Farber DL, Collman RG, Shaked A, Fouser LA, Weiner DB, Tessier PA, Friedman JR, Kiyono H, Bushman FD, Chang KM, Artis D. Innate lymphoid cells promote anatomical containment of lymphoid-resident commensal bacteria. *Science* 2012; **336**: 1321-1325 [PMID: 22674331 DOI: 10.1126/science.1222551]

56 **Tachiiri A**, Imamura R, Wang Y, Fukui M, Umemura M, Suda T. Genomic structure and inducible expression of the IL-22 receptor alpha chain in mice. *Genes Immun* 2003; **4**: 153-159 [PMID: 12618864]

57 **Lerman G**, Sharon M, Leibowitz-Amit R, Sidi Y, Avni D. The crosstalk between IL-22 signaling and miR-197 in human keratinocytes. *PLoS One* 2014; **9**: e107467 [PMID: 25208211 DOI: 10.1371/journal.pone.0107467]

58 **Mareninova OA**, Hermann K, French SW, O'Konski MS, Pandol SJ, Webster P, Erickson AH, Katunuma N, Gorelick FS, Gukovsky I, Gukovskaya AS. Impaired autophagic flux mediates acinar cell vacuole formation and trypsinogen activation in rodent models of acute pancreatitis. *J Clin Invest* 2009; **119**: 3340-3355 [PMID: 19805911 DOI: 10.1172/JCI38674]

59 **Fortunato F**, Bürgers H, Bergmann F, Rieger P, Büchler MW, Kroemer G, Werner J. Impaired autolysosome formation correlates with Lamp-2 depletion: role of apoptosis, autophagy, and necrosis in pancreatitis. *Gastroenterology* 2009; **137**: 350-360, 360.e1-5 [PMID: 19362087 DOI: 10.1053/j.gastro.2009.04.003]

60 **Yu H**, Jove R. The STATs of cancer--new molecular targets come of age. *Nat Rev Cancer* 2004; **4**: 97-105 [PMID: 14964307 DOI: 10.1038/nrc1275]

61 **Abdulla A**, Awla D, Thorlacius H, Regnér S. Role of neutrophils in the activation of trypsinogen in severe acute pancreatitis. *J Leukoc Biol* 2011; **90**: 975-982 [PMID: 21810937 DOI: 10.1189/jlb.0411195]

62 **Narushima Y**, Unno M, Nakagawara K, Mori M, Miyashita H, Suzuki Y, Noguchi N, Takasawa S, Kumagai T, Yonekura H, Okamoto H. Structure, chromosomal localization and expression of mouse genes encoding type III Reg, RegIII alpha, RegIII beta, RegIII gamma. *Gene* 1997; **185**: 159-168 [PMID: 9055810 DOI: 10.1016/S0378-1119(96)00589-6]

63 **Keim V**, Iovanna JL, Rohr G, Usadel KH, Dagorn JC. Characterization of a rat pancreatic secretory protein associated with pancreatitis. *Gastroenterology* 1991; **100**: 775-782 [PMID: 1704329]

64 **Iovanna J**, Orelle B, Keim V, Dagorn JC. Messenger RNA sequence and expression of rat pancreatitis-associated protein, a lectin-related protein overexpressed during acute experimental pancreatitis. *J Biol Chem* 1991; **266**: 24664-24669 [PMID: 1722211]

65 **Zhang H**, Kandil E, Lin YY, Levi G, Zenilman ME. Targeted inhibition of gene expression of pancreatitis-associated proteins exacerbates the severity of acute pancreatitis in rats. *Scand J Gastroenterol* 2004; **39**: 870-881 [PMID: 15513386 DOI: 10.1080/00365520410006477]

66 **Christa L**, Carnot F, Simon MT, Levavasseur F, Stinnakre MG, Lasserre C, Thepot D, Clement B, Devinoy E, Brechot C. HIP/PAP is an adhesive protein expressed in hepatocarcinoma, normal Paneth, and pancreatic cells. *Am J Physiol* 1996; **271**: G993-1002 [PMID: 8997243]

67 **McKie AT**, Simpson RJ, Ghosh S, Peters TJ, Farzaneh F. Regulation of pancreatitis-associated protein (HIP/PAP) mRNA levels in mouse pancreas and small intestine. *Clin Sci* (Lond) 1996; **91**: 213-218 [PMID: 8795446 DOI: 10.1042/cs0910213]

68 **Masciotra L**, Lechêne de la Porte P, Frigerio JM, Dusetti NJ, Dagorn JC, Iovanna JL. Immunocytochemical localization of pancreatitis-associated protein in human small intestine. *Dig Dis Sci* 1995; **40**: 519-524 [PMID: 7895535 DOI: 10.1007/BF02064359]

69 **Dieckgraefe BK**, Stenson WF, Korzenik JR, Swanson PE, Harrington CA. Analysis of mucosal gene expression in inflammatory bowel disease by parallel oligonucleotide arrays. *Physiol Genomics* 2000; **4**: 1-11 [PMID: 11074008]

70 **Ozturk M**, de la Monte SM, Gross J, Wands JR. Elevated levels of an exocrine pancreatic secretory protein in Alzheimer disease brain. *Proc Natl Acad Sci USA* 1989; **86**: 419-423 [PMID: 2463628 DOI: 10.1073/pnas.86.2.419]

71 **Duplan L**, Michel B, Boucraut J, Barthellémy S, Desplat-Jego S, Marin V, Gambarelli D, Bernard D, Berthézène P, Alescio-Lautier B, Verdier JM. Lithostathine and pancreatitis-associated protein are involved in the very early stages of Alzheimer's disease. *Neurobiol Aging* 2001; **22**: 79-88 [PMID: 11164279 DOI: 10.1016/S0197-4580(00)00182-2]

72 **Christa L**, Simon MT, Brezault-Bonnet C, Bonte E, Carnot F, Zylberberg H, Franco D, Capron F, Roskams T, Bréchot C. Hepatocarcinoma-intestine-pancreas/pancreatic associated protein (HIP/PAP) is expressed and secreted by proliferating ductules as well as by hepatocarcinoma and cholangiocarcinoma cells. *Am J Pathol* 1999; **155**: 1525-1533 [PMID: 10550309 DOI: 10.1016/S0002-9440(10)65468-5]

73 **Xie MJ**, Motoo Y, Iovanna JL, Su SB, Ohtsubo K, Matsubara F, Sawabu N. Overexpression of pancreatitis-associated protein (PAP) in human pancreatic ductal adenocarcinoma. *Dig Dis Sci* 2003; **48**: 459-464 [PMID: 12757156 DOI: 10.1023/A:1022520212447]

74 **Rosty C**, Christa L, Kuzdzal S, Baldwin WM, Zahurak ML, Carnot F, Chan DW, Canto M, Lillemoe KD, Cameron JL, Yeo CJ, Hruban RH, Goggins M. Identification of hepatocarcinoma-intestine-pancreas/pancreatitis-associated protein I as a biomarker for pancreatic ductal adenocarcinoma by protein biochip technology. *Cancer Res* 2002; **62**: 1868-1875 [PMID: 11912167]

75 **Lin YY**, Viterbo D, Mueller CM, Stanek AE, Smith-Norowitz T, Drew H, Wadgaonkar R, Zenilman ME, Bluth MH. Small-interference RNA gene knockdown of pancreatitis-associated proteins in rat acute pancreatitis. *Pancreas* 2008; **36**: 402-410 [PMID: 18437087 DOI: 10.1097/MPA.0b013e31815f3933]

76 **Shigekawa M**, Hikita H, Kodama T, Shimizu S, Li W, Uemura A, Miyagi T, Hosui A, Kanto T, Hiramatsu N, Tatsumi T, Takeda K, Akira S, Takehara T. Pancreatic STAT3 protects mice against caerulein-induced pancreatitis *via* PAP1 induction. *Am J Pathol* 2012; **181**: 2105-2113 [PMID: 23064197 DOI: 10.1016/j.ajpath.2012.08.038]

77 **Gironella M**, Folch-Puy E, LeGoffic A, Garcia S, Christa L, Smith A, Tebar L, Hunt SP, Bayne R, Smith AJ, Dagorn JC, Closa D, Iovanna JL. Experimental acute pancreatitis in PAP/HIP knock-out mice. *Gut* 2007; **56**: 1091-1097 [PMID: 17409121 DOI: 10.1136/gut.2006.116087]

78 **Murray PJ**, Wynn TA. Protective and pathogenic functions of macrophage subsets. *Nat Rev Immunol* 2011; **11**: 723-737 [PMID: 21997792 DOI: 10.1038/nri3073]

79 **Nakamichi I**, Habtezion A, Zhong B, Contag CH, Butcher EC, Omary MB. Hemin-activated macrophages home to the pancreas and protect from acute pancreatitis *via* heme oxygenase-1 induction. *J Clin Invest* 2005; **115**: 3007-3014 [PMID: 16239966 DOI: 10.1172/JCI24912]

80 **Mikami Y**, Takeda K, Shibuya K, Qiu-Feng H, Egawa S, Sunamura

M, Matsuno S. Peritoneal inflammatory cells in acute pancreatitis: Relationship of infiltration dynamics and cytokine production with severity of illness. *Surgery* 2002; **132**: 86-92 [PMID: 12110800 DOI: 10.1067/msy.2002.125171]

81 **Malleo G**, Mazzon E, Siriwardena AK, Cuzzocrea S. Role of tumor necrosis factor-alpha in acute pancreatitis: from biological basis to clinical evidence. *Shock* 2007; **28**: 130-140 [PMID: 17529903 DOI: 10.1097/shk.0b013e3180487ba1]

82 **Kaufmann P**, Tilz GP, Lueger A, Demel U. Elevated plasma levels of soluble tumor necrosis factor receptor (sTNFRp60) reflect severity of acute pancreatitis. *Intensive Care Med* 1997; **23**: 841-848 [PMID: 9310801 DOI: 10.1007/s001340050420]

83 **Granell S**, Pereda J, Gómez-Cambronero L, Cassinello N, Sabater L, Closa D, Sastre J. Circulating TNF-alpha and its soluble receptors during experimental acute pancreatitis. *Cytokine* 2004; **25**: 187-191 [PMID: 15164724 DOI: 10.1016/j.cyto.2003.10.011]

84 **Shen Y**, Cui N, Miao B, Zhao E. Immune dysregulation in patients with severe acute pancreatitis. *Inflammation* 2011; **34**: 36-42 [PMID: 20405190 DOI: 10.1007/s10753-010-9205-4]

85 **Surbatovic M**, Radakovic S. Tumor necrosis factor-α levels early in severe acute pancreatitis: is there predictive value regarding severity and outcome? *J Clin Gastroenterol* 2013; **47**: 637-643 [PMID: 23470643 DOI: 10.1097/MCG.0b013e31828a6cfc]

86 **Eyerich S**, Wagener J, Wenzel V, Scarponi C, Pennino D, Albanesi C, Schaller M, Behrendt H, Ring J, Schmidt-Weber CB, Cavani A, Mempel M, Traidl-Hoffmann C, Eyerich K. IL-22 and TNF-α represent a key cytokine combination for epidermal integrity during infection with Candida albicans. *Eur J Immunol* 2011; **41**: 1894-1901 [PMID: 21469124 DOI: 10.1002/eji.201041197]

87 **Ni J**, Hu G, Xiong J, Shen J, Shen J, Yang L, Tang M, Zhao Y, Ying G, Yu G, Hu Y, Xing M, Wan R, Wang X. Involvement of interleukin-17A in pancreatic damage in rat experimental acute necrotizing pancreatitis. *Inflammation* 2013; **36**: 53-65 [PMID: 22990529 DOI: 10.1007/s10753-012-9519-5]

88 **Dai SR**, Li Z, Zhang JB. Serum interleukin 17 as an early prognostic biomarker of severe acute pancreatitis receiving continuous blood purification. *Int J Artif Organs* 2015; **38**: 192-198 [PMID: 25907530 DOI: 10.5301/ijao.5000406]

89 **Sonnenberg GF**, Nair MG, Kirn TJ, Zaph C, Fouser LA, Artis D. Pathological versus protective functions of IL-22 in airway inflammation are regulated by IL-17A. *J Exp Med* 2010; **207**: 1293-1305 [PMID: 20498020 DOI: 10.1084/jem.20092054]

90 **Liang SC**, Tan XY, Luxenberg DP, Karim R, Dunussi-Joannopoulos K, Collins M, Fouser LA. Interleukin (IL)-22 and IL-17 are coexpressed by Th17 cells and cooperatively enhance expression of antimicrobial peptides. *J Exp Med* 2006; **203**: 2271-2279 [PMID: 16982811]

91 **Warren RB**, Griffiths CE. The future of biological therapies. *Semin Cutan Med Surg* 2010; **29**: 63-66 [PMID: 20430310 DOI: 10.1016/j.sder.2010.02.004]

92 **Wang X**, Ota N, Manzanillo P, Kates L, Zavala-Solorio J, Eidenschenk C, Zhang J, Lesch J, Lee WP, Ross J, Diehl L, van Bruggen N, Kolumam G, Ouyang W. Interleukin-22 alleviates metabolic disorders and restores mucosal immunity in diabetes. *Nature* 2014; **514**: 237-241 [PMID: 25119041 DOI: 10.1038/nature13564]

93 ClinicalTrials.gov. A service of the U.S. National Institutes of Health. Available from: URL: https://clinicaltrials.gov

P- Reviewer: Carrasco C, Poma EM, Vujasinovic M
S- Editor: Gong XM **L- Editor**: A **E- Editor**: Jiao XK

World Journal of Gastrointestinal Pathophysiology

Submit a Manuscript: http://www.wjgnet.com/esps/
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.4291/wjgp.v7.i1.117

World J Gastrointest Pathophysiol 2016 February 15; 7(1): 117-124
ISSN 2150-5330 (online)

MINIREVIEWS

Small bowel neuroendocrine tumors: From pathophysiology to clinical approach

Sofia Xavier, Bruno Rosa, José Cotter

Sofia Xavier, Bruno Rosa, José Cotter, Department of Gastroenterology, Centro Hospitalar do Alto Ave, 4835-044 Guimarães, Portugal

José Cotter, ICVS/3B's Associate Laboratory, University of Minho, Campus de Gualtar, 4710-057 Braga, Portugal

José Cotter, Life and Health Sciences Research Institute, School of Health Sciences, University of Minho, Campus de Gualtar, 4710-057 Braga, Portugal

Author contributions: Xavier S performed the literature search, designed the text structure and wrote the text; Rosa B and Cotter J suggested the theme to be reviewed, and made the several critical corrections and revisions, including English editing, until the submitted version was achieved.

Conflict-of-interest statement: All the authors hereby declare that they do not have any conflict-of-interest (including but not limited to commercial, personal, political, intellectual, or religious interests) related to the work submitted herein.

Correspondence to: Dr. Sofia Xavier, Department of Gastroenterology, Centro Hospitalar do Alto Ave, Rua dos Cutileiros, Creixomil, 4835-044 Guimarães, Portugal. smaxavier@gmail.com
Telephone: +351-253-540330
Fax: +351-253-513592

Received: June 28, 2015
Peer-review started: July 5, 2015
First decision: August 16, 2015
Revised: September 9, 2015
Accepted: December 16, 2015

Article in press: December 18, 2015
Published online: February 15, 2016

Abstract

Neuroendocrine tumors (NETs), defined as epithelial tumors with predominant neuroendocrine differentiation, are among the most frequent types of small bowel neoplasm. They represent a rare, slow-growing neoplasm with some characteristics common to all forms and others attributable to the organ of origin. The diagnosis of this subgroup of neoplasia is not usually straight-forward for several reasons. Being a rare form of neoplasm they are frequently not readily considered in the differential diagnosis. Also, clinical manifestations are nonspecific lending the clinician no clue that points directly to this entity. However, the annual incidence of NETs has risen in the last years to 40 to 50 cases per million probably not due to a real increase in incidence but rather due to better diagnostic tools that have become progressively available. Being a rare malignancy, investigation regarding its pathophysiology and efforts toward better understanding and classification of these tumors has been limited until recently. Clinical societies dedicated to this matter are emerging (NANETS, ENETS and UKINETS) and several guidelines were published in an effort to standardize the nomenclature, grading and staging systems as well as diagnosis and management of NETs. Also, some investigation on the genetic behavior of small bowel NETs has been recently released, shedding some light on the pathophysiology of these tumors, and pointing some new directions on the possible treating options. In this review we focus on the current status of the overall knowledge about small bowel NETs, focusing on recent breakthroughs and its potential application on clinical practice.

Key words: Neuroendocrine tumors; Gastrointestinal tumors; Small bowel neoplasms; Carcinoid; Diagnostic markers

Core tip: Annual incidence of neuroendocrine tumors (NETs) has risen in the last years to 40 to 50 cases per million probably due to better diagnostic tools. Recurrent loss of chromosomes 11 and 18 and gains of chromosomes 4, 5, 19 and 20 have been shown in NETs. Several cancer-related pathways were implied in NETs associated mutations, including PI3K/Akt/mTOR and TGF-β pathways. Genes involved in secretory activity were conserved in NETs, however alterations in transcription factors associated with neurodevelopmental process were reported. Studies suggest that miRNA may have a role in ileal NETs development and progression.

Xavier S, Rosa B, Cotter J. Small bowel neuroendocrine tumors: From pathophysiology to clinical approach. *World J Gastrointest Pathophysiol* 2016; 7(1): 117-124 Available from: URL: http://www.wjgnet.com/2150-5330/full/v7/i1/117.htm DOI: http://dx.doi.org/10.4291/wjgp.v7.i1.117

INTRODUCTION

Neuroendocrine tumors (NETs), defined as epithelial tumors with predominant neuroendocrine differentiation, are among the most frequent types of small bowel neoplasm. They represent a rare, slow-growing neoplasm with some characteristics common to all forms and others attributable to the organ of origin. The diagnosis of this subgroup of neoplasia is not usually straight-forward for several reasons. Being a rare form of neoplasm they are frequently not readily considered in the differential diagnosis. Also, clinical manifestations are nonspecific lending the clinician no clue that points directly to this entity. However, the annual incidence of NETs has risen in the last years to 40 to 50 cases per million probably not due to a real increase in incidence but rather due to better diagnostic tools that have become progressively available.

Being a rare malignancy, investigation regarding its pathophysiology and efforts toward better understanding and classification of these tumors has been limited until recently. Clinical societies dedicated to this matter are emerging (NANETS, ENETS and UKINETS) and several guidelines were published in an effort to standardize the nomenclature, grading and staging systems as well as diagnosis and management of NETs. Also, some investigation on the genetic behavior of small bowel NETs has been recently released, shedding some light on the pathophysiology of these tumors, and pointing some new directions on the possible treating options.

In this review we focus on the current status of the overall knowledge about small bowel NETs, focusing on recent breakthroughs and its potential application on clinical practice.

EPIDEMIOLOGY

Duodenal NETs comprise 1%-3% of all primary duodenal tumors and 2.8% of all carcinoid tumors according to the PAN-SEER Registry (1973-1999)[1]. According to SEER Program, the age-adjusted annual incidence of NETs arising from jejunum and ileum is 0.67 per 100000 and for appendix NETs it is 0.16 per 100000[2]. However, data extracted from large autopsies series indicates that the incidence of small bowel NETs may be up to 0.7%[3]. Also, time-trend analyses have shown a rise in the incidence of all these forms of NETs. This is probably not due to a real increase in the number of cases, but rather due to an increased diagnosis efficacy.

CLASSIFICATION, GRADING AND STAGING

NETs are rare neoplasms and can arise in most organs of the body. Some of their features are shared by all NETs, while others are attributable to their organ of origin[4]. Most of the studies regarding NETs have focused on the most frequent locations, such as pancreatic and gastrointestinal, limiting extensive knowledge of other less common forms of the disease.

All these features have contributed to the emergence of several nomenclatures, grading and classification systems. Although most of these systems proved themselves useful, the lack of a standard classification system has hindered the normalization of NETs classification and consequently, scientific community wide consensus. Since some of these systems are now firmly established and no clinical data favors one system over the others, it may be unpractical to adopt a single system, rejecting the remaining. Thus, while ENETS[5,6] favor the use of World Health Organization classification system, NANETS[7] propose that some basic data elements (proliferative rate, extent of local spread, immunohistochemical markers) should be specified and documented on pathological reports and that a specified system of nomenclature, grading and staging should be used. This can assure that the basic data are recorded, allowing retrospective comparison of NETs regardless of the specific classification system used.

CLINICAL FEATURES

Regarding clinical features there are several differences between duodenal and most distal NETs of jejunum and ileum.

Duodenal NETs are usually diagnosed in the sixth decade with a slight male predominance[8,9]. There are five main forms: Duodenal gastrinoma, the most common type; duodenal somatostatinoma; non-functioning duodenal NETs; duodenal gangliocytic paraganglioma and poorly differentiated neuroendocrine duodenal carcinomas[4,8]. Some authors also consider periampullary NETs as a different category given their different clinical, histological and growth behavior[8]. Most duodenal NETs

are small, single lesions, usually limited to the mucosa and submucosa. Regional lymph node metastases may be found in up to 60% of cases, while liver metastasis usually occur in less than 10%[8]. Since about 90% of duodenal NETs are not associated with clinical syndrome, most diagnoses are made accidentally during a routine workup or the patient develops symptoms attributable to the mass itself[8]. Most frequently reported presenting symptoms include pain, jaundice (more frequent in peri-ampullary NETS), nausea, vomits, diarrhea, obstruction, active bleeding or anemia[8,9]. In the minority of duodenal NETs that cause a functional syndrome the two main presentations are Zollinger-Ellison syndrome (ZES) and carcinoid syndrome[6].

ZES in duodenal gastrinomas usually presents with abdominal pain, diarrhea and reflux esophagitis[10]. Its diagnosis requires the demonstration of inappropriate hypergastrinemia and there are several conditions that can cause hypergastrinemia and complicate the diagnosis of a ZES. If the fasting serum gastrin is 1000 ng/L or greater and gastric pH is less than 2.5 the diagnosis is established if the patient is normocalcemic, has a normal renal function and doesn't have pyloric obstruction[11]. Duodenal NETs presenting with ZES can be sporadic or associated to MEN1 syndrome. While sporadic forms usually result from single lesions, MEN1 display multiple lesions[8,10].

Carcinoid syndrome is usually present in patients with liver metastasis[5,12] and is caused by excessive secretion of endogenous substances, more frequently serotonin. Patients can present with flushing, diarrhea, cough, whezzing and carcinoid heart disease[12]. Carcinoid heart disease is a right sided cardiac insufficiency, and in about 35% of patients that develop this condition death arises not due to tumor progression but rather to heart failure[12]. Regarding prognosis, patients with well-differentiated duodenal NETs have a global 5-year survival rate of nearly 85%[13]. The stage of disease at diagnosis highly influence prognosis, with 10-year survival of 95% for patients with local disease and 10% for those with distant metastases[9].

NETs of jejunum and ileum are usually diagnosed in the sixth/seventh decade[14] but, in opposition to duodenal NETs, have no gender preference. Most of them are nonfunctioning tumors but about 20% of patients show liver metastases and may present with carcinoid syndrome. At diagnosis, lesions are commonly > 2 cm, with invasion of muscularis propria and metastasis to regional lymph nodes[4]. Multiple lesions may be found in up to 40% of cases[4]. Clinical manifestations include abdominal pain, bowel obstruction, diarrhea, weight loss and bleeding[15]. The prognosis of these NETs is generally unfavorable when compared with other location tumors of comparable size since they have a higher tendency to grow and spread before the diagnosis is firmed[14,15]. The 5-year survival correlates with the stage of disease at diagnosis, being of 65% for patients with localized disease and only 36% for those with distant metastases[5].

PATHOPHYSIOLOGY

Small bowel tumors correspond to 1%-2% of all gastrointestinal malignancies, and NETs are only one of the subtypes of these rare neoplasms. Being an unusual form of oncologic disease, research towards better understanding these tumors has been scarce.

Phenotypically these neoplasms are composed by neuroendocrine cells which, in the gastrointestinal tract, are scattered through the mucosa. These cells get their name from their ability to express some proteins classically attributable to neural cells, as neuron-specific enolase and synaptophysin, and also to their capacity to produce hormones, such as somatostatin, substance P and vasoactive intestinal peptide. The tumorigenesis of NETs has not yet been elucidated, however recent efforts to clarify the genetic alterations in these tumors have been made.

Recently, two exome/genome-wide tumor DNA sequencing for the small intestine (SI) NET have been reported. The first one, from Banck *et al*[16], matched germline DNA of 48 small bowel NETs, with samples consisting of well differentiated primary tumors. More than 20000 genes were sequenced and the data from tumor was compared to normal tissue to identify genetic alterations. This study found point mutations, termed single nucleotide variants (SNV), at an average rate of 0.1 SNV per 10^6 nucleotides. This classifies small bowel NETs as a genomically stable cancer, with low mutation rates. SNV were found in 197 genes, most of which were known for being cancer genes. Those included *VHL*, *BRAF*, *FGFR2*, *MEN1*, *MLF1*, *SRC*, *SMAD* and *FANCD2* among others. Banck *et al*[16] also looked for somatic copy number alterations, that consist of large deletions or amplifications that can cause the inactivation of tumor suppressor genes or the excessive expression of oncogenes. This analyses revealed a recurrent loss of chromosomes 11 and 18 and gains of chromosomes 4, 5, 19 and 20. Furthermore, with resource to bioinformatic analysis, several cancer-related pathways were implied in these mutations, including PI3K/Akt/mTOR and TGF-β pathways. The involvement of mTOR pathway support the findings of recent clinical trials in which mTOR inhibitors seem active in the treatment of some SI NETs[17]. Also, TGF-β pathway had previously been implied as a regulator in small bowel NETs. A study based on immunohistochemistry staining of 104 NETs showed enhanced expression of TGF-β in all but 1 tumor[18]. Also, cell lines studies suggest that small bowel NET neoplastic cells were induced to proliferate by TGF-β1 but the same effect was not reproducible in normal small bowel cells[19]. This suggests that TGF-β pathway targeting therapies might be of use in NETs management, as previously pointed out in some studies[20].

Francis *et al*[21] published a multicenter study of whole exome sequencing of 29 small bowel NETs and whole genome sequencing of 15 primary small bowel NETs, focusing on analysis of small insertion and deletion termed indels. They found recurrent heterozygous

inactivating indels in the cell cycle inhibitor gene *CDKN1B* in 8% of small bowel NETs. With the release of this information, Banck *et al*[16] reviewed their results targeting *CDKN1B* gene, coming to similar results. This finding suggests that cell cycle inhibitory drugs may be of interest in the treatment of a subset of patients. Curiously, germline mutations in *CDKN1B* are known to cause MEN-4, a cancerous syndrome with known association to endocrine NETs, and *CDKN1B* mutations have been implied in other cancers, like colorectal, breast and prostate.

In an effort to understand the expression profiles and regulatory networks involved in NETs, Kidd *et al*[22] re-analysed two small intestinal tumor transcriptomes. They reported that in small bowel NETs the genes involved in secretory activity were conserved; however alterations in transcription factors associated with neurodevelopmental process were reported, suggesting that abnormalities of this process may be relevant in the neoplastic evolution. The study group was also able to confirm the loss of SDHD expression, a finding usually associated with benign tumors.

Another recent breakthrough in the understanding of NETs pathophysiology is the study of microRNAs (miRNAs). MiRNAs consist of small (19-25 nucleotides), non-coding RNAs with the capability to regulate the expression of at least one-third of protein-coding genes. Their deregulation has been associated with the development of cancer, and some subsets of neoplasm have been extensively studied in the field of miRNAs with results that point to the influence of deregulated miRNAs in the staging, prognosis and response to therapy[23]. They have the capacity to regulate gene expression, to influence neighbor cells and even to be packed and transported extracellularly, entering the bloodstream, and modulating cells at distant site in an hormone-like action[24].

This subtype of RNA can be extracted from several body fluids, is long-living *in vivo* and very stable *in vitro* which enables miRNA profiling techniques to be extremely sensitive, objective and standardized, characteristics that make miRNAs potential biomarkers[25]. Also, they can act as potential therapeutic targets with preclinical models pointing to the efficacy of miRNA based therapies[26].

Regarding SI NETs, two recent miRNA expression profile studies showed that miRNA deregulation played a role in small bowel NETs tumorigenesis. The first one associated miRNA-133a downregulation with progression from primary to metastatic disease. This suggests that this miRNA may have a role in ileal NETs development and progression, being a potential marker of diagnosis and/or prognosis[27]. Interestingly, miRNA-133a is located to chromosome 18, which was shown to be recurrently lost in whole genome studies. In the second study, the expression of miRNAs in well-differentiated small bowel NETs was evaluated, with the goal to provide new disease biomarkers. They reported that five miRNAs were upregulated (miRNA-96,-182,-183,-196 and-200) and four were downregulated (miRNA-31, -129-5p, -133a and-215) during tumor progression[28].

Although these findings might be promising, there is still much to be understood regarding the mechanisms behind NETs development. Nevertheless, this recent knowledge has allowed to guide the scientific community, with the emergence of clinical trials with therapies targeting the genetic alterations described so far.

BIOMARKERS AND OTHER LABORATORY TESTS

Several circulating tumor markers have been evaluated for the diagnosis and follow-up of NETs. Currently, chromogranin A (CgA) is the most important of these markers, and current guidelines recommend measurement of serum CgA at diagnosis[5,6,29]. It consists of a polypeptide widely expressed in secretory granules of neuroendocrine cells. Plasma CgA is elevated in 60%-100% of patients with NET, either functioning or nonfunctioning, with a sensitivity and specificity for detecting NET of 70% to 100%[29]. However, CgA levels are influenced by the assay used[30] and are highly influenced by several common conditions, and false elevation of this marker has been reported in patients using proton pump inhibitors, with renal or liver failure and even in chronic gastritis[31]. Other proposed markers are plasma neuron-specific enolase, urinary 5-HIAA (as a marker of carcinoid syndrome) and a variety of other secreted amines such as chromogranin B and C, substance P, neurotensin, among others[29].

In an effort to find better NETs biomarkers, Modlin *et al*[32] recently published a multi-transcript molecular signature for PCR blood analysis which may facilitate future diagnosis of NETs. They analyzed transcripts of 3 microarray datasets (NETs peripheral blood, NETs tissue and adenocarcinoma) and found 51 significantly elevated transcript markers. Based on that, gene-based classifiers were created and were able to detect NETs with high sensitivity (85%-98%), specificity (93%-97%), positive predictive value (95%-96%) and negative predictive value (87%-98%). The transcript marker was similarly effective in recognizing pancreatic and gastrointestinal NETs, as well as, metastases. Moreover, the gene-based classifier was significantly more accurate than CgA and, in patients with low CgA, the transcript markers were elevated in 91% of cases[32]. This may reflect the future utility of genetic markers as diagnostic tools of NETs, however further investigation needs to be done to validate this hypothesis.

DIAGNOSTIC IMAGING

For duodenal NETs diagnosis, upper gastrointestinal endoscopy with biopsies (or endoscopic removal of the lesion for histopathological assessment whenever feasible) is the most sensitive diagnostic test. This

Table 1 Duodenal neuroendocrine tumors treatment

Duodenal NETs – surgical treatment	
⩽ 1 cm	Local ressection (if possible)
⩾ 2 cm OR lymph nodes metastasis	Surgical ressection
Potentially resectable hepatic metastases without distant metastases and no other significant comorbility	Palliative surgery
Duodenal NETs – farmacological treatment	
Functional duodenal NETs	Hormone suppression treatment
Well-differentiated NETs	Systemic chemotherapy if advanced metastatic disease
Poorly differentiated tumors	Combination chemotherapy – variable duration disease remission mTOR, tyrosine kinase and VEGF inhibitors – phase 3 trials with promising results
Metastatic or inoperable disease	Peptide receptor radionuclide therapy When all other treatment options fail If positive octreoscan

NETs: Neuroendocrine tumors; VEGF: Vascular endothelial growth factor.

can be coupled with endoscopic ultrasound in order to locally stage the disease, by evaluating the depth of involvement and the presence of local lymph nodes metastases[6,33]. Endoscopic ultrasound may be complemented with fine-needle aspiration (FNA) to obtain cells in deeper layers such as the submucosa, for histological diagnosis. Regarding jejunal and ileal NETs, ileocolonoscopy can make the diagnosis in more distal lesions but until recently, most of the small bowel extent was not accessible to direct mucosal visualization. With the evolution of enteroscopic diagnostic tools, physicians have now at their disposal new, promising diagnostic tools like capsule enteroscopy (CE) and balloon-assisted enteroscopy. CE proved itself useful in the study of suspected small bowel neoplasms, due to its high diagnostic yield and non-invasiveness, obtaining high quality endoscopic imaging even in the absence of bowel preparation[34]. Also, CE seems better than CT and enteroclysis in detecting primary NETs and has a similar diagnostic yield as somatostatin receptor scintigraphy (SRS) with the advantage that the first one can differentiate between intestinal and mesenterial localization[35]. In a review of our center epidemiology on small bowel tumors diagnosed by CE, 1510 CE performed between 2006-2014 were reviewed and included those classified as having suspect small bowel tumors with ⩾ 10 mm dimension. Lesions suspect of SI tumor were identified in 19 EC (1.3%), and histological confirmation of primary small bowel neoplasm was obtained for 6 cases, of which 2 patients were diagnosed with NET - one duodenal and another ileal - (unpublished data).

After the diagnosis, thorax X-ray, Helical CT or MRI of the abdomen and pelvis coupled with SRS should be done to assess disease extent and search for distant metastasis[5,6,31]. SRS is an imaging diagnostic test in which a somatostatin analog, octreotide, is radiolabeled and administered to the patient. Since the majority of NETs express one or more subtypes of somatostatin receptors, this technique allows detection of local and distant disease. In patients with advanced disease, especially liver metastases, bone scintigraphy and MRI of the spine should also be done to exclude bone metastasis[5,6].

PET scan using its classic tracer ^{18}F-deoxyglucose is not effective in the diagnosis of well differentiated NETs. However, using this diagnostic technique with specific neuroendocrine tracers has shown good results, with better detection of small primary tumors and lymph node metastases than CT, MRI and even SRS[36,37].

Moreover, for patients with carcinoid syndrome it is mandatory to perform an echocardiography to evaluate the presence and severity of carcinoid heart disease[5].

TREATMENT

Concerning treatment, clinical societies state that curative surgery should be aimed for in patients with duodenal, jejunal and ileal NETs whenever possible[5,6,29,38].

For small (⩽ 1 cm) duodenal NETs local endoscopic resection is an option but larger duodenal NETs (⩾ 2 cm) or the presence of lymph node metastases should be treated surgically[6]. Palliative surgery should be offered for those patients with potentially resectable hepatic metastases without distant metastases and no other medical conditions that can markedly compromise life expectancy. In the minority of duodenal NETs that display functional hormonal syndromes, specific treatment for hormone excess suppression should be given to the patient[6] (Table 1).

Jejunal and ileal NETs have a greater propensity to metastasize and are more frequently multiple lesions therefore, even in small tumors, surgery should involve search for additional tumors by inspection and palpation[29] as well as wide lymphadenectomy[5]. In patients with liver metastases curative surgery should still be attempted, and intraoperative ultrasonography should be performed for detection of all liver metastases[5]. Palliative surgery should still be considered to prevent complications attributable to the tumor mass in patients not suitable for curative resection[5].

In jejunal and ileal NETs with carcinoid syndrome, somatostatin analogs effectively reduce symptoms in 40%-80% of patients. Also, this therapy proved capable

Table 2 Jejunal and ileal neuroendocrine tumors treatment

Jejunal and ileal NETs – surgical treatment	
Without metastasis, all sizes	Surgical resection with wide lymphadenectomy + search for other lesions
With liver metastases	Attempt curative surgery; intraoperative ultrasonography should be performed for detection of all liver metastases If patient not suitable for curative resection, palliative surgery should be considered to prevent complications attributable to the tumor mass
Jejunal and ileal NETs – farmacological treatment	
Functional jejunal-ileal NETs	1st line: Somatostatin analogs (symptomatic treatment and tumor growth stabilization) 2nd line: Interferon-α
Well-differentiated NETs	Systemic chemotherapy not recommended
Poorly differentiated tumors	Combination chemotherapy – variable duration disease remission mTOR, tyrosine kinase and VEGF inhibitors – phase 3 trials with promising results
Metastatic or inoperable disease	Peptide receptor radionuclide therapy When all other treatment options fail If positive Octreoscan

NETs: Neuroendocrine tumors; VEGF: Vascular endothelial growth factor.

to induce stabilization of tumor growth in up to 50% of cases thus, somastatin analogs are clearly indicated in functional jejunal-ileal NETs[5]. Interferon-α can be used with the same purpose but its toxicity profile make this a second-line treatment option[5,29] (Table 2).

Systemic chemotherapy showed poor results in the treatment of small bowel NETs and is not recommended for well-differentiated NETs of midgut and hindgut[5] and, in well-differentiated duodenal NETs it is reserved for those patients with advanced metastatic disease[6]. However, in patients with poorly differentiated tumors, combination chemotherapy has been shown to induce disease remission with variable duration[39,40]. Consequently, for this group of small bowel NETs chemotherapy constitutes a treatment option[41,42]. Clinical trials using mTOR, tyrosine kinase and VEGF inhibitors in the treatment of NETs are currently being developed, showing promising results. One trial used patients with metastatic or unresectable NETs on stable doses of octreoctide and randomized them to association treatment with either VEGF inhibitor bevacizumab or pegylated (PEG) interferon α-2b for 18 wk. Bevacizumab therapy showed greater reduction of blood flow and longer progression free survival compared to PEG-Interferon therapy. However this trial had a limited number of patients and a larger phase 3 confirmatory study is underway[43]. A phase 3 study compared the association of octreotide long acting-repeatable (LAR) with Everolimus, a mTOR inhibitor, versus its association with placebo. Median progression-free survival was 16.4 mo in the everolimus plus octreotide LAR group and 11.3 mo in the placebo plus octreotide LAR group[17]. These findings point to the survival benefit of everolimus in the treatment of NETs and although it cannot be considered as standard treatment, it can be considered for patients without other treatment options[29].

For patients with metastatic or inoperable disease who have exhausted all other treatment options, peptide receptor radionuclide therapy should be considered if Octreoscan is positive. This treatment uses the somatostatin receptor present on the neoplasic lesion to deliver radio-labeled peptides directly to it. The majority of clinical centers use either ^{90}yttrium or ^{177}lutetium but a recent study tested the combination of both radioisotopes with an improved overall survival compared to single radioisotope treatment[44]. Still, further studies on this new treatment field need to be done.

CONCLUSION

NETs are an unusual neoplastic disease with several unanswered questions regarding its pathophysiology, diagnosis and treatment. Recent breakthroughs have redirected our approach in therapy clinical trials and may bring better diagnostic tools and even new prognostic markers. Still much work has to be done in order to fully understand this disease.

REFERENCES

1 **Modlin IM**, Lye KD, Kidd M. A 5-decade analysis of 13,715 carcinoid tumors. *Cancer* 2003; **97**: 934-959 [PMID: 12569593 DOI: 10.1002/cncr.11105]

2 **Yao JC**, Hassan M, Phan A, Dagohoy C, Leary C, Mares JE, Abdalla EK, Fleming JB, Vauthey JN, Rashid A, Evans DB. One hundred years after "carcinoid": epidemiology of and prognostic factors for neuroendocrine tumors in 35,825 cases in the United States. *J Clin Oncol* 2008; **26**: 3063-3072 [PMID: 18565894 DOI: 10.1200/JCO.2007.15.4377]

3 **Moertel CG**, Sauer WG, Dockerty MB, Baggenstoss AH. Life history of the carcinoid tumor of the small intestine. *Cancer* 1961; **14**: 901-912 [PMID: 13771655 DOI: 10.1002/1097-0142(196109/10)14:5<901::AID-CNCR2820140502>3.0.CO;2-Q]

4 **Klöppel G**, Perren A, Heitz PU. The gastroenteropancreatic neuroendocrine cell system and its tumors: the WHO classification. *Ann N Y Acad Sci* 2004; **1014**: 13-27 [PMID: 15153416 DOI: 10.1196/annals.1294.002]

5 **Eriksson B**, Klöppel G, Krenning E, Ahlman H, Plöckinger U, Wiedenmann B, Arnold R, Auernhammer C, Körner M, Rindi G, Wildi S. Consensus guidelines for the management of patients with digestive neuroendocrine tumors--well-differentiated jejunal-ileal tumor/carcinoma. *Neuroendocrinology* 2008; **87**: 8-19 [PMID: 18097129 DOI: 10.1159/000111034]

6 **Jensen RT**, Rindi G, Arnold R, Lopes JM, Brandi ML, Bechstein WO, Christ E, Taal BG, Knigge U, Ahlman H, Kwekkeboom DJ, O'Toole D. Well-differentiated duodenal tumor/carcinoma (excluding gastrinomas). *Neuroendocrinology* 2006; **84**: 165-172 [PMID:

17312376 DOI: 10.1159/000098008]
7 **Klimstra DS**, Modlin IR, Coppola D, Lloyd RV, Suster S. The pathologic classification of neuroendocrine tumors: a review of nomenclature, grading, and staging systems. *Pancreas* 2010; **39**: 707-712 [PMID: 20664470 DOI: 10.1097/MPA.0b013e3181ec124e]
8 **Hoffmann KM**, Furukawa M, Jensen RT. Duodenal neuroendocrine tumors: Classification, functional syndromes, diagnosis and medical treatment. *Best Pract Res Clin Gastroenterol* 2005; **19**: 675-697 [PMID: 16253893 DOI: 10.1016/j.bpg.2005.05.009]
9 **Kirshbom PM**, Kherani AR, Onaitis MW, Hata A, Kehoe TE, Feldman C, Feldman JM, Tyler DS. Foregut carcinoids: a clinical and biochemical analysis. *Surgery* 1999; **126**: 1105-1110 [PMID: 10598194 DOI: 10.1067/msy.2099.101430]
10 **Roy PK**, Venzon DJ, Shojamanesh H, Abou-Saif A, Peghini P, Doppman JL, Gibril F, Jensen RT. Zollinger-Ellison syndrome. Clinical presentation in 261 patients. *Medicine* (Baltimore) 2000; **79**: 379-411 [PMID: 11144036 DOI: 10.1097/00005792-200011000-00004]
11 **Kaltsas GA**, Besser GM, Grossman AB. The diagnosis and medical management of advanced neuroendocrine tumors. *Endocr Rev* 2004; **25**: 458-511 [PMID: 15180952 DOI: 10.1210/er.2003-0014]
12 **Norheim I**, Oberg K, Theodorsson-Norheim E, Lindgren PG, Lundqvist G, Magnusson A, Wide L, Wilander E. Malignant carcinoid tumors. An analysis of 103 patients with regard to tumor localization, hormone production, and survival. *Ann Surg* 1987; **206**: 115-125 [PMID: 2440390 DOI: 10.1097/00000658-198708000-00001]
13 **Soga J**. Endocrinocarcinomas (carcinoids and their variants) of the duodenum. An evaluation of 927 cases. *J Exp Clin Cancer Res* 2003; **22**: 349-363 [PMID: 14582691 DOI: 10.1016/j.oraloncology.2003.09.017]
14 **Burke AP**, Thomas RM, Elsayed AM, Sobin LH. Carcinoids of the jejunum and ileum: an immunohistochemical and clinicopathologic study of 167 cases. *Cancer* 1997; **79**: 1086-1093 [PMID: 9070484 DOI: 10.1002/(SICI)1097-0142(19970315)79: 6<1086: : AID-CNCR5>3.0.CO; 2-E]
15 **Strodel WE**, Talpos G, Eckhauser F, Thompson N. Surgical therapy for small-bowel carcinoid tumors. *Arch Surg* 1983; **118**: 391-397 [PMID: 6830429 DOI: 10.1001/archsurg.1983.01390040003001]
16 **Banck MS**, Kanwar R, Kulkarni AA, Boora GK, Metge F, Kipp BR, Zhang L, Thorland EC, Minn KT, Tentu R, Eckloff BW, Wieben ED, Wu Y, Cunningham JM, Nagorney DM, Gilbert JA, Ames MM, Beutler AS. The genomic landscape of small intestine neuroendocrine tumors. *J Clin Invest* 2013; **123**: 2502-2508 [PMID: 23676460 DOI: 10.1172/jci67963]
17 **Pavel ME**, Hainsworth JD, Baudin E, Peeters M, Hörsch D, Winkler RE, Klimovsky J, Lebwohl D, Jehl V, Wolin EM, Oberg K, Van Cutsem E, Yao JC. Everolimus plus octreotide long-acting repeatable for the treatment of advanced neuroendocrine tumours associated with carcinoid syndrome (RADIANT-2): a randomised, placebo-controlled, phase 3 study. *Lancet* 2011; **378**: 2005-2012 [PMID: 22119496 DOI: 10.1016/S0140-6736(11)61742-X]
18 **Gilbert JA**, Adhikari LJ, Lloyd RV, Rubin J, Haluska P, Carboni JM, Gottardis MM, Ames MM. Molecular markers for novel therapies in neuroendocrine (carcinoid) tumors. *Endocr Relat Cancer* 2010; **17**: 623-636 [PMID: 20385747 DOI: 10.1677/ERC-09-0318]
19 **Kidd M**, Modlin IM, Pfragner R, Eick GN, Champaneria MC, Chan AK, Camp RL, Mane SM. Small bowel carcinoid (enterochromaffin cell) neoplasia exhibits transforming growth factor-beta1-mediated regulatory abnormalities including up-regulation of C-Myc and MTA1. *Cancer* 2007; **109**: 2420-2431 [PMID: 17469181 DOI: 10.1002/cncr.22725]
20 **Kidd M**, Schimmack S, Lawrence B, Alaimo D, Modlin IM. EGFR/TGFα and TGFβ/CTGF Signaling in Neuroendocrine Neoplasia: Theoretical Therapeutic Targets. *Neuroendocrinology* 2013; **97**: 35-44 [PMID: 22710195 DOI: 10.1159/000334891]
21 **Francis JM**, Kiezun A, Ramos AH, Serra S, Pedamallu CS, Qian ZR, Banck MS, Kanwar R, Kulkarni AA, Karpathakis A, Manzo V, Contractor T, Philips J, Nickerson E, Pho N, Hooshmand SM, Brais LK, Lawrence MS, Pugh T, McKenna A, Sivachenko A, Cibulskis K, Carter SL, Ojesina AI, Freeman S, Jones RT, Voet D, Saksena G, Auclair D, Onofrio R, Shefler E, Sougnez C, Grimsby J, Green L, Lennon N, Meyer T, Caplin M, Chung DC, Beutler AS, Ogino S, Thirlwell C, Shivdasani R, Asa SL, Harris CR, Getz G, Kulke M, Meyerson M. Somatic mutation of CDKN1B in small intestine neuroendocrine tumors. *Nat Genet* 2013; **45**: 1483-1486 [PMID: 24185511 DOI: 10.1038/ng.2821]
22 **Kidd M**, Modlin IM, Drozdov I. Gene network-based analysis identifies two potential subtypes of small intestinal neuroendocrine tumors. *BMC Genomics* 2014; **15**: 595 [PMID: 25023465 DOI: 10.1186/1471-2164-15-595]
23 **Di Leva G**, Croce CM. miRNA profiling of cancer. *Curr Opin Genet Dev* 2013; **23**: 3-11 [PMID: 23465882 DOI: 10.1016/j.gde.2013.01.004]
24 **Sato-Kuwabara Y**, Melo SA, Soares FA, Calin GA. The fusion of two worlds: non-coding RNAs and extracellular vesicles--diagnostic and therapeutic implications (Review). *Int J Oncol* 2015; **46**: 17-27 [PMID: 25338714 DOI: 10.3892/ijo.2014.2712]
25 **Nelson PT**, Wang WX, Wilfred BR, Tang G. Technical variables in high-throughput miRNA expression profiling: much work remains to be done. *Biochim Biophys Acta* 2008; **1779**: 758-765 [PMID: 18439437 DOI: 10.1016/j.bbagrm.2008.03.012]
26 **Fassan M**, Baffa R. MicroRNAs and targeted therapy: small molecules of unlimited potentials. *Curr Opin Genet Dev* 2013; **23**: 75-77 [PMID: 23523049 DOI: 10.1016/j.gde.2013.02.009]
27 **Ruebel K**, Leontovich AA, Stilling GA, Zhang S, Righi A, Jin L, Lloyd RV. MicroRNA expression in ileal carcinoid tumors: downregulation of microRNA-133a with tumor progression. *Mod Pathol* 2010; **23**: 367-375 [PMID: 20037573 DOI: 10.1038/modpathol.2009.161]
28 **Li SC**, Essaghir A, Martijn C, Lloyd RV, Demoulin JB, Oberg K, Giandomenico V. Global microRNA profiling of well-differentiated small intestinal neuroendocrine tumors. *Mod Pathol* 2013; **26**: 685-696 [PMID: 23328977 DOI: 10.1038/modpathol.2012.216]
29 **Boudreaux JP**, Klimstra DS, Hassan MM, Woltering EA, Jensen RT, Goldsmith SJ, Nutting C, Bushnell DL, Caplin ME, Yao JC. The NANETS consensus guideline for the diagnosis and management of neuroendocrine tumors: well-differentiated neuroendocrine tumors of the Jejunum, Ileum, Appendix, and Cecum. *Pancreas* 2010; **39**: 753-766 [PMID: 20664473 DOI: 10.1097/MPA.0b013e3181ebb2a5]
30 **Stridsberg M**, Eriksson B, Oberg K, Janson ET. A comparison between three commercial kits for chromogranin A measurements. *J Endocrinol* 2003; **177**: 337-341 [PMID: 12740022 DOI: 10.1677/joe.0.1770337]
31 **Vinik AI**, Woltering EA, Warner RR, Caplin M, O'Dorisio TM, Wiseman GA, Coppola D, Go VL. NANETS consensus guidelines for the diagnosis of neuroendocrine tumor. *Pancreas* 2010; **39**: 713-734 [PMID: 20664471 DOI: 10.1097/MPA.0b013e3181ebaffd]
32 **Modlin IM**, Drozdov I, Kidd M. The identification of gut neuroendocrine tumor disease by multiple synchronous transcript analysis in blood. *PLoS One* 2013; **8**: e63364 [PMID: 23691035 DOI: 10.1371/journal.pone.0063364]
33 **Yoshikane H**, Tsukamoto Y, Niwa Y, Goto H, Hase S, Mizutani K, Nakamura T. Carcinoid tumors of the gastrointestinal tract: evaluation with endoscopic ultrasonography. *Gastrointest Endosc* 1993; **39**: 375-383 [PMID: 8514069 DOI: 10.1016/s0016-5107(93)70109-1]
34 **Rosa BJ**, Barbosa M, Magalhães J, Rebelo A, Moreira MJ, Cotter J. Oral purgative and simethicone before small bowel capsule endoscopy. *World J Gastrointest Endosc* 2013; **5**: 67-73 [PMID: 23424190 DOI: 10.4253/wjge.v5.i2.67]
35 **van Tuyl SA**, van Noorden JT, Timmer R, Stolk MF, Kuipers EJ, Taal BG. Detection of small-bowel neuroendocrine tumors by video capsule endoscopy. *Gastrointest Endosc* 2006; **64**: 66-72 [PMID: 16813805 DOI: 10.1016/j.gie.2006.01.054]
36 **Koopmans KP**, de Vries EG, Kema IP, Elsinga PH, Neels OC, Sluiter WJ, van der Horst-Schrivers AN, Jager PL. Staging of carcinoid tumours with 18F-DOPA PET: a prospective, diagnostic

accuracy study. *Lancet Oncol* 2006; **7**: 728-734 [PMID: 16945767 DOI: 10.1016/s1470-2045(06)70801-4]

37 **Orlefors H**, Sundin A, Garske U, Juhlin C, Oberg K, Skogseid B, Langstrom B, Bergstrom M, Eriksson B. Whole-body (11)C-5-hydroxytryptophan positron emission tomography as a universal imaging technique for neuroendocrine tumors: comparison with somatostatin receptor scintigraphy and computed tomography. *J Clin Endocrinol Metab* 2005; **90**: 3392-3400 [PMID: 15755858 DOI: 10.1210/jc.2004-1938]

38 **Öberg K**, Knigge U, Kwekkeboom D, Perren A. Neuroendocrine gastro-entero-pancreatic tumors: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. *Ann Oncol* 2012; **23** Suppl 7: vii124-vii130 [PMID: 22997445 DOI: 10.1093/annonc/mds295]

39 **Fjällskog ML**, Granberg DP, Welin SL, Eriksson C, Oberg KE, Janson ET, Eriksson BK. Treatment with cisplatin and etoposide in patients with neuroendocrine tumors. *Cancer* 2001; **92**: 1101-1107 [PMID: 11571721 DOI: 10.1002/1097-0142(20010901)92:53.0.CO;2-V]

40 **Mitry E**, Baudin E, Ducreux M, Sabourin JC, Rufié P, Aparicio T, Aparicio T, Lasser P, Elias D, Duvillard P, Schlumberger M, Rougier P. Treatment of poorly differentiated neuroendocrine tumours with etoposide and cisplatin. *Br J Cancer* 1999; **81**: 1351-1355 [PMID: 10604732 DOI: 10.1038/sj.bjc.6690325]

41 **Nilsson O**, Van Cutsem E, Delle Fave G, Yao JC, Pavel ME, McNicol AM, Sevilla Garcia MI, Knapp WH, Keleştimur F, Sauvanet A, Pauwels S, Kwekkeboom DJ, Caplin M. Poorly differentiated carcinomas of the foregut (gastric, duodenal and pancreatic). *Neuroendocrinology* 2006; **84**: 212-215 [PMID: 17312381 DOI: 10.1159/000098013]

42 **Ahlman H**, Nilsson O, McNicol AM, Ruszniewski P, Niederle B, Ricke J, Jensen R, Kos-Kudła B, Oberg K, O'Connor JM, Pavel ME, Vullierme MP. Poorly-differentiated endocrine carcinomas of midgut and hindgut origin. *Neuroendocrinology* 2008; **87**: 40-46 [PMID: 17940332 DOI: 10.1159/000109976]

43 **Yao JC**, Phan A, Hoff PM, Chen HX, Charnsangavej C, Yeung SC, Hess K, Ng C, Abbruzzese JL, Ajani JA. Targeting vascular endothelial growth factor in advanced carcinoid tumor: a random assignment phase II study of depot octreotide with bevacizumab and pegylated interferon alpha-2b. *J Clin Oncol* 2008; **26**: 1316-1323 [PMID: 18323556 DOI: 10.1200/JCO.2007.13.6374]

44 **Villard L**, Romer A, Marincek N, Brunner P, Koller MT, Schindler C, Ng QK, Mäcke HR, Müller-Brand J, Rochlitz C, Briel M, Walter MA. Cohort study of somatostatin-based radiopeptide therapy with [(90)Y-DOTA]-TOC versus [(90)Y-DOTA]-TOC plus [(177)Lu-DOTA]-TOC in neuroendocrine cancers. *J Clin Oncol* 2012; **30**: 1100-1106 [PMID: 22393097 DOI: 10.1200/JCO.2011.37.2151]

P- Reviewer: Saniabadi AR, Song HJ, Wlodarczyk M
S- Editor: Kong JX **L- Editor**: A **E- Editor**: Jiao XK

World Journal of Gastrointestinal Pathophysiology

Submit a Manuscript: http://www.wjgnet.com/esps/
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.4291/wjgp.v7.i1.125

World J Gastrointest Pathophysiol 2016 February 15; 7(1): 125-130
ISSN 2150-5330 (online)

MINIREVIEWS

Mesenteric ischemia: Pathogenesis and challenging diagnostic and therapeutic modalities

Aikaterini Mastoraki, Sotiria Mastoraki, Evgenia Tziava, Stavroula Touloumi, Nikolaos Krinos, Nikolaos Danias, Andreas Lazaris, Nikolaos Arkadopoulos

Aikaterini Mastoraki, Evgenia Tziava, Stavroula Touloumi, Nikolaos Krinos, Nikolaos Danias, Nikolaos Arkadopoulos, 4th Department of Surgery, Athens University, Medical School, ATTIKON University Hospital, 12462 Chaidari, Athens, Greece

Sotiria Mastoraki, Andreas Lazaris, Department of Vascular Surgery, Athens University, Medical School, ATTIKON University Hospital, 12462 Chaidari, Athens, Greece

Author contributions: All authors contributed significantly in all stages of this work, they have read and approved the manuscript and confirm that no conflict of interest exists.

Conflict-of-interest statement: None.

Correspondence to: Aikaterini Mastoraki, MD, PhD, 4th Department of Surgery, Athens University, Medical School, ATTIKON University Hospital, 1 Rimini Str, 12462 Chaidari, Athens, Greece. dr_kamast@yahoo.gr
Telephone: +30-6932-577710
Fax: +30-210-5326412

Received: June 24, 2015
Peer-review started: June 25, 2015
First decision: August 26, 2015
Revised: October 22, 2015
Accepted: January 8, 2016
Article in press: January 11, 2016
Published online: February 15, 2016

Abstract

Mesenteric ischemia (MI) is an uncommon medical condition with high mortality rates. MI includes inadequate blood supply, inflammatory injury and eventually necrosis of the bowel wall. The disease can be divided into acute and chronic MI (CMI), with the first being subdivided into four categories. Therefore, acute MI (AMI) can occur as a result of arterial embolism, arterial thrombosis, mesenteric venous thrombosis and non-occlusive causes. Bowel damage is in proportion to the mesenteric blood flow decrease and may vary from minimum lesions, due to reversible ischemia, to transmural injury, with subsequent necrosis and perforation. CMI is associated to diffuse atherosclerotic disease in more than 95% of cases, with all major mesenteric arteries presenting stenosis or occlusion. Because of a lack of specific signs or due to its sometime quiet presentation, this condition is frequently diagnosed only at an advanced stage. Computed tomography (CT) imaging and CT angiography contribute to differential diagnosis and management of AMI. Angiography is also the criterion standard for CMI, with mesenteric duplex ultrasonography and magnetic resonance angiography also being of great importance. Therapeutic approach of MI includes both medical and surgical treatment. Surgical procedures include restoration of the blood flow with arteriotomy, endarterectomy or anterograde bypass, while resection of necrotic bowel is always implemented. The aim of this review was to evaluate the results of surgical treatment for MI and to present the recent literature in order to provide an update on the current concepts of surgical management of the disease. Mesh words selected include MI, diagnostic approach and therapeutic management.

Key words: Acute mesenteric ischemia; Mesenteric ischemia; Chronic diagnostic approach; Therapeutic management; Surgical strategy

Core tip: Mesenteric ischemia (MI) is an uncommon medical condition with high mortality rates. MI includes inadequate blood supply, inflammatory injury and eventually necrosis of the bowel wall. Because of a lack of specific signs or due to its sometime quiet presentation, this condition is frequently diagnosed only at an advanced stage. Therapeutic approach refers to both medical and surgical treatment. Surgical procedures include restoration of the blood flow with arteriotomy, endarterectomy or anterograde bypass, while resection of necrotic bowel is always implemented.

Mastoraki A, Mastoraki S, Tziava E, Touloumi S, Krinos N, Danias N, Lazaris A, Arkadopoulos N. Mesenteric ischemia: Pathogenesis and challenging diagnostic and therapeutic modalities. *World J Gastrointest Pathophysiol* 2016; 7(1): 125-130 Available from: URL: http://www.wjgnet.com/2150-5330/full/v7/i1/125.htm DOI: http://dx.doi.org/10.4291/wjgp.v7.i1.125

INTRODUCTION

Mesenteric ischemia (MI) is an uncommon medical condition that accounts 0.1% of all hospital admissions, with high mortality rates ranging from 24%-94%[1]. MI includes inadequate blood supply, inflammatory injury and eventually necrosis of the bowel wall. The disease can be divided into acute and chronic MI (CMI), with the first being subdivided into four categories[2]. Therefore, acute MI (AMI) can occur as a result of arterial embolism, arterial thrombosis, mesenteric venous thrombosis (MVT) and non-occlusive causes (NOMI), such as hypo-perfusion due to low cardiac output or mesenteric arterial vasoconstriction[3]. Bowel damage is in proportion to the mesenteric blood flow decrease and may vary from minimum lesions, due to reversible ischemia, to transmural injury, with subsequent necrosis and perforation[4]. CMI is associated to diffuse atherosclerotic disease in more than 95% of cases, with all major mesenteric arteries presenting stenosis or occlusion. Other causes include fibromuscular dysplasia, vasculitis, Takayasu arteritis, malignancy and radiation. Because of a lack of specific signs or due to its sometime quiet presentation, this condition is frequently diagnosed only at an advanced stage[5]. Constant, diffuse, non-localized or periumbilical abdominal pain remains the most common symptom[6-8]. Postprandial pain, nausea and weight loss occur in patients with CMI[4]. While laboratory studies or plain films of abdomen are not indicative, computed tomography (CT) imaging and CT angiography contribute to differential diagnosis and management of AMI. Angiography is also the criterion standard for CMI, with mesenteric duplex ultrasonography (US) and magnetic resonance angiography (MRA) also being of great importance[9-11]. Therapeutic approach of MI includes both medical and surgical treatment. Papaverine, heparin, warfarin and thrombolytic drugs are the most common medications[12]. Surgical procedures include restoration of the blood flow with arteriotomy, endarterectomy or anterograde bypass, while resection of necrotic bowel is always implemented[13]. The aim of this review was to evaluate the results of surgical treatment for MI and to present the recent literature in order to provide an update on the current concepts of surgical management of the disease. Mesh words selected include MI, diagnostic approach and therapeutic management.

HISTOLOGY AND PATHOGENESIS

In the process of embryogenesis, segmental arteries regress until three major vessels remain: The celiac artery (CA), the superior mesenteric artery (SMA) and the inferior mesenteric artery (IMA) as well. Typically, the CA supplies the foregut, hepatobiliary system and spleen, the SMA supports the midgut including small intestine and proximal mid colon up to the splenic flexure and the IMA refers to the hind gut with regard to distal colon beginning from the splenic flexure, and rectum[14]. The venous system, mainly, parallels the arterial branches. Therefore, the superior mesenteric vein drains the small intestine and cecum, while the inferior mesenteric vein includes the descending and sigmoid colon and the rectum[15]. The gastrointestinal (GI) system presents significant collateral circulation at all levels that provides protection from ischemia and can compensate for approximately a 75% acute reduction in mesenteric blood flow for up to 12 h, without substantial injury[16].

AMI caused by arterial embolism accounts for 50% of acute ischemic conditions. The SMA is the visceral vessel the most susceptible to emboli due to its small take-off angle from the aorta and higher flow. Most often, emboli lodge about 6-8 cm beyond the arterial origin, distal to the origin of the middle colic artery[6,17]. Typical causes of emboli include mural thrombi after myocardial infarction, atrial thrombi associated with mitral stenosis, atrial fibrillation, septic emboli from valvular endocarditis, mycotic aneurysm and thrombi formed at the site of atheromatous plaques within the aorta or at the sites of vascular aortic prosthetic grafts interposed anywhere between the heart and the origin of SMA[8,17].

The thrombosis of mesenteric arteries typically occurs at their origin causing extensive infarction and often affects at least two of the major visceral vessels. It is mainly a complication of preexisting visceral atherosclerotic lesions and involves acute worsening of the already compromised blood flow. It may also be attributed to arterial aneurysm or other vascular pathologies, such as dissection, trauma, mesenteric aneurysm rupture, fibromuscular dysplasia or vasculitis[18,19]. In inflammatory vascular disease, smaller vessels are commonly affected. On the other hand, NOMI results from hypoperfusion with secondary severe and prolonged visceral vasoconstriction. The most common initiating conditions involve systemic shock

due to decreased cardiac output following a myocardial infarction or congestive heart failure, septic shock or hypovolemia. Additionally, NOMI can be the result of compression by intra-abdominal tumors[6]. A secondary clinical entity of MI occurs due to mechanical obstruction, such as internal hernia with strangulation and volvulus.

Finally, MVT includes primary disorder with no identifiable predisposing factor and secondary MVT as well. The most common cause of secondary MVT is hypercoagulability. Additionally, in more than 95% of patients, diffuse atherosclerotic disease decreases the blood flow to the bowel and causes CMI. Other causes refer to fibromuscular dysplasia, vasculitis including Takayasu arteritis, giant cell arteritis, polyarteritis nodosa, systemic lupus erythematosus, thromboangiitis obliterans, malignancy and radiation. Subsequent complications conclude to thrombotic and embolic phenomena leading to manifestations of AMI[15,20,21].

Current pathophysiologic understanding of visceral perfusion suggests a pivotal role for the visceral circulation in the cardiovascular homeostasis regulation. GI perfusion is usually impaired early in situations including critical illness, major surgery and exercise, all of which are characterized by increased demands on the circulation to maintain tissue oxygen delivery[16,18,22,23]. This relative hypoperfusion often outlasts the period of the hypovolemic insult or low-flow state. Tissue damage results either from ischemic or reperfusion injury. Within 3-4 h after the onset of ischemia, there is necrosis of the mucosal villi and within 6 h, transmural, mural or mucosal infarction can be observed. The splenic flexure is more susceptible to ischemic injury as it is the separation point of distribution between the SMA and the IMA. In the early stages, the intestinal wall appears congested and later it becomes edematous, friable and hemorrhagic. Without treatment, patients present with bowel hemorrhage within 1-4 d while enteric bacteria can cause gangrene, which leads to perforation and sometimes severe sepsis[24,25].

CLINICAL PRESENTATION

With regard to clinical presentation, acute and chronic MI differs significantly dependent on the underlying etiology[26]. AMI's most typical symptom is abdominal pain that is disproportionate to physical examination findings. As ischemia is the pathologic process, the pain initially is visceral, diffused, non-localized and it may be moderate to severe, constant, sometimes colicky and occasionally unresponsive to opioid analgesics. Other common signs are nausea and vomiting while diarrhea progressing to constipation may be also present. Examination findings early in the course of the disease are limited and non-specific, including minimal abdominal tenderness. If ischemia is attributed to embolic disease, the pain is severe and abrupt, as occlusion is rapidly installed and collateral circulation is completely absent[13,22]. On the other hand, AMI due to arterial thrombosis has a significantly more gradual progression of ischemia and infarction and less severe presentation, because the artery is usually already partially blocked and a collateral supply has been established[18]. Once the ischemia has progressed transmurally, signs of peritonitis and septicemia are encountered[27]. Bowel necrosis, septic shock and death are common complications in AMI. On the contrary, CMI typically causes postprandial abdominal pain, generally epigastric or periumbilical, nausea and weight loss. Examination findings include signs of malnutrition, pain disproportionate to clinical features and abdominal bruit[5,28].

DIAGNOSTIC MODALITIES

Due to the unclear manifestation of AMI, this condition is often misdiagnosed causing serious morbidity and high mortality. Laboratory studies are non-specific. Initially, complete blood cell count may be normal but leukocytosis and/or leftward shift may be observed later in the clinical course. The levels of amylase and lactate dehydrogenase may be increased[29]. Metabolic acidosis is a common but nonspecific disorder. In addition, prothrombin time and activated partial thromboplastine time should be evaluated and especially when MVT occurs, patients should be examined for protein C and S and antithrombin Ⅲ deficiencies, abnormalities in lupus anticoagulant, anticardiolipin antibody and platelet aggregation[16,17,30].

In the absence of physical signs of peritonitis imaging studies are implemented. The majority of patients firstly undergo CT scanning in order to exclude other causes with similar clinical features and it presents a sensitivity of 71%-96% and a specificity of 92%-94% for AMI. CT findings include intramural (pneumatosis intestinalis) as well as portal vein gas, focal edematous bowel wall, mesenteric edema, abnormal gas patterns, streaking of mesentery and solid organ infarction. Arterial occlusion may present lack of enhancement of the vessels, but unlike embolic infarction, thrombosis of the SMA occurs more commonly in the origin of the vessel[31,32]. In MVT, CT scans may demonstrate an enlarged mesenteric or portal vein with sharp definition of the venous wall and low density within the vessel. Magnetic resonance imaging (MRI) along with MRA yields similar findings to those of CT scanning in AMI[15,21].

Angiography has been traditionally the most reliable method to assess the presence and the extent of occlusive disease. Anteroposterior views demonstrate collateral pathways and lateral projections depict the origins of visceral branches. Patients with thrombosis demonstrate complete lack of the visualization of the SMA and its branches, whereas those with embolism of the SMA present filling of the proximal SMA only with a sharp cutoff of the artery. Angiography is now superseded by CT angiography, as it is noninvasive and easily available[33]. However, if CT scanning is inconclusive and there is strong clinical suspicion of AMI, angiography is performed to verify the diagnosis. In addition, as

CT findings of NOMI are nonspecific, angiography reveals diffuse stenosis of the mesenteric vessels in the absence of occlusive lesions and reduced delineation of intestinal parenchyma[34]. Duplex US is highly specific (92%-100%) but not as sensitive as angiography (70%-89%) as the examination cannot detect clots beyond the proximal main vessels.

In CMI laboratory evaluation may confirm anemia, leucopenia or lymphopenia secondary to chronic malnourishment, as well as, hypoalbuminemia and electrolyte abnormalities[28]. As regards imaging studies, arteriography remains the criterion standard. Occlusion of two visceral branches of the aorta with severe stenosis of the remaining visceral branch, usually the celiac or SMA is observed. Multi-sliced CT is a noninvasive technique, which can play a pivotal role in diagnosing vascular disease of the celiac trunk and SMA in chronic ischemia. In addition, MRI and MRA are very promising diagnostic modalities when combined with fast contrast-enhanced (CE) techniques[5,35]. While AMI is an emergency where CT scanning is the most appropriate diagnostic tool, CMI is best examined with CE-MRA, coupled with measurements of flow. With this functional approach, MRA is the only modality that can completely assess abdominal vascular disease. Mesenteric duplex (US) is helpful to assess vascular patency, following visceral bypass grafting or endovascular stenting when combined with angiography to estimate potential existence of severe stenosis. However, the sensitivity is limited owing to intraperitoneal gas, obesity and previous abdominal surgical interventions[36,37].

THERAPEUTIC APPROACH

AMI high mortality rates indicate the importance of urgent medical treatment. Maintenance of hemodynamic stability and adequate oxygen saturation as well as correction of any electrolyte imbalance is of outmost importance. Vasopressors should be avoided. Blood products can be provided and 2-4 units of red blood cells should be available. Administration of broad spectrum antibiotics starts early to prevent and treat sepsis and should cover the colonic flora**.** Nasogastric tube decompression, correction of any acid/base abnormalities, bladder catheterization and intravenous fluid administration are always implemented preoperatively. Pain control is mainly accomplished with opioids[8,12,30].

Different drug treatment protocols have been suggested according to the subtypes of AMI. Targeted infusion of papaverine through angiography catheter at the affected vessels relieves the reactive vasospasm and therefore is proposed for all arterial forms of AMI as well as NOMI. Subsequent continuous administration of 30-60 mg/h papaverine after angiography with appropriate dose adjustment in accordance with clinical response for at least 24 h is warranted. Caution should be taken though as significant hypotension may occur if the catheter invades aorta. Relevant therapeutic intervention remains the treatment modality of choice for NOMI[12,17,30]. Finally, VEGF has been recently thoroughly proposed as a future potential therapeutic approach to MI[37].

In case of embolic AMI, infusion of thrombolytics within 8 h of symptoms onset is recommended for selected patients. As the main complication is GI bleeding, absolute contraindications for intense thrombolysis are the presence of peritonitis signs or bowel necrosis. Recent investigations suggest the tenecteplase or reteplase infusion angiographically to lyse thrombi instead of alteplase, as they appear to cause less non-intracranial bleeding[38-40]. Moreover, Ⅳ administration of anticoagulants to prevent further extension of thrombus in MVT or post-revascularization in arterial occlusive AMI has been advocated[41]. Heparin inhibits further thrombogenesis and prevents additional clot accumulation with particular caution to the possibility of GI bleeding. Conversion to oral warfarin with suitable dose adjustment is always indicated and should be continued for at least 6 mo[21,42].

Recent knowledge has generated a multidisciplinary surgical management of AMI. Initial aim is to restore intestinal oxygenation and to minimize or prevent severe complications such as peritonitis and gangrenous bowel perforation. Thus, surgical treatment of AMI with signs of peritonitis mainly includes exploratory laparotomy with meticulous assessment of bowel viability[6,30]. Resection of infarcted intestine is strongly indicated. In addition, intraoperative Doppler US and Ⅳ infusion of fluorescein and bowel examination under Wood lamp illumination can differentiate poorly perfused bowel. Resection of necrotic bowel plays a pivotal role for patient resuscitation while an attempt for anastomosis remains controversial. A second look operation is strongly suggested along with clinical examination and diagnostic imaging. Intense screening of intestinal revascularization is always implemented[26,43].

More specific, in case of embolic AMI an attempt of reperfusion remains of vital importance[6]. Surgical team can determine the location of the blockage by palpation and proceed to transverse arteriotomy proximal to the occlusion, using a balloon-tipped Fogarty catheter in order to extract the clot. The embolectomy can be performed most expeditiously by exposing the SMA at the base of the transverse mesocolon. The arteriotomy can be sealed primarily or vein-patched. Alternative approach refers to bypass technique with venous or arterial graft[17,30,44]. In case of thrombotic occlusion with absence of gangrenous bowel, revascularization is attempted either with antegrade or retrograde aorto-mesenteric bypass or with trans-aortic endarterectomy. In case of spontaneous dissection of the SMA before the onset of intestinal infarction successful percutaneous stent placement has been reported[39,45,46].

Significant incidence of AMI has also been referred after aortic aneurysm repair. Prevalence of clinically evident bowel ischemia after endovascular abdominal aortic reconstruction is comparable to open surgical

approach. However, small bowel ischemia higher prevalence in patients with endovascular repair has been detected and is associated with extremely high mortality. The direct pathologic evidence and the patterns of segmental, skipped, or patchy ischemia in most patients imply that micro-embolization plays a pivotal role[47,48].

CMI's management is mostly surgical. Due to the high rates of thrombosis, medical management as a sole treatment is warranted only in patients whose surgical risk outweighs potential benefits. Additionally, conservative treatment, such as bowel resting, smoking cessation and administration of vasodilator drugs, will not ameliorate disease progression[49]. Medicines used in CMI includes heparin and warfarin to prevent an acute thrombotic/embolic event, intra-arterial papaverine as a vasodilator prior to surgery to reduce risk of arterial spasm and nitrate therapy to provide short-term relief. Parenteral nutrition is warranted because of the long period of malnutrition[5,28].

Open revascularization (OR) and endovascular revascularization (ER) are the alternative modalities of treatment in patients with CMI[50-52]. ER appears to have lower postoperative mortality and morbidity rates and shorter intensive care unit and hospital stay. Therefore, ER has been proposed for high risk surgical candidates or those with short life expectancy due to its minimally invasive nature[35,52,53]. In ER, a short stump of the patent artery is necessary to gain wire access. Excessive endovascular manipulation can result in arterial dissection, perforation, or embolization. Moreover, severely calcified or long lesions and small-diameter mesenteric arteries are also associated with an increased risk of distal embolization and restenosis. Nevertheless, OR presents early as well as long term symptomatic relief and significantly lower restenosis rates compared to ER. Thus, the majority of the patients should be treated with conventional reconstruction especially if ER has previously attempted unsuccessfully. In conclusion, OR is still predominantly proposed although ER had recently gained popularity as an alternative therapeutic approach[37,52,54].

Surgical management includes transaortic endarterectomy of the celiac or SMA, antegrade bypass from the supraceliac aorta and retrograde bypass from the infrarenal aorta or the common iliac artery. Bypasses may be achieved using vein or prosthetic material. Primary stenting is favored in highly calcified, thrombotic, occlusive, or dissected lesions. Little is known about the impact of stent placement in terms of distal embolization. An embolic protection device may be considered in the presence of a large thrombus burden. However, there is limited evidence showing its efficacy during ER for CMI[37,50,51].

REFERENCES

1 **Roussel A**, Castier Y, Nuzzo A, Pellenc Q, Sibert A, Panis Y, Bouhnik Y, Corcos O. Revascularization of acute mesenteric ischemia after creation of a dedicated multidisciplinary center. *J Vasc Surg* 2015; **62**: 1251-1256 [PMID: 26243208 DOI: 10.1016/j.jvs.2015.06.204]

2 **Corcos O**, Nuzzo A. Gastro-intestinal vascular emergencies. *Best Pract Res Clin Gastroenterol* 2013; **27**: 709-725 [PMID: 24160929 DOI: 10.1016/j.bpg.2013.08.006]

3 **Bobadilla JL**. Mesenteric ischemia. *Surg Clin North Am* 2013; **93**: 925-940, ix [PMID: 23885938 DOI: 10.1016/j.suc.2013.04.002]

4 **van den Heijkant TC**, Aerts BA, Teijink JA, Buurman WA, Luyer MD. Challenges in diagnosing mesenteric ischemia. *World J Gastroenterol* 2013; **19**: 1338-1341 [PMID: 23538325 DOI: 10.3748/wjg.v19.i9.1338]

5 **Pecoraro F**, Rancic Z, Lachat M, Mayer D, Amann-Vesti B, Pfammatter T, Bajardi G, Veith FJ. Chronic mesenteric ischemia: critical review and guidelines for management. *Ann Vasc Surg* 2013; **27**: 113-122 [PMID: 23088809 DOI: 10.1016/j.avsg.2012.05.012]

6 **Vokurka J**, Olejnik J, Jedlicka V, Vesely M, Ciernik J, Paseka T. Acute mesenteric ischemia. *Hepatogastroenterology* 2008; **55**: 1349-1352 [PMID: 18795686]

7 **Tadros M**, Majumder S, Birk JW. A review of ischemic colitis: is our clinical recognition and management adequate? *Expert Rev Gastroenterol Hepatol* 2013; **7**: 605-613 [PMID: 24070152 DOI: 10.1586/17474124.2013]

8 **Safioleas MC**, Moulakakis KG, Papavassiliou VG, Kontzoglou K, Kostakis A. Acute mesenteric ischaemia, a highly lethal disease with a devastating outcome. *Vasa* 2006; **35**: 106-111 [PMID: 16796010]

9 **Palma Baro A**, Caldevilla Bernardo D, Parrondo Muiños C. [Mesenteric ischemia: update of new diagnostic techniques for an old disease, and review of radiological signs]. *Semergen* 2013; **39**: 279-281 [PMID: 23834979 DOI: 10.1016/j.semerg.2012.03.004]

10 **Seidel SA**, Bradshaw LA, Ladipo JK, Wikswo JP, Richards WO. Noninvasive detection of ischemic bowel. *J Vasc Surg* 1999; **30**: 309-319 [PMID: 10436451]

11 **Zwolak RM**. Can duplex ultrasound replace arteriography in screening for mesenteric ischemia? *Semin Vasc Surg* 1999; **12**: 252-260 [PMID: 10651454]

12 **Kozuch PL**, Brandt LJ. Review article: diagnosis and management of mesenteric ischaemia with an emphasis on pharmacotherapy. *Aliment Pharmacol Ther* 2005; **21**: 201-215 [PMID: 15691294]

13 **Bingol H**, Zeybek N, Cingöz F, Yilmaz AT, Tatar H, Sen D. Surgical therapy for acute superior mesenteric artery embolism. *Am J Surg* 2004; **188**: 68-70 [PMID: 15219487]

14 **Rosenblum JD**, Boyle CM, Schwartz LB. The mesenteric circulation. Anatomy and physiology. *Surg Clin North Am* 1997; **77**: 289-306 [PMID: 9146713]

15 **Kumar S**, Sarr MG, Kamath PS. Mesenteric venous thrombosis. *N Engl J Med* 2001; **345**: 1683-1688 [PMID: 11759648]

16 **Ha C**, Magowan S, Accortt NA, Chen J, Stone CD. Risk of arterial thrombotic events in inflammatory bowel disease. *Am J Gastroenterol* 2009; **104**: 1445-1451 [PMID: 19491858 DOI: 10.1038/ajg.2009.81]

17 **Herbert GS**, Steele SR. Acute and chronic mesenteric ischemia. *Surg Clin North Am* 2007; **87**: 1115-1134, ix [PMID: 17936478]

18 **Schoots IG**, Koffeman GI, Legemate DA, Levi M, van Gulik TM. Systematic review of survival after acute mesenteric ischaemia according to disease aetiology. *Br J Surg* 2004; **91**: 17-27 [PMID: 14716789]

19 **Dewitte A**, Biais M, Coquin J, Fleureau C, Cassinotto C, Ouattara A, Janvier G. [Diagnosis and management of acute mesenteric ischemia]. *Ann Fr Anesth Reanim* 2011; **30**: 410-420 [PMID: 21481561 DOI: 10.1016/j.annfar.2011.02.013]

20 **Abu-Daff S**, Abu-Daff N, Al-Shahed M. Mesenteric venous thrombosis and factors associated with mortality: a statistical analysis with five-year follow-up. *J Gastrointest Surg* 2009; **13**: 1245-1250 [PMID: 19296183 DOI: 10.1007/s11605-009-0833-7]

21 **Alvi AR**, Khan S, Niazi SK, Ghulam M, Bibi S. Acute mesenteric venous thrombosis: improved outcome with early diagnosis and prompt anticoagulation therapy. *Int J Surg* 2009; **7**: 210-213 [PMID: 19332155 DOI: 10.1016/j.ijsu.2009.03.002]

22 **Schoots IG**, Levi MM, Reekers JA, Lameris JS, van Gulik TM. Thrombolytic therapy for acute superior mesenteric artery occlusion. *J Vasc Interv Radiol* 2005; **16**: 317-329 [PMID: 15758127]

23 **Sanchez LD**, Tracy JA, Berkoff D, Pedrosa I. Ischemic colitis in marathon runners: a case-based review. *J Emerg Med* 2006; **30**: 321-326 [PMID: 16677987]
24 **Paterno F**, Longo WE. The etiology and pathogenesis of vascular disorders of the intestine. *Radiol Clin North Am* 2008; **46**: 877-885, v [PMID: 19103137 DOI: 10.1016/j.rcl.2008.06.005]
25 **Acosta S**. Surgical management of peritonitis secondary to acute superior mesenteric artery occlusion. *World J Gastroenterol* 2014; **20**: 9936-9941 [PMID: 25110423 DOI: 10.3748/wjg.v20.i29.9936]
26 **Chang JB**, Stein TA. Mesenteric ischemia: acute and chronic. *Ann Vasc Surg* 2003; **17**: 323-328 [PMID: 12704549]
27 **Zachariah SK**. Adult necrotizing enterocolitis and non occlusive mesenteric ischemia. *J Emerg Trauma Shock* 2011; **4**: 430-432 [PMID: 21887043 DOI: 10.4103/0974-2700.83881]
28 **Char D**, Hines G. Chronic mesenteric ischemia: diagnosis and treatment. *Heart Dis* 2001; **3**: 231-235 [PMID: 11975799]
29 **Lange H**, Jäckel R. Usefulness of plasma lactate concentration in the diagnosis of acute abdominal disease. *Eur J Surg* 1994; **160**: 381-384 [PMID: 7948358]
30 **Park WM**, Gloviczki P, Cherry KJ, Hallett JW, Bower TC, Panneton JM, Schleck C, Ilstrup D, Harmsen WS, Noel AA. Contemporary management of acute mesenteric ischemia: Factors associated with survival. *J Vasc Surg* 2002; **35**: 445-452 [PMID: 11877691]
31 **Horton KM**, Fishman EK. Multi-detector row CT of mesenteric ischemia: can it be done? *Radiographics* 2001; **21**: 1463-1473 [PMID: 11706217]
32 **Cikrit DF**, Harris VJ, Hemmer CG, Kopecky KK, Dalsing MC, Hyre CE, Fischer JM, Lalka SG, Sawchuk AP. Comparison of spiral CT scan and arteriography for evaluation of renal and visceral arteries. *Ann Vasc Surg* 1996; **10**: 109-116 [PMID: 8733861]
33 **Barmase M**, Kang M, Wig J, Kochhar R, Gupta R, Khandelwal N. Role of multidetector CT angiography in the evaluation of suspected mesenteric ischemia. *Eur J Radiol* 2011; **80**: e582-e587 [PMID: 21993179 DOI: 10.1016/j.ejrad.2011.09.015]
34 **Aschoff AJ**, Stuber G, Becker BW, Hoffmann MH, Schmitz BL, Schelzig H, Jaeckle T. Evaluation of acute mesenteric ischemia: accuracy of biphasic mesenteric multi-detector CT angiography. *Abdom Imaging* 2009; **34**: 345-357 [PMID: 18425546 DOI: 10.1007/s00261-008-9392-8]
35 **Loffroy R**, Steinmetz E, Guiu B, Molin V, Kretz B, Gagnaire A, Bouchot O, Cercueil JP, Brenot R, Krausé D. Role for endovascular therapy in chronic mesenteric ischemia. *Can J Gastroenterol* 2009; **23**: 365-373 [PMID: 19440568]
36 **Luther B**, Meyer F, Nowak T, Kempf U, Krasniqi H. [Chronically progressive occlusive disease of intestinal arteries - short overview from a vascular surgical perspective]. *Zentralbl Chir* 2011; **136**: 229-236 [PMID: 21462103]
37 **Crafts TD**, Jensen AR, Blocher-Smith EC, Markel TA. Vascular endothelial growth factor: therapeutic possibilities and challenges for the treatment of ischemia. *Cytokine* 2015; **71**: 385-393 [PMID: 25240960 DOI: 10.1016/j.cyto.2014.08.005]
38 **Wang MQ**, Liu FY, Duan F, Wang ZJ, Song P, Fan QS. Acute symptomatic mesenteric venous thrombosis: treatment by catheter-directed thrombolysis with transjugular intrahepatic route. *Abdom Imaging* 2011; **36**: 390-398 [PMID: 20652243 DOI: 10.1007/s00261-010-9637-1]
39 **Sivamurthy N**, Rhodes JM, Lee D, Waldman DL, Green RM, Davies MG. Endovascular versus open mesenteric revascularization: immediate benefits do not equate with short-term functional outcomes. *J Am Coll Surg* 2006; **202**: 859-867 [PMID: 16735198]
40 **Yanar F**, Agcaoglu O, Sarici IS, Sivrikoz E, Ucar A, Yanar H, Aksoy M, Kurtoglu M. Local thrombolytic therapy in acute mesenteric ischemia. *World J Emerg Surg* 2013; **8**: 8 [PMID: 23394456 DOI: 10.1186/1749-7922-8-8]
41 **Wichman HJ**, Cwikiel W, Keussen I. Interventional treatment of mesenteric venous occlusion. *Pol J Radiol* 2014; **79**: 233-238 [PMID: 25089163 DOI: 10.12659/PJR.890990]
42 **Yanar F**, Ağcaoğlu O, Gök AF, Sarıcı IS, Ozçınar B, Aksakal N, Aksoy M, Ozkurt E, Kurtoğlu M. The management of mesenteric vein thrombosis: a single institution's experience. *Ulus Travma Acil Cerrahi Derg* 2013; **19**: 223-228 [PMID: 23720109 DOI: 10.5505/tjtes.2013.47542]
43 **Wain RA**, Hines G. Surgical management of mesenteric occlusive disease: a contemporary review of invasive and minimally invasive techniques. *Cardiol Rev* 2008; **16**: 69-75 [PMID: 18281908 DOI: 10.1097/CRD.0b013e31815f98a4]
44 **Kuhelj D**, Kavcic P, Popovic P. Percutaneous mechanical thrombectomy of superior mesenteric artery embolism. *Radiol Oncol* 2013; **47**: 239-243 [PMID: 24133388 DOI: 10.2478/raon-2013-0029]
45 **Jun KW**, Kim MH, Park KM, Chun HJ, Hong KC, Jeon YS, Cho SG, Kim JY. Mechanical thrombectomy-assisted thrombolysis for acute symptomatic portal and superior mesenteric venous thrombosis. *Ann Surg Treat Res* 2014; **86**: 334-341 [PMID: 24949327 DOI: 10.4174/astr.2014.86.6.334]
46 **Luther B**, Meyer F, Mamopoulos A, Zapenko A, Doerbecker R, Wullstein C, Kroeger K, Katoh M. [Options and Limitations in Endovascular Therapy for Acute and Chronic Mesenteric Arterial Occlusions]. *Zentralbl Chir* 2015; **140**: 486-492 [PMID: 25401371 DOI: 10.1055/s-0034-1383234]
47 **Hechtman JF**, Lento PA, Scordi-bello I. Ischemic bowel due to embolization from an isolated mobile thrombus of the ascending aorta: a case report and review of the literature. *J Thromb Thrombolysis* 2011; **32**: 238-241 [PMID: 21416131 DOI: 10.1007/s11239-011-0581-x]
48 **Shimamoto T**, Komiya T. Clinical dilemma in the surgical treatment of organ malperfusion caused by acute type A aortic dissection. *Gen Thorac Cardiovasc Surg* 2014; **62**: 398-406 [PMID: 24771569 DOI: 10.1007/s11748-014-0406-x]
49 **Keese M**, Schmitz-Rixen T, Schmandra T. Chronic mesenteric ischemia: time to remember open revascularization. *World J Gastroenterol* 2013; **19**: 1333-1337 [PMID: 23539677 DOI: 10.3748/wjg.v19.i9.1333]
50 **Oderich GS**, Bower TC, Sullivan TM, Bjarnason H, Cha S, Gloviczki P. Open versus endovascular revascularization for chronic mesenteric ischemia: risk-stratified outcomes. *J Vasc Surg* 2009; **49**: 1472-1479.e3 [PMID: 19497510 DOI: 10.1016/j.jvs.2009.02.006]
51 **Oderich GS**, Tallarita T, Gloviczki P, Duncan AA, Kalra M, Misra S, Cha S, Bower TC. Mesenteric artery complications during angioplasty and stent placement for atherosclerotic chronic mesenteric ischemia. *J Vasc Surg* 2012; **55**: 1063-1071 [PMID: 22322121 DOI: 10.1016/j.jvs.2011.10.122]
52 **Assar AN**, Abilez OJ, Zarins CK. Outcome of open versus endovascular revascularization for chronic mesenteric ischemia: review of comparative studies. *J Cardiovasc Surg* (Torino) 2009; **50**: 509-514 [PMID: 19455085]
53 **Sundermeyer A**, Zapenko A, Moysidis T, Luther B, Kröger K. Endovascular treatment of chronic mesenteric ischemia. *Interv Med Appl Sci* 2014; **6**: 118-124 [PMID: 25243077 DOI: 10.1556/IMAS.6.2014.3.4]
54 **Shirasu T**, Hosaka A, Okamoto H, Shigematsu K, Takeda Y, Miyata T, Watanabe T. Bowel necrosis following endovascular revascularization for chronic mesenteric ischemia: a case report and review of the literature. *BMC Gastroenterol* 2013; **13**: 118 [PMID: 23865626 DOI: 10.1186/1471-230X-13-118]

P- Reviewer: Srivastava M, Woo SY
S- Editor: Ji FF **L- Editor**: A **E- Editor**: Jiao XK

World Journal of Gastrointestinal Pathophysiology

Submit a Manuscript: http://www.wjgnet.com/esps/
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.4291/wjgp.v7.i1.131

World J Gastrointest Pathophysiol 2016 February 15; 7(1): 131-137
ISSN 2150-5330 (online)

MINIREVIEWS

Role of nitric oxide in the pathogenesis of Barrett's-associated carcinogenesis

Gen Kusaka, Kaname Uno, Katsunori Iijima, Tooru Shimosegawa

Gen Kusaka, Kaname Uno, Katsunori Iijima, Tooru Shimosegawa, Division of Gastroenterology, Tohoku University Hospital, Sendai, Miyagi 981-8574, Japan

Author contributions: All authors contributed to this paper.

Conflict-of-interest statement: None.

Correspondence to: Gen Kusaka, MD, PhD, Division of Gastroenterology, Tohoku University Hospital, 1-1 Seiryo-cho, Aoba-ku, Sendai, Miyagi 981-8574, Japan. gkusaka@med.tohoku.ac.jp
Telephone: +81-22-7177171
Fax: +81-22-7177174

Received: June 25, 2015
Peer-review started: June 28, 2015
First decision: August 26, 2015
Revised: October 17, 2015
Accepted: November 10, 2015
Article in press: November 11, 2015
Published online: February 15, 2016

Abstract

Barrett's esophagus (BE), a premalignant condition to Barrett's adenocarcinoma (BAC), is closely associated with chronic inflammation due to gastro-esophageal reflux. Caudal type homeobox 2 (CDX2), a representative marker of BE, is increased during the metaplastic and neoplastic transformation of BE. Nitric oxide (NO) has been proposed to be a crucial mediator of Barrett's carcinogenesis. We previously demonstrated that CDX2 might be induced directly under stimulation of large amounts of NO generated around the gastro-esophageal junction (GEJ) by activating epithelial growth factor receptor in a ligand-independent manner. Thus, we reviewed recent developments on the role of NO in Barrett's carcinogenesis. Notably, recent studies have reported that microbial communities in the distal esophagus are significantly different among groups with a normal esophagus, reflux esophagitis, BE or BAC, despite there being no difference in the bacterial quantity. Considering that microorganism components can be one of the major sources of large amounts of NO, these studies suggest that the bacterial composition in the distal esophagus might play an important role in regulating NO production during the carcinogenic process. Controlling an inflammatory reaction due to gastro-esophageal reflux or bacterial composition around the GEJ might help prevent the progression of Barrett's carcinogenesis by inhibiting NO production.

Key words: Barrett's esophagus; Nitric oxide; Epithelial growth factor receptor; Caudal type homeobox 2; Microbiome

Core tip: Barrett's carcinogenesis is closely associated with chronic inflammation caused by gastro-esophageal reflux of gastric acid, bile acid and intraluminal microorganisms. Caudal type homeobox 2 (CDX2) is a crucial biomarker of Barrett's esophagus. We previously demonstrated that a high amount of nitric oxide (NO) at the gastro-esophageal junction (GEJ) might directly induce CDX2 through phosphorylation of epidermal growth factor receptor (EGFR) in a ligand-independent manner. Together with a possible effect of anti-EGFR-targeting drugs in inhibiting Barrett's adenocarcinoma, future research for controlling NO production around the GEJ might provide a new insight for developing a management strategy of Barrett's carcinogenesis.

Kusaka G, Uno K, Iijima K, Shimosegawa T. Role of nitric oxide in the pathogenesis of Barrett's-associated carcinogenesis. *World J Gastrointest Pathophysiol* 2016; 7(1): 131-137 Available from: URL: http://www.wjgnet.com/2150-5330/full/v7/i1/131.htm DOI: http://dx.doi.org/10.4291/wjgp.v7.i1.131

INTRODUCTION

Barrett's esophagus (BE) is defined as the premalignant condition which squamous epithelium is replaced by columnar epithelium, a well-known precursor of Barrett's adenocarcinoma (BAC)[1-3]. Because the incidence of Barrett's-associated neoplasm has increased sharply, it is important to elucidate its risk factors during the surveillance period. Many studies on the origin of Barrett's mucosa have proposed several theories, such as the differentiation of the stem cells in esophageal squamous tissues and a phenotypic switch and formation of metaplastic epithelium by gastro-esophageal refluxant[4-6].

Caudal type homeobox 2 (CDX2) is a member of the homeobox family of genes involved in the embryonic development of the alimentary tract and in the differentiation, proliferation, adhesion and apoptosis of the intestinal epithelium[7-9]. It is well-known that CDX2 is induced during the metaplastic and neoplastic transformation of BE, while it is not expressed in normal esophageal squamous epithelium and non-metaplastic gastric epithelium[8-11]. Thus, it has been reported that an immune-stain of CDX2 could become a useful biomarker of Barrett's-associated neoplasms[12-14]. Previous studies have revealed that reactive oxygen species (ROS) produced by the stimulation of acid, bile salts or major noxious agents in refluxed gastric juice might take a critical role for the induction of CDX2 during the sequential process of the metaplastic and neoplastic transformation of BE[7,11,15-28]. However, this mechanism is not fully understood.

ROLE OF NITRIC OXIDE IN BARRETT'S CARCINOGENESIS

Nitric oxide (NO) is a crucial regulatory mediator which has cytotoxic and cytostatic effects, depending on the environment[16,26]. A large amount of NO production is involved in cytotoxic processes, such as apoptosis, angiogenesis and DNA damage during cancer development[19]. In fact, it was reported that NO might work as a principle mediator in the induction of CDX2 during Barrett's carcinogenesis[7] and that reactive nitrogen oxide species might contribute to the progression from BE to BAC because of genotoxicity, such as DNA damage, strand breakage or modification[21,29,30].

From the point of view of NO generation, NO is classified into endogenous and exogenous[27] (Figure 1). Endogenous NO is derived from L-arginine by a family of related enzymes, which includes three different isoforms of NO synthase (NOS)[27,31]. Endothelial NOS (eNOS) in endothelial cells and a neuronal NOS (nNOS) in neuronal cells were reported to be physiological mediators of NO production at low concentrations[27]. Inducible NO (iNOS) is an inflammation-induced enzyme, which can mediate harmful production of high amounts of NO[27,32]. The induction of iNOS might be critically involved in Barrett's carcinogenesis because its expression was shown to be gradually overexpressed during the sequential development toward BAC, but not in normal esophageal/gastric mucosa[19,27,32].

Exogenous NO is generated within the upper-gastrointestinal tract lumen from the reduction of salivary nitrite to NO in a reaction with acid refluxant[33-35]. After ingesting nitrate-containing food, human salivary nitrite concentrations increase to 2-5 mmol/L, which maintained for a few hours by entero-salivary recirculation of the dietary nitrate[35,36]. Swallowed saliva is the main source of nitrate. The gastric juice containing the acidity and ascorbic acid content might convert the salivary nitrite to NO at the gastro-esophageal junction (GEJ)[21,37]. Iijima *et al*[21] investigated the serum and saliva nitrate concentrations within the upper gastrointestinal tract lumen in healthy volunteers of *Helicobacter pylori*-negative under basal conditions and after nitrate ingestion using custom-made sensors. After ingesting 2 mmol of potassium nitrate, the nitrate concentration of the serum, salivary and intra-lumin increased from 30 μmol/L to 95 μmol/L, from 36 μmol/L to 252 μmol/L, and from 4.7 μmol/L to 23.2 μmol/L, respectively. The highest concentrations of NO within the upper gastrointestinal tract lumen were observed at the site of the neutral pH of esophageal fell to the acidic pH of gastric. This suggested that the luminal generation of NO from nitrate ingestion through salivary nitrite was maximal at the GEJ. Thus, the site of generation of luminal NO would move to the oral side of the distal esophagus in patients with gastro-esophageal reflux[38].

Additionally, luminal NO with a high concentration due to both exogenous and endogenous NO production around the GEJ is rapidly diffused across epithelial membranes, resulting in damage to the tissues surrounding tissues the GEJ[29,30,39-41]. Such a large amount of NO also causes the production of peroxynitrite, a representative genotoxic mediator, and can contribute to the development of Barrett's carcinogenesis *via* a rapid reaction with superoxide in an inflammatory/tumor microenvironment[20,21,26,27,37]. Of the plasma membranous receptors of the esophageal tissue, we previously demonstrated that during Barrett's carcinogenesis, a large amount of NO might directly activate epithelial growth factor receptor (EGFR)[42], a 170-kDa glycoprotein which is a member of the receptor tyrosine kinase family and is involved in cell proliferation, differentiation, angiogenesis, invasion and tumor progression[1,3,43-46]. Previous studies demonstrated that overexpression of EGFR was observed during histological progression in BE tissue, as well as during tumor infiltration and metastasis[1,45]. Although it was previously indicated that ROS might indirectly enhance the CDX2 expression by the exposure of bile

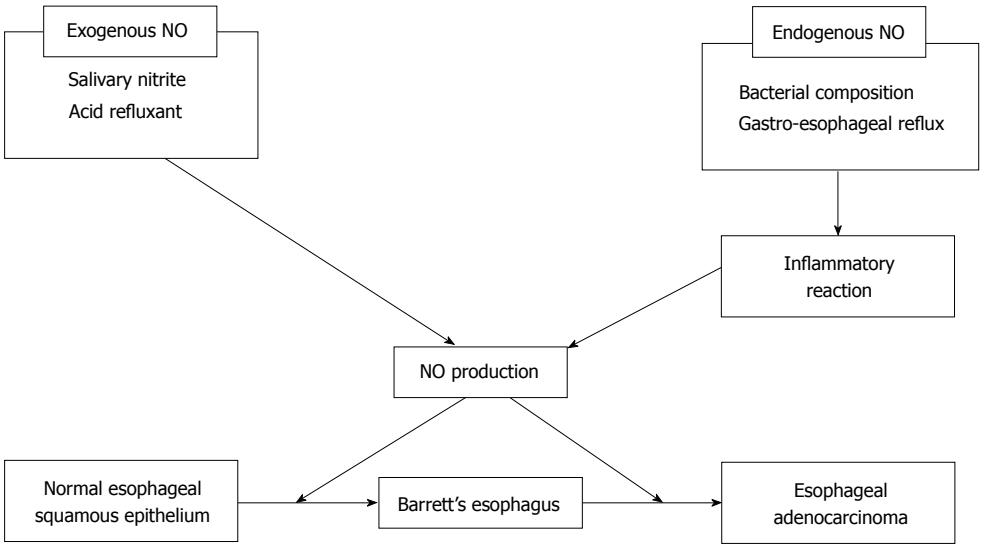


Figure 1 The process of nitric oxide generation. Exogenous NO is generated within the upper-gastrointestinal tract lumen from the reduction of salivary nitrite to NO in a reaction with acid refluxant. Endogenous NO is generated in chronic inflammation caused by gastro-esophageal reflux and bacterial composition. NO: Nitric oxide.

acid through EGFR activation in ligand-dependent and -independent manners[3,8,15,46], we investigated the direct role of NO through activation of EGFR during Barrett's carcinogenesis[42]. When we compared immune-staining of EGFR, CDX2 and nitrotyrosine, a marker of intracellular protein damaged by peroxynitrite, in normal human esophageal squamous epithelium specimens with those in BE specimens, we revealed the stronger expression of EGFR, CDX2 and nitrotyrosine in BE compared with those in normal human esophageal squamous epithelium with a positive correlation with BE progression. Second, we also investigated the effect of peroxynitrite, NOC7 which is NO donor and SIN-1 which is stimulant of peroxynitrite on the expression of phosphorylated EGFR and CDX2 by western blotting and reverse transcription-polymerase chain reaction. This showed that the phosphorylated EGFR and CDX2 were induced in dose- and time-dependent manners after expose to peroxynitrite, NOC7 and SIN-1. The expression of phosphorylated EGFR and CDX2 induced after expose to peroxynitrite, NOC7 and SIN-1 was diminished by EGFR-specific inhibitors AG1428 and EGFR-siRNA. Additionally, the induction of phosphorylated EGFR and CDX2 after exposure to NOC7 was diminished by carboxy-PTIO which is the NO scavenger in a dose-dependent manner. Taken together, these results suggest that the expression of CDX2 might be induced directly by the effect of extrinsic NO through the activation of EGFR in the progression of BE. Accordingly, these results indicate that the stimulation of high concentration of NO on the normal esophageal epithelium might directly induce the expression of CDX2 through EGFR activation and result in the progression of BE[7,8,27].

Notably, EGFR2 (HER2) is regarded as a useful target in controlling Barrett's cancer development, despite the uncertain treatment effect of HER2-targetting anti-cancer medications[47,48]. While researchers reported that the clinical trials targeting HER2 in the treatment of BAC were result it was not promising, another study showed that targeting erbB-2 in HER2-positive patients with adenocarcinoma at GEJ had better outcomes[49,50]. Gefitinib is another small molecule tyrosine kinase inhibitor (TKI) and the most common HER2-targetting anti-cancer medication that has been used to treat several malignancies. It is a representative oral TKI and inhibits EGF-induced phosphorylation and downstream signaling pathways. Janmaat *et al*[51] reported a phase Ⅱ study of 36 patients who had failed first-line treatment for advanced esophageal squamous cell carcinoma (ESCC) and esophageal adenocarcinoma (EAC) and were administered gefitinib at 500 mg/d. In this report, gefitinib had modest activity as a second-line treatment with only 2.8% of the patients with a partial response (PR), 27.8% with stable disease and 47.8% with disease progression. The progression-free survival time and the median overall survival time were 59 d and 164 d, respectively. Javle *et al*[52] reported a phase Ⅰ/Ⅱ study of 6 patients who had locally advanced unresectable EAC. This study used a combination of gefitinib and chemoradiotherapy. The protocol were composed of administration of gefitinib orally at 250 mg/d for 1 year, oxaliplatin intravenously at 85 or 100 mg/m^2 on days 1, 15, 29, and preoperative radiotherapy. They reported that this combination study was tolerable, but had limited efficacy. The median overall survival time and the disease-free survival time were 10.8 and 8.4 mo, respectively. Ilson *et al*[53] reported a phase Ⅱ study of the treatment effect of Erlotinib, another oral TKI, in 30 patients who had failed first-line treatment for ESCC and EAC. The patients had a 6.7% PR and 10.3 mo of median overall survival. These results supported an important role for EGFR in Barrett's cancer development. Targeted inhibition of EGFR activity and inhibition of EGFR activity by controlling NO production around the

GEJ might be a new clue to improve the management of Barrett's-associated neoplasm.

POSSIBLE ORIGIN OF NO IN SWALLOWED MICROORGANISMS

Several studies have reported that the bacterial composition in the distal esophagus might play an important role in the carcinogenic process. Human microbiota is estimated to be composed of approximately 10^{14} bacterial cells. The relationship the bacteria and host can be commensal and pathogenic[54]. Human microbiota in the gastrointestinal tract are essential for human survival, aiding digestion, assisting in the synthesis of vitamins and maintaining a normal physiological environment[54,55]. The host provides these microbiota safe housing and nutrition in return for the benefits from the microbes. An imbalance in this relationship may cause chronic inflammation and inflammation-associated malignancies.

Previous report mentioned that the normal distal esophagus have 6 phyla and 140 species of bacterial compositions[56]. The impairment of the mucosal barrier in the distal esophagus by reflux of gastric juice might disrupt the microenvironmental homeostasis, resulting in progression of Barrett's-associated neoplasm with remarkable changes in esophageal bacterial biota[56-58]. In fact, it was reported that the characteristics of bacterial communities differed between groups with normal esophagus, reflux esophagitis (RE), BE or BAC, despite there being no significant differences in the amount of bacteria[55,58].

By investigating the composition of bacteria in the distal esophagus of 3 groups (group with normal esophagus, group with RE and group with BE) from endoscopic biopsy specimens using a histological examination and DNA extraction, Liu *et al*[55] reported that *Fusobacterium* was found not in the group with a normal esophagus, but in the group with RE and BE, and that the most common bacterium was *Streptococcus* in the groups with normal esophagus and RE and *Veillonella* in the group with BE. However, the total amount of the detected bacterial DNA was not different between the groups[55]. Narikiyo *et al*[59] investigated the bacteria in esophageal carcinomatous tissues, the surrounding non-cancerous tissues and saliva from healthy people to determine the correlation between microorganisms and inflammation/carcinogenesis of the esophagus. They showed that *Treponema denticola* and *Streptococcus anginosus* (*S. anginosus*) were detected in the saliva from patients with BAC. Both of these bacteria were in low numbers in the samples from healthy people. They also reported that *S. angiosus* and *Streptoccus mitis* infections induced inflammatory cytokines, including IL-8 and GROα, and suggested these bacteria might play a significant role in Barrett's carcinogenesis through chronic inflammation. Yang *et al*[54] reported a dramatic alteration of the esophageal microbiome, such as Gram-positive bacteria were decreased and Gram-negative bacteria were increased in patients with esophagitis and BE. In fact, Gram-negative bacteria have lipopolysaccharides (LPS) in their outer membrane, which can promote the induction of pro-inflammatory cytokines, including IL-1β, IL-8, COX-2 and iNOS, by activating Toll-like receptor 4 and its downstream cascade, which are related to proliferation, differentiation and migration[60,61]. In addition, LPS may also cause the relaxation of the lower esophageal sphincter by activating iNOS and delaying gastric employing by increasing the intra-gastric pressure through COX-2 activation[62,63]. This suggests that the alteration of Gram-negative bacteria in bacterial composition in the distal esophagus might contribute to the development of inflammation-associated Barrett's carcinogenesis. Thus, interventions with the microorganism in the distal esophagus provided novel insights for new preventive/treatment strategy of BE.

PERSPECTIVE

Many studies have contributed to a better understanding of the role of NO in Barrett's carcinogenesis. There may be three ways to control NO production in Barrett's mucosa, such as: (1) diminishing acidity of the gastro-esophageal refluxant by using a proton-pump inhibitor[64]; (2) medical intervention of intramucosal microorganisms; and (3) medical inhibition of iNOS activity. Recent studies have indicated a significant role for esophageal microbiology in Barrett's carcinogenesis because of the differences in the bacterial communities between groups with normal esophagus, RE, BE or BAC[55,58]. Therefore, it is likely that the eradication of these bacteria by antibiotics or probiotics might be effective in reducing Barrett's cancer risk, although limited data of suitable antibiotics combinations for the microbiome have been reported. Yang *et al*[54] reported that Gram-negative bacteria were increased in the patients with esophagitis and BE. LPS from Gram-negative bacteria can up-regulate gene expression of inflammatory cytokines, including COX-2 and iNOS, by activating the NF-κB pathway[54]. Several studies have suggested that NF-κB inhibitors, including nonsteroidal anti-inflammatory drugs (NSAIDs), sulfasalazine and immunosuppressive agents, could be effective in reducing Barrett's inflammation. Therefore, they were regarded as a potential medication for preventing Barrett's carcinogenesis[61]. Moreover, Hansel *et al*[62] demonstrated that the prodrug L-N^6-(1-iminoethyl)lysine 5-tetrazole amide, an iNOS inhibitor, reduced the levels of NO in exhaled breath in healthy volunteers and patients with mild asthma without remarkable side-effects. These results suggest the therapeutic potential of iNOS inhibitors to reduce the risk of Barrett's cancer. Additionally, we previously demonstrated that the interaction between iNOS and COX-2 activities might play a crucial role in LPS-induced inflammation in a rat-model[65]. Therefore, controlling COX-2 activity can also regulate iNOS-derived NO

production in an inflammation reaction. Therefore, COX-2 inhibitors might have therapeutic potential in controlling the risk of neoplastic progression in patients with BE. Moreover, Calatayud *et al*[66] reported that the delay of gastric employing with an increase of the intra-gastric pressure from LPS stimulation could be blocked by down-regulating COX-2 activity using the selective COX-2 inhibitor NS398. In fact, in a meta-analysis of anti-cancerous effects of NSAIDs and aspirin for 1813 patients with EAC, Corley *et al*[67] suggested that the preventive role of COX-2 inhibitors in Barrett's carcinogenesis, although their clinical relevance against the development of Barrett's -associated neoplasm was unproven[67-69]. Accordingly, controlling enzymatic activity of iNOS and microorganisms in Barrett's tumor microenvironment may provide important insight into Barrett's carcinogenesis.

CONCLUSION

Barrett's carcinogenesis was associated with chronic inflammation as a consequence of gastro-esophageal reflux and microbiota in the gastrointestinal tract. This review focused on the role of NO in controlling inflammation during the Barrett's carcinogenic process. The control of NO production in chronic inflammation caused by gastro-esophageal reflux and microbiota might contribute to prevent Barrett's cancer development. Further studies are required to assess the therapeutic potential of controlling NO production.

REFERENCES

1 **Cronin J**, McAdam E, Danikas A, Tselepis C, Griffiths P, Baxter J, Thomas L, Manson J, Jenkins G. Epidermal growth factor receptor (EGFR) is overexpressed in high-grade dysplasia and adenocarcinoma of the esophagus and may represent a biomarker of histological progression in Barrett's esophagus (BE). *Am J Gastroenterol* 2011; **106**: 46-56 [PMID: 21157443 DOI: 10.1038/ajg.2010.433]

2 **Shaheen N**, Ransohoff DF. Gastroesophageal reflux, Barrett esophagus, and esophageal cancer: clinical applications. *JAMA* 2002; **287**: 1982-1986 [PMID: 11960541 DOI: 10.1001/jama.287.15.1982]

3 **Li Y**, Wo JM, Ray MB, Jones W, Su RR, Ellis S, Martin RC. Cyclooxygenase-2 and epithelial growth factor receptor up-regulation during progression of Barrett's esophagus to adenocarcinoma. *World J Gastroenterol* 2006; **12**: 928-934 [PMID: 16521222 DOI: 10.3748/wjg.v12.i6.928]

4 **Zhang HY**, Spechler SJ, Souza RF. Esophageal adenocarcinoma arising in Barrett esophagus. *Cancer Lett* 2009; **275**: 170-177 [PMID: 18703277 DOI: 10.1016/j.canlet.2008.07.006]

5 **Abdel-Latif MM**, Duggan S, Reynolds JV, Kelleher D. Inflammation and esophageal carcinogenesis. *Curr Opin Pharmacol* 2009; **9**: 396-404 [PMID: 19596608 DOI: 10.1016/j.coph.2009.06.010]

6 **Souza RF**, Krishnan K, Spechler SJ. Acid, bile, and CDX: the ABCs of making Barrett's metaplasia. *Am J Physiol Gastrointest Liver Physiol* 2008; **295**: G211-G218 [PMID: 18556417 DOI: 10.1152/ajpgi.90250.2008]

7 **Vaninetti N**, Williams L, Geldenhuys L, Porter GA, Guernsey DL, Casson AG. Regulation of CDX2 expression in esophageal adenocarcinoma. *Mol Carcinog* 2009; **48**: 965-974 [PMID: 19415720 DOI: 10.1002/mc.20549]

8 **Rahman FB**, Kadowaki Y, Ishihara S, Tobita H, Imaoka H, Fukuhara H, Aziz MM, Furuta K, Amano Y, Kinoshita Y. Fibroblast-derived HB-EGF promotes Cdx2 expression in esophageal squamous cells. *Lab Invest* 2010; **90**: 1033-1048 [PMID: 20351696 DOI: 10.1038/labinvest.2010.71]

9 **Takahashi O**, Hamada J, Abe M, Hata S, Asano T, Takahashi Y, Tada M, Miyamoto M, Kondo S, Moriuchi T. Dysregulated expression of HOX and ParaHOX genes in human esophageal squamous cell carcinoma. *Oncol Rep* 2007; **17**: 753-760 [PMID: 17342311]

10 **McColl KE**. When saliva meets acid: chemical warfare at the oesophagogastric junction. *Gut* 2005; **54**: 1-3 [PMID: 15591495 DOI: 10.1136/gut.2004.047126]

11 **Burnat G**, Rau T, Elshimi E, Hahn EG, Konturek PC. Bile acids induce overexpression of homeobox gene CDX-2 and vascular endothelial growth factor (VEGF) in human Barrett's esophageal mucosa and adenocarcinoma cell line. *Scand J Gastroenterol* 2007; **42**: 1460-1465 [PMID: 17852856 DOI: 10.1080/00365520701452209]

12 **Johnson DR**, Abdelbaqui M, Tahmasbi M, Mayer Z, Lee HW, Malafa MP, Coppola D. CDX2 protein expression compared to alcian blue staining in the evaluation of esophageal intestinal metaplasia. *World J Gastroenterol* 2015; **21**: 2770-2776 [PMID: 25759548 DOI: 10.3748/wjg.v21.i9.2770]

13 **Freund JN**, Domon-Dell C, Kedinger M, Duluc I. The Cdx-1 and Cdx-2 homeobox genes in the intestine. *Biochem Cell Biol* 1998; **76**: 957-969 [PMID: 10392709 DOI: 10.1139/bcb-76-6-957]

14 **Silberg DG**, Swain GP, Suh ER, Traber PG. Cdx1 and cdx2 expression during intestinal development. *Gastroenterology* 2000; **119**: 961-971 [PMID: 11040183 DOI: 10.1053/gast.2000.18142]

15 **Avissar NE**, Toia L, Hu Y, Watson TJ, Jones C, Raymond DP, Matousek A, Peters JH. Bile acid alone, or in combination with acid, induces CDX2 expression through activation of the epidermal growth factor receptor (EGFR). *J Gastrointest Surg* 2009; **13**: 212-222 [PMID: 18854960 DOI: 10.1007/s11605-008-0720-7]

16 **Clemons NJ**, McColl KE, Fitzgerald RC. Nitric oxide and acid induce double-strand DNA breaks in Barrett's esophagus carcinogenesis via distinct mechanisms. *Gastroenterology* 2007; **133**: 1198-1209 [PMID: 17919494 DOI: 10.1053/j.gastro.2007.06.061]

17 **Feagins LA**, Zhang HY, Zhang X, Hormi-Carver K, Thomas T, Terada LS, Spechler SJ, Souza RF. Mechanisms of oxidant production in esophageal squamous cell and Barrett's cell lines. *Am J Physiol Gastrointest Liver Physiol* 2008; **294**: G411-G417 [PMID: 18063706 DOI: 10.1152/ajpgi.00373.2007]

18 **Rantanen TK**, Räsänen JV, Sihvo EI, Ahotupa MO, Färkkilä MA, Salo JA. The impact of antireflux surgery on oxidative stress of esophageal mucosa caused by gastroesophageal reflux disease: 4-yr follow-up study. *Am J Gastroenterol* 2006; **101**: 222-228 [PMID: 16454822 DOI: 10.1111/j.1572-0241.2006.00420.x]

19 **Clemons NJ**, Shannon NB, Abeyratne LR, Walker CE, Saadi A, O'Donovan ML, Lao-Sirieix PP, Fitzgerald RC. Nitric oxide-mediated invasion in Barrett's high-grade dysplasia and adenocarcinoma. *Carcinogenesis* 2010; **31**: 1669-1675 [PMID: 20584750 DOI: 10.1093/carcin/bgq130]

20 **Iijima K**, Grant J, McElroy K, Fyfe V, Preston T, McColl KE. Novel mechanism of nitrosative stress from dietary nitrate with relevance to gastro-oesophageal junction cancers. *Carcinogenesis* 2003; **24**: 1951-1960 [PMID: 12970071 DOI: 10.1093/carcin/bgg168]

21 **Iijima K**, Henry E, Moriya A, Wirz A, Kelman AW, McColl KE. Dietary nitrate generates potentially mutagenic concentrations of nitric oxide at the gastroesophageal junction. *Gastroenterology* 2002; **122**: 1248-1257 [PMID: 11984511 DOI: 10.1053/gast.2002.32963]

22 **Jenkins GJ**, D'Souza FR, Suzen SH, Eltahir ZS, James SA, Parry JM, Griffiths PA, Baxter JN. Deoxycholic acid at neutral and acid pH, is genotoxic to oesophageal cells through the induction of ROS: The potential role of anti-oxidants in Barrett's oesophagus. *Carcinogenesis* 2007; **28**: 136-142 [PMID: 16905748 DOI: 10.1093/carcin/bgl147]

23 **Dvorak K**, Payne CM, Chavarria M, Ramsey L, Dvorakova B, Bernstein H, Holubec H, Sampliner RE, Guy N, Condon A, Bernstein

C, Green SB, Prasad A, Garewal HS. Bile acids in combination with low pH induce oxidative stress and oxidative DNA damage: relevance to the pathogenesis of Barrett's oesophagus. *Gut* 2007; **56**: 763-771 [PMID: 17145738 DOI: 10.1136/gut.2006.103697]

24 **Jenkins GJ**, Cronin J, Alhamdani A, Rawat N, D'Souza F, Thomas T, Eltahir Z, Griffiths AP, Baxter JN. The bile acid deoxycholic acid has a non-linear dose response for DNA damage and possibly NF-kappaB activation in oesophageal cells, with a mechanism of action involving ROS. *Mutagenesis* 2008; **23**: 399-405 [PMID: 18515815 DOI: 10.1093/mutage/gen029]

25 **Morelli MP**, Cascone T, Troiani T, De Vita F, Orditura M, Laus G, Eckhardt SG, Pepe S, Tortora G, Ciardiello F. Sequence-dependent antiproliferative effects of cytotoxic drugs and epidermal growth factor receptor inhibitors. *Ann Oncol* 2005; **16** Suppl 4: iv61-iv68 [PMID: 15923432 DOI: 10.1093/annonc/mdi910]

26 **Vaninetti NM**, Geldenhuys L, Porter GA, Risch H, Hainaut P, Guernsey DL, Casson AG. Inducible nitric oxide synthase, nitrotyrosine and p53 mutations in the molecular pathogenesis of Barrett's esophagus and esophageal adenocarcinoma. *Mol Carcinog* 2008; **47**: 275-285 [PMID: 17849424 DOI: 10.1002/mc.20382]

27 **Bae JD**, Jung KH, Ahn WS, Bae SH, Jang TJ. Expression of inducible nitric oxide synthase is increased in rat Barrett's esophagus induced by duodenal contents reflux. *J Korean Med Sci* 2005; **20**: 56-60 [PMID: 15716603 DOI: 10.3346/jkms.2005.20.1.56]

28 **Marchetti M**, Caliot E, Pringault E. Chronic acid exposure leads to activation of the cdx2 intestinal homeobox gene in a long-term culture of mouse esophageal keratinocytes. *J Cell Sci* 2003; **116**: 1429-1436 [PMID: 12640028 DOI: 10.1242/jcs.00338]

29 **Ara N**, Iijima K, Asanuma K, Yoshitake J, Ohara S, Shimosegawa T, Yoshimura T. Disruption of gastric barrier function by luminal nitrosative stress: a potential chemical insult to the human gastro-oesophageal junction. *Gut* 2008; **57**: 306-313 [PMID: 17965057 DOI: 10.1136/gut.2007.128074]

30 **Ito H**, Iijima K, Ara N, Asanuma K, Endo H, Asano N, Koike T, Abe Y, Imatani A, Shimosegawa T. Reactive nitrogen oxide species induce dilatation of the intercellular space of rat esophagus. *Scand J Gastroenterol* 2010; **45**: 282-291 [PMID: 20001645 DOI: 10.3109/00365520903469956]

31 **Ruano MJ**, Hernández-Hernando S, Jiménez A, Estrada C, Villalobo A. Nitric oxide-induced epidermal growth factor-dependent phosphorylations in A431 tumour cells. *Eur J Biochem* 2003; **270**: 1828-1837 [PMID: 12694196]

32 **Wilson KT**, Fu S, Ramanujam KS, Meltzer SJ. Increased expression of inducible nitric oxide synthase and cyclooxygenase-2 in Barrett's esophagus and associated adenocarcinomas. *Cancer Res* 1998; **58**: 2929-2934 [PMID: 9679948]

33 **Benjamin N**, O'Driscoll F, Dougall H, Duncan C, Smith L, Golden M, McKenzie H. Stomach NO synthesis. *Nature* 1994; **368**: 502 [PMID: 8139683 DOI: 10.1038/368502a0]

34 **Lundberg JO**, Weitzberg E, Lundberg JM, Alving K. Intragastric nitric oxide production in humans: measurements in expelled air. *Gut* 1994; **35**: 1543-1546 [PMID: 7828969 DOI: 10.1136/gut.35.11.1543]

35 **McKnight GM**, Smith LM, Drummond RS, Duncan CW, Golden M, Benjamin N. Chemical synthesis of nitric oxide in the stomach from dietary nitrate in humans. *Gut* 1997; **40**: 211-214 [PMID: 9071933 DOI: 10.1136/gut.40.2.211]

36 **Pannala AS**, Mani AR, Spencer JP, Skinner V, Bruckdorfer KR, Moore KP, Rice-Evans CA. The effect of dietary nitrate on salivary, plasma, and urinary nitrate metabolism in humans. *Free Radic Biol Med* 2003; **34**: 576-584 [PMID: 12614846 DOI: 10.1016/S0891-5849(02)01353-9]

37 **Mukaida H**, Toi M, Hirai T, Yamashita Y, Toge T. Clinical significance of the expression of epidermal growth factor and its receptor in esophageal cancer. *Cancer* 1991; **68**: 142-148 [PMID: 2049734]

38 **Suzuki H**, Iijima K, Scobie G, Fyfe V, McColl KE. Nitrate and nitrosative chemistry within Barrett's oesophagus during acid reflux. *Gut* 2005; **54**: 1527-1535 [PMID: 16227357 DOI: 10.1136/gut.2005.066043]

39 **Ishiyama F**, Iijima K, Asanuma K, Ara N, Yoshitake J, Abe Y, Koike T, Imatani A, Ohara S, Shimosegawa T. Exogenous luminal nitric oxide exacerbates esophagus tissue damage in a reflux esophagitis model of rats. *Scand J Gastroenterol* 2009; **44**: 527-537 [PMID: 19172433 DOI: 10.1080/00365520802699260]

40 **Endo H**, Iijima K, Asanuma K, Ara N, Ito H, Asano N, Uno K, Koike T, Imatani A, Shimosegawa T. Exogenous luminal nitric oxide exposure accelerates columnar transformation of rat esophagus. *Int J Cancer* 2010; **127**: 2009-2019 [PMID: 20131319 DOI: 10.1002/ijc.25227]

41 **Asanuma K**, Iijima K, Sugata H, Ohara S, Shimosegawa T, Yoshimura T. Diffusion of cytotoxic concentrations of nitric oxide generated luminally at the gastro-oesophageal junction of rats. *Gut* 2005; **54**: 1072-1077 [PMID: 15860569 DOI: 10.1136/gut.2004.063107]

42 **Kusaka G**, Uno K, Iijima K, Endo H, Asano N, Koike T, Imatani A, Shimosegawa T. The role of nitric oxide in the induction of caudal-type homeobox 2 through epidermal growth factor receptor in the development of Barrett's esophagus. *Scand J Gastroenterol* 2012; **47**: 1148-1158 [PMID: 22834965 DOI: 10.3109/00365521.2012.703232]

43 **Lee HC**, An S, Lee H, Woo SH, Jin HO, Seo SK, Choe TB, Yoo DH, Lee SJ, Hong YJ, Park MJ, Rhee CH, Park IC, Hong SI. Activation of epidermal growth factor receptor and its downstream signaling pathway by nitric oxide in response to ionizing radiation. *Mol Cancer Res* 2008; **6**: 996-1002 [PMID: 18567803 DOI: 10.1158/1541-7786.MCR-08-0113]

44 **Andl CD**, Mizushima T, Nakagawa H, Oyama K, Harada H, Chruma K, Herlyn M, Rustgi AK. Epidermal growth factor receptor mediates increased cell proliferation, migration, and aggregation in esophageal keratinocytes in vitro and in vivo. *J Biol Chem* 2003; **278**: 1824-1830 [PMID: 12435727 DOI: 10.1074/jbc.M209148200]

45 **Hanawa M**, Suzuki S, Dobashi Y, Yamane T, Kono K, Enomoto N, Ooi A. EGFR protein overexpression and gene amplification in squamous cell carcinomas of the esophagus. *Int J Cancer* 2006; **118**: 1173-1180 [PMID: 16161046 DOI: 10.1002/ijc.21454]

46 **Goldman A**, Chen HD, Roesly HB, Hill KA, Tome ME, Dvorak B, Bernstein H, Dvorak K. Characterization of squamous esophageal cells resistant to bile acids at acidic pH: implication for Barrett's esophagus pathogenesis. *Am J Physiol Gastrointest Liver Physiol* 2011; **300**: G292-G302 [PMID: 21127259 DOI: 10.1152/ajpgi.00461.2010]

47 **Gowryshankar A**, Nagaraja V, Eslick GD. HER2 status in Barrett's esophagus & amp; esophageal cancer: a meta analysis. *J Gastrointest Oncol* 2014; **5**: 25-35 [PMID: 24490040 DOI: 10.3978/j.issn.2078-6891.2013.039]

48 **Xu Y**, Sheng L, Mao W. Role of epidermal growth factor receptor tyrosine kinase inhibitors in the treatment of esophageal carcinoma and the suggested mechanisms of action. *Oncol Lett* 2013; **5**: 19-24 [PMID: 23255886 DOI: 10.3892/ol.2012.994]

49 **Clemons NJ**, Phillips WA, Lord RV. Signaling pathways in the molecular pathogenesis of adenocarcinomas of the esophagus and gastroesophageal junction. *Cancer Biol Ther* 2013; **14**: 782-795 [PMID: 23792587 DOI: 10.4161/cbt.25362]

50 **Rubenstein JH**, Dahlkemper A, Kao JY, Zhang M, Morgenstern H, McMahon L, Inadomi JM. A pilot study of the association of low plasma adiponectin and Barrett's esophagus. *Am J Gastroenterol* 2008; **103**: 1358-1364 [PMID: 18510610 DOI: 10.1111/j.1572-0241.2008.01823.x]

51 **Janmaat ML**, Gallegos-Ruiz MI, Rodriguez JA, Meijer GA, Vervenne WL, Richel DJ, Van Groeningen C, Giaccone G. Predictive factors for outcome in a phase II study of gefitinib in second-line treatment of advanced esophageal cancer patients. *J Clin Oncol* 2006; **24**: 1612-1619 [PMID: 16575012 DOI: 10.1200/JCO.2005.03.4900]

52 **Javle M**, Pande A, Iyer R, Yang G, LeVea C, Wilding G, Black J, Nava H, Nwogu C. Pilot study of gefitinib, oxaliplatin, and radiotherapy for esophageal adenocarcinoma: tissue effect predicts clinical response. *Am J Clin Oncol* 2008; **31**: 329-334 [PMID: 18845990 DOI: 10.1097/COC.0b013e318161dc04]

53 **Ilson DH**, Kelsen D, Shah M, Schwartz G, Levine DA, Boyd J, Capanu M, Miron B, Klimstra D. A phase 2 trial of erlotinib in patients with previously treated squamous cell and adenocarcinoma of the esophagus. *Cancer* 2011; **117**: 1409-1414 [PMID: 21425140 DOI: 10.1002/cncr.25602]

54 **Yang L**, Francois F, Pei Z. Molecular pathways: pathogenesis and clinical implications of microbiome alteration in esophagitis and Barrett esophagus. *Clin Cancer Res* 2012; **18**: 2138-2144 [PMID: 22344232 DOI: 10.1158/1078-0432.CCR-11-0934]

55 **Liu N**, Ando T, Ishiguro K, Maeda O, Watanabe O, Funasaka K, Nakamura M, Miyahara R, Ohmiya N, Goto H. Characterization of bacterial biota in the distal esophagus of Japanese patients with reflux esophagitis and Barrett's esophagus. *BMC Infect Dis* 2013; **13**: 130 [PMID: 23496929 DOI: 10.1186/1471-2334-13-130]

56 **Pei Z**, Bini EJ, Yang L, Zhou M, Francois F, Blaser MJ. Bacterial biota in the human distal esophagus. *Proc Natl Acad Sci USA* 2004; **101**: 4250-4255 [PMID: 15016918 DOI: 10.1073/pnas.0306398101]

57 **Yang L**, Chaudhary N, Baghdadi J, Pei Z. Microbiome in reflux disorders and esophageal adenocarcinoma. *Cancer J* 2014; **20**: 207-210 [PMID: 24855009 DOI: 10.1097/PPO.0000000000000044]

58 **Blackett KL**, Siddhi SS, Cleary S, Steed H, Miller MH, Macfarlane S, Macfarlane GT, Dillon JF. Oesophageal bacterial biofilm changes in gastro-oesophageal reflux disease, Barrett's and oesophageal carcinoma: association or causality? *Aliment Pharmacol Ther* 2013; **37**: 1084-1092 [PMID: 23600758 DOI: 10.1111/apt.12317]

59 **Narikiyo M**, Tanabe C, Yamada Y, Igaki H, Tachimori Y, Kato H, Muto M, Montesano R, Sakamoto H, Nakajima Y, Sasaki H. Frequent and preferential infection of Treponema denticola, Streptococcus mitis, and Streptococcus anginosus in esophageal cancers. *Cancer Sci* 2004; **95**: 569-574 [PMID: 15245592 DOI: 10.1111/j.1349-7006.2004.tb02488.x]

60 **Pikarsky E**, Porat RM, Stein I, Abramovitch R, Amit S, Kasem S, Gutkovich-Pyest E, Urieli-Shoval S, Galun E, Ben-Neriah Y. NF-kappaB functions as a tumour promoter in inflammation-associated cancer. *Nature* 2004; **431**: 461-466 [PMID: 15329734 DOI: 10.1038/nature02924]

61 **Yamamoto Y**, Gaynor RB. Therapeutic potential of inhibition of the NF-kappaB pathway in the treatment of inflammation and cancer. *J Clin Invest* 2001; **107**: 135-142 [PMID: 11160126 DOI: 10.1172/JCI11914]

62 **Hansel TT**, Kharitonov SA, Donnelly LE, Erin EM, Currie MG, Moore WM, Manning PT, Recker DP, Barnes PJ. A selective inhibitor of inducible nitric oxide synthase inhibits exhaled breath nitric oxide in healthy volunteers and asthmatics. *FASEB J* 2003; **17**: 1298-1300 [PMID: 12738811 DOI: 10.1096/fj.02-0633fje]

63 **Buchholz BM**, Chanthaphavong RS, Bauer AJ. Nonhemopoietic cell TLR4 signaling is critical in causing early lipopolysaccharide-induced ileus. *J Immunol* 2009; **183**: 6744-6753 [PMID: 19846874 DOI: 10.4049/jimmunol.0901620]

64 **Islami F**, Kamangar F, Boffetta P. Use of proton pump inhibitors and risk of progression of Barrett's esophagus to neoplastic lesions. *Am J Gastroenterol* 2009; **104**: 2646-2648 [PMID: 19806109 DOI: 10.1038/ajg.2009.369]

65 **Uno K**, Iuchi Y, Fujii J, Sugata H, Iijima K, Kato K, Shimosegawa T, Yoshimura T. In vivo study on cross talk between inducible nitric-oxide synthase and cyclooxygenase in rat gastric mucosa: effect of cyclooxygenase activity on nitric oxide production. *J Pharmacol Exp Ther* 2004; **309**: 995-1002 [PMID: 14988416 DOI: 10.1124/jpet.103.061283]

66 **Calatayud S**, García-Zaragozá E, Hernández C, Quintana E, Felipo V, Esplugues JV, Barrachina MD. Downregulation of nNOS and synthesis of PGs associated with endotoxin-induced delay in gastric emptying. *Am J Physiol Gastrointest Liver Physiol* 2002; **283**: G1360-G1367 [PMID: 12433667 DOI: 10.1152/ajpgi.00168.2002]

67 **Corley DA**, Kerlikowske K, Verma R, Buffler P. Protective association of aspirin/NSAIDs and esophageal cancer: a systematic review and meta-analysis. *Gastroenterology* 2003; **124**: 47-56 [PMID: 12512029 DOI: 10.1053/gast.2003.50008]

68 **Heath EI**, Canto MI, Piantadosi S, Montgomery E, Weinstein WM, Herman JG, Dannenberg AJ, Yang VW, Shar AO, Hawk E, Forastiere AA. Secondary chemoprevention of Barrett's esophagus with celecoxib: results of a randomized trial. *J Natl Cancer Inst* 2007; **99**: 545-557 [PMID: 17405999 DOI: 10.1093/jnci/djk112]

69 **Gatenby PA**, Ramus JR, Caygill CP, Winslet MC, Watson A. Aspirin is not chemoprotective for Barrett's adenocarcinoma of the oesophagus in multicentre cohort. *Eur J Cancer Prev* 2009; **18**: 381-384 [PMID: 19620873 DOI: 10.1097/CEJ.0b013e]

P- Reviewer: Miheller P, Shimoyama S
S- Editor: Ji FF **L- Editor**: A **E- Editor**: Jiao XK

World Journal of Gastrointestinal Pathophysiology

Submit a Manuscript: http://www.wjgnet.com/esps/
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.4291/wjgp.v7.i1.138

World J Gastrointest Pathophysiol 2016 February 15; 7(1): 138-149
ISSN 2150-5330 (online)

ORIGINAL ARTICLE

Basic Study

Differential expression of pancreatic protein and chemosensing receptor mRNAs in NKCC1-null intestine

Emily M Bradford, Kanimozhi Vairamani, Gary E Shull

Emily M Bradford, Department of Internal Medicine, Division of Gastroenterology, University of Kentucky, Lexington, KY 40536-0298, United States

Kanimozhi Vairamani, Gary E Shull, Department of Molecular Genetics, Biochemistry and Microbiology, University of Cincinnati College of Medicine, Cincinnati, OH 45267-0524, United States

Author contributions: Bradford EM and Shull GE conceived the study, analyzed the data, and wrote the manuscript; Vairamani K analyzed and compiled microarray data; Bradford EM performed all of the experiments.

Supported by National Institutes of Health to Gary E Shull, No. DK050594.

Institutional review board statement: Because human subjects or tissues were not used in this study, approval from the institutional review board was not required. Ethical issues relating to the animal protocol were reviewed and approved by the Institutional Animal Care and Use Committee of the University of Cincinnati.

Institutional animal care and use committee statement: All procedures involving animals were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Cincinnati (protocol number: 06-06-22-02).

Conflict-of-interest statement: The authors have no conflict of interest related to this manuscript.

Data sharing statement: The microarray data have been deposited in the National Center for Biotechnology Gene Expression Omnibus and can be freely accessed as described in Methods. The Slc12a2 (NKCC1) knockout mouse model has been deposited in a publically available repository and can be accessed as described in Methods.

Correspondence to: Gary E Shull, PhD, Professor, Department of Molecular Genetics, Biochemistry and Microbiology, University of Cincinnati College of Medicine, 231 Albert Sabin Way, Cincinnati, OH 45267-0524, United States. shullge@ucmail.uc.edu
Telephone: +1-513-5580056
Fax: +1-513-5591885

Received: June 29, 2015
Peer-review started: July 2, 2015
First decision: September 22, 2015
Revised: October 10, 2015
Accepted: December 17, 2015
Article in press: December 18, 2015
Published online: February 15, 2016

Abstract

AIM: To investigate the intestinal functions of the NKCC1 Na^+-K^+-2Cl cotransporter (*SLC12a2* gene), differential mRNA expression changes in NKCC1-null intestine were analyzed.

METHODS: Microarray analysis of mRNA from intestines of adult wild-type mice and gene-targeted NKCC1-null mice (n = 6 of each genotype) was performed to identify patterns of differential gene expression changes. Differential expression patterns were further examined by Gene Ontology analysis using the online Gorilla program, and expression changes of selected genes were verified using northern blot analysis and quantitative real time-polymerase chain reaction. Histological staining and immunofluorescence were performed to identify cell types in which upregulated pancreatic digestive enzymes were expressed.

RESULTS: Genes typically associated with pancreatic function were upregulated. These included lipase, amylase, elastase, and serine proteases indicative of pancreatic exocrine function, as well as insulin and regenerating islet genes, representative of endocrine function. Northern blot analysis and immunohistochemistry showed that differential expression of exocrine pancreas mRNAs was specific to the duodenum and localized to a subset of goblet cells. In addition, a major pattern of changes involving differential expression of olfactory receptors that function in chemical sensing, as well as other chemosensing G-protein coupled receptors, was observed. These changes in chemosensory receptor expression may be related to the failure of intestinal function and dependency on parenteral nutrition observed in humans with *SLC12a2* mutations.

CONCLUSION: The results suggest that loss of NKCC1 affects not only secretion, but also goblet cell function and chemosensing of intestinal contents *via* G-protein coupled chemosensory receptors.

Key words: *SLC12a2*; Chemosensory; Chemosensitivity; Gastrointestinal; Dyspepsia

Core tip: The NKCC1 Na^+-K^+-$2Cl^-$ cotransporter is a major mechanism of Cl^- uptake in support of secretion. To investigate its intestinal functions we analyzed mRNA expression changes in NKCC1-null intestines. Differentially expressed genes included digestive enzymes and a large number of olfactory and other G-protein coupled receptors that function in chemical sensing. This suggests that loss of NKCC1 affects not only secretion, but also digestion and chemosensing of components of the intestinal contents. The results likely have relevance to recent evidence showing that mutations in the human *NKCC1* gene cause unexplained food intolerance and failure of intestinal function requiring parenteral nutrition.

Bradford EM, Vairamani K, Shull GE. Differential expression of pancreatic protein and chemosensing receptor mRNAs in NKCC1-null intestine. *World J Gastrointest Pathophysiol* 2016; 7(1): 138-149 Available from: URL: http://www.wjgnet.com/2150-5330/full/v7/i1/138.htm DOI: http://dx.doi.org/10.4291/wjgp.v7.i1.138

INTRODUCTION

The NKCC1 Na^+-K^+-$2Cl^-$ cotransporter, encoded by the *SLC12A2* gene, is expressed in all mammalian tissues and is particularly prominent in epithelial cells of the gastrointestinal tract[1]. Detailed immunohistochemical studies show that it is localized to basolateral membranes of intestinal secretory epithelia[1], where it mediates uptake of much of the Cl^- that is then secreted through the apical cystic fibrosis transmembrane conductance regulator (CFTR). In addition to secretory epithelia, NKCC1 is expressed in all segments of the gastrointestinal tract and throughout the crypt and villus epithelium, including both absorptive cells and goblet cells[1]. Thus, loss or inhibition of NKCC1 could have wide-ranging effects on gastrointestinal function. In fact, a National Institutes of Health web site (http://www.genome.gov/27551936) on Rare or Undiagnosed Diseases reports that humans with *SLC12A2* mutations have "exercise intolerance, dilated cardiomyopathy, and episodic hypoglycemia progressing to unexplained failure of intestinal function/food tolerance with subsequent parenteral nutrition dependency". The basis for the severe intestinal phenotype in humans is not known, but since it does not mimic the Cystic Fibrosis intestinal phenotype, it is unlikely to be due entirely to the secretory defect.

When anion secretion is impaired, the intestinal lumen can become desiccated, which leads to constipation, obstructions, and intussusception. Mice lacking CFTR usually develop lethal intestinal obstructions by 6 wk of age and require treatment with an osmotic laxative for normal survival[2], but the intestinal phenotype of *Nkcc1*$^{-/-}$ mice is much less severe[3]. In the original study, a small percentage of the *Nkcc1*$^{-/-}$ mice on a mixed genetic background developed intestinal impactions[3]. However, *Nkcc1*$^{-/-}$ mice on the FVB/N background (Swiss mice carrying the Fv1b sensitizing allele) that were used for the current study do not develop impactions, and the intestinal tract appears histologically normal.

Loss of NKCC1 in mice causes a defect in intestinal anion secretion as measured by reductions in the bumetanide-sensitive component in short circuit currents in duodenum, jejunum, and cecum[3-5]. However, compensatory pathways for basolateral Cl^- uptake have been identified in duodenum and colon[5,6], providing an explanation of why the loss of NKCC1 does not cause a profound defect in anion secretion in the intestinal tract. To gain a better understanding of the functions of NKCC1 and the molecular consequences of NKCC1-deficiency in the intestine, we performed microarray analyses of mRNA from *Nkcc1*$^{-/-}$ small intestine. The observed alterations in the expression of mRNAs encoding digestive enzymes and receptors that mediate chemical sensing could be involved in intestinal pathologies resulting from the inhibition or loss of NKCC1.

MATERIALS AND METHODS

NKCC1-null mutant mouse model

Nkcc1$^{-/-}$ mice were generated as previously described[3] and inbred onto the FVB/N background for at least 10 generations. FVB/N are Swiss mice, derived from the HFSF/N strain, which carry the Fv1b (Friend Leukemia Virus strain B) sensitizing allele. These *Nkcc1*$^{-/-}$ mice are available from the Jackson Labs Mutant Mouse Regional Resource Center and can be identified by searching

Table 1 Primer sequences used for quantitative real-time polymerase chain reaction and northern blot analyses

Name	Sequence
Pdx1	F: ACCAAAGCTCACGCGTGGAAA
	R: TGATGTGTCTCTCGGTCAAGTT
Insulin 1	F: TTGCCCTCTGGGAGCCCAAA
	R: CAGATGCTGGTGCAGCACTG
Insulin 2	F: TCTTCCTCTGGGAGTCCCAC
	R: CAGATGCTGGTGCAGCACTG
Glut2	F: CATTGCTGGAAGAAGCGTATCAG
	R: GAGACCTTCTGCTCAGTCGACG
Amylase	F: TTAACGATAATAAATGTAATGGAGAA
	R: CCCTGCTACTCCAATGTCAA
Trypsin	F: TGAGCAGTTTGTCAATTCTGCC
	R: GCATGATGTCGTTGTTCAGGG
Elastase	F: GTAGTTGCAGCCCAGAGAGG
	R: TCTGCTATGTCACAGGCTGG
Gp2	F: TCCCTGCCAGAATCACACGGT
	R: GGAGGTCCTGCCTACAGACACA
L32	F: GATCTAGCGGCCGCCATCTGTTTACGGCATCATG
	R: TAGATCGCGGCCGCCGCTCCCATAACCGATGTTGG

Forward and reverse primers for quantitative real-time polymerase chain reaction and northern blot probes are listed 5′ to 3′.

(http://www.jax.org/mmrrc/) with the gene symbol *Slc12a2* (strain name: FVB.Cg-Slc12a2tm1Ges). Wild-type (WT) FVB/N mice were originally obtained from Jackson Labs and bred in house. All experimental pairs were derived by breeding of NKCC1 heterozygous breeding pairs, were between 8 and 12 wk of age, and were age- and gender-matched. Mouse numbers (*n*) are indicated in methods or figure legends. Mice were maintained in a specific pathogen-free barrier facility, with access to food and water *ad libitum*, and all experiments were approved by the University of Cincinnati Animal Care and Use Committee.

Isolation of intestinal tissues

Mice were anesthetized by intraperitoneal injection of Avertin (2.5% solution of Tribromethanol) at 500 mg/kg, followed by cervical dislocation after the mice were anesthetized. Intestinal segments were removed and flushed with ice-cold phosphate-buffered saline. Some samples were frozen in liquid nitrogen and used for extraction of total RNA using Tri-Reagent (Molecular Research Center) and others were processed for immunolocalization studies as described in detail below.

Northern blot analysis and real-time polymerase chain reaction

Northern blot analysis was performed using 10 mg of total RNA per sample, and blots were hybridized with ^{32}P-labeled cDNA as previously described[7]. Primer sequences are given in Table 1. Quantification of band intensity, including background subtraction and normalization to the ribosomal L32 subunit mRNA, was done using ImageQuant software (Amersham Biosciences).

For quantitative real-time polymerase chain reaction (PCR), 4 μg of total RNA was reversed transcribed using oligo d(T) and SuperScript Ⅱ reverse transcriptase (Invitrogen). The cDNA was diluted 5-fold in water. Primer sequences are given in Table 1. For each primer set, optimization using serially-diluted standards and melting curve analysis was performed. Real-time PCR was run on a DNA Engine Opticon Ⅱ (MJ Research) thermalcycler using iQ SYBR Green (BioRad). For each gene, samples were run at least in duplicate, and mean values were normalized to the expression of *L32*. Standard curves were prepared for each gene on every run. All pairwise comparisons were done using a student's *t*-test; *P*-values of < 0.05 were considered significant.

Morphology and immunofluorescence

Intestinal segments were fixed in 10% neutral buffered formalin overnight and embedded in paraffin. Goblet cells were identified with hemotoxylin and eosin (H and E) or Alcian Blue/Periodic Acid-Schiff (AB/PAS) staining as described previously[8]. For immunofluorescence, sections ($n = 4$ WT and 3 NKCC1-null mutant) were deparaffinized in Citrisolve, rehydrated through successive ethanol washes, and boiled for 5 min in a sodium citrate buffer. Sections were incubated with primary antibodies for 12 h at room temperature, washed, and incubated with either another primary antibody or the appropriate secondary antibodies for 12 h at room temperature. Polyclonal antibodies against amylase (Sigma), trypsin (Abcam), elastase (Abcam), and intestinal trefoil factor 3 (TFF3) (ITF-Santa Cruz Biotechnology) were used at a 1:100 dilution; all secondary antibodies (Alexa Fluor, Molecular Probes) were used at a 1:200 dilution, and DAPI was included in the mounting medium (Vector Laboratories) for staining of nuclei. For localization studies using both fluorescence and AB/PAS, immunohistochemistry was done first and the slides were photographed. The slides were then washed, AB/PAS staining was performed, and the same areas were imaged. For colocalization studies, images were merged in Adobe Photoshop.

Microarray analysis

Microarray analyses were performed by the University of Cincinnati Genomics and Microarray Laboratory core facility exactly as described previously[8] using the Duke University Murine Operon v.3.0 spotted Array, which consisted of the Operon mouse oligonucleotide library, version 3.0, comprised of 70-mer oligonucleotides representing mouse genes. For analysis of NKCC1-null small intestine, total RNA was isolated from the entire small intestine of 8-wk old WT and NKCC1-null mutant (KO) mice ($n = 6$ of each genotype) on the FVB/N background, and cDNA was generated and labeled with Cy3 or Cy5 fluorophores and hybridized with the microarrays. The fold-changes and statistical significance for the microarray analysis were calculated exactly as described previously[8]. Briefly, Cy3 and Cy5 labeling

was alternated between WT and KO samples (dye-flip controls) to control for the influence of fluorophore labeling on probe hybridization. Values are represented as fold-changes (ratio of fluorescence intensity values), with positive values indicating upregulation and negative values indication downregulation in KO relative to WT. The complete set of microarray data as well a list of the significantly changed genes and more detailed methods have been deposited in the National Center for Biotechnology Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/) and are available through GEO accession number GSE19117. The statistical analysis of the data has been reviewed by Dr. Mario Medvedovic, Director of the Division of Biostatistics and Bioinformatics, Department of Environmental Health, University of Cincinnati.

Gene Ontology analysis

Gene Onlology analysis was performed using the online GOrilla program[9] (Gene Ontology enRIchment anaLysis and visuaLizAtion tool) (http://cbl-gorilla.cs.technion.ac.il/). Two analysis options were used: (1) Single Rank List, with the entire gene set ranked according to *P* values; and (2) Two Unranked Lists, in which a target list of genes with a specific range of *P* values is compared against the list of all genes analyzed (13551 total). The Single Rank option avoids setting an arbitrary cutoff of *P* values and was useful for identifying GO categories that were highly enriched at the top end of the significance range. In the Two List analysis, significance and enrichment was calculated based on the number of genes in each GO category that appear in the target list and background list. Enrichment (N, B, n, b) is defined as: N is the total number of genes, B is the total number of genes associated with a specific GO term, n is the number of genes in the top of the user's input list or in the target set when appropriate, b is the number of genes in the intersection; Enrichment = (b/n)/(B/N). The program calculates statistical probabilities using the hypergeometric distribution[9].

Expression atlas data

RNA Seq mRNA expression data for human duodenum and pancreas was obtained from the European Bioinformatics Institute (EBI) Expression Atlas (http://www.ebi.ac.uk/gxa/home). This data base allowed an evaluation of the relative expression levels of the various pancreatic genes that were upregulated in the duodenum of *Nkcc1*$^{-/-}$ mice. The data were available through baseline experiments, Uhlen's lab, in which 32 human tissues are represented.

RESULTS

Upregulation of pancreatic genes in NKCC1-null small intestine

Microarray analysis was performed using small intestine RNA from the *Nkcc1*$^{-/-}$ and WT mice. Among the top 30 genes exhibiting differential expression in the *Nkcc1*-null intestine, all were upregulated, with induction ranging from 3.5-fold to 15.8-fold (Table 2). A pattern of genes typically associated with the exocrine pancreas, namely the digestive enzymes and components of the zymogen granules, was immediately apparent. Cpa1 is a pancreatic carboxypeptidase that was upregulated 15.8-fold. Elastases 1, 2, and 3B were all up-regulated and exhibited high fluorescence intensities. Several serine proteases, including trypsins 1, 2, 4, and 10, as well as chymotrypsins A, B and C, were also up-regulated. Amylase, another digestive enzyme produced by the pancreas, was increased 8.7-fold. Besides digestive enzymes, a number of the highly upregulated genes encode proteins that are associated with zymogen granules (see Discussion). These included glycoprotein 2 (Gp2, 3.9-fold), pancreatic protein disulfide isomerase (Pdia2, 4.5-fold), and syncollin (Sycn, 3.0-fold; $P < 0.001$, data not shown).

In addition to enzymes typical of the exocrine pancreas, genes commonly expressed in the endocrine pancreas were also upregulated. These included Reg1 and Reg2 (regenerating islet or pancreatic stone proteins)[10], which were upregulated 4.6- and 3.6-fold (Table 2), insulin 1 (Ins1, 1.92-fold; $P < 0.003$), and pancreatic-duodenal homeobox 1 (Pdx1; 2.53-fold, $P < 0.002$), which is heavily involved in regulation of β-cell function[11]. Glut2, which is the most abundant facilitative glucose transporter in the intestine[12], was also upregulated (1.65-fold, $P < 0.007$).

Northern blot analysis and quantitative RT-PCR of several genes validated the results of the microarray analysis (Figure 1). The expression of elastase (4.7-fold), amylase (1.9-fold), glycoprotein 2 (3.4-fold), and trypsin (6.0-fold) were all significantly up-regulated in the duodenum (Figure 1A and B). The Northern blot data indicated that the up-regulation of these genes observed in microarray analyses of the *Nkcc1*$^{-/-}$ small intestine occurs primarily in the duodenum. It should be noted that expression of these genes in the duodenum appeared to be robust and did not require long exposure times to be detected, even in the WT samples. RT-PCR confirmed upregulation of Pdx1, Ins1, and Glut2 in the *Nkcc1*$^{-/-}$ small intestine (Figure 1C).

Morphology of the NKCC1-deficient gastrointestinal tract

In previous studies the stomach, intestine, liver, and pancreas of *Nkcc1*$^{-/-}$ mice appeared histologically normal[3], and no apparent differences in the morphology of the gastrointestinal tract was observed in the current study. Based on the results of the microarray analysis and histology, it is evident that the basic population of cells in the intestine of the *Nkcc1*$^{-/-}$ mice is not significantly altered. Expression of the Paneth cell markers matrix metalloproteinase 7 and lysozyme were not significantly changed, and neither were the expression of the major goblet cell markers mucin 2 and TFF3. The expression

Table 2 Genes with the highest levels of differential expression in the *Nkcc1*-null intestine

Gene symbol	Description	Average intensity	Fold change	*P* value
Cpa1	Carboxypeptidase A1, pancreatic	1811	15.86	0.00001
Elane	Elastase 2	2118	12.05	0.00001
Glb1l3	Galactosidase, beta 1 like 3	234	10.27	0.0001
Try4	Trypsin 4	2251	9.91	0.00001
Rnase1	Ribonuclease 1, pancreatic RNase A	592	9.28	0.00001
Cpb1	Carboxypeptidase B1	866	9.15	0.00001
Amy2a5	Amylase 2, pancreatic	691	8.75	0.00001
Try10	Trypsin 10	2306	8.33	0.00001
Ctrc	Chymotrypsin C (caldecrin)	397	7.79	0.0003
Ctrb1	Chymotrypsinogen B1	3360	7.66	0.00001
Pnlip	Pancreatic lipase	1938	7.24	0.00001
Prss1	Protease, serine 1 (trypsin 1)	1481	6.93	0.00001
Cel	Bile salt activated lipase, pancreatic	356	6.73	0.00001
Cela3b	Elastase 3B, pancreatic	1576	6.69	0.00001
Prss2	Protease, serine 2 (trypsin II)	716	6.52	0.0001
Pnliprp1	Pancreatic lipase related protein 1	574	5.83	0.0001
Muc6	Mucin 6, gastric	152	5.78	0.0053
Tff2	Trefoil factor 2	1391	5.77	0.0200
Cela1	Elastase 1, pancreatic	549	4.84	0.0002
V1rh10	Vomeronasal 1 receptor, H10	107	4.66	0.0008
Calb3	Calbindin D9K	609	4.64	0.00001
Reg1	Lithostathine 1 (pancreatic stone protein 1)	5648	4.56	0.0008
Pdia2	Protein disulfide isomerase, pancreatic	292	4.47	0.0004
Akp3	Alkaline phosphatase 3, intestine	366	4.02	0.00001
Gp2	Glycoprotein 2 (zymogen granule membrane)	377	3.92	0.0001
Cep350	Centrosomal protein 350	141	3.92	0.0003
Bmper	BMP-binding endothelial regulator	318	3.78	0.0011
Olfr846	Olfactory receptor 846	432	3.66	0.0001
Reg2	Lithostathine 2 (pancreatic stone protein 2)	253	3.58	0.0383
Ctrl	Chymotrypsin like; chymotrypsin A	1431	3.49	0.0030

Differential expression is presented as fold-changes in *Nkcc1*$^{-/-}$ intestine relative to wild-type intestine. Average intensity (average of Cy3 and Cy5 fluorescence intensities) is roughly indicative of relative expression levels. *P* values are less than or equal to the numbers shown.

of sucrase-isomaltase (alpha glucosidase), associated with terminally-differentiated enterocytes, was also not significantly different. Collectively, these data support the histological assessment that the basic structure and cell populations in the gut of the mutant mice are not significantly changed.

Immunolocalization of pancreatic enzymes in the duodenum

Immunofluorescence of amylase, trypsin, and elastase identified cytoplasmic staining of a subset of cells in the crypts and along the villi. Morphologically, these cells were clearly goblet cells. Co-immunofluorescence experiments using an antibody against intestinal TFF3, which is a goblet-cell specific marker, demonstrated that amylase, elastase and trypsin co-localize with Tff3 in goblet cells (Figure 2). Although mucus is known to bind antibodies non-specifically, in sections containing only the primary or secondary antibody, the goblet cells excluded all background fluorescence, indicating that the staining for each antibody was specific.

Interestingly, all amylase-positive cells contained TFF3; however, not all TFF3-positive cells were amylase-positive. This indicates that only a subset of goblet cells produce pancreatic-type peptidases and auxiliary zymogen granule proteins. Immunolocalization of amylase in cells that were also stained with AB/PAS provided further evidence that expression was occurring in a subset of goblet cells, as there were AB/PAS-positive goblet cells that lacked amylase (Figure 3). In some cases, it was possible to identify an amylase or elastase-positive cloud or cap over the lumenal border of the cells, and above the mucus, suggesting that these proteins are secreted from a separate compartment, or from separate granules, than TFF3-labeled mucins.

Gene Ontology analysis of differentially expressed genes in Nkcc1$^{-/-}$ *intestine*

The differentially expressed genes were subjected to Gene Ontology analysis using the online GOrilla program[9], and significant GO categories were identified (Table 3). Using the single rank option (see Methods), the GO categories included those dealing with digestion and closely related protease and triglyceride lipase activities, which were highly enriched in the top end of the significance range. Sensory perception and the closely related olfactory receptor/G-protein coupled receptor activities were also enriched using the single rank option; these categories included genes spanning a much greater significance range; this was particularly apparent for olfactory receptor activities. Similar results were obtained using the two list option and a

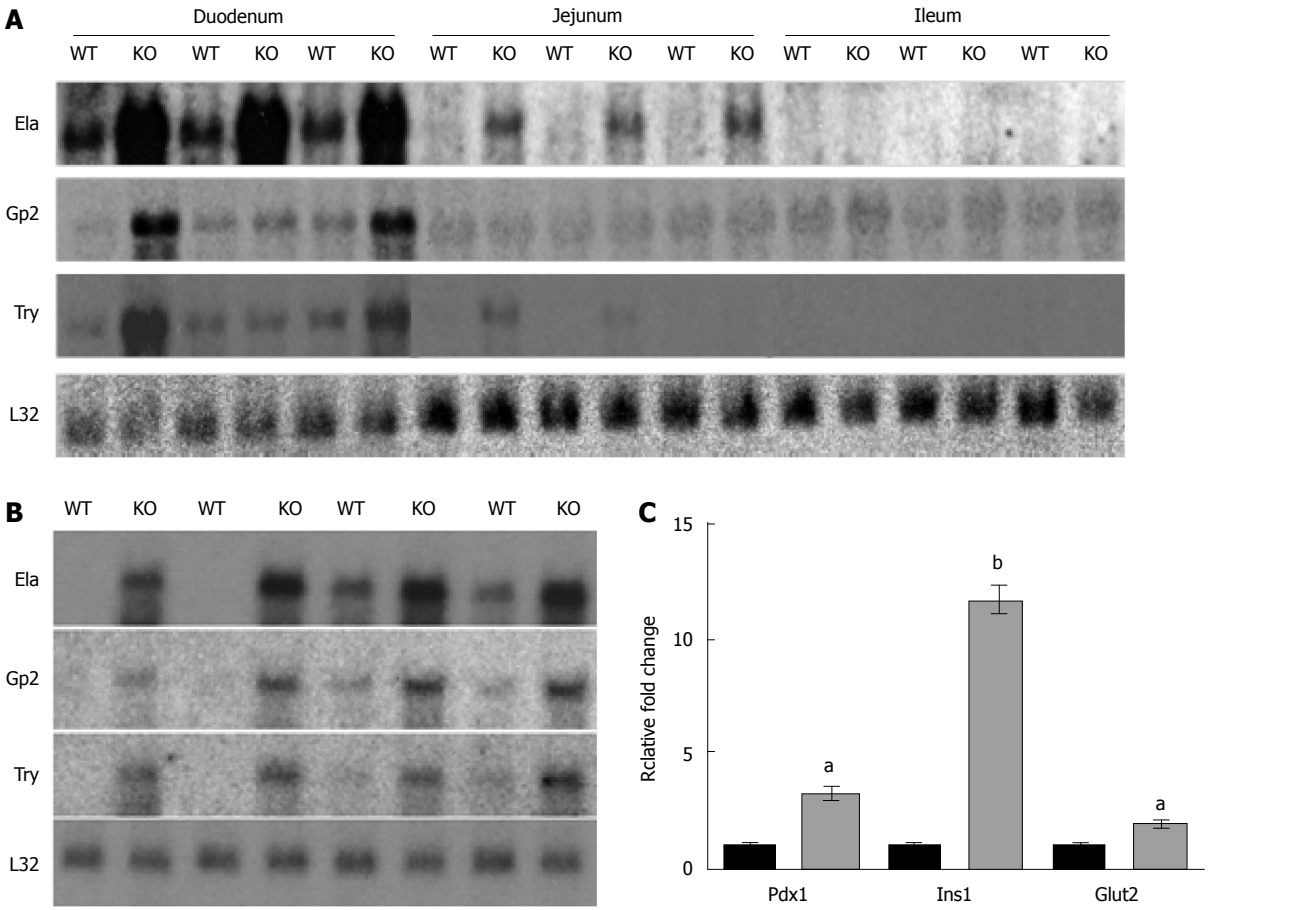


Figure 1 Northern blot and polymerase chain reaction analysis of duodenal gene expression. A: Northern blots show upregulation of Ela, Gp2, and Try primarily in the duodenum of *Nkcc1*$^{-/-}$ (KO) mice relative to WT; *n* = 3 WT and 3 KO for each segment; B: Duodenal tissue from a separate cohort of mice confirms upregulation of elastase (4.7-fold, *P* = 0.002), glycoprotein 2 (3.5-fold, *P* = 0.02) and trypsin (6.0-fold, *P* = 0.002); *n* = 4 WT and 4 KO; C: Quantitative real-time polymerase chain reaction analysis of dx1, Ins1, and Glut2 shows increased expression of these genes in *Nkcc1*$^{-/-}$ duodenum. Expression levels were normalized using the L32 ribosomal subunit mRNA; *n* = 4 WT (black bars) and 4 KO (grey bars). $^{a}P < 0.01$ *vs* WT, $^{b}P < 0.002$ *vs* WT. Gp2: Glycoprotein 2; Ela: Elastase; Try: Trypsin; WT: Wild-type; KO: *Nkcc1*$^{-/-}$; Pdx1: Pancreatic duodenal homeobox 1; Ins1: Insulin1; Glut2: Glucose transporter type 2.

significance cutoff of $P < 0.025$, which analyzed the top 390 genes out of 13551 associated with known GO categories.

The most statistically significant GO categories included genes encoding the pancreatic enzymes that were discussed above, along with genes for additional peptidases and lipases, most of which are directed at digestion of food in the intestinal tract (Table 4). Most of these genes were upregulated; the few that were modestly down-regulated were peptidases with either no apparent role in digestion (*Otud4*, *Dpep3*, and *Hpn*) or that were expressed at very low levels for a digestive enzyme, such as pepsinogen 5.

The most intriguing changes involved altered expression of receptors involved in chemical sensing (Table 5). These included some G-protein coupled receptors (GPCRs) that are known to be involved in chemical sensing in the intestinal tract, such as Gpbar1[13] and Ffar2[14], as well as a large number of G-protein receptors of the olfactory receptor family and several of the vomeronasal receptor family. As discussed below, this suggests that the loss of NKCC1 affects chemosensing in the intestinal tract, and also suggests that olfactory and vomeronasal receptors play a major role in this process.

DISCUSSION

The results of this study show that the loss of NKCC1 in small intestine leads to upregulation of digestive enzymes, many of which are typical of the exocrine pancreas, and to differential expression of a large number of genes encoding chemosensing receptors. As noted in the Introduction, loss-of-function mutations in the *SLC12A2* (NKCC1) gene lead to unexplained failure of intestinal function/food tolerance (http://www.genome.gov/27551936). Although it is possible that impaired secretion contributes to this phenotype, considerable compensation for loss of NKCC1-mediated basolateral Cl^--uptake occurs in the intestine[5,6] and Cystic Fibrosis patients and mice exhibit a more profound secretory defect than NKCC1-null mice. This suggests that factors other than impaired secretion account for the severe intestinal phenotype in humans with *Nkcc1* mutations.

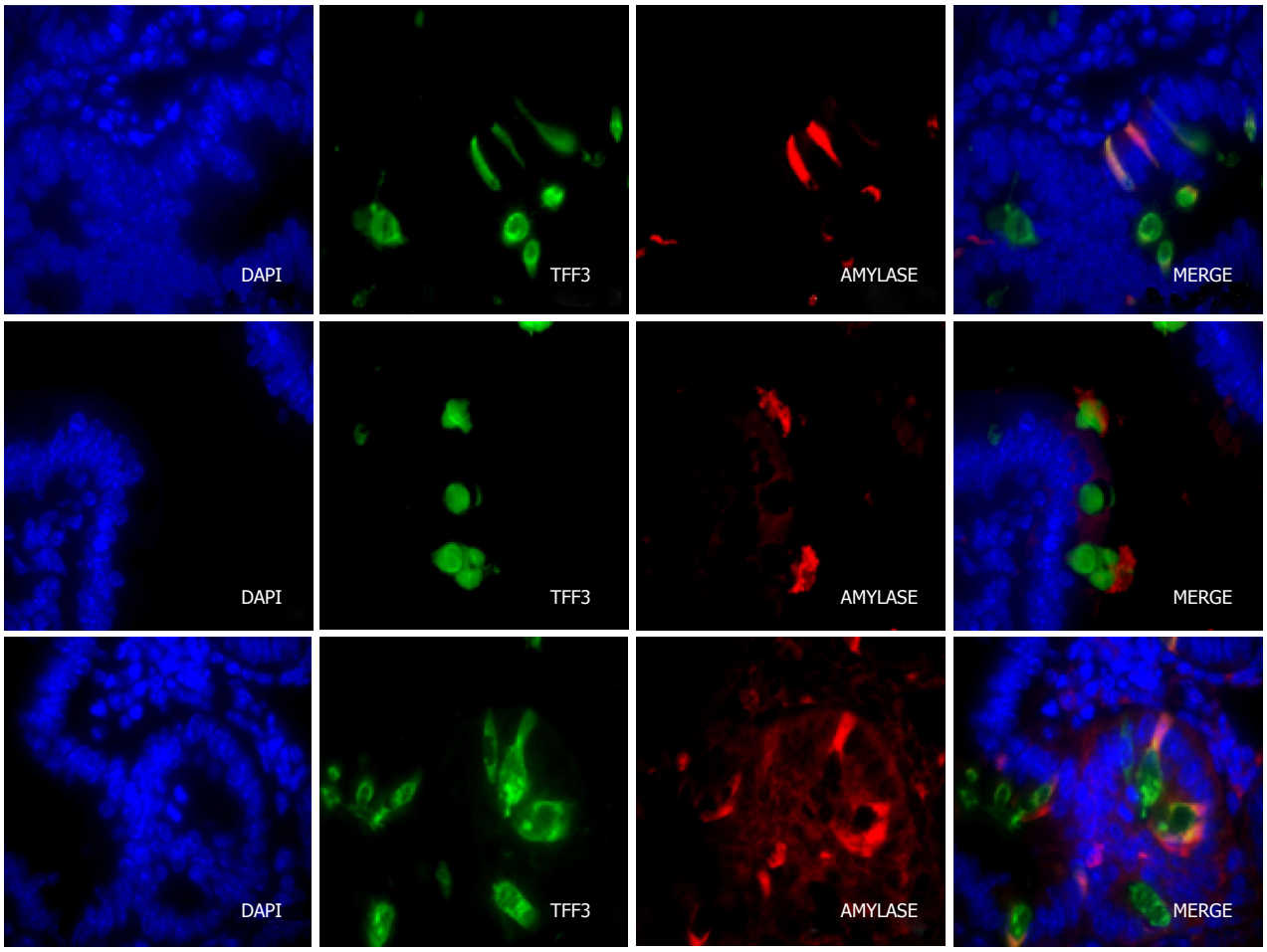


Figure 2 Colocalization of peptidases in goblet cells of the duodenum. DAPI (blue) identifies nuclei and TFF3 (intestinal trefoil factor, green fluorescence) identifies goblet cells. Elastase, amylase and trypsin are identified by red fluorescence. The merged images show colocalization of each of these proteases and TFF3. Staining patterns were assessed in 4 wild-type and 3 *Nkcc1*$^{-/-}$ mice. The images shown are for NKCC1-null mice. Control sections with only primary or only secondary antibodies do not identify goblet cells (not shown).

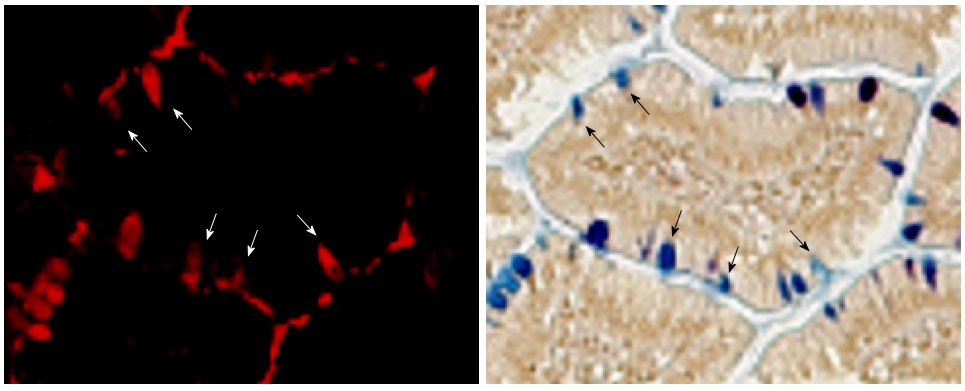


Figure 3 Amylase is expressed in a subset of goblet cells. Immunofluorescence of amylase (top, red) identifies many, but not all, goblet cells that are clearly stained with AB/PAS (bottom). The same sections (slides) from NKCC1-null intestine was used for immunofluorescence, imaged, and then stained with AB/PAS. Arrows indicate the same cell in both images. AB/PAS: Alcian Blue/Periodic Acid-Schiff.

The most striking result of the microarray analyses, although perhaps of limited clinical importance (discussed below), was the increased expression of digestive enzymes that are normally expressed at high levels in the pancreas and the restriction of their expression to a subset of goblet cells. Because it is critical to prevent premature release of digestive enzymes within the pancreas, many digestive enzymes are produced and packaged as inactive forms in zymogen granules, which release the enzymes upon secretion into the duodenum. Several genes that encode proteins involved in zymogen granule function (Gp2, Sync, and Pdia2),

Table 3 Significantly enriched Gene Ontology categories

GO category	*P*-value	Enrichment	(*N*, B, *n*, b)
Single ranked list			
GO:0008236 Serine-type peptidase activity	3.38E-14	35.6	(13551, 135, 31, 11)
GO:0008233 Peptidase activity	7.66E-11	12.6	(13551, 451, 31, 13)
GO:0007586 Digestion	1.82E-10	209.8	(13551, 19, 17, 5)
GO:0007600 Sensory perception	3.45E-8	1.67	(13551, 817, 1270, 128)
GO:0006508 Proteolysis	4.01E-8	7.65	(13551, 743, 31, 13)
GO:0004984 Olfactory receptor activity	7.11E-8	1.82	(13551, 552, 1270, 94)
GO:0004806 Triglyceride lipase activity	7.01E-7	271.0	(13551, 10, 15, 3)
GO:0004930 GPCR activity	3.86E-5	2.41	(13551, 512, 352, 32)
Two unranked lists (target and background)			
GO:0008236 Serine-type peptidase activity	6.03E-8	4.63	(13551, 135, 390, 18)
GO:0007586 Digestion	5.79E-7	12.8	(13551, 19, 390, 7)
GO:0004930 GPCR activity	1.30E-5	2.24	(13551, 512, 390, 33)
GO:0004984 Olfactory receptor activity	5.86E-5	2.08	(13551, 552, 390,3 3)
GO:0004806 Triglyceride lipase activity	1.24E-4	13.9	(13551, 10, 390, 4)
GO:0008233 Peptidase activity	2.71E-4	2.08	(13551, 451, 390, 27)

GO categories were identified using both the Single rank and Two unranked list options of the GOrilla Gene Ontology program[9]. *N* is the total number of genes, B is the total number of genes associated with a specific GO term, *n* is the number of genes in the target set, b is the number of genes in the intersection. Enrichment = (b/*n*)/(B/*N*). GPCR: G-protein coupled receptor; GO: Gene Ontology.

Table 4 Digestive enzymes and peptidases altered in small intestine of *Nkcc1*$^{-/-}$ mice

Gene symbol	Description	Average intensity	Fold change	*P*-value
Cpa1	Carboxypeptidase A1, pancreatic	1811	15.86	0.00001
Elane	Elastase 2	2118	12.05	0.00001
Try4	Trypsin 4	2251	9.91	0.00001
Cpb1	Carboxypeptidase B1 (tissue)	866	9.15	0.00001
Try10	Trypsin 10	2306	8.33	0.00001
Ctrc	Chymotrypsin C (caldecrin)	397	7.79	0.0003
Ctrb1	Chymotrypsinogen B	3360	7.66	0.00001
Pnlip	Pancreatic lipase	1938	7.24	0.00001
Prss1	Protease, serine 1 (trypsin 1)	1481	6.93	0.00001
Cel	Carboxyester lipase, pancreatic	356	6.73	0.00001
Cela3b	Elastase 3B, pancreatic	1576	6.69	0.00001
Prss2	Trypsin Ⅱ, anionic	716	6.52	0.0001
Pnliprp1	Pancreatic lipase related protein 1	574	5.83	0.0001
Cela1	Elastase 1, pancreatic	549	4.84	0.0002
2210010C04Rik	RIKEN cDNA 2210010C04 gene	125	3.89	0.0005
Ctrl	Chymotrypsin like; chymotrypsin A	1431	3.49	0.0030
Klk1	Kallikrein 1	278	1.82	0.0180
Klk1b26	Kallikrein 1-related petidase b26	988	1.80	0.0030
Pnliprp2	Pancreatic lipase related protein 2	833	1.74	0.0181
Klk1b21	Kallikrein 21	386	1.66	0.0036
Cyp7b1	Cytochrome P450 7B1	100	1.53	0.0229
Prcp	Prolyl carboxypeptidase (angiotensinase C)	566	1.51	0.0047
Amz1	Archaelysin family metallopeptidase 1	887	1.35	0.0121
Otud4	OTU domain containing 4	524	-1.34	0.0221
Pga5	Pepsinogen 5, group Ⅰ	168	-1.42	0.0198
Dpep3	Dipeptidase 3	301	-1.48	0.0197
Hpn	Serine protease hepsin	206	-1.99	0.0211

Differential expression is presented as fold-changes in *Nkcc1*$^{-/-}$ intestine relative to wild-type intestine. Average intensity (average of Cy3 and Cy5 fluorescence intensities) is roughly indicative of relative expression levels. *P* values are less than or equal to the numbers shown.

were upregulated. Glycoprotein 2 is the most abundant protein on zymogen granules and is thought to regulate exocytosis at the apical pole of the granule[15]. Syncollin is found on the luminal surface of zymogen granules, and syncollin-deficient mice exhibit impaired exocytosis of granules from pancreatic acinar cells[16]. Pancreatic protein disulfide isomerase plays a major role in forming the disulfide linkages of secretory proteins and has been localized to zymogen granule membranes[17]. These expression patterns are consistent with increased synthesis and packaging of zymogen granule proteins in the *Nkcc1*$^{-/-}$ intestine.

During embryonic development, the endoderm gives rise to the pancreas, liver, and the epithelial

Table 5 G-protein coupled, olfactory, and vomeronasal receptors

Gene symbol	Description	Average intensity	Fold change	*P*-value
V1rh10	Vomeronasal 1 receptor, H10	107	4.66	0.0008
Olfr846	Olfactory receptor 846	432	3.66	0.0001
Olfr491	Olfactory receptor 491	79	3.18	0.0051
Olfr419	Olfactory receptor 419	477	3.12	0.0004
Olfr827	Olfactory receptor 827	122	2.65	0.0001
Olfr992	Olfactory receptor 992	300	2.53	0.0005
Olfr506	Olfactory receptor 506	143	2.48	0.0049
Olfr812	Olfactory receptor 812	53	2.37	0.0048
Gpr63	G protein-coupled receptor 63	42	2.08	0.0023
Olfr1080	Olfactory receptor 1080	66	1.90	0.0107
V1rh1	Vomeronasal 1 receptor, H2	60	1.88	0.0071
Olfr344	Olfactory receptor 344	50	1.87	0.0057
Olfr1340	Olfactory receptor 1340	79	1.83	0.0123
Olfr378	Olfactory receptor 378	67	1.81	0.0072
Olfr643	Olfactory receptor 643	77	1.72	0.0080
Olfr1000	Olfactory receptor 996	475	1.72	0.0127
Olfr1134	Olfactory receptor 1134	139	1.71	0.0101
Olfr906	Olfactory receptor 906	134	1.68	0.0038
Olfr736	Olfactory receptor 736	92	1.67	0.0166
Olfr433	Olfactory receptor 433	189	1.65	0.0039
Olfr78	Olfactory receptor 78	125	1.62	0.0087
Olfr90	Olfactory receptor 90	106	1.56	0.0235
Prokr1	Prokineticin receptor 1	196	1.56	0.0096
Olfr1494	Olfactory receptor 1494	457	1.54	0.0180
Olfr1462	Olfactory receptor 1462	260	1.52	0.0183
Olfr444	Olfactory receptor 444	252	1.52	0.0154
Nmur1	Neuromedin U receptor 1	196	1.51	0.0177
Olfr1366	Olfactory receptor 1366	89	1.50	0.0207
Olfr804	Olfactory receptor 804	404	1.48	0.0175
Gpbar1	G protein-coupled bile acid receptor 1	129	1.47	0.0216
Gprc5a	G protein-coupled receptor, C5A	936	1.46	0.0142
Olfr740	Olfactory receptor 739	172	1.43	0.0164
Olfr1156	Olfactory receptor 152	156	1.43	0.0211
Rxfp4	Relaxin family peptide receptor 4	397	1.37	0.0132
Igf2r	Insulin-like growth factor 2 receptor	1089	-1.39	0.0158
Olfr641	Olfactory receptor 641	652	-1.39	0.0211
Olfr48	Olfactory receptor 140	99	-1.48	0.0143
Vmn1r210	Vomeronasal 1 receptor 210	192	-1.54	0.0127
Grm2	Glutamate receptor, metabotropic 2	179	-1.63	0.0028
Vmn1r5	Vomeronasal 1 receptor 5	124	-1.64	0.0071
Ffar2	Free fatty acid receptor 2	203	-1.85	0.0092

Differential expression is presented as fold-changes in *Nkcc1*$^{-/-}$ intestine relative to wild-type intestine. Average intensity (average of Cy3 and Cy5 fluorescence intensities) is roughly indicative of relative expression levels. *P* values are less than or equal to the numbers shown.

lining of the gastrointestinal tract. This requires the expression of various transcription factors, as well as interactions between the epithelium and surrounding mesenchyme[18,19]. It has become clear that the relative plasticity of the pancreas, liver, and intestine is high, and that certain populations of cells within these organs can undergo transdifferentiation. Although this initially seemed to be an interesting possibility, it is unlikely that transdifferentiation is occurring in the *Nkcc1*$^{-/-}$ small intestine. Several transcription factors, including Pdx1 and pancreas transcription factor 1 (Ptf1a) have been shown to mediate plasticity between the pancreas and small intestine. Expression of Pdx1 is a key regulatory step in the development of both the pancreas and the duodenum[20], and inactivation of Ptf1a drives pancreatic precursor cells toward intestinal cell fates[21]. Although expression of pancreatic genes was upregulated, Pdx1 was only modestly increased and expression of Ptf1a was not changed (data not shown). Furthermore, when we examined relative expression levels of the pancreatic enzymes in human pancreas and duodenum that are available through the EBI Expression Atlas (see methods) it was clear that the pancreatic enzymes are expressed at several orders of magnitude higher levels in pancreas than in duodenum. Thus, the apparently robust expression of pancreatic enzymes in the *Nkcc1*$^{-/-}$ intestine is likely to be far below the levels of expression occurring in the pancreas. Similarly, although insulin (Ins1) was mildly induced in *Nkcc1*$^{-/-}$ intestine, the EBI expression Atlas data indicates that insulin expression in human pancreas is far greater than that in duodenum (relative transcript levels 551 in pancreas *vs* 0.3 in duodenum). Although we obtained an antibody against human insulin, which readily and specifically identified

pancreatic islet cells in the mouse (data not shown), we were unable to detect insulin in *Nkcc1*$^{-/-}$ intestine, consistent with exceedingly low levels of expression.

The reason for the increased expression of pancreatic enzymes in goblet cells is unclear. Histological analyses gave no evidence for an increase in goblet cells in *Nkcc1*$^{-/-}$ intestine, and the expression levels of Tff3 (intestinal trefoil factor) and Muc2 (mucin 2), which are expressed at very high levels in goblet cells, were not significantly changed. However, NKCC1 has been shown to be expressed at high levels on basolateral membranes of goblet cells[1], and there is evidence that it affects mucous secretion[22]. Thus, it is possible that the absence of NKCC1 specifically in goblet cells has a direct effect on the expression of pancreatic enzymes. Whether this is due to subtle effects of NKCC1-deficiency on the differentiation of goblet cells is unclear.

Despite the changes in mRNA expression patterns between the WT and *Nkcc1*$^{-/-}$ intestine, previous studies of two different NKCC1 knockouts revealed no significant histological changes between the two genotypes in the adult intestine[3,4]. Furthermore, detailed morphometric analyses of the intestinal tracts of 8-wk-old WT and *Nkcc1*$^{-/-}$ mice, the same age as used in the current study, revealed no significant differences in cell structure[3]. We cannot, of course, rule out subtle changes in cell differentiation resulting from changes during early development or during regular turnover of the epithelium. However, the normal epithelial cell structure and relatively modest changes in expression of two of the major transcription factors involved in development of the pancreas and intestine, argue against defects in development and/or epithelial cell differentiation as the basis of the observed expression changes. Also, as discussed below, NKCC1 serves a direct role in the process of chemical sensing in olfactory neurons, suggesting that the changes in expression of chemosensing receptors is a direct response to the absence NKCC1 rather than being secondary to developmental defects.

Although less dramatic than the changes in pancreatic genes, the significant changes in expression of a large number of G-protein coupled chemosensing receptors suggest that the loss of NKCC1 may cause major impairments in the sensing of nutrients and other constituents of the intestinal contents. It is clear from many studies that GPCRs play a major role in intestinal chemosensing[23]. In fact, some of the GPCRs identified as differentially expressed in our microarray analyses have been shown to play important roles in chemosensing in the gut. Gpbar1 (1.47-fold increase) is expressed in enteroendocrine L-cells, where it senses bile acids and transduces a signal that leads to secretion of glucagon-like peptide 1 (GLP-1) and peptide tyrosine-tyrosine, thereby regulating glucose homeostasis[13,24]. Ffar2 (-1.85-fold decrease) is expressed in enteroendocrine cells and serves as a sensor of short-chain fatty acids[14]. Gpr63 (2.1-fold increase), which was expressed at relatively low levels, has been identified as a receptor for several lipids, including N-Arachidonylethanolamine[25] and both sphingosine 1-phosphate and dioleoylphasphatidic acid[26]. Grm2 (-1.63-fold decrease) is one of the metabotropic glutamate receptors that is involved in nutrient sensing through taste receptors[27]. Several of the differentially expressed GPCRs serve as receptors for peptide hormones that affect gut function. These include Rxfp4 (1.37-fold increase), which is the receptor for the orexigenic (appetite-stimulating) insulin-like peptide 5[28], and Nmur1 (1.51-fold increase), which is a receptor for neuromedin U and functions in glucose and appetite homeostasis[29].

The GPCRs that are known to play major roles in chemical sensing include taste receptors, olfactory receptors, and volmeronasal receptors. Taste receptors are known to function in the intestinal tract and to play major roles in nutrient sensing and subsequent signaling events[27,30-32]. There is less information about the role of olfactory receptors in chemosensing in the intestinal tract, but recent evidence indicates that olfactory receptors are expressed on colonic epithelial tissues, where they may detect bacterial metabolites in the luminal contents[33]. In addition, specific olfactory receptors have been shown to be differentially expressed in the duodenum of obesity-prone rats fed a high-fat diet[34]. The authors speculated that these receptors may be involved in sensing and responding to dietary fat. Specific olfactory receptors have also been identified in human enterochromaffin cells and when stimulated can lead to the release of serotonin[35,36]. Vomeronasal receptors also function in chemosensing[37], but as far as we are aware there is no previous evidence for their expression and function in the intestinal tract. Nevertheless, V1rh10 was the most highly upregulated gene among the G-protein coupled receptors (4.66-fold), indicating that it's expression is responsive to the loss of NKCC1.

Previous studies have shown that NKCC1 plays an important role in signaling through olfactory receptors. Odorant-induced Cl^- currents, which are required for signaling, were reduced in olfactory neurons of *Nkcc1*$^{-/-}$ mice[38], indicating that NKCC1 is an important mechanism for Cl^--uptake in olfactory neurons. However, later studies provided strong evidence for additional Cl^--loading mechanisms that can provide some compensation for the loss of NKCC1[39,40], and physiological studies indicated that WT and *Nkcc1*$^{-/-}$ mice have similar sensitivities to several specific odorants[41]. In more recent studies, however, loss of NKCC1 caused defects in the sensitivity to a complex mixture of odorants, and RNA Seq analysis of WT and *Nkcc1*$^{-/-}$ olfactory epithelium revealed significant differential expression changes involving a large number of olfactory receptors[42]. Thus, loss of NKCC1 leads to differential expression changes involving chemosensing receptors in both the olfactory epithelium[42] and in the small intestine, as indicated in the current study.

In summary, the microarray analysis of *Nkcc1*$^{-/-}$ small intestine indicates that the loss of NKCC1 leads both to increased expression of pancreatic enzymes in a subset of goblet cells and to differential expression of chemosensing GPCRs, including a large number of olfactory receptors. Because a number of GPCRs with well-established roles in chemosensing in the intestine were among the receptors that were changed, it seems likely that the differential expression of olfactory receptors is also indicative of altered sensing of components of the luminal contents. This finding raises the possibility that the episodic hypoglycemia and failure of intestinal function/food tolerance observed in human patients with NKCC1 (*SLC12A2* gene) mutations may be due to chronic deficiencies in their ability to properly sense and respond to nutrients and other constituents of the intestinal lumen. Finally, it should also be noted that loop diuretics that inhibit NKCC1 have been associated with an increased incidence of intestinal dysfunction, including constipation[43] and indications of a poor outcome following ischemic colitis[44]. It is therefore possible that inhibition of NKCC1 by these common therapeutic agents could lead to intestinal dysfunction similar to that observed in NKCC1 genetic deficiencies. Whether this is due to developmental abnormalities involving one or more cell types in the intestinal epithelium and/or is a more direct response to impaired chemosensing is unclear.

COMMENTS

Background

In the intestine, the basolateral NKCC1 Na^+-K^+-$2Cl^-$ cotransporter is the major mechanism of chloride uptake in support of secretion; however, it is not restricted to secretory epithelia and may serve other functions as well. To gain insights regarding novel functions of NKCC1 in the intestine, mRNA expression patterns in wild-type and NKCC1-null intestine were analyzed.

Research frontiers

This study was designed to determine changes in gene expression occurring in the intestinal tract of mice lacking NKCC1. Analysis of those patterns provided evidence that NKCC1 plays an important role in chemical sensing in the intestinal tract, a relatively new area of investigation.

Innovations and breakthroughs

This study demonstrates for the first time that mRNA levels for a large number of olfactory receptors and other G-protein coupled receptors involved in chemical sensing in the intestinal tract are altered in response to the loss of NKCC1.

Applications

The effects of *NKCC1* ablation on the expression of large numbers of chemosensory receptors raises the possibility that the failure of intestinal function in response to genetic mutations in the human *NKCC1* gene and side effects of loop diuretic treatment in the intestine could be due in part to impaired chemical sensing.

Terminology

The *Slc12a2* gene, encoding the NKCC1 Na^+-K^+-$2Cl^-$ cotransporter, was genetically ablated in the mouse model used in this study. The corresponding human gene is *SLC12A2*; mutations in human *SLC12A2* gene have been shown to cause failure of intestinal function.

Peer-review

This study aimed to analyze the mRNA expression changes in the intestine of *NKCC1*$^{-/-}$ mice. The methods of this study, methods were properly used to identify cell types, and results were also presented clearly.

REFERENCES

1 **Jakab RL**, Collaco AM, Ameen NA. Physiological relevance of cell-specific distribution patterns of CFTR, NKCC1, NBCe1, and NHE3 along the crypt-villus axis in the intestine. *Am J Physiol Gastrointest Liver Physiol* 2011; **300**: G82-G98 [PMID: 21030607 DOI: 10.1152/ajpgi.00245.2010]

2 **Clarke LL**, Gawenis LR, Franklin CL, Harline MC. Increased survival of CFTR knockout mice with an oral osmotic laxative. *Lab Anim Sci* 1996; **46**: 612-618 [PMID: 9001172]

3 **Flagella M**, Clarke LL, Miller ML, Erway LC, Giannella RA, Andringa A, Gawenis LR, Kramer J, Duffy JJ, Doetschman T, Lorenz JN, Yamoah EN, Cardell EL, Shull GE. Mice lacking the basolateral Na-K-2Cl cotransporter have impaired epithelial chloride secretion and are profoundly deaf. *J Biol Chem* 1999; **274**: 26946-26955 [PMID: 10480906 DOI: 10.1074/jbc.274.38.26946]

4 **Grubb BR**, Lee E, Pace AJ, Koller BH, Boucher RC. Intestinal ion transport in NKCC1-deficient mice. *Am J Physiol Gastrointest Liver Physiol* 2000; **279**: G707-G718 [PMID: 11005757]

5 **Walker NM**, Flagella M, Gawenis LR, Shull GE, Clarke LL. An alternate pathway of cAMP-stimulated Cl secretion across the NKCC1-null murine duodenum. *Gastroenterology* 2002; **123**: 531-541 [PMID: 12145806]

6 **Gawenis LR**, Bradford EM, Alper SL, Prasad V, Shull GE. AE2 Cl-/HCO3- exchanger is required for normal cAMP-stimulated anion secretion in murine proximal colon. *Am J Physiol Gastrointest Liver Physiol* 2010; **298**: G493-G503 [PMID: 20110461 DOI: 10.1152/ajpgi.00178]

7 **Woo AL**, Gildea LA, Tack LM, Miller ML, Spicer Z, Millhorn DE, Finkelman FD, Hassett DJ, Shull GE. In vivo evidence for interferon-gamma-mediated homeostatic mechanisms in small intestine of the NHE3 Na+/H+ exchanger knockout model of congenital diarrhea. *J Biol Chem* 2002; **277**: 49036-49046 [PMID: 12370192]

8 **Bradford EM**, Sartor MA, Gawenis LR, Clarke LL, Shull GE. Reduced NHE3-mediated Na+ absorption increases survival and decreases the incidence of intestinal obstructions in cystic fibrosis mice. *Am J Physiol Gastrointest Liver Physiol* 2009; **296**: G886-G898 [PMID: 19164484 DOI: 10.1152/ajpgi.90520]

9 **Eden E**, Navon R, Steinfeld I, Lipson D, Yakhini Z. GOrilla: a tool for discovery and visualization of enriched GO terms in ranked gene lists. *BMC Bioinformatics* 2009; **10**: 48 [PMID: 19192299 DOI: 10.1186/1471-2105-10-48]

10 **Qiu L**, List EO, Kopchick JJ. Differentially expressed proteins in the pancreas of diet-induced diabetic mice. *Mol Cell Proteomics* 2005; **4**: 1311-1318 [PMID: 15961380 DOI: 10.1074/mcp.M500016-MCP200]

11 **Kaneto H**, Matsuoka TA, Miyatsuka T, Kawamori D, Katakami N, Yamasaki Y, Matsuhisa M. PDX-1 functions as a master factor in the pancreas. *Front Biosci* 2008; **13**: 6406-6420 [PMID: 18508668]

12 **Thorens B**. GLUT2, glucose sensing and glucose homeostasis. *Diabetologia* 2015; **58**: 221-232 [PMID: 25421524 DOI: 10.1007/s00125-014-3451-1]

13 **Ullmer C**, Alvarez Sanchez R, Sprecher U, Raab S, Mattei P, Dehmlow H, Sewing S, Iglesias A, Beauchamp J, Conde-Knape K. Systemic bile acid sensing by G protein-coupled bile acid receptor 1 (GPBAR1) promotes PYY and GLP-1 release. *Br J Pharmacol* 2013; **169**: 671-684 [PMID: 23488746 DOI: 10.1111/bph.12158]

14 **Nøhr MK**, Pedersen MH, Gille A, Egerod KL, Engelstoft MS, Husted AS, Sichlau RM, Grunddal KV, Poulsen SS, Han S, Jones RM, Offermanns S, Schwartz TW. GPR41/FFAR3 and GPR43/FFAR2 as cosensors for short-chain fatty acids in enteroendocrine cells vs FFAR3 in enteric neurons and FFAR2 in enteric leukocytes. *Endocrinology* 2013; **154**: 3552-3564 [PMID: 23885020 DOI: 10.1210/en.2013-1142]

15 **Parker EM**, Zaman MM, Freedman SD. GP2, a GPI-anchored protein in the apical plasma membrane of the pancreatic acinar cell, co-immunoprecipitates with src kinases and caveolin. *Pancreas* 2000; **21**: 219-225 [PMID: 11039464]

16 **Wäsle B**, Turvey M, Larina O, Thorn P, Skepper J, Morton AJ, Edwardson JM. Syncollin is required for efficient zymogen granule exocytosis. *Biochem J* 2005; **385**: 721-727 [PMID: 15462671]

17 **Bruneau N**, Lombardo D, Levy E, Bendayan M. Roles of molecular chaperones in pancreatic secretion and their involvement in intestinal absorption. *Microsc Res Tech* 2000; **49**: 329-345 [PMID: 10820517]

18 **Montgomery RK**, Mulberg AE, Grand RJ. Development of the human gastrointestinal tract: twenty years of progress. *Gastroenterology* 1999; **116**: 702-731 [PMID: 10029630]

19 **Stainier DY**. No organ left behind: tales of gut development and evolution. *Science* 2005; **307**: 1902-1904 [PMID: 15790841]

20 **Offield MF**, Jetton TL, Labosky PA, Ray M, Stein RW, Magnuson MA, Hogan BL, Wright CV. PDX-1 is required for pancreatic outgrowth and differentiation of the rostral duodenum. *Development* 1996; **122**: 983-995 [PMID: 8631275]

21 **Kawaguchi Y**, Cooper B, Gannon M, Ray M, MacDonald RJ, Wright CV. The role of the transcriptional regulator Ptf1a in converting intestinal to pancreatic progenitors. *Nat Genet* 2002; **32**: 128-134 [PMID: 12185368]

22 **Dolganov GM**, Woodruff PG, Novikov AA, Zhang Y, Ferrando RE, Szubin R, Fahy JV. A novel method of gene transcript profiling in airway biopsy homogenates reveals increased expression of a Na+-K+-Cl- cotransporter (NKCC1) in asthmatic subjects. *Genome Res* 2001; **11**: 1473-1483 [PMID: 11544191]

23 **Reimann F**, Tolhurst G, Gribble FM. G-protein-coupled receptors in intestinal chemosensation. *Cell Metab* 2012; **15**: 421-431 [PMID: 22482725 DOI: 10.1016/j.cmet.2011.12.019]

24 **Thomas C**, Gioiello A, Noriega L, Strehle A, Oury J, Rizzo G, Macchiarulo A, Yamamoto H, Mataki C, Pruzanski M, Pellicciari R, Auwerx J, Schoonjans K. TGR5-mediated bile acid sensing controls glucose homeostasis. *Cell Metab* 2009; **10**: 167-177 [PMID: 19723493 DOI: 10.1016/j.cmet.2009.08.001]

25 **Yin H**, Chu A, Li W, Wang B, Shelton F, Otero F, Nguyen DG, Caldwell JS, Chen YA. Lipid G protein-coupled receptor ligand identification using beta-arrestin PathHunter assay. *J Biol Chem* 2009; **284**: 12328-12338 [PMID: 19286662 DOI: 10.1074/jbc.M806516200]

26 **Niedernberg A**, Tunaru S, Blaukat A, Ardati A, Kostenis E. Sphingosine 1-phosphate and dioleoylphosphatidic acid are low affinity agonists for the orphan receptor GPR63. *Cell Signal* 2003; **15**: 435-446 [PMID: 12618218]

27 **Xiao W**, Feng Y, Holst JJ, Hartmann B, Yang H, Teitelbaum DH. Glutamate prevents intestinal atrophy via luminal nutrient sensing in a mouse model of total parenteral nutrition. *FASEB J* 2014; **28**: 2073-2087 [PMID: 24497581 DOI: 10.1096/fj.13-238311]

28 **Grosse J**, Heffron H, Burling K, Akhter Hossain M, Habib AM, Rogers GJ, Richards P, Larder R, Rimmington D, Adriaenssens AA, Parton L, Powell J, Binda M, Colledge WH, Doran J, Toyoda Y, Wade JD, Aparicio S, Carlton MB, Coll AP, Reimann F, O'Rahilly S, Gribble FM. Insulin-like peptide 5 is an orexigenic gastrointestinal hormone. *Proc Natl Acad Sci USA* 2014; **111**: 11133-11138 [PMID: 25028498 DOI: 10.1073/pnas.1411413111]

29 **Dalbøge LS**, Pedersen SL, Secher T, Holst B, Vrang N, Jelsing J. Neuromedin U inhibits food intake partly by inhibiting gastric emptying. *Peptides* 2015; **69**: 56-65 [PMID: 25895852 DOI: 10.1016/j.peptides.2015.04.010]

30 **Mace OJ**, Lister N, Morgan E, Shepherd E, Affleck J, Helliwell P, Bronk JR, Kellett GL, Meredith D, Boyd R, Pieri M, Bailey PD, Pettcrew R, Foley D. An energy supply network of nutrient absorption coordinated by calcium and T1R taste receptors in rat small intestine. *J Physiol* 2009; **587**: 195-210 [PMID: 19001049 DOI: 10.1113/jphysiol.2008.159616]

31 **Wang JH**, Inoue T, Higashiyama M, Guth PH, Engel E, Kaunitz JD, Akiba Y. Umami receptor activation increases duodenal bicarbonate secretion via glucagon-like peptide-2 release in rats. *J Pharmacol Exp Ther* 2011; **339**: 464-473 [PMID: 21846840 DOI: 10.1124/jpet.111.184788]

32 **Rønnestad I**, Akiba Y, Kaji I, Kaunitz JD. Duodenal luminal nutrient sensing. *Curr Opin Pharmacol* 2014; **19**: 67-75 [PMID: 25113991 DOI: 10.1016/j.coph.2014.07.010]

33 **Kaji I**, Karaki S, Kuwahara A. Taste sensing in the colon. *Curr Pharm Des* 2014; **20**: 2766-2774 [PMID: 23886384]

34 **Primeaux SD**, Braymer HD, Bray GA. High fat diet differentially regulates the expression of olfactory receptors in the duodenum of obesity-prone and obesity-resistant rats. *Dig Dis Sci* 2013; **58**: 72-76 [PMID: 23053893 DOI: 10.1007/s10620-012-2421-z]

35 **Braun T**, Voland P, Kunz L, Prinz C, Gratzl M. Enterochromaffin cells of the human gut: sensors for spices and odorants. *Gastroenterology* 2007; **132**: 1890-1901 [PMID: 17484882]

36 **Kidd M**, Modlin IM, Gustafsson BI, Drozdov I, Hauso O, Pfragner R. Luminal regulation of normal and neoplastic human EC cell serotonin release is mediated by bile salts, amines, tastants, and olfactants. *Am J Physiol Gastrointest Liver Physiol* 2008; **295**: G260-G272 [PMID: 18556422 DOI: 10.1152/ajpgi.00056.2008]

37 **Chamero P**, Leinders-Zufall T, Zufall F. From genes to social communication: molecular sensing by the vomeronasal organ. *Trends Neurosci* 2012; **35**: 597-606 [PMID: 22658923 DOI: 10.1016/j.tins.2012.04.011]

38 **Reisert J**, Lai J, Yau KW, Bradley J. Mechanism of the excitatory Cl- response in mouse olfactory receptor neurons. *Neuron* 2005; **45**: 553-561 [PMID: 15721241]

39 **Nickell WT**, Kleene NK, Gesteland RC, Kleene SJ. Neuronal chloride accumulation in olfactory epithelium of mice lacking NKCC1. *J Neurophysiol* 2006; **95**: 2003-2006 [PMID: 16319203]

40 **Nickell WT**, Kleene NK, Kleene SJ. Mechanisms of neuronal chloride accumulation in intact mouse olfactory epithelium. *J Physiol* 2007; **583**: 1005-1020 [PMID: 17656441]

41 **Smith DW**, Thach S, Marshall EL, Mendoza MG, Kleene SJ. Mice lacking NKCC1 have normal olfactory sensitivity. *Physiol Behav* 2008; **93**: 44-49 [PMID: 17719611]

42 **Haering C**, Kanageswaran N, Bouvain P, Scholz P, Altmüller J, Becker C, Gisselmann G, Wäring-Bischof J, Hatt H. Ion transporter NKCC1, modulator of neurogenesis in murine olfactory neurons. *J Biol Chem* 2015; **290**: 9767-9779 [PMID: 25713142 DOI: 10.1074/jbc.M115.640656]

43 **Fosnes GS**, Lydersen S, Farup PG. Constipation and diarrhoea - common adverse drug reactions? A cross sectional study in the general population. *BMC Clin Pharmacol* 2011; **11**: 2 [PMID: 21332973 DOI: 10.1186/1472-6904-11-2]

44 **Mosli M**, Parfitt J, Gregor J. Retrospective analysis of disease association and outcome in histologically confirmed ischemic colitis. *J Dig Dis* 2013; **14**: 238-243 [PMID: 23419044 DOI: 10.1111/1751-2980.12045]

P- Reviewer: Koh TW, Wang YN
S- Editor: Ji FF **L- Editor**: A **E- Editor**: Jiao XK

World Journal of Gastrointestinal Pathophysiology

Submit a Manuscript: http://www.wjgnet.com/esps/
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.4291/wjgp.v7.i1.150

World J Gastrointest Pathophysiol 2016 February 15; 7(1): 150-159
ISSN 2150-5330 (online)

ORIGINAL ARTICLE

Basic Study

Neurophysiological mechanisms of bradykinin-evoked mucosal chloride secretion in guinea pig small intestine

Mei-Hua Qu, Wan-Sheng Ji, Ting-Kun Zhao, Chun-Yan Fang, Shu-Mei Mao, Zhi-Qin Gao

Mei-Hua Qu, Ting-Kun Zhao, Chun-Yan Fang, Shu-Mei Mao, Zhi-Qin Gao, Key Lab of Applied Pharmacology in Universities of Shandong, Weifang Medical University, Weifang 261053, Shandong Province, China

Wan-Sheng Ji, Department of Gastroenterology, Affiliated Hospital of Weifang Medical University, Weifang 261053, Shandong Province, China

Author contributions: Qu MH and Gao ZQ designed the study; Qu MH, Ji WS, Zhao TK, Fang CY, and Mao SM perform the experiments and data analysis; Qu MH wrote the paper.

Supported by Grants from the Shandong Provincial Natural Science Foundation, China, No. ZR2009CL047 and No. ZR2011HM043; the Scientific Research Foundation for Returned Scholars (Ministry of Education of China, 2011, 43th); Shandong Province Higher Educational Science and Technology Program, No. J14LK15; and Excellent Teachers International Training Cooperation Program of Shandong Province (for Yang XY and Qu MH).

Institutional animal care and use committee statement: All procedures involving animals were reviewed and approved by the Institutional Animal Care and Use Committee of the Weifang Medical University.

Conflict-of-interest statement: The authors have no conflicts of interest to declare.

Data sharing statement: No additional data are available.

Correspondence to: Mei-Hua Qu, PhD, MD, Key Lab of Applied Pharmacology in Universities of Shandong, Department of Pharmacology, Weifang Medical University, 7166 Baotong West Street, Weifang 261053, Shandong Province, China. qumeihua@hotmail.com
Telephone: +86-536-8462466
Fax: +86-536-8462466

Received: February 28, 2015
Peer-review started: March 26, 2015
First decision: April 20, 2015
Revised: April 29, 2015
Accepted: September 2, 2015
Article in press: September 7, 2015
Published online: February 15, 2016

Abstract

AIM: To investigate the mechanism for bradykinin (BK) to stimulate intestinal secretomotor neurons and intestinal chloride secretion.

METHODS: Muscle-stripped guinea pig ileal preparations were mounted in Ussing flux chambers for the recording of short-circuit current (*Isc*). Basal *Isc* and *Isc* stimulated by BK when preincubated with the BK receptors antagonist and other chemicals were recorded using the Ussing chamber system. Prostaglandin E2 (PGE_2) production in the intestine was determined by enzyme immunologic assay (EIA).

RESULTS: Application of BK or B2 receptor (B2R) agonist significantly increased the baseline *Isc* compared to the control. B2R antagonist, tetrodotoxin and scopolamine (blockade of muscarinic receptors) significantly suppressed the increase in *Isc* evoked by BK. The BK-evoked *Isc* was suppressed by cyclooxygenase (COX)-1 or COX-2 specific inhibitor as well as nonselective COX inhibitors. Preincubation of submucosa/mucosa preparations with BK for 10 min significantly increased PGE_2 production and this was abolished by the COX-1 and COX-2 inhibitors.

The BK-evoked *Isc* was suppressed by nonselective EP receptors and EP4 receptor antagonists, but selective EP1 receptor antagonist did not have a significant effect on the BK-evoked *Isc*. Inhibitors of PLC, PKC, calmodulin or CaMK Ⅱ failed to suppress BK-induced PGE_2 production.

CONCLUSION: The results suggest that BK stimulates neurogenic chloride secretion in the guinea pig ileum by activating B2R, through COX increasing PGE_2 production. The post-receptor transduction cascade includes activation of PLC, PKC, CaMK, IP_3 and MAPK.

Key words: Brandykinin; Ussing chamber; Brandykinin receptor; Cyclooxygenase; Prostaglandin E; Chloride secretion

Core tip: Bradykinin (BK) can stimulate intestinal chloride secretion and the firing of intestinal secretomotor neurons in the small intestine, but the mechanism is not well understood. In this study, muscle-stripped guinea pig ileal preparations were mounted in Ussing flux chambers for the recording of short-circuit current (*Isc*). BK agonist and BK antagonist were added to check the *Isc* change. Inhibitors of the signal transductors were pre-incubated with the tissue for 10 min before evoking with BK, and the *Isc* change was recorded. The change of prostaglandin E2 (PGE_2) secretion was detected by ELISA after treatment with BK for 3 h. Results suggest that BK stimulates neurogenic chloride secretion in the guinea pig ileum by activating B_2 receptors on secretomotor neurons, activating cyclooxygenase-1, and stimulating PGE_2 production. The post-receptor transduction cascade includes activation of PLC, PKC, CaMK, IP_3, and MAPK.

Qu MH, Ji WS, Zhao TK, Fang CY, Mao SM, Gao ZQ. Neurophysiological mechanisms of bradykinin-evoked mucosal chloride secretion in guinea pig small intestine. *World J Gastrointest Pathophysiol* 2016; 7(1): 150-159 Available from: URL: http://www.wjgnet.com/2150-5330/full/v7/i1/150.htm DOI: http://dx.doi.org/10.4291/wjgp.v7.i1.150

INTRODUCTION

Bradykinin (BK) is a nonapeptide that belongs to a group of structurally related 9-11 amino acid peptides (kinins), which are produced by kallikrein-mediated enzymatic cleavage of kininogen at the site of tissue injury and inflammation[1]. BK is formed in plasma and tissues in response to infection, tissue trauma, or inflammatory alterations, such as an increase in vascular permeability, edema formation, and pain. BK is widely distributed in the central and peripheral nervous systems, including the enteric nervous system[2,3]. Two subtypes of BK receptors, namely, BK receptor type 1 (B1R) and BK receptor type 2 (B2R), are identified based on their amino acid sequence and pharmacological properties[4,5]. BK receptors belong to the family of G-protein-coupled receptors with seven transmembrane helices. BK and kallidin are ligands for the constitutively expressed B2R, whereas *des*-Arg^9-BK (in rodents) and *des*-Arg^{10}-kallidin (in human) are ligands for the inducible B1R[6,7].

Previously, we demonstrated that B2R is expressed on a majority of the ganglion cells in the myenteric and submucosal plexuses in the small intestine of guinea pigs[8-10]. Exposing neurons in the guinea pig small intestinal myenteric or submucosal plexus to BK *in vitro* evokes slow activation of depolarization of the membrane potential and enhanced excitability characterized by increased firing frequency during intraneuronal injection of depolarizing current pulses in both AH- and S-type neurons and the appearance of anodal break excitation at the offset of hyperpolarizing current pulses in AH neurons[8,9]. The results suggested that BK acts *via* B2R on myenteric and submucosal neurons to stimulate the formation of prostaglandins. The eletrophysiologic data recorded using "sharp" microelectrodes suggested that BK might act in the enteric nervous system as a paracrine mediator to alter neural control of secretory and motility functions at the organ level.

This work aimed to investigate how the involvement of BK as an excitatory neuromodulator on submucosal secretomotor neurons at the cellular neurophysiological level translates to the physiology of intestinal secretion at the level of the integrated system[11,12].

MATERIALS AND METHODS

Tissue preparation

The animal protocol was designed to minimize pain or discomfort to the animals. The animals were acclimatized to laboratory conditions (23 ℃, 12 h/12 h light/dark, 50% humidity, *ad libitum* access to food and water) for two weeks prior to experimentation. Adult male Hartley-strain guinea pigs (300-350 g) were stunned by a sharp blow to the head and exsanguinated from the cervical vessels according to a protocol approved by Weifang Medical University Laboratory Animal Care and Use Committee. The tissue preparations were essentially conducted as described[13,14]. Briefly, segments of the small intestine were removed, flushed with ice-cold Krebs solution, and opened along the mesenteric border. The "muscle-stripped" preparations were obtained by removing the longitudinal and circular muscle layers together with the myenteric plexus by microdissection. The submucosal plexus remained intact with the mucosa. About 4-6 of the flat-sheet preparations were obtained from the ileum of each animal for mounting in Ussing flux chambers. The Krebs solution was composed of 120, 6, 2.5, 1.2, 1.35, 14.4, and 11.5 mM of NaCl, KCl, $CaCl_2$, $MgCl_2$, NaH_2PO_4, $NaHCO_3$, and glucose,

respectively.

Ussing flux chambers

Ussing flux chambers were equipped with a pair of Ag/AgCl electrodes *via* Krebs-agar bridges connected to Calomel half-cells for the measurement of transmural potential difference (PD). A second pair of electrodes was connected to an automated voltage clamp apparatus, which compensated for the solution resistance between the PD-sensing bridges. The flat-sheet preparations were mounted between halves of Ussing chambers, which had a total cross-sectional area of 0.64 cm^2. The tissues were bathed on both sides with 10 mL of Krebs solution and maintained at 37 ℃ by circulation from a temperature-controlled water bath. The necessary current to change the transepithelial PD by 2.5 mV was used to monitor tissue conductance and calculated according to the Ohm's law, as a determinant of tissue viability. Short-circuit current (*Isc*) was monitored by a voltage-clamp apparatus (DVC-1000, World Precision Instruments, Sarasota, FL). The mounted "muscle-stripped" preparations were balanced at 37 ℃ and bubbled with 95% O_2/5% CO_2 for 30 min before the preparations were treated with BK. Pharmacological agents were applied by addition to the bathing solution. BK (10 nM) was added to the serosal compartment of the chamber. To test the effect of other pharmacological agents on BK-evoked chloride secretion, pharmacological agents were added 10 min before the preparations were stimulated by BK (10 nM). The changes in *Isc* were calculated as ⊿*Isc*, and the effects of pharmacological agents were normalized to the cross-sectional area of the preparations. Each inhibitor was pre-incubated with the tissue for 10 min, and the *Isc* showed no change.

Prostaglandin E2 release

For these studies, all utensils and solutions were sterilized in 75% ethanol or autoclaved. The submucosa/mucosa preparations were prepared by carefully removing the longitudinal and circular muscle layers, as well as the myenteric plexus. The weight of each piece of preparations was recorded. The preparations were incubated in 2 mL of Dulbecco's modified Eagle's medium containing 100 U/mL penicillin G, 100 μg/mL streptomycin sulfate, 0.25 μg/mL amphotericin B, and glucose (12 mM) in a humidified 5% CO_2 incubator at 37 ℃. Preparations were pre-incubated for 30 min at 37 ℃ in the presence of FR122047, NS398, U73122, BisI, calmodulin inhibitor W7, or KT5720. Subsequently, 100 nM BK was added to each culture well, and a further 10 min or 3 h incubation was performed. The supernatant of each well was collected for prostaglandin E_2 (PGE_2) enzyme immunologic assay (EIA) (Cayman chemical, Ann Arbor, Michigan 48108 United States). EIA was preformed following the protocol provided by the manufacturer. Three independent experiments were performed, and the data were analyzed with Sigma plot software. Preparations without any treatment were used as controls.

Chemicals

BK acetate, B1R agonist BK fragment 1-8 acetate hydrate, B2R agonist [Hyp3]-BK, B2R antagonist HOE-140, tetrodotoxin (TTX), N-nitro-L-arginine methyl ester (L-NAME), cyclooxygenase (COX)-2 inhibitor NS398, COX-1 inhibitor FR122047, nonspecific COX inhibitors indomethacin and piroxicam, selective IP_3 receptor antagonist 2-APB, MAPK inhibitor PD98059, and tyrosine protein kinase inhibitor genistein were purchased from Sigma (St. Louis, MO). KT5720, W7, and nonselective VIP receptor antagonist VIP_{6-28} (human, bovine, porcine, rat) were obtained from BACHEM Bioscience, Inc., King of Prussia. U73122 (1-[6-[[(17β)-3-methoxyestra-1,3,5(100-trien-17-yl] amino]hexyl]-^{1}H-pyrrole-2,5-dione), bisindolylmaleimide I (2-[1-(3-dimethylaminopropyl)indol-3-yl]-3-(indol-3-yl)maleimide, BisI), and KN-62 were purchased from Tocris (Ellisville, MO). Stock solutions were prepared in Krebs solution or deionized H_2O, except for piroxicam, U73122, and KN-62, which were solubilized in DMSO and stored at -20 ℃. AH6809, SC-19220, and GW627368X were from Cayman Chemical (Ann Arbor, MI). The volume added to 10 mL of the bath solutions did not exceed 10 μL. Pharmacological agents were applied by addition to the Krebs' bathing solution.

Statistical analysis

Data are presented as mean ± SE. Student's *t*-test was used for the statistical analysis of significance of differences in the means with $P < 0.05$ considered significant. The statistical methods of this study were reviewed by Xiangyun Li from the Department of Statistics, Weifang Medical University.

RESULTS

BK-evoked increase in Isc is mediated by B2R

Baseline *Isc* and tissue conductance for submucosal/mucosal preparations from the guinea pig ileum were similar to those described previously (Fang *et al*[14], 2006). The addition of BK to the bathing solution on the submucosal side of the preparations evoked a rapid increase in *Isc*, and BK (10 nM), applied to the submucosal side of the submucosa/mucosa preparations, increased baseline *Isc* by 54.3 ± 5.4 μA/cm^2 (Figure 1). The B1R agonist, BK fragment 1-8 (1 μM), failed to elicit any changes in baseline *Isc*. The B2R agonist, [Hyp^3]-BK (1 μM), increased baseline *Isc* by 42.1 ± 5.7 μA/cm^2. Pre-incubation with the B2R antagonist, HOE-140 (1 μM), for 10 min significantly suppressed BK-evoked increase in *Isc* from 54.3 ± 5.4 to -1.1 ± 1.1 μA/cm^2 ($P < 0.01$). The results suggest that BK-evoked increase in *Isc* is mediated through B2R, but not B1R.

BK-evoked increase in Isc is mediated mainly by the enteric nervous system and by an increase of acetylcholine release

Stimulation of *Isc* by BK (10 nM) was significantly decreased in the presence of 1 μM of TTX from 57.8 ±

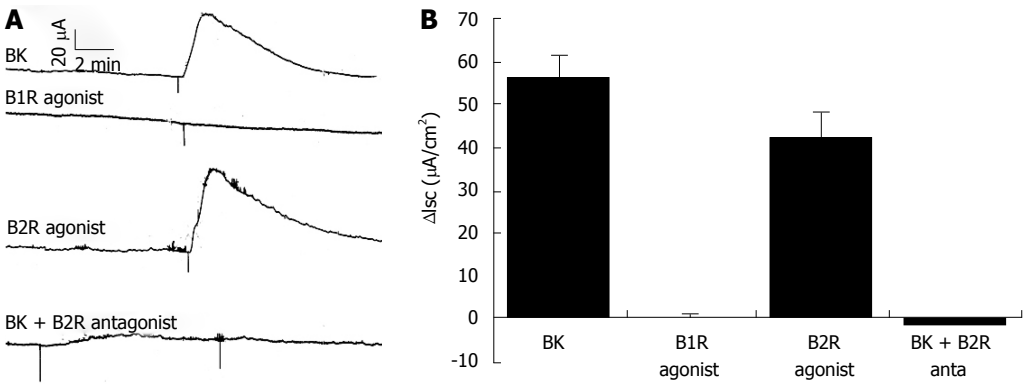


Figure 1 Bradykinin-evoked increase in short-circuit current is mediated through B2R but not B1R. A: Application of BK (10 nM) to the serosal side of the ileal preparation of guinea pigs ($P < 0.001$); B: Quantitative data showing the effect of B1 and B2 receptor agonists on baseline *Isc* and the effect of B2 receptor antagonist on BK-evoked *Isc*. Values are expressed as mean ± SE; $n = 6$-12 animals. BK: Bradykinin; *Isc*: Increase in short-circuit current.

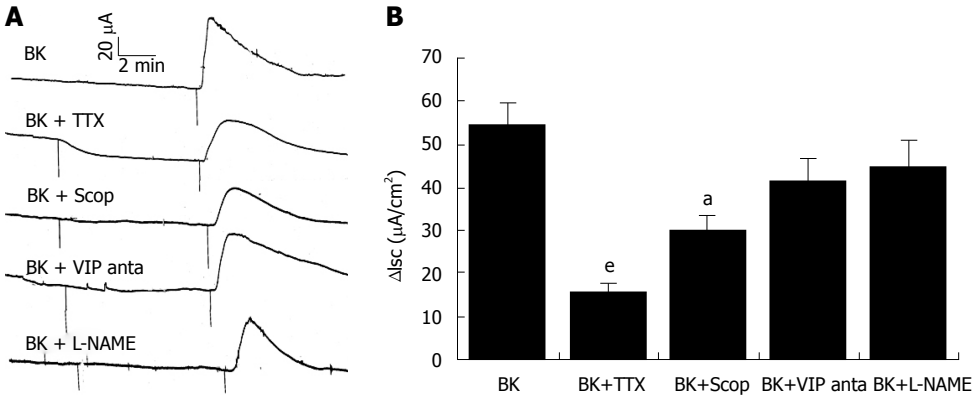


Figure 2 Bradykinin-evoked increase in short-circuit current is mediated mainly by the enteric nervous system and by an increase of acetylcholine release. A: Effect of tetrodotoxin (TTX; 1 μM), a muscarinic receptor antagonist, scopolamine (Scop; 1 μM), a VIP receptor antagonist (VIP 6-28; 1 μM) or an inhibitor of nitric oxide synthase, L-NAME (100 μM), on BK-evoked *Isc*; B: Quantitative data showing the effect of TTX, scopolamine, VIP antagonist and L-NAME on BK-evoked response in *Isc*. The vertical axis represents the changes of *Isc*. Values are expressed as mean ± SE, $n = 6$ animals. $^{a}P < 0.01$, $^{e}P < 0.001$ (*vs* BK alone). TTX: Tetrodotoxin; Scop: Scopolamine; VIP: Vasoactive intestinal peptide; BK: Bradykinin; *Isc*: Increases in short-circuit current.

7.1 μA/cm² to 15.6 ± 1.9 μA/cm² ($n = 6$; $P < 0.01$), which suggested that BK-evoked secretion was largely mediated by activation of the submucosal secretomotor neurons (Figure 2). The nonselective muscarinic acetylcholine antagonist, scopolamine; the vasoactive intestinal peptide (VIP) receptor antagonist, VIP_{6-28}; and the nitric-oxide synthase inhibitor, L-NAME; were used as pharmacological tools to test the kind of enteric neurons involved in BK-evoked secretory responses in the small intestine of guinea pigs. Pretreatment with scopolamine (1 μM) significantly suppressed the 10 nM BK-evoked increase of *Isc* from 54.3 ± 5.4 μA/cm² to 30.2 ± 9.4 μA/cm² ($n = 6$; $P < 0.01$). In the presence of VIP_{6-28} (1 μM) or L-NAME (100 μM), BK (10 nM) increased the baseline *Isc* to 41.5 ± 5.1 μA/cm² ($n = 6$; $P > 0.05$) or 44.6 ± 6.0 μA/cm² ($n = 6$; $P > 0.05$), respectively; neither showed a significant effect on 10 nM BK-evoked increase of *Isc*.

BK-evoked increase in Isc is mediated by the stimulation of prostaglandin release

The specific COX-2 inhibitor NS398 (10 μM), specific COX-1 inhibitor FR122047 (1 μM), and nonspecific COX inhibitors indomethacin (1 μM) and piroxicam (1 μM) were used to test a hypothesis that BK-evoked increase in *Isc* is mediated by prostaglandins, and the results are shown in Figure 3. Pretreatment with NS398 (10 μM) and FR122047 (1 μM) suppressed the 10 nM BK-evoked increases in *Isc* from 59.69 ± 8.45 μA/cm² to 22.8 ± 5.9 μA/cm² ($n = 6$; $P < 0.01$) and 25.3 ± 6.6 μA/cm² ($n = 6$; $P < 0.01$), respectively. The nonspecific COX inhibitors, indomethacin or piroxicam abolished BK-evoked increase in *Isc* ($n = 6$; $P < 0.01$). Suppression of BK-evoked secretory responses by the COX inhibitors suggested that the responses to BK occurred secondary to the release of prostaglandins and their excitatory action on submucosal secretomotor neurons or directly on intestinal epithelial cells.

Involvement of prostaglandins in BK-evoked secretory responses was further supported by the finding that BK (10 nM) stimulated the synthesis and release of PGE_2 from the submucosal preparations of the guinea pig ileum when compared with the preparations that were not treated with BK (Figure 4). Both FR122047 (1

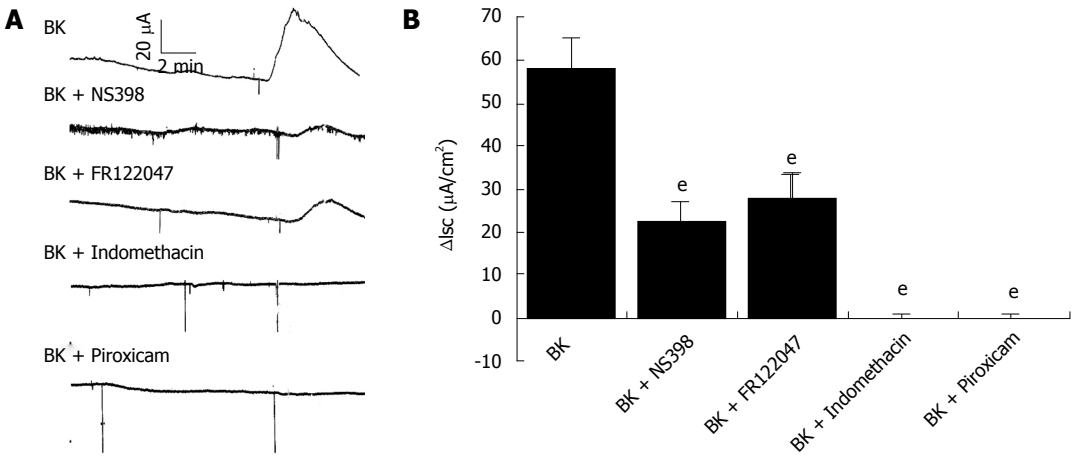


Figure 3 Cyclooxygenase inhibitors suppress bradykinin-evoked increase in short-circuit current. A: Pretreatment with the COX-2 inhibitor NS398, COX1 inhibitor FR122047 (1 μM), non-specific COX inhibitors indomethacin or piroxicam suppressed BK-evoked *Isc*; B: Quantitative data showing the effect of COX inhibitors on BK-evoked increase in *Isc*. Values are expressed as mean ± SE, n = 6 animals. $^{e}P < 0.01$ *vs* BK alone. COX: Cyclooxygenase; BK: Bradykinin; *Isc*: Increase in short-circuit current.

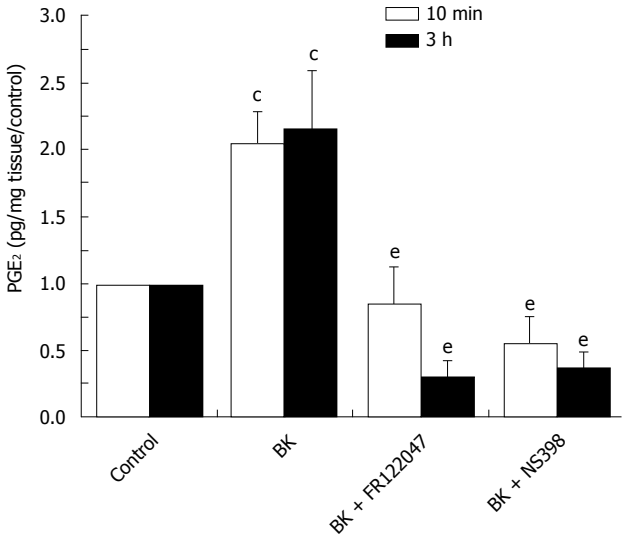


Figure 4 Bradykinin-induced prostaglandin E2 production in submucosal preparations is mediated by both COX-1 and COX-2. Preincubation of submucosal preparations with BK (10 nM) for 10 min or 3 h significantly increased PGE2 production and this was suppressed by the COX-1 inhibitor FR122047 (1 μM) or the COX-2 inhibitor NS398 (10 μM). Values are expressed as mean ± SE, n = 3 independent samples, each assayed in duplicate. $^{c}P < 0.01$ BK *vs* control; $^{e}P < 0.01$ pretreated with agents *vs* BK alone. BK: Bradykinin; PGE2: Prostaglandin E2.

μM) and NS398 (10 μM) suppressed BK-evoked PGE_2 production after incubation with BK for 10 min and 3 h, respectively (Figure 4).

Furthermore, BK-evoked increase in *Isc* was significantly decreased by pretreatment with prostaglandin EP receptor antagonists (Figure 5). Pretreatment with EP1, EP2, EP3-Ⅲ, and DP1 receptor antagonist AH6809 (10 μM) reduced BK-evoked ⊿*Isc* from 69.5 ± 8.3 μA/cm^2 to 24.2 ± 5.5 μA/cm^2 (n = 6, $P < 0.01$), and pretreatment with GW627368X (a potent and selective competitive antagonist of the EP4 receptor, 10 μM) suppressed the ⊿*Isc* from 69.5 ± 8.3 μA/cm^2 to 21.8 ± 5.1 μA/cm^2 (n = 6, $P < 0.01$) (Figure 5). Meanwhile, pretreatment with SC-19220 (a selective EP1 receptor antagonist, 10 μM) did not significantly affect the ⊿*Isc* evoked by BK (n = 6, P = 0.13). All of the above information suggests that BK-evoked increase in ⊿*Isc* was mediated by the stimulation of PGE_2 release.

Signal transduction mechanisms of BK-evoked increase in Isc

Our earlier studies on the mechanisms of post-receptor signal transduction using electrophysiological methods suggested that BK caused excitation of submucosal secretomotor neurons by activation of B2R on submucosal neurons, stimulation of a signal transduction pathway involving phospholipase C (PLC), elevation of intraneuronal IP_3, and elevation of free cytosolic Ca^{2+}[9]. These findings suggested that stimulation of PLC and intracellular IP_3 would also underlie a component of the stimulatory action of BK on ⊿*Isc* at the tissue level. We followed the earlier electrophysiological studies at the cellular level by using the same pharmacological tools to test the hypothesis at the tissue level in Ussing chamber studies.

U73122 was used to inhibit PLC activity. Exposure to 10 μM U73122 for 10 min prior to the application of 10 nM BK resulted in the suppression of BK-evoked responses from 57.3 ± 6.2 μA/cm^2 for BK alone to 9.4 ± 4.3 μA/cm^2 (n = 6; $P < 0.01$) (Figure 6). Pre-exposure of the preparations to the vehicle (DMSO) alone had no significant effect on BK-evoked responses (data not shown). The protein kinase C (PKC) inhibitor bisindolylmaleimide Ⅰ (Bis Ⅰ, 10 μM) decreased the BK-evoked responses to 6.77 ± 5.13 μA/cm^2 (n = 6-12; $P < 0.01$).

Results of our previous work on the cellular neurophysiology of secretomotor neurons suggested that stimulation of calmodulin-dependent protein kinase is involved in B2R-mediated excitation of secretomotor neurons[9]. We used W-7, a membrane permeable calmodulin inhibitor, and KN-62, a selective calmodulin-dependent protein kinase inhibitor, as pharmacological

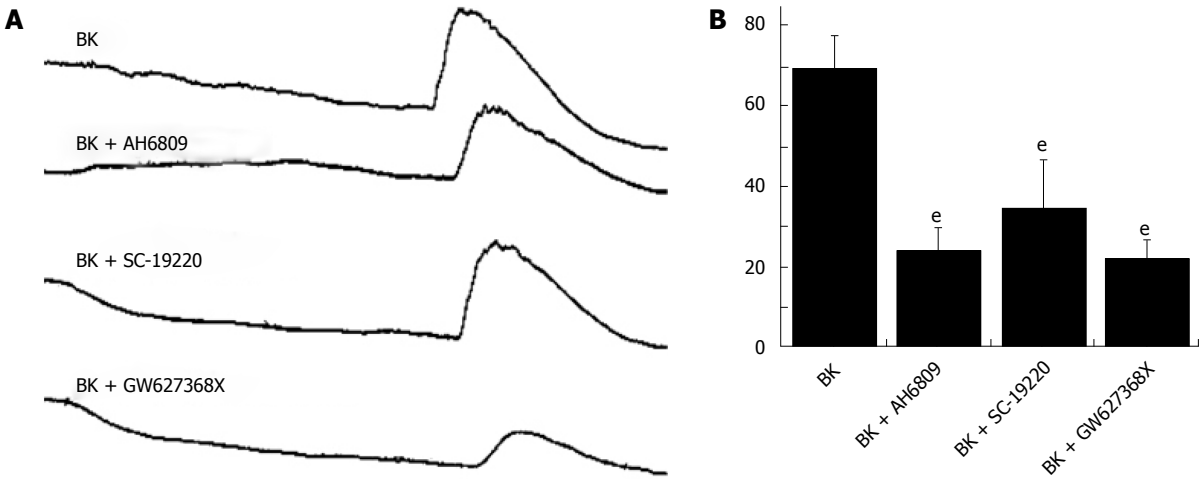


Figure 5 Bradykinin-evoked increase in short-circuit current is mainly mediated by the prostaglandin E2 receptors. A: Pretreatment with EP receptors antagonist AH6809, SC-19220 (a selective antagonist of EP1 receptor), or GW627368X (a selective antagonist of EP4 receptor) suppressed BK-evoked *Isc* ($P < 0.01$); B: Quantitative data showing the effect of AH6809, Sc-19220 and GW627368X on BK-evoked response in *Isc*. The vertical axis represents the changes of *Isc*. Values are expressed as mean ± SE, n = 6-12 animals. $^{e}P < 0.01$ (compared to BK alone). BK: Bradykinin; PGE_2: Prostaglandin E2; *Isc*: Increase in short-circuit current.

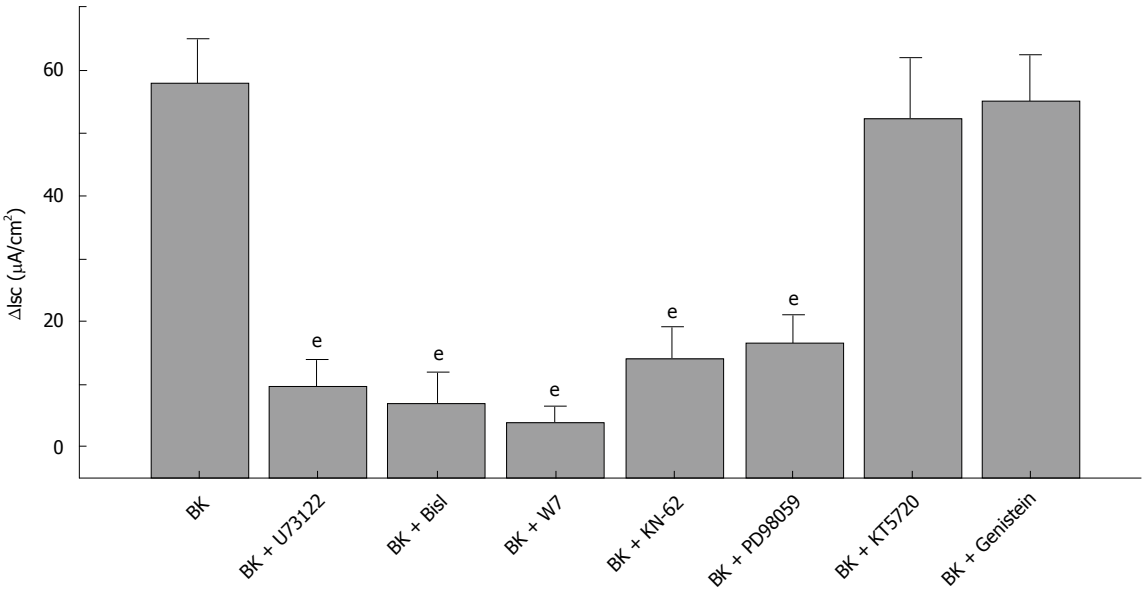


Figure 6 Post-receptor signal transduction for bradykinin-evoked increase in baseline increase in short-circuit current. The BK-evoked *Isc* was suppressed in the presence of the PLC inhibitor U73122, the PKC inhibitor Bis-Ⅰ, the calmodulin inhibitor W7, the calmdulin kinase Ⅱ inhibitor KN-62 (10 μM), or the MAPK pathway inhibitor PD 98059 ($^{e}P < 0.01$ *vs* BK alone). Inhibitor of PKA (KT5720 1 μM) or inhibitor of tyrosine protein kinase (genistein 10 μM) did not have a significant effect on BK-evoked increase in *Isc*. *Isc*: Increase in short-circuit current; BK: Bradykinin; PLC: Phospholipase C; PKC: Protein kinase C; Bis-Ⅰ: Bisindolymaleimide Ⅰ.

tools to test the hypothesis that stimulation of *Isc* by BK involved post-receptor activation of calmodulin-dependent protein kinase. Pretreatment with W-7 (50 μM) and KN-62 (10 μM) for 10 min reduced the responses to 10 nM BK from 57.3 ± 6.2 μA/cm^2 to 3.6 ± 2.5 μA/cm^2 and 14.1 ± 4.9 μA/cm^2 (n = 6; $P < 0.01$), respectively (Figure 6). Meanwhile, pretreatment with 2-APB (10 μM), an IP_3 inhibitor, reduced the response to 3.2 ± 4.0 μA/cm^2 (n = 6; $P < 0.01$). The MAPK pathway inhibitor PD98059 was used to test the downstream signal cascade of PKC. Pre-incubation with PD98059 (10 μM) for 10 min prior to the BK (10 nM) exposure significantly reduced BK-evoked increase of *Isc* to 16.7 ± 4.1 μA/cm^2 (n = 6; $P < 0.01$) (Figure 6).

The protein kinase A (PKA) inhibitor, KT5720, was used to address the question of whether the cAMP/PKA signaling pathway is involved in B2R-evoked secretory response. No suppression of BK-evoked increase of *Isc* occurred in the 6 preparations that were incubated for 10 min in 10 μM KT5720 (Figure 6). The tyrosine protein kinase inhibitor, genistein, was used to verify whether tyrosine protein kinase plays a role in BK-evoked secretory response. The results showed that pretreatment with genistein (10 μM) for 10 min failed to block BK-evoked *Isc* (Figure 6).

To test whether the similar signal transduction mechanisms are also involved in BK-induced PGE_2 production, the submucosa/mucosa preparations were

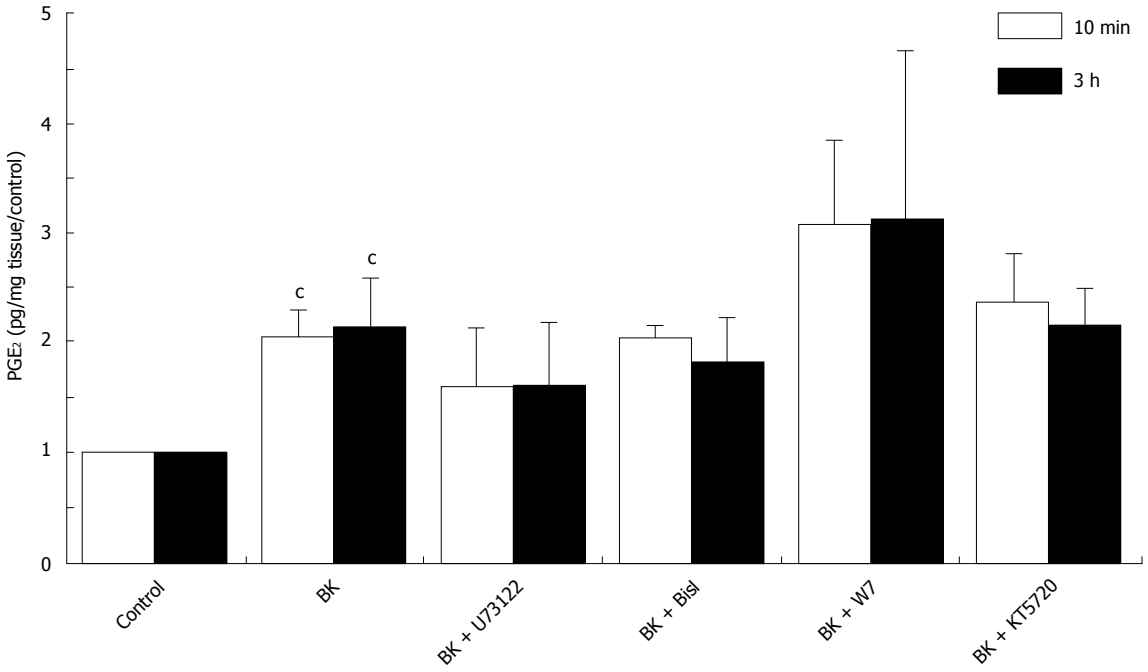


Figure 7 Inhibitors of PLC (U73122; 10 μM), protein kinase C (Bisl; 10 μM), calmodulin (W7; 50 μM), or PKA (KT5720; 1 μM) fail to suppress bradykinin-induced PGE2 production. Values are expressed as mean ± SE, *n* = 3 independent samples, each assayed in duplicate. $^{c}P < 0.01$ BK *vs* control. BK: Bradykinin; PGE_2: Prostaglandin E2.

pretreated with the inhibitors of PLC (U73122), PKC (Bis Ⅰ), CaM (W7), or PKA (KT5720) for 30 min before exposure to BK (10 nM). These inhibitors did not significantly affect BK-induced PGE_2 production (Figure 7).

DISCUSSION

BK stimulates Cl^- secretion by the guinea pig and rabbit ileum[15], rabbit colon[16], rat colon[17], human colon, monolayers of human HCA-7 cells[18,19], and T-84 cells[20]. However, the detailed mechanism of the action of kinins on intestinal secretion is not fully understood, and results are controversial. Diener *et al*[21] have found evidence for the involvement of enteric cholinergic nerves in BK-stimulated secretory response in the rat descending colon. However, also using descending rat colon, another group[22] did not find significant inhibition of BK-induced secretion in the presence of TTX (1 μM) and atropine (1 μM). Furthermore, Manning *et al*[23] reported that BK-induced Cl^- secretion by the guinea pig ileum was not TTX-sensitive and thought to occur by a direct action in the mucosa. The reasons for these differences are unknown but could be caused by the degree that the submucosal plexus has been stripped away from the mucosa.

By using guinea pig ileum submucosa/mucosa preparations, we found that BK-induced intestinal secretion was mainly mediated by the enteric nervous system. The neurotoxin TTX inhibited responses to BK by 73%, hence suggesting that the enteric nervous system plays a pivotal role. Secretomotor neurons in the submucosal plexus are the final common motor pathways from the integrative networks of the enteric nervous system to the intestinal secretory glands. They transmit the signals for autonomic minute-to-minute regulation of mucosal secretion and liquidity of the intestinal contents in concert with submucosal vasodilation and increased blood flow in support of the stimulated secretion[24-27]. Enhanced mucosal secretion, after elevation of excitability in secretomotor neurons, increases the liquidity of the luminal contents and might lead to neurogenic secretory diarrhea. Our previous studies found the expression of B2R mRNA and protein in submucosal neurons. BK increased excitatory in both AH- and S-type neurons in the submucosal plexus[9,10]. The S-type neurons are primarily secretomotor neurons that receive excitatory synaptic input from their AH-type neighbors[24]. Although both cholinergic and VIPergic secretomotor neurons are excited by BK, only the cholinergic muscarinic receptor antagonist, scopolamine, attenuated the secretory responses to BK. The VIP receptor antagonist, VIP_{6-28}, had no significant effect. BK stimulated *Isc* in a TTX- and scopolamine-sensitive manner and therefore correlated with the slow excitatory action of BK on cholinergic secretomotor neurons, which we found at the cellular level. However, direct effects on epithelial cells cannot be ruled out, which is evidenced by the inability of TTX to abolish BK-stimulated secretory responses, and BK stimulates ion transport in cultured monolayers of epithelial cells[28]. The presence of B_2 receptors on intestinal epithelial cells[29] also suggests a potential direct action of BK on epithelial cells.

BK-stimulated intestinal Cl^- secretion depends on the formation of prostaglandins. Inhibition of prostaglandin formation by indomethacin or piroxicam or blocking

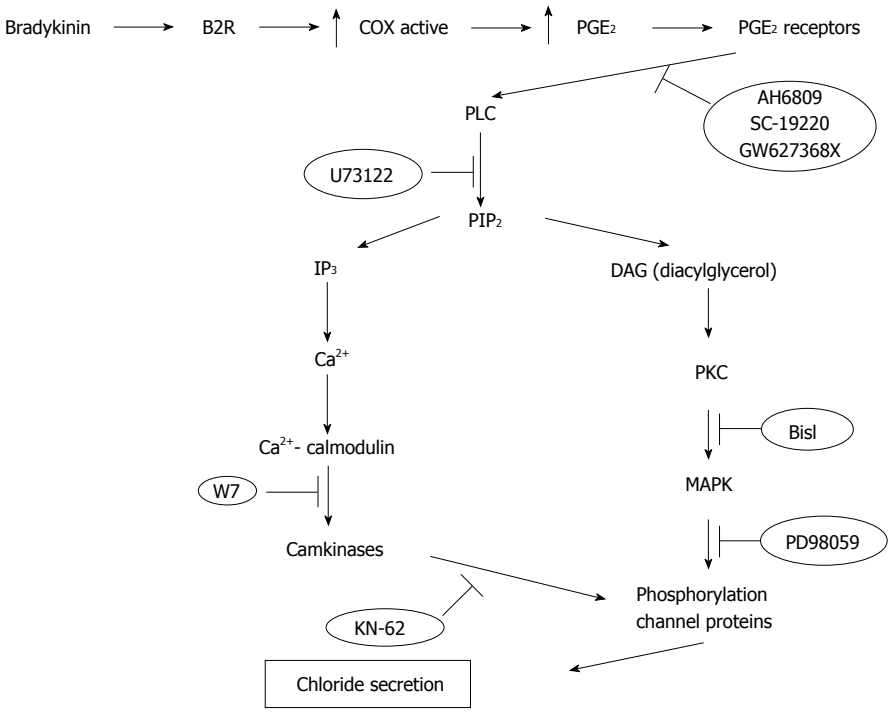


Figure 8 Overview of metabotropic signal transduction cascade for bradykinin evoked chloride secretion in the submucosal plexus of the guinea pig small intestine. Bradykinin activates B2R receptor, G protein, phospholipase C, PIP2 hydrolysis, IP3 and DAG generation, Ca^{2+}-Camodulin, PKC and CaMkinases, cation channel open, and chloride secretion. Termination of chloride secretion is postulated to result from activation of the intraneutonal phosphatase, calcineurin. The blocking agents for each of the steps are included in the figure. PLC: Phospholipase C; PKC: Protein kinase C; PGE_2: Prostaglandin E2.

the prostaglandin receptors suppresses the secretory responses that are evoked by B2R activation in the small intestine of guinea pigs. This observation is in agreement with previous studies in the rat, rabbit, and human intestine[28] where products of the COX pathway were implicated in BK-stimulated ion transport. However, the isoforms of COX that are involved in the process are not clear. COX enzymes have two isoforms, namely, COX-1 and COX-2. COX-1 is constitutively expressed throughout the gastrointestinal tract. COX-2 was initially considered an inducible form that has been highly expressed at sites of inflammation, but subsequently constitutive expression of COX-2 in normal digestive tract has been demonstrated[5,30]. Both COX-1 and COX-2 inhibitors significantly suppressed BK-induced secretory response in the small intestine of guinea pigs, thus suggesting that the constitutive COX-1, as well as constitutive COX-2, is involved in the production of prostaglandins, which is further supported by the observation that BK-stimulated PGE_2 production from submucosa/mucosa preparations was greatly suppressed by COX-1 and COX-2 inhibitors. Several cellular elements of the intestine can produce prostaglandins upon activation of B2R. Our studies and those from others[29] suggest that enteric neurons, fibroblasts, and enterocytes are likely the sources.

We found that the same pharmacological agents, which suppress BK-evoked slow excitatory responses in secretomotor neurons by selective inhibition of individual steps in the PLC-IP_3-Ca^{2+}-PKC signal transduction pathway, also suppressed the BK-evoked increase in *Isc*. These inhibitory effects of the events in the PLC-PKC signal transduction cascade on BK-evoked stimulation of *Isc* are consistent with the inhibition of intracellular post-B2R signaling in the secretomotor neurons. Nevertheless, the agents that suppressed post-B2R signal transduction in secretomotor neurons might act also to suppress the signal transduction in enterocytes. By contrast, the agents that suppressed BK-evoked increase in *Isc* did not significantly affect BK-induced PGE_2 production in the submucosa/mucosa preparations, thereby suggesting that the PLC-IP_3-Ca^{2+}-PKC signal transduction pathway is activated after prostaglandins are released.

In conclusion, the data presented here are consistent with the possibility that BK stimulates secretory responses in the intestine *via* activating B_2 receptors in submucosal secretomotor neurons and in epithelial cells, as shown in Figure 8. Activation of B2R causes the release of prostaglandins through both COX-1 and COX-2 dependent pathways. Once released, prostaglandins excite submucosal cholinergic secretomotor neurons and indirectly stimulate Cl^- secretion. Prostaglandins may also act directly in epithelial cells to stimulate ion transport. PLC, IP_3, Ca^{2+}, PKC, and MAP kinase all play a role in the signal transduction of BK-stimulated secretory responses.

COMMENTS

Background

Bradykinin (BK) is widely distributed in the central and peripheral nervous

systems, including the enteric nervous system. BK can stimulate intestinal chloride secretion and the firing of intestinal secretomotor neurons in the small intestine, but the mechanism is not well understood.

Research frontiers

The present work aimed to investigate how the involvement of BK as an excitatory neuromodulator on submucosal secretomotor neurons at the cellular neurophysiological level translates to the physiology of intestinal secretion at the level of the integrated system.

Innovations and breakthroughs

This study demonstrates that BK stimulates intestinal chloride secretion responses *via* activating B2 receptors in submucosal secretomotor neurons and in epithelial cells. Activation of B2 receptors causes the release of prostaglandins through both cyclooxygenase (COX)-1 and COX-2 dependent pathways. Once released, prostaglandins excite submucosal cholinergic secretomotor neurons and indirectly stimulate Cl^- secretion. Prostaglandins may also act directly in epithelial cells to stimulate ion transport. Phospholipase C (PLC), IP_3, Ca^{2+}, protein kinase C (PKC), and MAP kinase (MAPK) all play a role in the signal transduction of BK-stimulated secretory responses.

Applications

The authors studied the mechanism of how BK stimulates the intestinal chloride secretion. The results suggest that BK stimulates neurogenic chloride secretion in the guinea pig ileum by activating B2 receptors on secretomotor neurons, activating COX, and stimulating prostaglandin E2 (PGE_2) production. The post-receptor transduction cascade includes the activation of PLC, PKC, CaMK, IP_3, and MAPK. This study gives a better understanding of the BK-evoked secretion pathway.

Peer-review

In this manuscript, the authors reported the BK-evoked mucosal secretion in the guinea pig small intestine. By using a list of inhibitors, antagonists, and agonists, the authors concluded that BK-evoked chloride secretion depends on the engagement on the B2 receptor, the activation of COX, production of PGE_2, and downstream signaling cascade. Understanding the mechanism may provide a valuable insight into the regulation of mucosal secretion and other intestinal functions. The manuscript is well-organized, and conclusions were precisely based on obtained data.

REFERENCES

1 **Couture R**, Lindsey, CJ. Brain kallikrein-kinin system: from receptors to neuronal pathways and physiological functions. In: Quirion R, Bjo¨rklund A, Ho¨kfelt TŽ. Handbook of Chemical Neuroanatomy. Peptide Receptors, Part I, 2000: 241-300 [DOI: 10.1016/S0924-8196(00)80009-3]

2 **Raidoo DM**, Bhoola KD. Pathophysiology of the kallikrein-kinin system in mammalian nervous tissue. *Pharmacol Ther* 1998; **79**: 105-127 [PMID: 9749879]

3 **Walker K**, Perkins M, Dray A. Kinins and kinin receptors in the nervous system. *Neurochem Int* 1995; **26**: 1-16; discussion 17-26 [PMID: 7787759 DOI: 10.1016/0197-0186(94)00114-A]

4 **Souza DG**, Lomez ES, Pinho V, Pesquero JB, Bader M, Pesquero JL, Teixeira MM. Role of bradykinin B2 and B1 receptors in the local, remote, and systemic inflammatory responses that follow intestinal is chemia and reperfusion injury. *J Immunol* 2004; **172**: 2542-2548 [PMID: 14764727 DOI: 10.4049/jimmunol.172.4.2542]

5 **Stadnicki A**, Pastucha E, Nowaczyk G, Mazurek U, Plewka D, Machnik G, Wilczok T, Colman RW. Immunolocalization and expression of kinin B1R and B2R receptors in human inflammatory bowel disease. *Am J Physiol Gastrointest Liver Physiol* 2005; **289**: G361-G366 [PMID: 15805101 DOI: 10.1152/ajpgi.00369.2004]

6 **Chan SK**, Rudd JA. Role of bradykinin B2 receptors in the modulation of the peristaltic reflex of the guinea pig isolated ileum. *Eur J Pharmacol* 2006; **539**: 108-115 [PMID: 16650846 DOI: 10.1016/j.ejphar.2006.04.002]

7 **Zelawski W**, Machnik G, Nowaczyk G, Plewka D, Lorenc Z, Sosada K, Stadnicki A. Expression and localisation of kinin receptors in colorectal polyps. *Int Immunopharmacol* 2006; **6**: 997-1002 [PMID: 16644486 DOI: 10.1016/j.intimp.2006.01.016]

8 **Hu HZ**, Liu S, Gao N, Xia Y, Mostafa R, Ren J, Zafirov DH, Wood JD. Actions of bradykinin on electrical and synaptic behavior of neurones in the myenteric plexus of guinea-pig small intestine. *Br J Pharmacol* 2003; **138**: 1221-1232 [PMID: 12711622 DOI: 10.1038/sj.bjp.0705180]

9 **Hu HZ**, Gao N, Liu S, Ren J, Wang X, Xia Y, Wood JD. Action of bradykinin in the submucosal plexus of guinea pig small intestine. *J Pharmacol Exp Ther* 2004; **309**: 320-327 [PMID: 14718600 DOI: 10.1124/jpet.103.059188]

10 **Hu HZ**, Gao N, Liu S, Ren J, Xia Y, Wood JD. Metabotropic signal transduction for bradykinin in submucosal neurons of guinea pig small intestine. *J Pharmacol Exp Ther* 2004; **309**: 310-319 [PMID: 14718601 DOI: 10.1124/jpet.103.059204]

11 **Qu MH**, Wang XY, Sun XH, Liu SM, Wang GD, Zou F, Xia Y, Wood JD. Enteric Neurophysiological Mechanisms of Action for Bradykinin-Evoked Mucosal Chloride Secretion in Guinea Pig Small Intestine. *Gastroenterology* 2008; **134**: A687

12 **Qu MH**, Wang XY, Sun XH, Liu SM, Wang GD, Zou F, Xia Y, Wood JD. Synaptic Activation of Trpc Channels By Metabotropic Purinergic P2Y1 Receptors in the Submucosal Plexus of the Guinea-Pig Small Intestine. *Gastroenterology* 2007; **132**: A18

13 **Fei G**, Wang YZ, Liu S, Hu HZ, Wang GD, Qu MH, Wang XY, Xia Y, Sun X, Bohn LM, Cooke HJ, Wood JD. Stimulation of mucosal secretion by lubiprostone (SPI-0211) in guinea pig small intestine and colon. *Am J Physiol Gastrointest Liver Physiol* 2009; **296**: G823-G832 [PMID: 19179625 DOI: 10.1152/ajpgi.90447.2008]

14 **Fang X**, Hu HZ, Gao N, Liu S, Wang GD, Wang XY, Xia Y, Wood JD. Neurogenic secretion mediated by the purinergic P2Y1 receptor in guinea-pig small intestine. *Eur J Pharmacol* 2006; **536**: 113-122 [PMID: 16566916 DOI: 10.1016/j.ejphar.2006.02.040]

15 **Musch MW**, Kachur JF, Miller RJ, Field M, Stoff JS. Bradykinin-stimulated electrolyte secretion in rabbit and guinea pig intestine. Involvement of arachidonic acid metabolites. *J Clin Invest* 1983; **71**: 1073-1083 [PMID: 6406543 DOI: 10.1172/JCI110857]

16 **Phillips JA**, Hoult JR. Secretory effects of kinins on colonic epithelium in relation to prostaglandins released from cells of the lamina propria. *Br J Pharmacol* 1988; **95**: 701-712 [PMID: 3207989 DOI: 10.1111/j.1476-5381.1988.tb11696.x]

17 **Stewart JM**, Gera L, Chan DC, Bunn PA, York EJ, Simkeviciene V, Helfrich B. Bradykinin-related compounds as new drugs for cancer and inflammation. *Can J Physiol Pharmacol* 2002; **80**: 275-280 [PMID: 12025961 DOI: 10.1139/y02-030]

18 **Cuthbert AW**, Margolius HS. Kinins stimulate net chloride secretion by the rat colon. *Br J Pharmacol* 1982; **75**: 587-598 [PMID: 7066606 DOI: 10.1111/j.1476-5381.1982.tb09178.x]

19 **Cuthbert AW**, Kirkland SC, MacVinish LJ. Kinin effects on ion transport in monolayers of HCA-7 cells, a line from a human colonic adenocarcinoma. *Br J Pharmacol* 1985; **86**: 3-5 [PMID: 2413939 DOI: 10.1111/j.1476-5381.1985.tb09428.x]

20 **Baird AW**, Skelly MM, O'Donoghue DP, Barrett KE, Keely SJ. Bradykinin regulates human colonic ion transport in vitro. *Br J Pharmacol* 2008; **155**: 558-566 [PMID: 18604228 DOI: 10.1038/bjp.2008.288]

21 **Diener M**, Bridges RJ, Knobloch SF, Rummel W. Indirect effects of bradykinin on ion transport in rat colon descendens: mediated by prostaglandins and enteric neurons. *Naunyn Schmiedebergs Arch Pharmacol* 1988; **337**: 69-73 [PMID: 3368015 DOI: 10.1007/bf00169479]

22 **Tien XY**, Wallace LJ, Kachur JF, Won-Kim S, Gaginella TS. Neurokinin A increases short-circuit current across rat colonic mucosa: a role for vasoactive intestinal polypeptide. *J Physiol* 1991; **437**: 341-350 [PMID: 1653854 DOI: 10.1113/jphysiol.1991.sp018599]

23 **Manning DC**, Snyder SH, Kachur JF, Miller RJ, Field M. Bradykinin receptor-mediated chloride secretion in intestinal function. *Nature* 1982; **299**: 256-259 [PMID: 6125894 DOI: 10.1038/299256a0]

24 **Cooke HJ**. Neurotransmitters in neuronal reflexes regulating intestinal secretion. *Ann N Y Acad Sci* 2000; **915**: 77-80 [PMID:

11193603 DOI: 10.1111/j.1749-6632.2000.tb05225.x]
25 Cooke HJ, Christofi F. Enteric neural regulation of mucosal secretion, In: Barrett KE., Ghishan FK., Johnson LR, Merchant JL, Said HM, Wood JD. Cellular neurophysiology of enteric neurons. In: Johnson LR, Barrett KE, Ghishan FK, Merchant JL, Said HM, and Wood JD, editors. Physiology of the Gastrointestinal Tract, 4th Ed. San Diego: Academic Press, 2006: 629-664
26 **Wood JD**. Enteric nervous system: reflexes, pattern generators and motility. *Curr Opin Gastroenterol* 2008; **24**: 149-158 [PMID: 18301264 DOI: 10.1097/MOG.0b013e3282f56125]
27 **Morrissey NK**, Bellenger CR, Baird AW. Bradykinin stimulates prostaglandin E2 production and cyclooxygenase activity in equine nonglandular and glandular gastric mucosa in vitro. *Equine Vet J* 2008; **40**: 332-336 [PMID: 18331972 DOI: 10.2746/042516408X293556]
28 **Porcher C**, Horowitz B, Ward SM, Sanders KM. Constitutive and functional expression of cyclooxygenase 2 in the murine proximal colon. *Neurogastroenterol Motil* 2004; **16**: 785-799 [PMID: 15601429 DOI: 10.1111/j.1365-2982.2004.00568.x]
29 **Zaika O**, Mamenko M, O'Neil RG, Pochynyuk O. Bradykinin acutely inhibits activity of the epithelial Na+ channel in mammalian aldosterone-sensitive distal nephron. *Am J Physiol Renal Physiol* 2011; **300**: F1105-F1115 [PMID: 21325499 DOI: 10.1152/ajprenal.00606.2010]
30 **Bernardini N**, Colucci R, Mattii L, Segnani C, Fornai M, de Giorgio R, Barbara G, Castagna M, Nardini V, Dolfi A, Del Tacca M, Blandizzi C. Constitutive expression of cyclooxygenase-2 in the neuromuscular compartment of normal human colon. *Neurogastroenterol Motil* 2006; **18**: 654-662 [PMID: 16918730 DOI: 10.1111/j.1365-2982.2006.00795.x]

P- Reviewer: Liao GX
S- Editor: Yu J **L- Editor**: A **E- Editor**: Jiao XK

World Journal of Gastrointestinal Pathophysiology

Submit a Manuscript: http://www.wjgnet.com/esps/
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.4291/wjgp.v7.i1.160

World J Gastrointest Pathophysiol 2016 February 15; 7(1): 160-170
ISSN 2150-5330 (online)

ORIGINAL ARTICLE

Clinical Trials Study

Hypoallergenic formula with *Lactobacillus rhamnosus GG* for babies with colic: A pilot study of recruitment, retention, and fecal biomarkers

Nicole Y Fatheree, Yuying Liu, Michael Ferris, Melissa Van Arsdall, Valarie McMurtry, Marcela Zozaya, Chunyan Cai, Mohammad H Rahbar, Manouchehr Hessabi, Ta Vu, Christine Wong, Juleen Min, Dat Q Tran, Fernando Navarro, Wallace Gleason, Sara Gonzalez, J Marc Rhoads

Nicole Y Fatheree, Yuying Liu, Melissa Van Arsdall, Juleen Min, Dat Q Tran, Fernando Navarro, Wallace Gleason, Sara Gonzalez, J Marc Rhoads, Department of Pediatrics, the University of Texas Health Science Center at Houston Medical School, Houston, TX 77030, United States

Chunyan Cai, Mohammad H Rahbar, Manouchehr Hessabi, Biostatistics/Epidemiology/Research Design Core, Center for Clinical and Translational Sciences, the University of Texas Health Science Center at Houston, Houston, TX 77030, United States

Chunyan Cai, Mohammad H Rahbar, Manouchehr Hessabi, Division of Epidemiology, Human Genetics and Environmental Sciences, the University of Texas School of Public Health at Houston, Houston, TX 77030, United States

Michael Ferris, Valarie McMurtry, Marcela Zozaya, Department of Pediatrics, Louisiana State University Health Science Center and the Research Institute for Children, New Orleans, LA 70112, United States

Ta Vu, Christine Wong, Investigational Drug Services Pharmacy, Memorial Hermann Hospital, Houston, TX 77030, United States

Author contributions: The first two authors contributed equally to this work and are both considered first authors.. Fatheree NY, Liu Y, Rahbar MH and Rhoads JM were integral to the clinical protocol and study design; Fatheree NY, Van Arsdall M, Navarro F, Gleason W and Rhoads JM collected clinical data; Gonzalez S supported research team by providing nutritional expertise; Liu Y provided essential expertise for analyzing cytokines and fecal calprotectin; Min J and Tran DQ conducted research on Treg analysis; Tu V and Wong C provided investigative pharmaceutical expertise during research; Ferris M, McMurty V and Zozoya M provided essential experience and expertise with high throughput sequence analysis of 16S rRNA gene sequences of fecal microbiota; Chunyan C, Rahbar MH and Hessabi M performed data management and statistical analyses; Fatheree NY, Liu Y, Ferris M and Rhoads JM wrote the paper; Rhoads JM has primary responsibility for content; all authors read and approved the final manuscript.

Supported by An Investigator Initiated Trial grant from Mead Johnson Nutrition which had no influence on the design or conduct of the study, data management and analysis, writing of the report, or the decision to submit the manuscript for publication.

Institutional review board statement: The study was reviewed and approved by the University of Texas Health Science Center at Houston Institutional Review Board.

Clinical trial registration statement: This registration policy applies to prospective, randomized, controlled trials only, NCT01279265.

Informed consent statement: All study participants, or their legal guardian, provided informed written consent prior to study enrollment.

Conflict-of-interest statement: Not declared.

Data sharing statement: No additional data are available.

Correspondence to: J Marc Rhoads, MD, Department of Pediatrics, the University of Texas Health Science Center at Houston Medical School, 6431 Fannin St., MSB 3.137, Houston, TX 77030, United States. j.marc.rhoads@uth.tmc.edu
Telephone: +1-713-5007642

Fax: +1-713-5005750

Received: March 27, 2015
Peer-review started: March 31, 2015
First decision: June 18, 2015
Revised: September 25, 2015
Accepted: November 3, 2015
Article in press: November 4, 2015
Published online: February 15, 2016

Abstract

AIM: To investigate recruitment, retention, and estimates for effects of formula supplementation with *Lactobacillus rhamnosus GG* (LGG) on inflammatory biomarkers and fecal microbial community in infants with colic.

METHODS: A prospective, double-blind, placebo-controlled trial was conducted in otherwise healthy infants with colic. We screened 74 infants and randomized and analyzed results in 20 infants [9 receiving LGG (LGG+) and 11 not receiving LGG (LGG-)]. LGG was incorporated in the formula (Nutramigen®) (minimum of 3×10^7 CFU/d) in the LGG+ group. Fecal microbiota and inflammatory biomarkers, including fecal calprotectin (FC), plasma cytokines, circulating regulatory T cells (Tregs), and crying + fussing time were analyzed to determine optimal time points and effect sizes for a larger trial.

RESULTS: Recruitment in this population was slow, with about 66% of eligible infants willing to enroll; subject retention was better (75%). These rates were influenced by parents' reluctance to volunteer their infant for a clinical trial and by their tendency to change formulas. The maximal difference of crying + fussing time was observed at day 14, comparing the 2 groups, with a mean difference of -91 (95%CI: -76, 259) min (P = NS). FC showed no significant difference, but the optimal time to determine a potential effect was at day 90 [with a mean difference of 121 (95%CI: -48, 291) μg/g stool], observing a lower level of FC in the LGG+ group. The fecal microbial communities were chaotic, as determined by Shannon's diversity index and not apparently influenced by the probiotic. No significant change was observed in plasma inflammatory cytokines or Tregs, comparing LGG+ to LGG- groups.

CONCLUSION: Designing future colic trials involving a probiotic-supplemented formula for infants in the United States will require consideration for difficult enrollment. Infants with colic have major variations in feal microbiota and calprotectin, both of which improve with time, with optimal time points for measurement at days 14 and 90 after treatment.

Key words: Barr diary; Regulatory T cells; Cytokines; Crying; Fussing; Probiotic; Inflammation; Biomarker; Newborn; Intestine

Core tip: The "dysbiosis" theory proposes that newborns with abnormal colonization are predisposed to having gut inflammation and colic. Probiotics may reduce crying and diversify the fecal microbiota. A prospective, double-blind, placebo-controlled trial was conducted in healthy infants with colic. After 75% screen failure or dropouts, 20 infants were analyzed (9 receiving formula with *Lactobacillus GG* and 11 not receiving *Lactobacillus rhamnosus GG* in their formula). We found that: (1) recruitment/retention indicate future randomized controlled trials should enroll 80 patients with an optimal timepoint for observing a potential difference in crying at 14 d; (2) microbial communities were chaotic in infants with colic, even more so than reported in Dutch infants; and (3) our study was the first to analyze cytokine levels and circulating Tregs in infants with colic.

Fatheree NY, Liu Y, Ferris M, Van Arsdall M, McMurtry V, Zozaya M, Cai C, Rahbar MH, Hessabi M, Vu T, Wong C, Min J, Tran DQ, Navarro F, Gleason W, Gonzalez S, Rhoads JM. Hypoallergenic formula with *Lactobacillus rhamnosus GG* for babies with colic: A pilot study of recruitment, retention, and fecal biomarkers. *World J Gastrointest Pathophysiol* 2016; 7(1): 160-170 Available from: URL: http://www.wjgnet.com/2150-5330/full/v7/i1/160.htm DOI: http://dx.doi.org/10.4291/wjgp.v7.i1.160

INTRODUCTION

Colic has been defined as inconsolable crying and fussing, for greater than 3 h daily for more than 3 d per week in infants from 3 wk to 3 mo of age[1]. There have been many theories to explain the occurrence of colic, including bacterial overgrowth[1], the "fourth trimester" theory in which the baby is wishes to remain *in utero*[2], parental depression[3], excessive intestinal gas[1], and milk protein allergy[4]. Savino was the first to propose that an abnormal microbiota ("dysbiosis") might be an important pathophysiological mechanism, by demonstrating a reduced abundance of *Lactobacilli* and increased abundance of *E. coli* in the stools of infants with colic[5].

Our previous studies showed that intestinal inflammation was a feature of colic[6]. This led to the hypothesis that there may be an association of dysbiosis and intestinal inflammation, both of which may improve with probiotic treatment. The probiotic *Lactobacillus rhamnosus GG* (LGG) has been shown to reduce diarrhea in children with acute infectious enteritis[7] and to facilitate the development of a more diverse fecal microbiota[8]. Others have hypothesized that early colonization of the immature small intestine with lactobacillus would reduce gut inflammation and symptoms in infants with colic. However, previous studies have focused on breast-fed babies.

In the current studies, our two major aims were: (1) to investigate the feasibility of recruitment and retention of babies with colic randomized to receive a probiotic-containing formula; and (2) to determine effect size of LGG-supplemented formula on crying + fussing time, the intestinal microbiota, and the inflammatory biomarker calprotectin in infants with colic.

MATERIALS AND METHODS

Ethical review and approval

This protocol was approved by the Institutional Review Board of the University of Texas Health Science Center at Houston. Every 10 patients, a Data Safety Monitoring Board convened to review safety. A consort checklist is available as supporting information. The trial protocol was registered in http://www.clinicaltrials.gov NCT01279265.

Study design, population and randomization

This study was a prospective, double-blind, placebo-controlled trial in otherwise healthy infants with colic, designed as a pilot study for determining potential effects of treatment with casein-hydrolysate formula with LGG versus no LGG on selected biomarkers in infants with established colic. This formula (Nutramigen) was chosen because at the time it was the only formula that could be obtained in liquid form with or without probiotic, because it has been suggested to be beneficial in infants with colic[4], and because Food and Drug Administration-monitored safety and biomarker trials of direct probiotic supplementation in infants with colic had not been completed at this point. Partially of fully formula-fed infants age 3-13 wk old born full-term (> 37 wk gestation) were included if they fulfilled the colic definition of crying and fussing more than 3 h per day for at least 3 d weekly, documented at enrollment by at least 2 abnormal Barr diaries over a 3-d period[9]. Patients were actively recruited through university community clinics, 4 pediatric practices affiliated with our university, the pediatric gastroenterology clinic, and other local pediatricians *via* mailings, television coverage, and a website. Infants were excluded if they had failure to thrive, chronic lung disease, diarrhea, fever, and if they took a probiotic prior to enrollment.

Infants were randomly assigned by block randomization (groups of 4) to one of two formulas, either casein hydrolysate (Nutramigen®) with LGG (Enflora™) or casein hydrolysate without LGG (Nutramigen A+®). Initially, the protocol was to enroll children with or without colic to receive the above 2 formulas in white, unlabeled containers and to measure crying + fussing time and the biomarkers, but the protocol had to be changed because they were uncomfortable with this type of label. We changed the protocol so that containers were partially covered with a sticky label to ensure blinding. The randomization schedule was computer-generated, prepared by the study biostatistician and implemented by pharmacists in Department of Investigational Drugs Services at Memorial Hermann Hospital. Infants with partial breast-feeding were required to take at least 240 mL of formula per day to ensure at least 3.6×10^7 CFU's of LGG (if they were randomized to the LGG+ group). However, most infants were completely formula-fed (as shown in Table 1). The infants were required to take study formula for the entire 90 d of observation, with research visits on days 1, 14, 42, and 90. Patients were followed by telephone on a weekly basis. During each clinical visit, the medical history and clinical condition of each infant was evaluated by a pediatric gastroenterologist. Stool and blood were collected at baseline and follow up visits.

Barr Diary

Infant crying and fussing time was quantified using the Barr Diary, a well-validated instrument, as previously described[9] at each study visit.

Breath tests

Parents/guardians were asked to have the infant fast for a minimum of 3 h before the baseline visit and before visit 2. After two baseline samples were collected (separated by 15 min), the infant was fed 60 mL of glucose water, and at time = 45 min, exhaled air was collected and breath hydrogen and methane were measured using the Quintron Model SC Microlyzer™ (Quintron Instrument Co., Inc., Milwaukee, Wisconsin). In all infants, breath methane was negligible. A breath test was considered positive if the baseline hydrogen level was $\geqslant$ 20 parts per million (ppm) or if there was an increase from baseline of $\geqslant$ 12 ppm[6].

Research lab protocols

Fecal calprotectin (FC). Stool samples was prepared and analyzed by using a quantitative calprotectin ELISA kit according to manufacturer's instructions, as previously described[10]. The level of calprotectin was expressed as μg/g of stool weight.

Plasma cytokines and percentage of Tregs: Plasma cytokines were detected by using MSD Human ProInflammatory 7-Plex Ultra-Sensitive Kit (Meso Scale Discovery®, Gaithersburg, MD) which measures human IFN-γ, IL-1β, IL-6, IL-8, IL-10, IL-12p70, and TNF-α[10]. This inflammatory panel was chosen because LGG has been shown to prevent enterocyte apoptosis induced by IFN-γ, IL-1β, IL-6, IL-8, IL-10, IL-12p70, and TNF-α[11] Isolated peripheral blood mononuclear cells were stained with surface CD4 and intracellular FOXP3 antibodies and analyzed by using flow cytometry[10].

Fecal pyrosequencing analysis: Parents were instructed to collect a stool sample within 48 h of the visit and to store stools frozen. In the lab, stool samples were subdivided and stored at -80 ℃ until analyzed. DNA extraction, polymerase chain reaction-amplification, pyrosequencing and taxonomic identification of 16S rRNA

Table 1 Comparison of baseline characteristics of randomized patients in the two study groups

	LGG+ group		LGG- group		
Continuous variables	*n*	mean ±SD	*n*	mean ± SD	*P*
Age at the time randomized (d)	9	57 ± 30	11	68 ± 28	0.34[1]
Gestational age (wk)	9	38 ± 2	11	37 ± 2	0.20[1]
Birth weight (kg)	9	3.1 ± 0.8	11	2.9 ± 1.1	0.47[1]
Birth height (cm)	7	51.6 ± 1.8	9	47.2 ± 5.3	0.04[1]
Discrete variables	*n*	mean ± SD	*n*	mean ± SD	
Gender	9		11		
Female		4 (44.4)		4 (36.4)	1.0
Male		5 (55.6)		7 (63.6)	
Race	9		11		1.0
African-american		3 (33.3)		4 (36.4)	
Caucasian		6 (66.7)		7 (63.6)	
Ethnicity	9		11		1.0
Hispanic or latino		2 (22.2)		2 (18.2)	
Not hispanic or latino		7 (77.8)		9 (81.8)	
Partial breast feed	9		11		1.0
Yes		3 (33.3)		3 (27.3)	
No		6 (66.7)		8 (72.7)	
Formula type	9		11		
Earth's best		1 (11.1)		0 (0)	
Enfamil		1 (11.1)		0 (0)	
Enfamil gentleease		1 (11.1)		0 (0)	
Gerber good start		0 (0)		1 (9.1)	
Isomil		1 (11.1)		1 (9.1)	
Neocate		0 (0)		1 (9.1)	
Nutramigen ready mix		0 (0)		1 (9.1)	
Similac		2 (22.2)		0 (0)	
Similac advance		1 (11.1)		0 (0)	
Similac senstive		0 (0)		5 (45.5)	
Breast milk		2 (22.2)		2 (18.2)	

[1]Denotes *P* values obtained by non-parametric Wilcoxon rank sum test. All other *P* values are obtained by Fisher's exact test. The values in parentheses indicate percentage.

gene sequences in stool specimens were performed as previously described[12], using QIIME[13] to analyze microbial communities. A total of 185373 reads with an average length of 460 ± 62 bases were included in this study. The average number of reads per sample was 3783.

Statistical analysis

Sample size and power: This pilot study aimed to determine recruitment, retention, adverse events, and biomarkers but was not powered to detect differences in crying time between the two study arms. (Based on our previous study[6] showing 297 ± 142 min/d as a mean crying+fussing time in colicky infants, we determined that a sample size of 60 colicky infants (30 infants per study arm) would have been required to detect a mean difference of 100 min/d of crying + fussing time, with a power of 0.80 at the 5% level).

Baseline characteristics were compared between two groups using the two sample *t*-test or Wilcoxon rank sum test for continuous variables and the Fisher's exact test for categorical variables. For estimating the effect size of Barr diary crying time and FC, we used generalized Estimating Equation method with autoregressive covariance structure to account for potential correlation between measures at multiple visits during the follow up period. Time variable (*i.e.,* visits), treatment group indicator, as well as their interactions are included as covariates to estimate the differences in the outcomes between the two study arms (*i.e.,* LGG- values minus LGG+ values) over time. The adjusted means and the mean differences between LGG- *vs* LGG+ groups as well as their 95%CI were calculated. The Wilcoxon rank sum test was applied to compare the cytokines at baseline and at each of the follow up visits. All the above analyses were conducted using statistical software SAS 9.3 (SAS Institute, Cary, NC). Microbiota data were analyzed using Mann-Whitney *U*-test and one-way ANOVA using GraphPad Prism v5.0 for windows (GraphPad Software, San Diego, CA). Shannon's Diversity Index was calculated using a locally developed pyrosequencing pipeline[12].

RESULTS

Clinical characteristics

Enrollment took place from September 2011 to January 2013. Seventy-four infants with colic were screened based on the above inclusion and exclusion criteria. Forty-four (59%) infants were not included in the study because they did not come to the scheduled first clinic visit or because the parents/guardians chose not to participate. Thirty were enrolled, but 4 changed their mind after signing consent, and 6 were uncomfortable when they received cans of formula with a white label. Subsequently, we changed by taping over the label to hide only the probiotic part of the label (The study

Table 2 Longitudinal analysis of clinical variables by study group (LGG+ *vs* LGG-) at baseline and follow up visits

	Adjusted means (95%CI)			
	LGG+ group	LGG- group	Mean differences(95%CI)	*P* value
Crying + fussing time (min)				
Visit 1 (Baseline)	296 (210, 381)	337 (251, 422)	41 (-80, 161)	0.51
Visit 2	197 (117, 278)	289 (142, 436)	91 (-76, 259)	0.29
Visit 3	144 (54, 234)	199 (69, 328)	55 (-104, 213)	0.50
Visit 4	111 (65, 157)	133 (60, 205)	22 (-64, 107)	0.62
Fecal calprotectin (μg/g)				
Visit 1 (Baseline)	285 (199, 371)	294 (184, 404)	9 (-131, 149)	0.90
Visit 2	226 (182, 270)	305 (186, 423)	79 (-48, 205)	0.22
Visit 3	229 (113, 345)	250 (154, 347)	21 (-130, 172)	0.78
Visit 4	211 (80, 342)	332 (225, 440)	121 (-48, 291)	0.16

Longitudinal model: Barr Diary data = $\beta_0 + \beta_1 \times$ visit2 + $\beta_2 \times$ visit3 + $\beta_3 \times$ visit4 + β_4 group + $\beta_5 \times$ visit2 group + $\beta_6 \times$ visit3 group + $\beta_7 \times$ visit4 group. Here, visit2, visit3, visit4 are dummy variables; visit2 = 1 if at visit 2, 0 otherwise; visit3 = 1 if at visit 3, 0 otherwise; visit4 = 1 if at visit 4, 0 otherwise; group = 1 if in LGG group, 0 otherwise.

initially was designed to include 30 infants in each group, but it was closed because of lack of funds to continue). Twenty infants randomized to either the LGG+ group (*n* = 9) or to the placebo (LGG- group) (*n* = 11) group were able to be analyzed (Figure 1, Consort Diagram). Baseline characteristics including mean gestational age, age at enrollment, weight, and length showed no differences between the two groups (Table 1). Feeding method was exclusive formula feeding in 70%, although 3 in each group were receiving limited supplementation with breast milk. A wide range of formulas were given prior to enrollment, none of which contained a probiotic. Almost 5-h daily of crying and fussing provided evidence of severe symptoms in these infants.

Clinical course

Longitudinal analysis of outcome variables indicated that total crying + fussing times at baseline were comparable, and at each of the three treatment visits crying + fussing time decreased (Table 2). The maximal difference of crying + fussing time was observed at visit 2 (day 14) comparing the 2 groups, with a mean difference of -91 (95%CI: -76, 259) min, trending toward a shorter crying+fussing time in the LGG+ group. This difference was entirely the result of improved fussing time. After the immediate dropout following written consent of 4 infants, we observed a loss of 5 of the 20 children during follow-up. These children dropped out because of mild diarrhea (*n* = 1) or their parents' decision to change the formula (*n* = 4). No adverse events during the period of observation were deemed attributable to the study product (LGG). These findings indicate that in studies of babies with colic, dropout is a common problem, with formula-changing being the major reason.

FC

We previously demonstrated an elevated FC in babies with colic, compared to age-matched babies without colic[6]. Longitudinal analysis of FC at baseline and at follow up visits showed that the values were similar at baseline (Table 2), while the maximal mean difference in FC between the LGG+ and LGG- groups was seen at visit 4 (90 d of probiotic formula treatment), with a difference of -121 (-48, 291) μg/g stool, observing a statistically nonsignificant lower level of FC in the LGG+ group.

Fecal microbiota

Distribution of predominant bacterial taxa in colicky infants: No differences in fecal diversity at any of the visits were observed between the infants with LGG+ and LGG- formulae. At baseline, the most abundant bacterial phylum was Firmicutes (72%); followed by Proteobacteria (24%). Enterobacteriales was the most abundant order and Enterobacteriaceae the most abundant family in these infants. The genus level analysis at enrollment showed the major genera to be *Blautia*, *Escherichia/Shigella*, *Enterococcus*, *Streptococcus*, and *Coprobacillus* (Figure 2). There was no significant difference in averaged genus level microbial composition distribution between (LGG+)-fed colicky babies compared to (LGG-)-fed babies with regard to the major taxa. Minor differences might have been missed because of small sample size.

Abundance of *L. rhamnosus* in stool specimens: Previously, investigators reported that a 2 wk trial of LGG at similar doses resulted in positive LGG cultures of the stool of 85% of healthy infants during its administration and in half when measured 28 d later[14]. Here, *L. rhamnosus* abundance increased to about 5% of total bacteria after 14 d of LGG+ formula treatment (*P* = 0.006), which was significantly higher than baseline, and also higher than on visit 3 or visit 4 (*P* < 0.05) (Figure 3A).

Diversity of microbiota and changes over time: We had the opportunity to analyze the evolution of microbial communities of a set of dizygotic twins at the genus level, as shown in Figure 3B. One infant was randomized to the LGG- group, while the other

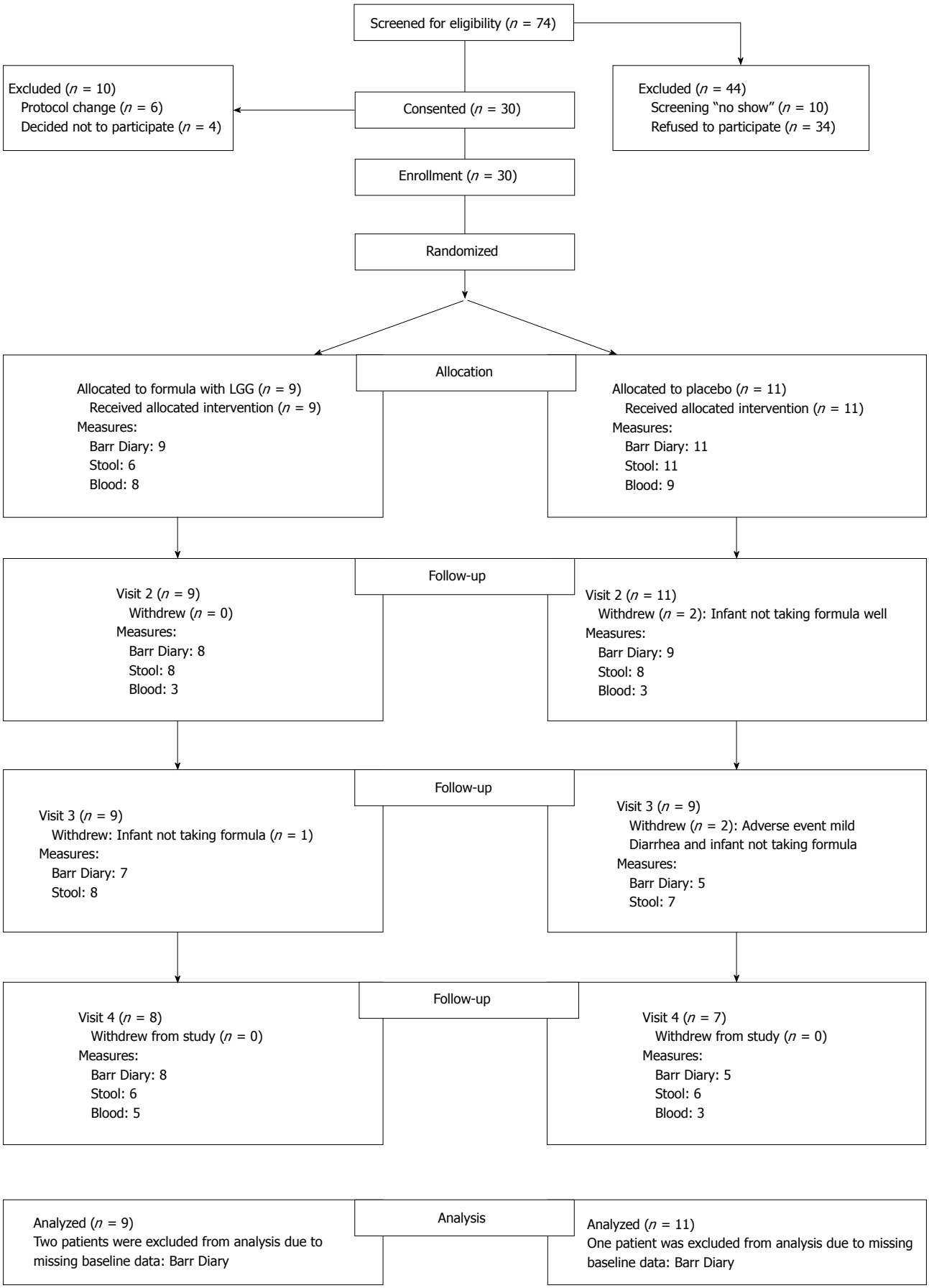


Figure 1 Participant flow diagram.

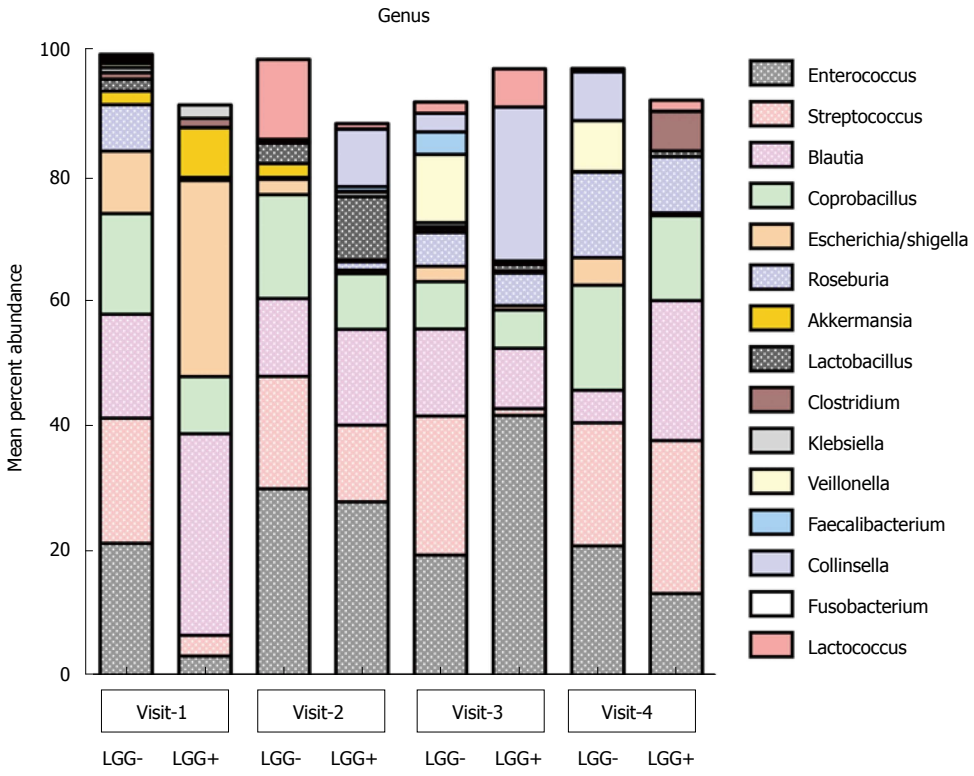


Figure 2 Fecal microbiota of infants with colic before and after treatment with *Lactobacillus rhamnosus GG vs* placebo. Average percent abundance of major bacterial groups at the genus level in colicky infants treated with LGG [LGG (+), *n* = 3] or placebo [LGG(-), *n* = 6]. Note that this was the subset of infants that had stools available for analysis at each of the 4 clinic visits. Visit 1 (day 1); visit 2 (day 14); visit 3 (day 42); and visit 4 (day 90). LGG: *Lactobacillus rhamnosus GG*.

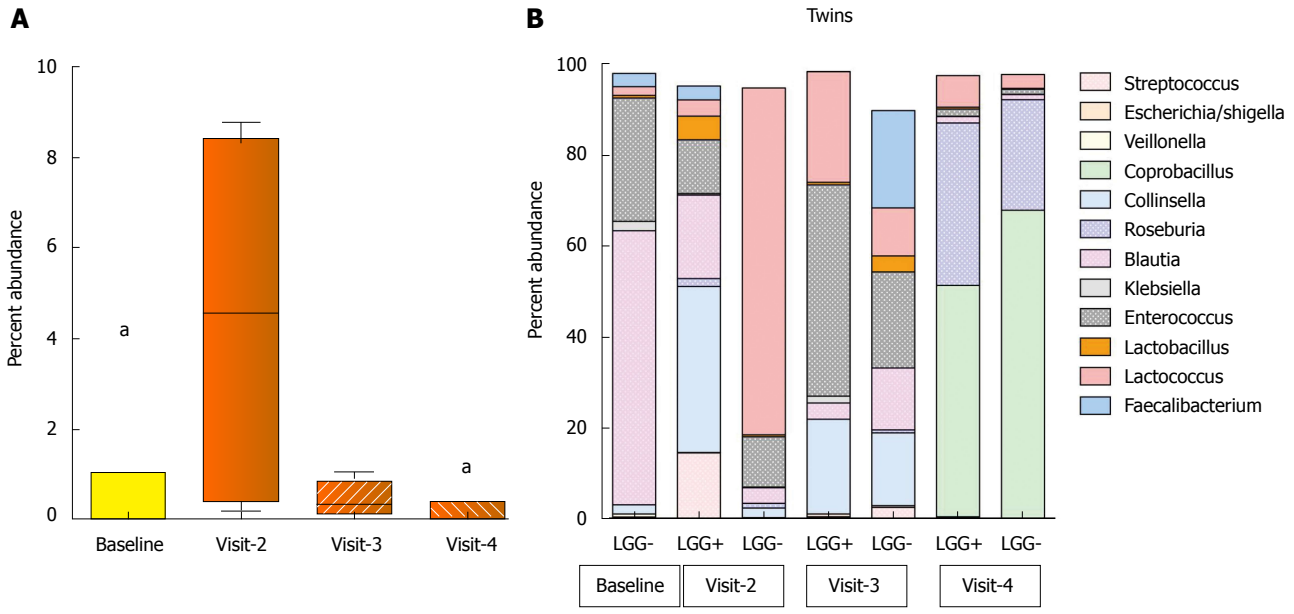


Figure 3 Abundance of *Lactobacillus rhamnosus* and shifts in composition of stool specimens. A: *Lactobacillus rhamnosus* 16S rDNA sequence abundance in colicky infants at baseline and 14, 42 and 90 d after treatment with LGG (visits 2, 3 and 4). The median, 25th-75th percentiles (boxes) and 10th-90th percentiles (whiskers) are represented. Black dots represent outliers. $^{a}P < 0.05$ compared to baseline and all other visits, respectively; B: Twins' bacterial distribution at genus level. The abundance of major bacterial genera in stools of twin infants with colic: baseline (visit 1) and 14, 42 and 90 d after assignment to formula +/- LGG (visits 2, 3 and 4). Only one stool sample was evaluable at visit 1. The percent of abundance of Lactobacillus was indicated as orange box which was increased in LGG+ compared to LGG- at visit 2 ($P < 0.05$). LGG: *Lactobacillus rhamnosus GG*.

was randomized to LGG+. Note that we were unable to obtain baseline sequence data for one twin despite two attempts, but data were available for both at each follow-up visit. At each of the treatment visits, we were able to compare the microbiota of the two infants, and we found that *Lactobacillus* was present in the

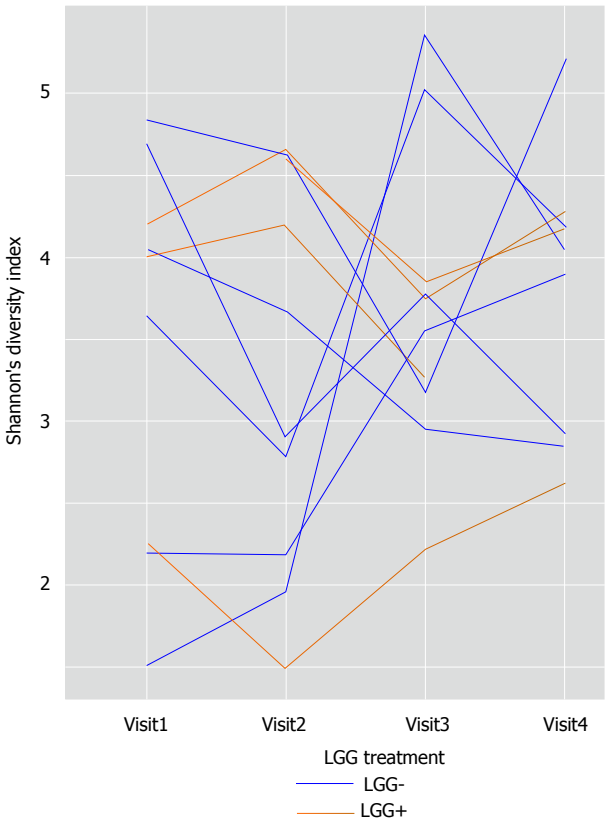


Figure 4 Shannon's diversity of stool samples, measured over time. LGG- infants' stools are shown in blue and LGG+ infants' stool results are shown in orange. Results are shown only for the patients for whom stools were available at each clinic visit. For two patients in the LGG+ group, one of the visits did not yield sequencing results. Note wide fluctuation at the various time points in infants in both groups. LGG: *Lactobacillus rhamnosus GG*.

twin babies' stools at all follow-up visits. It was most abundant in the LGG+ infant at the 2nd visit. However, lactobacillus abundance declined to low levels in both infants by visit 4. Comparing visits 2, 3 and 4 in these two infants, the stool composition fluctuated greatly. However, comparison of the microbiota of twin A and B revealed remarkable convergence of the community profile among the twins, most evident at visit 4.

Shannon's diversity index: varied wildly in all infants over time. Figure 4 shows data for all infants whose parents brought stools at each of the 4 clinic visits. There were no major differences noted between LGG+ and LGG- infants, because in both groups diversity fluctuated greatly from visit to visit. Thus, fecal microbial community was "chaotic" and did not show stabilization by the end of the 3-mo study, when the infants were 4-5 mo old.

Other clinical and basic lab testing

There were no major differences between the LGG+ and LGG- groups with respect to breath hydrogen levels (data not shown), plasma cytokine levels, or percentage of circulating Tregs (Table 3). With respect to breath hydrogen, 5 of the 20 infants (25%) had breath hydrogen increases of ⩾ 12 ppm above baseline at their initial sampling, while two infants (10%) had increased breath hydrogen at visit 2. However, there was no significant difference between the LGG+ and placebo groups with respect to breath hydrogen changes, and there was also no correlation between changes in breath hydrogen and crying time.

Table 3 Longitudinal analysis of cytokins by study group (LGG+ *vs* LGG-) at baseline and follow up visits

	Adjusted geometric means (95%CI)		*P*
	LGG+ group	LGG- group	
Cytokines			
IFN-γ (pg/mL)			
Visit 1 (Baseline)	0.7 (0.4, 1.3)	0.6 (0.3, 1.2)	0.84
Visit 2	1.2 (0.7, 2.0)	0.5 (0.3, 0.9)	0.04
Visit 4	0.4 (0.3, 0.6)	0.2 (0.1, 0.4)	0.07
IL-10 (pg/mL)			
Visit 1 (Baseline)	3.7 (2.3, 6.0)	4.2 (2.2, 8.1)	0.78
Visit 2	4.3 (3.2, 5.7)	3.8 (2.3, 6.4)	0.72
Visit 4	2.7 (1.7, 4.2)	2.0 (1.1, 3.7)	0.47
IL-12p70 (pg/mL)			
Visit 1 (Baseline)	0.2 (0.1, 0.4)	0.2 (0.1, 0.4)	0.85
Visit 2	0.3 (0.2, 0.4)	0.4 (0.3, 0.6)	0.29
Visit 4	0.2 (0.1, 0.5)	0.3 (0.2, 0.4)	0.57
IL-1β (pg/mL)			
Visit 1 (Baseline)	0.08 (0.05, 0.13)	0.19 (0.08, 0.43)	0.09
Visit 2	0.27 (0.08, 0.88)	0.15 (0.06, 0.40)	0.45
Visit 4	0.11 (0.05, 0.21)	0.16 (0.05, 0.49)	0.52
IL-6 (pg/mL)			
Visit 1 (Baseline)	0.3 (0.2, 0.5)	0.4 (0.3, 0.6)	0.36
Visit 2	0.3 (0.2, 0.6)	0.3 (0.2, 0.4)	0.66
Visit 4	0.2 (0.1, 0.4)	0.2 (0.1, 0.3)	0.63
IL-8 (pg/mL)			
Visit 1 (Baseline)	6.5 (4.8, 8.7)	7.0 (5.3, 9.4)	0.69
Visit 2	5.4 (4.1, 7.3)	9.5 (6.6, 13.7)	0.02
Visit 4	4.9 (3.2, 7.5)	4.6 (2.8, 7.8)	0.88
TNF-α (pg/mL)			
Visit 1 (Baseline)	4.5 (3.8, 5.3)	4.8 (3.7, 6.2)	0.69
Visit 2	5.4 (4.4, 6.5)	4.2 (3.2, 5.5)	0.15
Visit 4	4.4 (3.3, 5.9)	5.7 (4.7, 6.9)	0.14
Treg (%)			
Visit 1 (Baseline)	7.2 (6.5, 8.0)	6.7 (5.9, 7.5)	0.37
Visit 2	7.5 (6.8, 8.2)	8.5 (7.0, 10.3)	0.23
Visit 4	7.5 (6.1, 9.3)	9.2 (8.1, 10.5)	0.10

Longitudinal model: $\ln(\text{cytokines}) = \beta_0 + \beta_1 \times \text{visit2} + \beta_2 \times \text{visit4} + \beta_3 \times \text{group} + \beta_4 \times \text{visit2 group} + \beta_5 \times \text{visit4 group}$; Here, visit2, visit4 are dummy variables; visit2 = 1 if at visit 2, 0 otherwise; visit4 = 1 if at visit 4, 0 otherwise; group = 1 if in LGG group, 0 otherwise. The geometric mean is the anti-log of arithmetic mean of log-transformed value. *P* values are obtained using wald test to test whether the differences of cytokines between study groups are significant at each visit.

DISCUSSION

Recruitment and retention

Recently published in JAMA Pediatrics, Sung's systematic review of probiotics for colic emphasized that there remains "insufficient evidence to support probiotics to manage colic, especially in formula-fed infants"[15]. Our study which focused on this group, was not powered to determine efficacy of LGG+ formula, but we aimed to identify key information for future prospective randomized, controlled trials of treatments for infants

with colic in the United States. Although not focused on colic, a recent double blind placebo-controlled trial focused on preterm infants (gestational age 32-36 wk.) who were randomized to receive LGG (> 10^9 CFU/d), a combination prebiotic or placebo. Authors reported that these infants were more likely to be contented (as opposed to "excessive criers") during the first 2 mo of life if they received prebiotic or LGG as compared to placebo[16]. Barr diaries were not used in this study. In addition to Sung's trial, our results may be included in meta-analyses of probiotic supplementation for formula-fed infants with colic.

One key finding was that recruitment of babies with colic in this country is difficult. During the consent process, two-thirds of parents we interviewed declined to particiate in this trial. Possible reasons included the FDA- and IRB-mandated consent form (which contained the statement "very rare cases of blood infection, acidosis (acid in the blood), endocarditis (heart valve infection), and meningitis (swelling and irritation of the covering tissue around the brain) in patients who are already ill have been reported". Parents who declined to participate said that they preferred to try formulas, herbal remedies, and probiotics that are advertised to be beneficial for babies with colic or "sensitive" intestines. To the authors, this indicated their uncertainty associated with clinical research and the informed consent process.

After recruitment, retention was about 75%, with dropouts related to infants not taking the formula or to the observation of loose stools. Based on our previous study of crying + fussing time in colicky infants[6], we determined that a sample size of 60 colicky infants (30 infants per study arm) would detect a mean difference of 100 min/d of crying + fussing time between the 2 study groups. We attempted to recruit this number over 2 years, but were unable to do so. Although we cannot be certain that enrollment and dropout rates would be the same for similar trials, we can estimate that a future probiotic formula trial conducted in the United States would require recruitment of > 80 enrollees for 60 infants to complete the trial. With our recruitment numbers in the 4th largest United States city, with 5300 pediatric gastroenterology visits annually, we suggest that a 3-center, 2-year trial would be optimal. Note that smaller differences in crying + fussing time were found to be significant in the Savino *et al*[5] and Szajewska *et al*[17] studies, but they required numbers of infants similar to the 30 in each group that we herein recommend.

Safety

We found no major side effects or safety problems contributing to product concerns or patient dropouts.

Possible gut inflammation in colic

We reported previously in infants with colic elevated FC, suggesting a contribution of gut inflammation to this condition[18]. In the current study, we found that FC levels were high at virtually all time-points in the infants with colic, in both LGG+ and LGG- infants. The values of FC were consistently above the normal clinical range reported in adults (0-162 μg/g). An elevated FC in infants with colic and in normal infants at this age may reflect low-grade intestinal inflammation during this period of aggressive microbial colonization[19]. In addition, some of the infants were partially breast-fed, a condition arguably associated with a higher FC level[20], although there were only 3 such infants in each group. The difference in FC between groups was not significant. There could be a downward trend as a consequence of normal colonization, because FC levels in older children are substantially lower than those in infants[21]. Savino *et al*[22] recently published in abstract form a similar increase in FC in infants with colic.

While FC may be a helpful biomarker during the evolution of colic, plasma cytokines appear not to be informative. All of the cytokine levels in the infant plasma were detectable above the minimal detection level of the assay, and values were similar to those previously reported in other studies[23-25].

Gas production in colic

Our study provides evidence that excessive intestinal H_2 gas production is less prevalent in our population than was reported previously[1]. Glucose breath testing and/or elevated fasting breath H_2 in our study was abnormal in only 25% of our infants with colic. A previous study, in which infants with colic received the nonabsorbable sugar lactulose which raised breath H_2 values, showed that most babies did not have any symptomatic response to lactulose[26]. It is possible that excessive gas could be a contributing factor to colic in a subset of infants.

Microbial characterization of infant colic

Pyrosequencing of 16S rRNA in the stool showed several interesting findings. First, longitudinally hyper-variable microbiota profiles during the 3-mo study (very evident in the twin pair shown in Figure 1B) support the concept of a "chaotic" pattern of colonization described by Palmer *et al*[27] early in life. This chaotic pattern has been challenged, with Koenig *et al*[28] suggesting a revised concept of population shifts attributable to major changes in life events. However in our study, patients were followed by telephone on a weekly basis for 3 mo. There were no major changes in life events, such as diet, diarrhea, antibiotic administration, or probiotic ingestion that were reported (Figure 1B).

One unexpected observation of our study was that inclusion of LGG in the formula had little impact on the overall composition or microbial diversity of the stool. This finding was particularly evident comparing the microbiota of the twin infants. The twins' fecal microbial composition at 3 time-points fluctuated greatly, and there were *Lactobacilli* in both infants' stools at visits 2 (day 14) to 4

(day 90), even though only one received LGG. Previous studies have shown that among adult monozyotic twins, the average microbiota similarity between twins is significantly higher than between unrelated subjects[29]. We suggest that factors other than *L. rhamnosus* were responsible for shifts in the fecal microbiota pattern of the infants. Such factors may include exposure to other people, animals, or environments.

In summary, our study showed that future trials of probiotics in formula for infants with colic at concentrations similar to those of Nutramigen with LGG should aim to recruit around 80 infants and should focus on determining efficacy on crying time at 14 d and on FC at 90 d. Changes in the microbiota (and/or their metabolic products) might be optimally observed at 2-4 wk of treatment.

ACKNOWLEDGMENTS

The authors thank Drs. Mary Kay Koenig, Kuojen Tsao, and Cynthia Bell, for serving on the Data Safety Monitoring Committee. We would also like to acknowledge the University of Texas Pediatric Clinic's physicians and nursing staff, as well as the Memorial Hermann and Kelsey-Seybold clinics, and other local Houston pediatric clinics in Houston that provided referrals for this study. We thank Dr. Yen Pham for her assistance in project planning and IRB approval, Deborah Lake and Meredith Rayne in Media Relations for helping us recruit patients, and the pediatric phlebotomist, Shantel Beasley. Finally, we thank Elizabeth Donnachie from Gulf States Hemophilia Center at the University of Texas Health Science Center at Houston, who assisted in operating BD FACSCalibur.

COMMENTS

Background

Colic has been defined as inconsolable crying plus fussing time equaling greater than 3 h daily for more than 3 d per week in infants from 3 wk to 3 mo of age. There have been many theories to explain the occurrence of colic, including bacterial overgrowth, the "fourth trimester" theory in which the baby would prefer to stay in utero, parental depression, excessive intestinal gas, and milk protein allergy. The probiotic *Lactobacillus rhamnosus GG* (LGG) has been shown to reduce diarrheal volume and duration in children with acute infectious enteritis; it has also been shown to facilitate the development of more diverse fecal microbiota. Savino was the first to propose that an abnormal microbiota ("dysbiosis") might be an important pathophysiological mechanism.

Research frontiers

Colic has begun to be studied as an example of possible dysbiosis with inflammation of the gut. Recent studies have centered on possible gut developmental issues, including the transition of environment from the intrauterine "bubble" to the external world and exposure to many new bacterial communities. Manipulation of the gut microbiota could be a major advance toward reducing the crying and fussing times of these infants.

Innovations and breakthroughs

This study is one of the first to investigate the impact on a probiotic on the microbiota of colicky babies and fecal calprotectin, as a marker of inflammation, during early life.

Applications

To summarize the practical applications of your research findings, so that readers may understand the perspectives by which this study will affect the field and future research. Future trials of probiotic-supplemented formulas may benefit from our pilot trial. The authors demonstrated robust numbers for crying + fussing time, fecal calprotectin, and microbial diversity in this population.

Terminology

LGG is a health-promoting bacterial species which may be capable of reducing inflammation and regulating gut function. Microbiota is the term for bacterial community found in various locations such as skin and gut. The authors' focus is on the intestine, which has been shown to regulate diverse functions in the human body.

Peer-review

The topic is interesting and the manuscript well presented.

REFERENCES

1 **Moore DJ**, Robb TA, Davidson GP. Breath hydrogen response to milk containing lactose in colicky and noncolicky infants. *J Pediatr* 1988; **113**: 979-984 [PMID: 3193321 DOI: 10.1016/S0022-3476(88)80567-5]

2 **Karp H**. The fourth trimester and the calming reflex: novel ideas for nurturing young infants. *Midwifery Today Int Midwife* 2012; **(102)**: 25-26, 67 [PMID: 22856072]

3 **van den Berg MP**, van der Ende J, Crijnen AA, Jaddoe VW, Moll HA, Mackenbach JP, Hofman A, Hengeveld MW, Tiemeier H, Verhulst FC. Paternal depressive symptoms during pregnancy are related to excessive infant crying. *Pediatrics* 2009; **124**: e96-103 [PMID: 19564275 DOI: 10.1542/peds.2008-3100]

4 **Lucassen PL**, Assendelft WJ. Systematic review of treatments for infant colic. *Pediatrics* 2001; **108**: 1047-1048 [PMID: 11589211 DOI: 10.1542/peds.108.4.1047]

5 **Savino F**, Cordisco L, Tarasco V, Palumeri E, Calabrese R, Oggero R, Roos S, Matteuzzi D. Lactobacillus reuteri DSM 17938 in infantile colic: a randomized, double-blind, placebo-controlled trial. *Pediatrics* 2010; **126**: e526-e533 [PMID: 20713478 DOI: 10.1542/peds.2010-0433]

6 **Rhoads JM**, Fatheree NY, Norori J, Liu Y, Lucke JF, Tyson JE, Ferris MJ. Altered fecal microflora and increased fecal calprotectin in infants with colic. *J Pediatr* 2009; **155**: 823-828.e1 [PMID: 19628216 DOI: 10.1016/j.jpeds.2009.05.012]

7 **Szajewska H**, Mrukowicz JZ. Probiotics in the treatment and prevention of acute infectious diarrhea in infants and children: a systematic review of published randomized, double-blind, placebo-controlled trials. *J Pediatr Gastroenterol Nutr* 2001; **33** Suppl 2: S17-S25 [PMID: 11698781 DOI: 10.1097/00005176-200110002-00004]

8 **Azad MB**, Konya T, Maughan H, Guttman DS, Field CJ, Sears MR, Becker AB, Scott JA, Kozyrskyj AL. Infant gut microbiota and the hygiene hypothesis of allergic disease: impact of household pets and siblings on microbiota composition and diversity. *Allergy Asthma Clin Immunol* 2013; **9**: 15 [PMID: 23607879]

9 **Barr RG**, Rotman A, Yaremko J, Leduc D, Francoeur TE. The crying of infants with colic: a controlled empirical description. *Pediatrics* 1992; **90**: 14-21 [PMID: 1614771]

10 **Mangalat N**, Liu Y, Fatheree NY, Ferris MJ, Van Arsdall MR, Chen Z, Rahbar MH, Gleason WA, Norori J, Tran DQ, Rhoads JM. Safety and tolerability of Lactobacillus reuteri DSM 17938 and effects on biomarkers in healthy adults: results from a randomized masked trial. *PLoS One* 2012; **7**: e43910 [PMID: 22970150 DOI: 10.1371/journal.pone.0043910]

11 **Yan F**, Polk DB. Probiotic bacterium prevents cytokine-induced apoptosis in intestinal epithelial cells. *J Biol Chem* 2002; **277**: 50959-50965 [PMID: 12393915]

12 **Gupta RW**, Tran L, Norori J, Ferris MJ, Eren AM, Taylor CM, Dowd SE, Penn D. Histamine-2 receptor blockers alter the fecal

microbiota in premature infants. *J Pediatr Gastroenterol Nutr* 2013; **56**: 397-400 [PMID: 23254444 DOI: 10.1097/MPG.0b013e318282a8c2]

13 **Caporaso JG**, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, Fierer N, Peña AG, Goodrich JK, Gordon JI, Huttley GA, Kelley ST, Knights D, Koenig JE, Ley RE, Lozupone CA, McDonald D, Muegge BD, Pirrung M, Reeder J, Sevinsky JR, Turnbaugh PJ, Walters WA, Widmann J, Yatsunenko T, Zaneveld J, Knight R. QIIME allows analysis of high-throughput community sequencing data. *Nat Methods* 2010; **7**: 335-336 [PMID: 20383131 DOI: 10.1038/nmeth.f.303]

14 **Petschow BW**, Figueroa R, Harris CL, Beck LB, Ziegler E, Goldin B. Effects of feeding an infant formula containing Lactobacillus GG on the colonization of the intestine: a dose-response study in healthy infants. *J Clin Gastroenterol* 2005; **39**: 786-790 [PMID: 16145341 DOI: 10.1097/01.mcg.0000177245.53753.86]

15 **Sung V**, Collett S, de Gooyer T, Hiscock H, Tang M, Wake M. Probiotics to prevent or treat excessive infant crying: systematic review and meta-analysis. *JAMA Pediatr* 2013; **167**: 1150-1157 [PMID: 24100440]

16 **Pärtty A**, Luoto R, Kalliomäki M, Salminen S, Isolauri E. Effects of early prebiotic and probiotic supplementation on development of gut microbiota and fussing and crying in preterm infants: a randomized, double-blind, placebo-controlled trial. *J Pediatr* 2013; **163**: 1272-1277.e1-2 [PMID: 23915796 DOI: 10.1016/j.jpeds.2013.05.035]

17 **Szajewska H**, Gyrczuk E, Horvath A. Lactobacillus reuteri DSM 17938 for the management of infantile colic in breastfed infants: a randomized, double-blind, placebo-controlled trial. *J Pediatr* 2013; **162**: 257-262 [PMID: 22981952 DOI: 10.1016/j.jpeds.2012.08.004]

18 **Kapel N**, Campeotto F, Kalach N, Baldassare M, Butel MJ, Dupont C. Faecal calprotectin in term and preterm neonates. *J Pediatr Gastroenterol Nutr* 2010; **51**: 542-547 [PMID: 20818270]

19 **Schelonka RL**, Maheshwari A, Carlo WA, Taylor S, Hansen NI, Schendel DE, Thorsen P, Skogstrand K, Hougaard DM, Higgins RD. T cell cytokines and the risk of blood stream infection in extremely low birth weight infants. *Cytokine* 2011; **53**: 249-255 [PMID: 21145756 DOI: 10.1016/j.cyto.2010.11.003]

20 **Savino F**, Castagno E, Calabrese R, Viola S, Oggero R, Miniero R. High faecal calprotectin levels in healthy, exclusively breast-fed infants. *Neonatology* 2010; **97**: 299-304 [PMID: 19887860 DOI: 10.1159/000255161]

21 **Olafsdottir E**, Aksnes L, Fluge G, Berstad A. Faecal calprotectin levels in infants with infantile colic, healthy infants, children with inflammatory bowel disease, children with recurrent abdominal pain and healthy children. *Acta Paediatr* 2002; **91**: 45-50 [PMID: 11883817 DOI: 10.1111/j.1651-2227.2002.tb01638.x]

22 **Savino F,** De MA, Ceratto S, and Mostert M. Fecal Calprotectin During Treatment of Severe Infantile Colic With Lactobacillus reuteri DSM 17938: A Randomized, Double-Blind, Placebo-Controlled Trial. *Pediatrics* 2015; **135**: S5-S6 [DOI: 10.1542/peds.2014-3330H]

23 **Keir AK**, McPhee AJ, Andersen CC, Stark MJ. Plasma cytokines and markers of endothelial activation increase after packed red blood cell transfusion in the preterm infant. *Pediatr Res* 2013; **73**: 75-79 [PMID: 23095979 DOI: 10.1038/pr.2012.144]

24 **Mellgren K**, Hedegaard CJ, Schmiegelow K, Müller K. Plasma cytokine profiles at diagnosis in pediatric patients with non-hodgkin lymphoma. *J Pediatr Hematol Oncol* 2012; **34**: 271-275 [PMID: 22430582 DOI: 10.1097/MPH.0b013e3182431e02]

25 **Rovira-Vallbona E**, Moncunill G, Bassat Q, Aguilar R, Machevo S, Puyol L, Quintó L, Menéndez C, Chitnis CE, Alonso PL, Dobaño C, Mayor A. Low antibodies against Plasmodium falciparum and imbalanced pro-inflammatory cytokines are associated with severe malaria in Mozambican children: a case-control study. *Malar J* 2012; **11**: 181 [PMID: 22646809 DOI: 10.1186/1475-2875-11-181]

26 **Treem WR**, Hyams JS, Blankschen E, Etienne N, Paule CL, Borschel MW. Evaluation of the effect of a fiber-enriched formula on infant colic. *J Pediatr* 1991; **119**: 695-701 [PMID: 1658281 DOI: 10.1016/S0022-3476(05)80282-3]

27 **Palmer C**, Bik EM, DiGiulio DB, Relman DA, Brown PO. Development of the human infant intestinal microbiota. *PLoS Biol* 2007; **5**: e177 [PMID: 17594176 DOI: 10.1371/journal.pbio.0050177]

28 **Koenig JE**, Spor A, Scalfone N, Fricker AD, Stombaugh J, Knight R, Angenent LT, Ley RE. Succession of microbial consortia in the developing infant gut microbiome. *Proc Natl Acad Sci USA* 2011; **108** Suppl 1: 4578-4585 [PMID: 20668239]

29 **Tims S**, Derom C, Jonkers DM, Vlietinck R, Saris WH, Kleerebezem M, de Vos WM, Zoetendal EG. Microbiota conservation and BMI signatures in adult monozygotic twins. *ISME J* 2013; **7**: 707-717 [PMID: 23190729 DOI: 10.1038/ismej.2012.146]

P- Reviewer: Bourke B, Bian ZX, Silva MA
S- Editor: Tian YL **L- Editor**: A **E- Editor**: Jiao XK

World Journal of Gastrointestinal Pathophysiology

Submit a Manuscript: http://www.wjgnet.com/esps/
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.4291/wjgp.v7.i1.171

World J Gastrointest Pathophysiol 2016 February 15; 7(1): 171-180
ISSN 2150-5330 (online)

ORIGINAL ARTICLE

Observational Study

Efficacy and tolerability of hydrogen carbonate-rich water for heartburn

André-Michael Beer, Ralf Uebelhack, Ute Pohl

André-Michael Beer, Klinik Blankenstein, Ruhr-Universität Bochum, 45527 Hattingen, Germany

Ralf Uebelhack, Ute Pohl, Analyze and Realize GmbH, 13456 Berlin, Germany

Author contributions: Beer AM contributed to writing of the article; Uebelhack R was principal investigator and medical expert; Pohl U contributed to study conception and design, data acquisition as well as data analysis and interpretation; All authors contributed to editing, reviewing and final approval of the article.

Supported by Deutsche Heilbrunnen im Verband Deutscher Mineralbrunnen e. V., Kennedyallee 28, 53175 Bonn, Germany, www.vdm-bonn.de.

Institutional review board statement: The study protocol was approved by the relevant ethics committee [State Office of Health and Social Affairs Berlin (Landesamt für Gesundheit und Soziales Berlin)] and the competent authority [Federal Institute for Drugs and Medical Devices (Bundesinstitut für Arzneimittel und Medizinprodukte, Bonn)]. The presence of the approval document was verified by the editorial office. The document is available upon request.

Informed consent statement: Both the patients and the investigator signed the informed consent form before inclusion. The presence of the approval document was verified by the editorial office. The document is available upon request.

Conflict-of-interest statement: The preparation of the manuscript, and thus indirectly the authors Uebelkack R and Pohl U, were supported by Deutsche Heilbrunnen im Verband Deutscher Mineralbrunnen e. V., Kennedyallee 28, 53175 Bonn, Germany, www.vdm-bonn.de. The authors declare that besides this no conflict of interest exists.

Data sharing statement: No additional data are available.

Correspondence to: Dr. Ute Pohl, Analyze and Realize GmbH, Waldseeweg 6, 13456 Berlin, Germany. upohl@a-r.com
Telephone: +49-30-40008100
Fax: +49-30-40008100

Received: July 14, 2015
Peer-review started: July 17, 2015
First decision: August 31, 2015
Revised: November 17, 2015
Accepted: December 16, 2015
Article in press: December 18, 2015
Published online: February 15, 2016

Abstract

AIM: To investigate the efficacy and safety of mineral water with a high content of hydrogen carbonate in patients with heartburn.

METHODS: This open, single-center, single-arm clinical pilot study enrolled 50 patients, 18-64 years old, who had been suffering from heartburn at least twice a week for at least 3 mo before entering the study. Pharmacological treatment of heartburn was not permitted, and patients with severe organic diseases were excluded. After a run-in period of one week, the participants received 1.5 L of the test water for the following 6 wk; 300 mL with meals t.i.d., the remainder to be drunk throughout the day. During the trial, there were five visits at the study center (screening, baseline, two interim visits and the final visit). The efficacy endpoints included incidence and duration of heartburn episodes per week by patient's self-assessment (heartburn diary) as well as changes in symptom severity as per symptom specific questionnaires [Reflux Disease

Questionnaire (RDQ); Quality of Life in Reflux and Dyspepsia (QOLRAD); Gastrointestinal Quality of Life Index] and overall health-related quality of life per SF-12 (12-question short form) at each visit. At the end of the study, patients and investigators independently rated the overall efficacy of the test water on a 4-point Likert scale. Safety was assessed by evaluation of adverse events (AEs), vital signs (heart rate, blood pressure) and laboratory parameters. Changes from initial to final examinations were assessed by the non-parametric Wilcoxon test; categorical variables were compared using the χ^2 test, and for more than 5 categories, by the U-test.

RESULTS: Twenty-eight participants were men, 22 women. The mean age of the patients in the full analysis set/intention-to treat population (FAS/ITT) was 40.6 years. Forty-two participants completed the study according to the study protocol and formed the per-protocol set (PP population); 48 participants drank the water at least once as requested and were analyzed as ITT population. The occurrence of heartburn was statistically significantly reduced at wk 6 in both the ITT and the PP populations. At wk 6, the mean number of heartburn episodes/week decreased by 5.1 episodes ($P < 0.001$) and the mean duration of heartburn symptoms by 19 min (ITT) ($P = 0.002$). The frequency of heartburn symptoms was reduced in 89.6% of the patients ($P < 0.001$), and the duration of symptoms in 79.2% of patients (ITT) ($P < 0.001$). All dimensions of the RDQ (heartburn, regurgitation, gastro-esophageal reflux disease symptoms, dyspepsia) showed a significant improvement at 6 wk. Likewise, disease-specific quality of life improved significantly (QOLRAD, GIQLI). Overall, 89.4% of patients rated the efficacy of the test water as "good" or "very good", as did the investigators for 91.5% of the patients. There were no serious AEs. After 6 wk, systolic and diastolic blood pressure values decreased slightly but significantly [-3.5 and -3.0 mmHg, respectively ($P = 0.008$ and $P = 0,002$)]. Ninety-six percent of patients and investigators for the same percentage of patients rated the tolerability of the water as "good" or "very good".

CONCLUSION: The data demonstrate effectiveness of a hydrogen carbonate-rich mineral water in alleviating heartburn frequency and severity, thereby improving quality of life. The water has excellent tolerability.

Key words: Heartburn; Hydrogen carbonate-rich mineral water; Open clinical pilot study; Patients; Regurgitation; Gastroesophageal reflux disease symptoms; Dyspepsia; Blood pressure; Tolerability; Quality of life

Core tip: This open, single-center, single-arm clinical study investigated the efficacy and safety of mineral water with a high content of hydrogen carbonate in patients with heartburn. After 6 wk, the occurrence of heartburn was statistically significantly reduced both in the intention-to-treat (48 patients) and the per-protocol populations (42 patients). All dimensions of the Reflux Disease Questionnaire (heartburn, regurgitation, gastroesophageal reflux disease symptoms, dyspepsia) showed significant improvement at week 6. Likewise, disease-specific quality of life improved significantly, and there was a slight but significant decrease in blood pressure. The tolerability was rated good or very good by 96% of patients and investigators.

Beer AM, Uebelhack R, Pohl U. Efficacy and tolerability of hydrogen carbonate-rich water for heartburn. *World J Gastrointest Pathophysiol* 2016; 7(1): 171-180 Available from: URL: http://www.wjgnet.com/2150-5330/full/v7/i1/171.htm DOI: http://dx.doi.org/10.4291/wjgp.v7.i1.171

INTRODUCTION

Gastroesophageal reflux disease (GERD) with insufficiency of the lower esophageal sphincter can significantly affect a person's quality of life, even if endoscopy does not demonstrate any pathological findings [non-erosive reflux disease (NERD); early stage of GERD]. Symptoms include burning retrosternal and/or epigastric pains ("heartburn") and range from belching/regurgitation of food remains to an unpleasant, salty/soapy taste in the mouth to the point of vomiting.

Heartburn is one of the most frequent gastrointestinal symptoms affecting patients. According to a current review, the range of GERD (heartburn and/or regurgitation on at least 1 d/wk) prevalence estimates was 8.8%-25.9% in Europe, 18.1%-27.8% in North America, but only 2.5%-7.8% in East Asia[1].

The pathogenesis of the symptoms of NERD has not yet been finally clarified. The direct acidic effect is probably an essential factor, but possibly not the only one. Under discussion are the increased sensitivity of intra-esophageal pain receptors, increased diffusion of gastric acid into the esophageal epithelium and up-regulation of the peripheral pain receptors with resultant central sensitization of spinal neurons[2].

The treatment of NERD or GERD focuses on the neutralization of gastric acid using a multistage approach. The first stage involves lifestyle changes such as weight reduction and abstinence from certain foods or beverages. If mild symptoms persist in spite of these measures, calcium, magnesium or aluminum antacids are used to neutralize gastric acid. In a subsequent step, histamine-2 receptor blockers (ranitidine, famotidine) or proton-pump inhibitors (PPIs) (omeprazole, pantoprazole) are utilized[3].

An alternative that also meets patients' increasing desire for complementary- medicine approaches is the administration of hydrogen carbonate-rich mineral water (minimum content of hydrogen carbonate 1300 mg/L).

As part of a buffer reaction, the hydrogen carbonate anion binds the protons of the gastric acid in a similar way to pharmacological antacids ($H^+ + HCO_3^- \rightarrow H_2O + CO_2$). Additionally, a dilution effect is achieved by the volume of liquid in which the HCO_3^- anions are dissolved. Various waters containing hydrogen carbonate have displayed positive study results in functional dyspepsia[4-6].

Moreover, hydrogen carbonate-rich mineral water strengthens the natural protective mechanisms of the gastric mucosa with an improvement in mucosal secretion and blood flow. Finally, an influence of hydrogen carbonate on the homoeostasis of enterohormones has been discussed, whereby secretin and cholecystokinin secretion would be promoted and gastrin secretion inhibited; the overall equilibrium would be shifted towards acid inhibition[7].

Within this context, an open pilot clinical study on the efficacy and tolerability of a mineral water containing hydrogen carbonate was conducted in patients with mild heartburn symptoms. The aim was to test whether a mineral water with a high content of hydrogen carbonate leads to an improvement in the symptoms and the concomitant quality of life.

MATERIALS AND METHODS

A total of 50 patients aged 18 to 64 years were included in the open, single-arm, single-center clinical study over a time period of 7 mo. Before inclusion, the patients were extensively informed by the investigator of the benefits and risks of the trial and also asked to read the comprehensive patient information. In case of patient's decision to participate in the study, both the patient and the investigator signed the informed consent.

Inclusion criteria

For at least 3 mo before the start of the study, the participants had to have had heartburn symptoms that had not been treated with medication, involving at least two episodes a week according to information provided by the patient. As a further requirement, the participants should be drinking at least 1.3 L of fluids (water, tea, soft drinks) a day, to ensure a corresponding fluid intake.

Criteria for continuing the study after run-in

During the one-week run-in phase, the patients documented their episodes of heartburn and drinking habits in a daily diary. For further participation in the clinical trial, the patients had to have heartburn at least twice a week and consume at least 1.3 L of fluids (water, tea, soft drinks) per day.

Exclusion criteria

Patients with the following conditions were excluded: Serious organic diseases of the heart or gastrointestinal tract, history of surgical interventions of the esophagus, stomach and small intestine, irritable bowel syndrome, endocrinological diseases, serious renal insufficiency, a tendency towards formation of calcium or "infection" kidney stones, *E. coli* urinary tract infections, iron-deficiency anemia, persistent vomiting or a family history of gastrointestinal tumors. Further exclusion criteria were: Medication for acid blockade (intake of antacids and histamine-2 receptor blockers in the 2 d before and during the study, intake of PPIs in the 14 d before and during the study), *Helicobacter* eradication drugs (in the 3 mo before and during the study), acetylsalicylic acid and non-steroidal anti-inflammatory drugs during the study, expected non-compliance and pregnancy and breastfeeding.

Study conduct

A schematic of the study conduct can be seen in Figure 1. The duration of the study was 7 wk per patient. During this time, there were five visits to the study center: (1) visit 1 (V1, screening): Providing the patients with information on the aim and course of the study, written patient consent, taking the patient's history and recording drug intake, a physical examination involving measurement of heart rate and blood pressure, taking blood and urine samples, performing a pregnancy test where appropriate, issue of patient diary; (2) visit 2 (V2, 8 to 12 d after V1, baseline): Initial examination with documentation of new or changed concomitant diseases/concomitant medication, return and examination of the patient diary with regard to symptoms and fluid intake, measurement of blood pressure and heart rate, completion of the Reflux Disease Questionnaire (RDQ), Quality of Life in Reflux and Dyspepsia (QOLRAD), GIQLI and SF-12 questionnaires, issue of patient diary; (3) visit 3 and 4 (V3, V4, control visits 14 ± 3 d and 28 ± 5 d after V2): Documentation of new or changed concomitant medication, documentation of any adverse events (AEs), measurement of blood pressure and heart rate, completion of the questionnaires, return, examination and issue of patient diary; and (4) visit 5 (V5, final visit, 42 ± 5 d after V2): Documentation of new or changed concomitant medication, documentation of any AEs, measurement of blood pressure and heart rate, blood and urine samples, pregnancy test where appropriate, completion of the questionnaires, return and examination of patient diary, overall assessment of the efficacy and tolerability by investigator and patient, final examination, return of the test water after the visit.

The study protocol was approved by the relevant ethics committee [State Office of Health and Social Affairs Berlin (Landesamt für Gesundheit und Soziales Berlin)] and the competent authority [Federal Institute for Drugs and Medical Devices (Bundesinstitut für Arzneimittel und Medizinprodukte, Bonn)]. Implementation of the study was in line with the principles of the World Medical Association (Declaration of Helsinki), the guidelines for Good Clinical Practice [CPMP/ICH/135/95; Topic E6 (R1)], the German Medicinal Products Act (Arzneimittelgesetz) and the Ordinance on Good Clinical

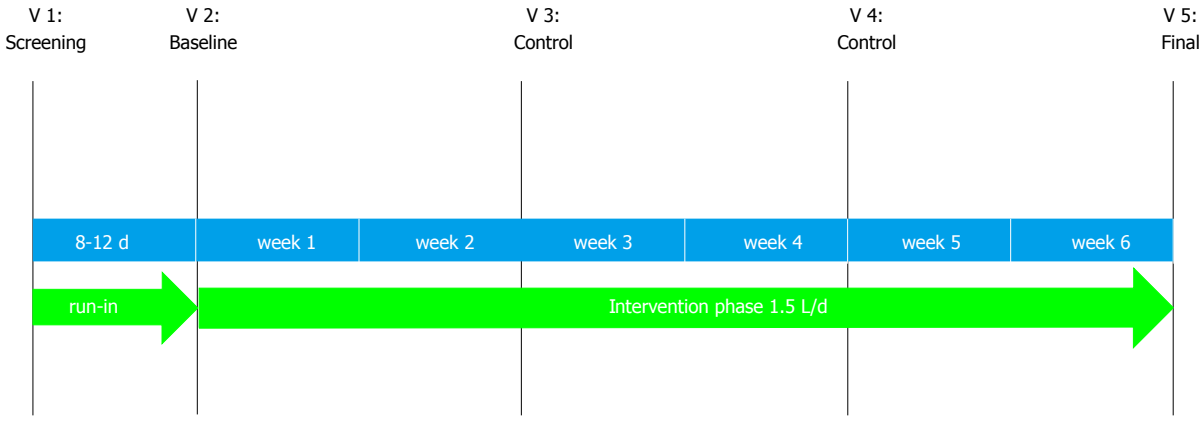


Figure 1 Study conduct. After a run-in phase without treatment, there was a 6-wk intervention phase, in which patients consumed 1.5 L of mineral water per day. The visit schedule is indicated. V: Visit.

Table 1 Components of the test mineral water

Cations	Content (mg/L)	Anions	Content (mg/L)
Lithium	0.14	Fluoride	0.15
Sodium	121.0	Chloride	39.0
Potassium	10.2	Bromide	0.11
Rubidium	0.01	Iodide	0.019
Magnesium	109.4	Nitrate	8.28
Calcium	331.0	Sulphate	34.8
Strontium	2.54	Hydrogen phosphate	0.1
Barium	0.055	Hydrogen carbonate	1775.0
Chromium	0.005	Undissociated substances	
Manganese	0.03	Metasilicic acid	37.2
Iron	0.068	Metaboric acid	1.1
Nickel	0.002	Metatitanic acid	0.002
Copper	0.002	Gaseous substances	
Silver	0.002	Free dissolved carbon dioxide	3500
Zinc	0.007		
Aluminium	0.004		
Lead	0.005		

Practice.

Investigational product

A mineral water with a high hydrogen carbonate content was examined (Table 1).

The mineral water is currently approved in Germany for use in supporting gastrointestinal function, improving calcium and magnesium supply, and supportive treatment of chronic urinary tract infections.

The study was registered in the EU Clinical Trials Register under the EudraCT No. 2013-001584-22.

Intervention

For a period of 6 wk (± 5 d), all the participants had to drink 1.5 L of the test water daily, divided into three times 300 mL with the main meals, the rest being drunk in several portions during the course of the day.

Test parameters - endpoints

Efficacy: Frequency and duration of heartburn episodes based on entries in the patient diaries, therapeutic course and subjective perception of general health using validated German versions of the RDQ, QOLRAD, GIQLI and SF-12 questionnaires, each time comparing visit 2 with visits 3, 4 and 5 (after 2, 4 and 6 wk); overall assessment by patients and investigators.

Tolerability: AEs/effects during the study according to information provided by the patients, changes in heart rate and blood pressure and changes in laboratory values in a comparison between visit 2 and visit 5; overall assessment by patients and investigators.

Test instruments

Heartburn diary: Here, the patients recorded each occurrence of heartburn and stated whether they had taken an agent against it as an exceptional measure. The number and duration of the heartburn episodes and the need for rescue medication were established on the basis of this information. The patient diary was kept throughout the study.

Drink diary: This questionnaire records daily what and how much the patients drank, and additionally, after visit 2, the daily intake of the investigational product. The drink diary was kept throughout the study.

RDQ: This questionnaire records the symptoms of the upper gastrointestinal tract[8], and comprises 12 questions in four dimensions (heartburn, regurgitation, GERD, dyspepsia) regarding the frequency and severity of the symptoms during the past week in each instance. The symptoms include a burning feeling and pains behind the sternum and in the middle of the upper abdomen, an acidic taste in the mouth and an unpleasant belching of the stomach contents. The frequency of symptoms is recorded on a 5-point scale ("did not occur at all" to "daily"), and the severity of symptoms on a 6-point scale ("did not occur at all" to "strong"). The questionnaire was used at visit 2, visit 3, visit 4 and visit 5.

QOLRAD[9]: This questionnaire comprises 25 questions in five dimensions (emotional stress, sleep distur-

bances, problems with eating and drinking, physical and social function, vitality) during the past week in each instance. The frequency and severity of symptoms are recorded on a seven-point scale in each instance ("constant" to "never" or "very strong" to "not at all"). The questionnaire was used at visit 2, visit 3, visit 4 and visit 5.

GIQLI[10]: This questionnaire evaluates the quality of life of patients with diseases of the gastrointestinal tract. It comprises 36 questions on symptoms of the disease, psychological wellbeing and physical and social function, mainly during the past 2 wk in each instance. There is also a question to determine the impact of the treatment. The answers are on a five-point scale. The questionnaire was used at visit 2, visit 3, visit 4 and visit 5.

The Short Form-12 Health Survey Questionnaire[11]: This questionnaire is a short form of the SF-36 Health Survey Questionnaire on health-related quality of life. With its 12 questions in eight dimensions it covers the subjective perception of health (physical functioning, physical role functioning, bodily pain, general health perceptions, vitality, social role functioning, emotional role functioning and mental health) during the past week in each instance (in the version used). The questionnaire was used at visit 2, visit 3, visit 4 and visit 5.

Overall subjective assessment: The efficacy of the investigational product was assessed by investigator and patients on a four-point Likert scale using the rating points "very good", "good", "moderate" and "poor".

AEs: According to information provided by the patient, the investigator recorded the nature, duration and degree of severity of adverse events. Furthermore, the causality between the test water and an adverse event was assessed as being "definite", "probable", "possible", "improbable", "none" or "not assessable".

Laboratory parameters: Fasting blood samples with - at visit 1 and visit 5 - determination of hemoglobin, hematocrit, erythrocytes, thrombocytes, leukocytes, alanine transaminase, aspartate aminotransferase, gamma-glutamyl transferase, alkaline phosphatase, bilirubin, creatinine, urea, uric acid and - only at visit 1 - of glycated hemoglobin and thyroid-stimulating hormone. Blood pressure and heart rate at every visit.

Overall assessment of tolerability: At visit 5, the tolerability of the investigational product was subjectively rated by the investigators and the patients using a four-point Likert scale with the rating points "very good", "good", "moderate", and "poor".

Compliance: Was checked on the basis of the unused test water returned at the end of the study. Deviation in the used quantity of water by 25% or more or total duration of use by over 5 d, was considered non-compliance.

Statistics analysis

Statistical evaluation was performed regarding the absolute and relative changes in the assessed parameters in the comparison between visit V2 and visit V5. All parameters for the examination of efficacy and tolerance as well as further parameters relevant to the study were examined and descriptively evaluated using the methods for exploratory data analysis. Metric data (continuous measurement data) were collected, and evaluation comprised descriptive statistical parameters (number of cases, average, standard deviation, median, extremes and quartiles). For ordinal or nominal data, the frequency distributions were evaluated.

The examination of changes between the initial, control and final examinations was performed using the non-parametric Wilcoxon test. For comparison of proportional values the Chi-Square test was used, and for comparison of more than five categories, the U-test. Parametric procedures were also used for the interpretation of quantitative results.

A type 1 error (alpha error) of 5.0% (two-way) or 2.5% (one-way) and a power of 80% were assumed. Ninety-five percent confidence intervals were determined. All *P*-values from statistical tests are to be understood as being exclusively exploratory. SPSS®, Version 22 for Windows™ was used for the evaluation.

RESULTS

The investigational product was assigned to a total of 50 patients. The following groups can be distinguished for the study evaluation: (1) The FAS/ITT population (Full Analysis Set; Intent-to-treat population) included all patients who had drunk the test water at least once and for whom efficacy data were available: $n = 48$; (2) The VCAS/PP population (Valid Case Analysis Set; Per-protocol population) included those patients who had drunk the test water in accordance with the study plan and for whom there were no major protocol violations: $n = 42$; and (3) The safety population for examination of tolerability included all patients who had used the investigational product at least once and for whom safety data were available: $n = 49$.

Demographics

A total of 28 males and 22 females were included. The mean age of the patients was 40.6 ± 11.5 in the FAS population. The ethnicity of all patients was "Caucasian".

Concomitant diseases/medication

The most common concomitant diseases were those of the gastrointestinal tract (54 items), followed by "surgical or other procedures" (39 items). The most

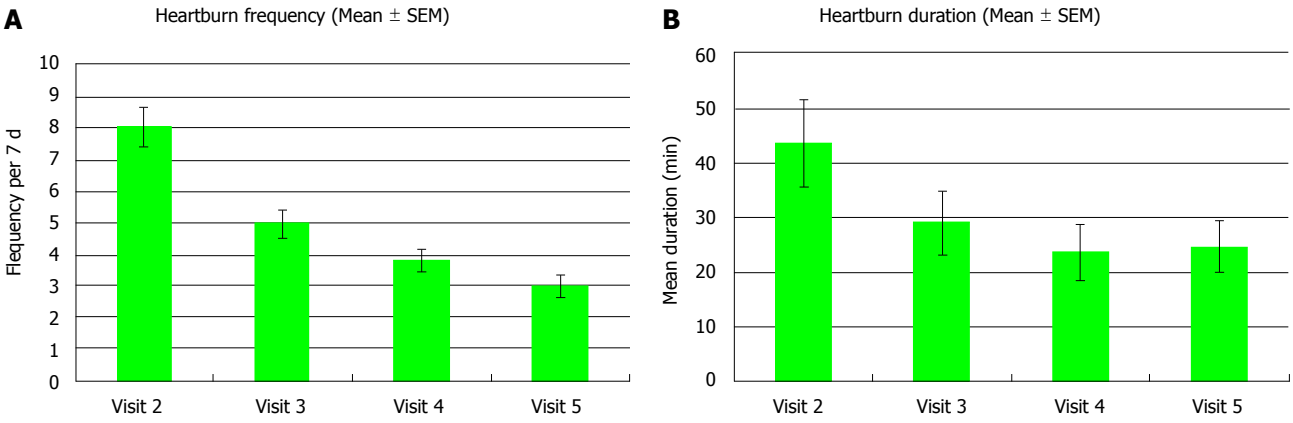


Figure 2 Development of heartburn symptoms in the course of the study. The data is from the full analysis set. A: Frequency of heartburn during the course of the study; B: Duration of heartburn during the course of the study.

Table 2 Overview of the changes with regard to heartburn (full analysis set)

Heartburn (n = 48)	V2 mean (SD) median	V5 mean (SD) median	V2-V5 mean (SD) median	P value	Proportion of patients displaying a decrease/an increase	P value
Frequency	8.06 (4.43) 8.0	3.00 (2.29) 2.5	5.06 (4.81) 5.0	< 0.001	89.6%/8.3%	< 0.001
Duration (min)	43.7 (55.9) 24.5	24.7 (32.2) 12.7	19.0 (40.7) 4.2	0.002	79.2%/20.8%	< 0.001

common concomitant medications stated were substances from the group "sexual hormones and their inhibitors" (7 items), "substances with influence on the renin-angiotensin system" and "substances with influence on lipid metabolism" (2 items each).

Efficacy - frequency and duration of heartburn (based on the patient diaries)

After 6 wk, the mean number of heartburn episodes per week in the FAS population had decreased by 5.1 ± 4.8 at visit 5 compared to 8.1 at visit 2 (P < 0.001) (Figure 2A). The qualitative change in the frequency of heartburn per week from visit 2 to visit 5 was statistically significant in both populations (FAS, VCAS) evaluated (P < 0.001). In the FAS population, after 6 wk of treatment, 43 patients reported less frequent heartburn episodes (89.6%), in one patient the frequency remained unchanged (2.1%) and in 4 patients more episodes were recorded (8.3%) (Table 2) (P < 0.001).

After 6 wk, the mean duration of heartburn per week had decreased significantly by 19 min at visit 5 compared to 43.7 min at visit 2 (P < 0.001) (Figure 2B). The duration of heartburn decreased in 38 patients of the FAS population (79.2%) (P < 0.001) (Table 2).

Efficacy - therapeutic course assessed per RDQ

Changes were observed for the following dimensions:

Heartburn: The mean heartburn score decreased by 3.6 ± 3.7 points (from 5.40 at visit 2 to 1.83 at visit 5). In the FAS population, the frequency of heartburn decreased in 63.8% of the patients. In 70.2% of the patients, the intensity of the heartburn decreased from visit 2 to visit 5. These differences were statistically significant.

Regurgitation: The mean regurgitation score decreased by 5.0 ± 4.8 points (from 7.83 at visit 2 to 2.80 at visit 5). In the FAS population, 76.1% of the patients experienced less frequent regurgitation. In 71.7% of the patients, the intensity of the regurgitation decreased from visit 2 to visit 5. These differences were statistically significant.

GERD: The term designates both the disease itself and one of the dimensions in the RDQ questionnaire. The mean GERD score decreased by 8.7 ± 7.0 points (from 13.35 at visit 2 to 4.67 at visit 5). In the FAS population, 91.3% of the patients experienced GERD symptoms less frequently. In 87.0% of the patients, the intensity of the symptoms decreased from visit 2 to visit 5. These differences were statistically significant.

Dyspepsia: The mean dyspepsia score decreased by 3.5 ± 4.2 points (from 5.82 at visit 2 to 2.29 at visit 5). In the FAS population, 66.7% of the patients experienced dyspeptic symptoms less frequently. In 62.2% of the patients, the intensity of symptoms decreased from visit 2 to visit 5. These differences were statistically significant.

Efficacy - therapeutic course assessed per QOLRAD

Changes were observed for the following dimensions:

Emotional stress: The mean score in the FAS population significantly (*i.e.*, improved) by 0.8 ± 1.1 points (from 5.76 at visit 2 to 6.59 at visit 5).

Sleep quality: The mean score in the FAS population increased significantly by 0.7 ± 1.0 points (from 5.76 at visit 2 to 6.48 at visit 5).

Problems with eating and drinking: The mean score in the FAS population increased significantly by 1.1 ± 1.2 points (from 5.26 at visit 2 to 6.36 at visit 5).

Physical/social functioning: The mean score in the FAS population increased significantly by 0.6 ± 0.8 points (from 6.14 at visit 2 to 6.77 at visit 5).

Vitality: The mean score in the FAS population increased significantly by 0.8 ± 1.1 points (from 5.38 at visit 2 to 6.21 at visit 5).

Efficacy - therapeutic course assessed per GIQLI

The total score in the FAS population increased significantly by 16.6 ± 16.2 points (from 110.5 at visit 2 to 127.0 at visit 5). Changes were observed for the following dimensions:

Symptoms: The mean score in the FAS population increased significantly by 10.9 ± 9.4 points (from 55.9 at visit 2 to 66.7 at visit 5).

Emotions: The mean score in the FAS population increased significantly by 2.2 ± 3.1 points (from 15.3 at visit 2 to 17.5 at visit 5).

Physical functioning: The mean score in the FAS population increased significantly by 2.6 ± 4.9 points (from 21.4 at visit 2 to 23.9 at visit 5).

Social function: The mean score in the FAS population increased significantly by 1.0 ± 2.2 points (from 14.1 at visit 2 to 15.1 at visit 5).

Efficacy - subjective perception of health (SF-12 questionnaire)

In the assessment per SF-12 questionnaire, statistically significant improvements regarding the following parameters took place between visit 2 and visit 5: (1) General state of health, quantitative ($P < 0.001$); (2) Being able to do only certain things ($P = 0.044$); (3) Pain during everyday activities ($P < 0.001$); and (4) Calm and relaxed ($P < 0.002$); Full of energy ($P < 0.003$).

Global assessment of efficacy

At the end of the study, 89.4% of the patients assessed the efficacy as being "good" or "very good". For 91.5% of the patients, the investigators assessed the efficacy as being "good" or "very good". Statistically significant differences between the patients' assessments and those of the investigators were not observed ($P = 1.00$).

Tolerability

In the course of the clinical trial, a total of two adverse events were documented by the investigators, namely moderate headache in 2 patients (4.0%). Neither of them was assessed by the investigators as being linked to the investigational product. The patients treated the headaches themselves with paracetamol and/or ibuprofen. No adverse effects were observed.

During the course of the study, the systolic blood pressure decreased by 3.5 mmHg ($P = 0.008$) and the diastolic blood pressure by 3.0 mmHg ($P = 0.002$). The heart rate also decreased from 71/min (visit 2) to 68.8/min (visit 5) ($P = 0.012$).

There were no relevant changes in the examined laboratory parameters.

Ninety-five point eight percent of the patients assessed the tolerability of the test water as being "very good" or "good", and for 95.7% of the patients, investigators assessed the patients' tolerability on the Likert scale as being "very good" or "good".

DISCUSSION

Our 6-wk intervention, with a daily consumption of 1.5 L of a hydrogen carbonate-rich mineral water, clearly reduced the symptoms in patients with heartburn and improved their quality of life. The frequency of heartburn episodes continuously decreased in the course of the study, while the duration of the episodes increased slightly at the final visit in comparison with the second control visit. One possible explanation could be that the duration of the episode is a very subjective parameter, and documentation by the patients may be somewhat less accurate with respect to the assessment of minor differences, as observed between visit 4 and visit 5.

The water was well tolerated, and no adverse effects were observed.

The questionnaires used to assess the subjective symptoms (RDQ) and quality of life (QOLRAD and GILQI) show an improvement of the symptoms and the symptom-associated limitations in quality of life.

Similar results have been reported by Gunasekaran *et al*[12], who studied the effect of GERD on health-related quality of life in 134 adolescent subjects. Using QOLRAD, the authors found a negative effect on the symptoms of GERD at baseline. After 8 wk of omeprazole administration, the values in all five dimensions improved significantly and to a clinically relevant extent, similar to - or even more pronounced than in - adults. In the present study with the hydrogen carbonate-rich mineral water, the symptom reduction was, in all dimensions, of clinical relevance (effect size $\geqslant 0.5$) and in a similar range as reported in the open-label study

with the proton pump inhibitor.

Interestingly, PPI treatment showed success in certain patients with typical reflux symptoms who could not be diagnosed with GERD *via* pH-impedance monitoring[13]. Similarly, such patients may also be responders to hydrogen carbonate-rich mineral water treatment - or possibly even to a placebo treatment.

Chen *et al*[13] used the GIQLI to compare the quality of life in 25 patients undergoing either laparoscopic or open cholecystectomy. They concluded that this questionnaire could be used as a good measure of the quality of life in patients before and after the intervention. The reported improvement in quality of life related to the gastrointestinal tract is comparable to that observed in the present study, both for the total score and for individual dimensions. This example from the literature demonstrates that it is possible to compare the success of different treatments by comparing GIQLI results.

"Lifestyle changes" are often recommended as part of the overall treatment concept, *e.g.*, small low-fat meals, which should not be eaten immediately before going to bed, weight reduction, an elevated upper-body position at night and abstinence from "triggers" such as coffee, chocolate, nicotine and alcohol[14]. However, natural mineral waters are rarely mentioned as part of this concept, even though more and more people are trying to alleviate their symptoms using natural remedies before seeking medical attention.

Nevertheless, the positive influence of hydrogen carbonate-rich water on heartburn and gastrointestinal symptoms in general has already been demonstrated several times. An Italian group administered 250 mL of a calcium- and hydrogen carbonate-rich mineral water (Ca^{2+} 486.6 mg/L; HCO_3^- 1750.7 mg/L) to patients with gastroesophageal reflux as demonstrated by pH metry. The group then established clear and lasting pH increases in the esophagus and stomach, with levels differing significantly from those in the control subjects who had been given tap water. The patients also reported a subjective improvement in their heartburn following administration of the water containing calcium hydrogen carbonate[4].

For a period of 30 d, Bertoni *et al*[5] administered water with high mineral content (hydrogen carbonate content 683 mg/L, 1.5 L/d) to a total of 18 patients with dyspeptic symptoms within the past 3 mo. The frequency and severity of the symptoms were assessed at the beginning of the study and after 30 d on a five-point Likert scale (0: Symptoms never occur; 4: Symptoms occur daily/are very marked). After the 30-d test phase, there was a significant improvement in the severity and frequency of dyspeptic symptoms, including epigastric pain and heartburn[5].

Another multicentric study examined the efficacy of a mineral-water cure in 1667 patients with functional dyspepsia. This involved an initial cycle during which the patients received 2 L of a hydrogen carbonate-rich mineral water daily for 21 d. One year later, 996 participants were re-examined and underwent a second treatment cycle. At the beginning of the study and after one year, each patient completed a questionnaire on symptoms, lifestyle and utilization of medicinal services. At the beginning of the study as well as after the two drinking cure periods, the gastrointestinal symptoms were assessed, and in 60 patients the secretion of gastric acid was also determined at these times. In all patients, a significant decrease in the frequency of symptoms was established at the end of the first and second drinking cure cycles. After the drinking cures, the secretion of gastric acid was also reduced in 87.5% of the patients who were examined in this regard[6].

In an animal model, it was demonstrated that water containing hydrogen carbonate (683 mg/L) can prevent alcohol-induced damage to the gastric mucosa, even with extremely high amounts of alcohol (23 mL of 100% ethanol/kg), although the water was only effective if administered 30 min before the alcohol load. With longer intervals, it was not possible to prevent the toxic effect[15].

Furthermore, back in 2001, Böhmer *et al*[7] reported in an overview that functional dyspeptic symptoms, including acid reflux, are improved by the appropriate waters. One postulated principle of action is that the HCO_3^- anions have a direct buffer effect on the protons of the gastric acid. Purely arithmetically speaking, a hydrogen carbonate-rich mineral water can deliver a similar buffer capacity to that achieved by over-the-counter antacids based on calcium carbonate/magnesium carbonate: 1.7 g hydrogen carbonate has a buffer capacity of 27.5 mEq, compared to 15.5 mEq for one antacid tablet.

Further advantages of mineral waters include the improved supply of fluids as well as the higher level of safety, which virtually excludes intoxication. Also, the cations dissolved in the mineral water in addition to the HCO_3^- (in the test water examined mainly Ca^{2+} and Mg^{2+}), have good bioavailability and contribute to supplying the organism with minerals. Additionally, since it is frequently stated that heartburn occurs postprandially, it appears that intake at mealtimes is favorable.

Even when heartburn symptoms are not attributable to a serious disease, they affect the quality of life of the concerned person - sometimes considerably. According to a British study of 924 patients with reflux symptoms, 50% complained of limitations both in their everyday working life and during leisure activities[16]. Among 108 clinical patients with GERD, 22.3% had regular sleep disturbances, 27.8% had problems with eating and drinking, and 11.2% had disorders that hindered professional activity[17].

Accordingly, the improvement in quality of life plays a considerable role in treatment. Treatment with PPIs, for example, reduced the symptoms' effect on quality of life in patients who responded to it. However, only 186 out of 482 patients treated with PPIs reported that their efficacy was complete or good[16].

Limitations

The present study comprises a number of limitations. The number of patients examined was relatively small, and there was no control group. Because of the open design, the participants knew that they were drinking water that might possibly reduce their symptoms - a placebo effect might have influenced the subjective assessments used for the evaluation of the defined endpoints, both in the diaries and in the questionnaires. Apart from the blood pressure measurement, which showed a significant reduction in both systolic and diastolic blood pressure, and the assessment of laboratory parameters, no further objective assessments, *e.g.*, pH measurements, were carried out.

Most of the remaining study conditions were not controlled, either. While the participants were not supposed to change their other eating habits, this was not checked within the study. Therefore, an influence of food on the results of the study cannot be excluded.

Additionally, the nature of the patient recruitment probably led to selection bias, since participants with a particular interest in health topics were included, as it was apparent from the advertising material that the test substance was a natural product.

A hydrogen carbonate-rich mineral water can qualitatively and quantitatively reduce heartburn symptoms and improve the subjective wellbeing of patients affected. Further studies of a randomized, placebo-controlled and blinded design should follow this open pilot study, which was mainly carried out to generate hypotheses for later confirmatory trials.

ACKNOWLEDGMENTS

The authors thank Margret I. Moré for critically reading and formatting the manuscript.

COMMENTS

Background

This open, single-center, single-arm clinical study investigated the efficacy and safety of mineral water with a high content of hydrogen carbonate in patients with heartburn. Previous studies have also demonstrated the positive influence of hydrogen carbonate-rich water on heartburn and gastrointestinal symptoms.

Research frontiers

Hydrogen carbonate-rich water as effective heartburn remedy deserves more scientific attention.

Innovations and breakthroughs

This study applies a whole range of different test instruments to measure and evaluate the effects of the mineral water on heartburn. Therefore it is an excellent standard for further studies on heartburn or gastroesophageal reflux disease (GERD). It is also a prerequisite for a larger randomized controlled study.

Applications

Hydrogen carbonate-rich water should become a standard treatment for any kind of mild heartburn problems in addition to life-style changes. It may be just as efficient or even more efficient than antacids, and may even offer advantages in more severe heartburn cases generally treated with proton pump inhibitors or histamine-2 receptor blockers. This remains to be tested.

Terminology

GERD: Gastroesophageal reflux disease; NERD: Non-erosive reflux disease, early stage of GERD. Both include symptoms of heartburn.

Peer-review

This is a well-designed and written paper that aimed to explore the efficacy of hydrogen carbonate-rich water in patients with heartburn showing a very nice reduction in heartburn perception and the duration of heartburn per week. The statistical analysis has been well-conducted.

REFERENCES

1 **El-Serag HB**, Sweet S, Winchester CC, Dent J. Update on the epidemiology of gastro-oesophageal reflux disease: a systematic review. *Gut* 2014; **63**: 871-880 [PMID: 23853213 DOI: 10.1136/gutjnl-2012-304269]

2 **Altomare A**, Guarino MP, Cocca S, Emerenziani S, Cicala M. Gastroesophageal reflux disease: Update on inflammation and symptom perception. *World J Gastroenterol* 2013; **19**: 6523-6528 [PMID: 24151376 DOI: 10.3748/wjg.v19.i39.6523]

3 **Lacy BE**, Talley NJ, Locke GR, Bouras EP, DiBaise JK, El-Serag HB, Abraham BP, Howden CW, Moayyedi P, Prather C. Review article: current treatment options and management of functional dyspepsia. *Aliment Pharmacol Ther* 2012; **36**: 3-15 [PMID: 22591037 DOI: 10.1111/j.1365-2036.2012.05128.x]

4 **Grassi M**, Fraioli A, Pappalardo G, Messina B, Belardinelli L, Guadalaxara A. Alkalizing activity of a calcium-bicarbonate-containing water, evaluated for pH, in patients with gastroesophageal reflux. *Clin Ter* 1993; **143**: 131-136 [PMID: 8222543]

5 **Bertoni M**, Olivieri F, manghetti M, Boccolini E, Bellomini MG, Blandizzi C, Bonino F, Del Tacca M. Effects of a bicarbonate-alkaline mineral water on gastric functions and functional dyspepsia: a preclinical and clinical study. *Pharmacol Res* 2002; **46**: 525-531 [PMID: 12457626 DOI: 10.1016/S1043661802002323]

6 **Gasbarrini G**, Candelli M, Graziosetto RG, Coccheri S, Di Iorio F, Nappi G. Evaluation of thermal water in patients with functional dyspepsia and irritable bowel syndrome accompanying constipation. *World J Gastroenterol* 2006; **12**: 2556-2562 [PMID: 16688801]

7 **Böhmer H**, Resch K, Waldow R. Dyspepsie: Hydrogencarbonathaltige Heilwässer unterstützen die Therapie mit Antazida. *Natura Med* 2001; **16**: 28-33

8 **Nocon M**, Kulig M, Leodolter A, Malfertheiner P, Willich SN. Validation of the Reflux Disease Questionnaire for a German population. *Eur J Gastroenterol Hepatol* 2005; **17**: 229-233 [PMID: 15674102 DOI: 10.1097/00042737-200502000-00015]

9 **Kulich KR**, Malfertheiner P, Madisch A, Labenz J, Bayerdörffer E, Miehlke S, Carlsson J, Wiklund IK. Psychometric validation of the German translation of the Gastrointestinal Symptom Rating Scale (GSRS) and Quality of Life in Reflux and Dyspepsia (QOLRAD) questionnaire in patients with reflux disease. *Health Qual Life Outcomes* 2003; **1**: 62 [PMID: 14613560 DOI: 10.1186/1477-7525-1-62]

10 **Eypasch E**, Williams JI, Wood-Dauphinee S, Ure BM, Schmülling C, Neugebauer E, Troidl H. Gastrointestinal Quality of Life Index: development, validation and application of a new instrument. *Br J Surg* 1995; **82**: 216-222 [PMID: 7749697 DOI: 10.1002/bjs.1800820229]

11 **Gandek B**, Ware JE, Aaronson NK, Apolone G, Bjorner JB, Brazier JE, Bullinger M, Kaasa S, Leplege A, Prieto L, Sullivan M. Cross-validation of item selection and scoring for the SF-12 Health Survey in nine countries: results from the IQOLA Project. International Quality of Life Assessment. *J Clin Epidemiol* 1998; **51**: 1171-1178 [PMID: 9817135]

12 **Gunasekaran T**, Tolia V, Colletti RB, Gold BD, Traxler B, Illueca M, Crawley JA. Effects of esomeprazole treatment for gastroesophageal

reflux disease on quality of life in 12- to 17-year-old adolescents: an international health outcomes study. *BMC Gastroenterol* 2009; **9**: 84 [PMID: 19922626 DOI: 10.1186/1471-230X-9-84]

13 **Chen L**, Tao SF, Xu Y, Fang F, Peng SY. Patients' quality of life after laparoscopic or open cholecystectomy. *J Zhejiang Univ Sci B* 2005; **6**: 678-681 [PMID: 15973772 DOI: 10.1631/jzus.2005.B0678]

14 **Oliver K**, Davies G. Heartburn: influence of diet and lifestyle. *Nutr Food Sci* 2007; **38**: 548-554 [DOI: 10.1108/00346650810920141]

15 **Nassini R**, Andrè E, Gazzieri D, De Siena G, Zanasi A, Geppetti P, Materazzi S. A bicarbonate-alkaline mineral water protects from ethanol-induced hemorrhagic gastric lesions in mice. *Biol Pharm Bull* 2010; **33**: 1319-1323 [PMID: 20686225]

16 **Jones R**, Liker HR, Ducrotté P. Relationship between symptoms, subjective well-being and medication use in gastro-oesophageal reflux disease. *Int J Clin Pract* 2007; **61**: 1301-1307 [PMID: 17590216 DOI: 10.1111/j.1742-1241.2007.01475.x]

17 **Lee SW**, Lien HC, Lee TY, Yang SS, Yeh HJ, Chang CS. Heartburn and regurgitation have different impacts on life quality of patients with gastroesophageal reflux disease. *World J Gastroenterol* 2014; **20**: 12277-12282 [PMID: 25232262 DOI: 10.3748/wjg.v20.i34.12277]

P- Reviewer: de Bortoli N
S- Editor: Kong JX **L- Editor**: A **E- Editor**: Jiao XK

World Journal of Gastrointestinal Pathophysiology

Submit a Manuscript: http://www.wjgnet.com/esps/
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.4291/wjgp.v7.i1.181

World J Gastrointest Pathophysiol 2016 February 15; 7(1): 181-185
ISSN 2150-5330 (online)

ORIGINAL ARTICLE

Prospective Study

High rate of *Helicobacter pylori* reinfection in Lithuanian peptic ulcer patients

Laimas Jonaitis, Gediminas Kiudelis, Paulius Slepavicius, Limas Kupcinskas

Laimas Jonaitis, Gediminas Kiudelis, Paulius Slepavicius, Limas Kupcinskas, Department of Gastroenterology, Medical Academy, Lithuanian University of Health Sciences, LT-50028 Kaunas, Lithuania

Author contributions: Jonaitis L, Kiudelis G and Kupcinskas L designed and planned the study, recruited the patients, collected all the data, and performed all the investigations; Jonaitis L performed statistical analysis; Jonaitis L, Slepavicius P prepared the manuscript; all authors were involved in drafting and revising the manuscript.

Institutional review board statement: The study was reviewed and approved by the Kaunas Regional Biomedical Research Ethics Committee (Protocol No. 8/2011).

Informed consent statement: All study participants, or their legal guardian, provided informed written consent prior to study enrollment.

Conflict-of-interest statement: Laimas Jonaitis, Gediminas Kiudelis, Paulius Slepavicius, Limas Kupcinskas declare that there are no conflicts of interest.

Data sharing statement: Technical appendix, statistical code, and dataset available from the corresponding author at laimasj@takas.lt. Participants gave informed consent for data sharing. No additional data are available.

Correspondence to: Laimas Jonaitis, MD, PhD, Department of Gastroenterology, Medical Academy, Lithuanian University of Health Sciences, Eiveniu 2, LT-50028 Kaunas, Lithuania. laimasj@takas.lt
Telephone: +370-37326264
Fax: +370-37331458

Received: May 27, 2015
Peer-review started: May 29, 2015
First decision: June 19, 2015
Revised: August 19, 2015
Accepted: November 30, 2015
Article in press: December 3, 2015
Published online: February 15, 2016

Abstract

AIM: To evaluate the frequency of *Helicobacter pylori* (*H. pylori*) reinfection in peptic ulcer patients during 9 years after *H. pylori* eradication.

METHODS: We invited 117 peptic ulcer patients in whom eradication of *H. pylori* was confirmed 1 year after eradication treatment both by histology and by rapid urease test. In total, 57 patients were available for the study procedures: 34 (59.6%) male, 23 (40.4%) female; mean age 52.3 ± 13.0 years. There were 45 (78.9%) patients with duodenal ulcer and 12 (21.1%) with gastric ulcer. *H. pylori* was diagnosed by a rapid urease test and histology if endoscopy was performed. If endoscopy was refused, *H. pylori* was diagnosed by the C14-urea breath test and serology. *H. pylori* was established if at least one of the tests was positive.

RESULTS: The mean follow-up was 8.9 ± 1.0 years (range, 6-12). *H. pylori* was established in 15 patients. In 2 *H. pylori*-negative patients, *H. pylori* was established during the follow-up period and eradicated. Therefore, we consider that reinfection occurred in 17 patients. In the *per protocol* analysis, reinfection was established in 17 of 57 (29.8%; 95%CI: 19.2-42.2) patients during the follow-up period. The annual rate of infection was 3.36%. If all non-responders were considered *H. pylori*-negative, reinfection would be 14.5% (17/117), the annual rate

being 1.63%. The mean age of patients with reinfection was 51.8 ± 14.0 years, and without reinfection was 52.5 ± 13.0 years, $P > 0.05$; the mean body mass index of patients with reinfection was 27.2 ± 4.1 kg/m^2, and without reinfection was 25.7 ± 4.2 kg/m^2, $P > 0.05$. There were no differences in the reinfection rates according the location of the peptic ulcer, the eradication regimen used, and smoking status.

CONCLUSION: The reinfection rate of *H. pylori* is relatively high in Lithuania and probably related to the high prevalence of *H. pylori*, what may reflect differences in the socioeconomic status between Western and Eastern European countries.

Key words: *Helicobacter pylori*; Reinfection; Prevalence; Peptic ulcer; Eradication

Core tip: The reinfection rate of *Helicobacter pylori* (*H. pylori*) varies according to geographical area. In regions with higher socioeconomic status and lower prevalence of *H. pylori* it is only 1.68% of cases. In developing areas, the reinfection rate could be much higher. Lithuania, as well as other Eastern and Central European countries, is in transition, and the prevalence of *H. pylori* is not as low as in Western regions, but not as high as in developing countries. According to our study, the *H. pylori* reinfection rate in Lithuania is relatively high (the annual rate being 3.36%), probably because of the high prevalence of *H. pylori*. This could indirectly reflect differences in the socioeconomic status between Western and Eastern European countries.

Jonaitis L, Kiudelis G, Slepavicius P, Kupcinskas L. High rate of *Helicobacter pylori* reinfection in Lithuanian peptic ulcer patients. *World J Gastrointest Pathophysiol* 2016; 7(1): 181-185 Available from: URL: http://www.wjgnet.com/2150-5330/full/v7/i1/181.htm DOI: http://dx.doi.org/10.4291/wjgp.v7.i1.181

INTRODUCTION

It is well established that *Helicobacter pylori* (*H. pylori*) infection is the main cause of chronic gastritis and peptic ulcer disease, and a definite risk factor for gastric cancer[1-4]. Consensuses from different parts of the world strongly recommend eradication of *H. pylori* to cure peptic ulcer disease and MALT lymphoma, and to decrease the risk of gastric cancer[5-7]. *H. pylori* eradication is recommended to alleviate the burden of functional dyspepsia and some other digestive and extragastric pathologies[8-13]. Current treatment modalities allow eradication of the *H. pylori* bacterium in up to 90% of cases (less if there is clarithromycin resistance). Nevertheless, in some cases, recrudescence or reinfection of *H. pylori* may occur. Reinfection is considered when *H. pylori* is found after confirmed *H. pylori* eradication. The confirmation of eradication must be performed not earlier than 6 mo after eradication treatment. It has been reported that in highly developed countries reinfection is rare and may account no more than 1.68% of cases[14]. In contrast, in developing areas the reinfection rate could be much higher and has been reported to reach 9.63%[14]. Central and Eastern European countries are areas of medium to high *H. pylori* prevalence. The reinfection rates could be expected to be in between the rates indicated above[8-32]. *H. pylori*-related diseases are common in these countries and *H. pylori* eradication treatment is widely applied. There are little data from Eastern and Central Europe about the reinfection of *H. pylori*. In the Maastricht consensus, the recommendation to regularly investigate the regional epidemiological status of *H. pylori* has been proposed, indicating that the data for prevalence, eradication rates, antibacterial resistance, and reinfection rates are important[13].

Therefore, we carried out a long-term follow-up study to evaluate the frequency of *H. pylori* reinfection in peptic ulcer patients in Lithuania after confirmation of *H. pylori* eradication.

MATERIALS AND METHODS

Patients

We included peptic ulcer patients from our previous 1-year follow-up studies[15,20]; 117 patients, who were *H. pylori*-negative 1 year after eradication treatment (therefore, considered to have true eradication), were invited to participate in the study by mail or telephone. Fifty seven patients responded and were available for the study procedures. Written informed consent from all participants and approval of the Kaunas Regional Biomedical Research Ethics Committee was obtained. During the visits, demographic and clinical data were obtained. The flowchart of the study is presented in Figure 1. The previous 1-wk eradication regimens of these patients contained omeprazole, amoxicillin, and clarithromycin [applied to 33 (58%) patients]; omeprazole, amoxicillin and metronidazole [applied to 12 (21%) patients]; omeprazole, clarithromycin and metronidazole [applied to 12 (21%) patients].

Diagnosis of H. pylori

According to the protocol of previous studies[15,20], the final *H. pylori* status was determined after 12 mo following eradication therapy by a rapid urease test (RUT) and histology. Patients were considered as *H. pylori*-negative if both tests were negative.

The mean follow-up period was 8.9 ± 1.0 years (range, 6-12) after the confirmation of the negative *H. pylori* status. At this time *H. pylori* was tested by RUT and histology if patients agreed to undergo endoscopy. If endoscopy was refused, *H. pylori* was tested by the ^{14}C-urea breath test (UBT) "Heliprobe"[18,24-26] and serology (the quantitative test "SureScreen Diagnostics

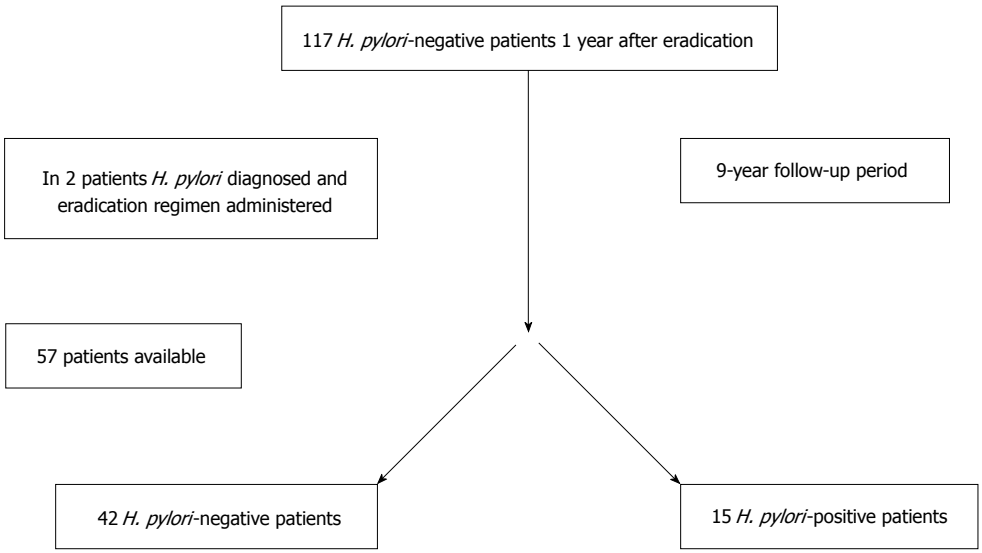


Figure 1 Flowchart of follow-up. *H. pylori*: *Helicobacter pylori*.

Table 1 Characteristics of patients with and without *Helicobacter pylori* reinfection *n* (%)

	Reinfection *n* = 17	No reinfection *n* = 40	*P*-value
Male	12 (70.6)	22 (55)	> 0.05
Smokers	7 (41.2)	19 (47.5)	> 0.05
Duodenal ulcer	13 (76.5)	32 (80)	> 0.05
Primary eradication regimen - 7 d triple therapy:			
Omeprazole, clarithromycin, amoxicillin	9 (52.9)	24 (60)	> 0.05
Omeprazole, metronidazole, amoxicillin	4 (33.3)	8 (20)	> 0.05
Omeprazole, clarithromycin, metronidazole	4 (33.3)	8 (20)	> 0.05

Ltd" which is CE Marked, Food and Drug Administration Approved[17]). *H. pylori*-positivity was established, if at least one of the tests was positive.

Forty three patients were tested by RUT and histology; 14 patients (those who refused endoscopy) were tested by UBT and by additional serology.

Statistical analysis

The data were analyzed and compared using the χ^2 or Student *t* test. Values of $P < 0.05$ were considered significant.

RESULTS

The 57 patients consisted of 34 (59.6%) males and 23 (40.4%) females, with a mean age of 52.3 ± 13.0 years. There were 45 (78.9%) patients with a duodenal ulcer and 12 (21.1%) with a gastric ulcer.

Endoscopy was performed in 43 (75.4%) patients. *H. pylori* was established in 15 patients, and 42 patients were *H. pylori*-negative. In 2 *H. pylori*-negative patients *H. pylori* had been established by RUT during the follow-up period (both had peptic ulcer relapse), and successful eradication treatment had been administered. Therefore, we may count 17 patients with *H. pylori* reinfection during follow-up.

In the *per protocol* analysis, reinfection was established in 17 (29.8%; 95%CI: 19.2-42.2) of 57 patients, the annual rate being 3.36%. If we consider that all non-responders were *H. pylori*-negative (the most optimistic analysis), the reinfection rate would be 14.5% (17/117), the annual rate being 1.63%.

The mean age was 51.8 ± 14.0 years in patients with reinfection, and 52.5 ± 13.0 years in patients without reinfection ($P > 0.05$); the mean body mass index in the 2 groups was 27.2 ± 4.1 kg/m^2 and 25.7 ± 4.2 kg/m^2, respectively ($P > 0.05$). A comparison of characteristics of patients with and without reinfection is presented in Table 1.

DISCUSSION

The reinfection rate of *H. pylori* could be mostly dependent on the prevalence of *H. pylori* in the specific region. It could be considered as an indirect indicator of the socioeconomic status of the region. Yan *et al*[14] analyzed the correlation between *H. pylori* recurrence rate and socioeconomic development [as represented by Human Development Index (HDI)], using data from 77 studies, which were considered reliable. Countries with very high HDI had a mean annual rate of 1.68%, which was significantly lower than that of high HDI countries at

6.05%, medium HDI countries at 7.04%, and low HDI countries at 9.63% (global annual rate being 2.82%)[14]. Lithuania is placed in the very high HDI category.

The studies indicate that low socioeconomic status was one of the major risk factors for a high prevalence of *H. pylori* infection[16,23]. Eastern and Central European countries are in a transitional area, where the prevalence of *H. pylori* is not as low as in Western regions, but not as high as in the developing countries[27]. There are no large epidemiological studies on the prevalence of *H. pylori* in this region, but there are data on the prevalence of *H. pylori* in specific groups of patients. The prevalence of *H. pylori* in middle-aged outpatients was 69%[21]. The prevalence of *H. pylori* in 22-year-old medical students established by serology was 30.4% and has decreased substantially during last 17 years[22].

In our study, in the *per protocol* analysis, *H. pylori* reinfection was established in 29.8% (17/57) (95%CI: 19.2-42.2) of patients during our 9-year follow-up, the annual rate being 3.36%. We also calculated the reinfection rates on the assumption that all non-responders were *H. pylori*-negatives: In this case reinfection would be 14.5% (17/117), the annual rate being 1.63%. In reality, the reinfection rate is probably in between these numbers, and could be considered a relatively high *H. pylori* reinfection rate. This may indicate that in Lithuania the decrease in the prevalence of *H. pylori* infection[21,22] is not as fast as was supposed, probably related to rather slow development of the socioeconomic status of the country, or the *H. pylori* prevalence is decreasing much slower than the speed of socioeconomic development. It is logical to believe that similar results could be found in neighboring countries.

The advantage of our study is that we established true reinfection, as all our patients were *H. pylori*-negative 1-year after the eradication regimen. We would like to stress that our study is the first to report on the reinfection rates of adult peptic ulcer patients from Central and Eastern Europe. In contrast to many South European countries, where the prevalence of antimicrobial drug resistance is significant, *H. pylori* susceptibility to the standard antibiotics in Lithuania remains high[19]. Therefore, high reinfection rates could be the most important issue in the management of *H. pylori* infection in the country.

There are some limitations of the study. The low number of responses (57 out of 117 patients) to follow-up investigations allowed us to speculate with the most pessimistic and most optimistic numbers, not being able exactly to determine the rates of *H. pylori* reinfection. Besides, we did not have epidemiological data of our patients, thus we could not examine the reasons for reinfection. Our investigated demographic and clinical characteristics were not predictive of reinfection.

In conclusion, the reinfection rate of *H. pylori* in a cohort of peptic ulcer patients in Lithuania was relatively high, and this may be related to the relatively high prevalence of *H. pylori* infection in the country, suggesting that socioeconomic differences between Western and Eastern European countries are probably still marked.

COMMENTS

Background

It is strongly recommended to eradicate *Helicobacter pylori* (*H. pylori*) in order to cure peptic ulcer disease and MALT lymphoma, to decrease risk of gastric cancer, and to alleviate the burden of functional dyspepsia and some other digestive and extragastric pathologies. At present, the eradication of *H. pylori* could be achieved in up to 90% of cases. Nevertheless, recrudescence or reinfection may occur over time.

Research frontiers

In the Maastricht consensus, the recommendation to regularly investigate the regional epidemiological status of *H. pylori* has been proposed, indicating that data of prevalence, eradication rates, antibacterial resistance, and reinfection rates are important. The reinfection of *H. pylori* has been reported to be not infrequent, especially in areas of high *H. pylori* prevalence. More studies are necessary to establish the rate of reinfection in different parts of the world. Factors which may contribute to the occurrence of *H. pylori* reinfection have to be elucidated.

Innovations and breakthroughs

There are few available data from Eastern and Central Europe on reinfection rates of *H. pylori*. This article presents a long-term follow-up study, which evaluates the frequency of *H. pylori* reinfection in Lithuanian peptic ulcer patients after confirmation of *H. pylori* eradication. These are probably the first data from Eastern-Central Europe regarding reinfection of *H. pylori* in peptic ulcer patients.

Applications

The results of the study add important scientific information on *H. pylori* reinfection rates in Central and Eastern Europe. This is important knowledge reflecting the current Maastricht consensus. It also encourages us to rethink the present epidemiological situation regarding the prevalence of *H. pylori* in Lithuania and the whole region. The reinfection rate of *H. pylori* in the cohort of peptic ulcer patients in Lithuania may be related to the relatively high prevalence of *H. pylori* infection in this region, suggesting that socioeconomic differences between Western and Eastern European countries are probably still marked.

Terminology

Reinfection of *H. pylori* is considered when a new strain of *H. pylori* is found after confirmed eradication. The confirmation of eradication must be performed not earlier than 6 mo after eradication treatment. Recrudescence of *H. pylori* is considered as a recurrence of the previous infection by the same *H. pylori* strain.

Peer-review

The subject of the present manuscript is interesting and important, as there are not many studies on the annual rate of *H. pylori* reinfection in this geographic area. However, some additions and clarifications should be performed to improve the manuscript.

REFERENCES

1 **Cover TL**, Blaser MJ. Helicobacter pylori in health and disease. *Gastroenterology* 2009; **136**: 1863-1873 [PMID: 19457415 DOI: 10.1053/j.gastro.2009.01.073]

2 **Fuccio L**, Zagari RM, Eusebi LH, Laterza L, Cennamo V, Ceroni L, Grilli D, Bazzoli F. Meta-analysis: can Helicobacter pylori eradication treatment reduce the risk for gastric cancer? *Ann Intern Med* 2009; **151**: 121-128 [PMID: 19620164 DOI: 10.7326/0003-4819-151-2-200907210-00009]

3 **Hopkins RJ**, Girardi LS, Turney EA. Relationship between Helicobacter pylori eradication and reduced duodenal and gastric

ulcer recurrence: a review. *Gastroenterology* 1996; **110**: 1244-1252 [PMID: 8613015]
4 **McColl KE**. Clinical practice. Helicobacter pylori infection. *N Engl J Med* 2010; **362**: 1597-1604 [PMID: 20427808]
5 **Malfertheiner P**, Megraud F, O'Morain CA, Atherton J, Axon AT, Bazzoli F, Gensini GF, Gisbert JP, Graham DY, Rokkas T, El-Omar EM, Kuipers EJ. Management of Helicobacter pylori infection--the Maastricht IV/ Florence Consensus Report. *Gut* 2012; **61**: 646-664 [PMID: 22491499]
6 **Chey WD**, Wong BC. American College of Gastroenterology guideline on the management of Helicobacter pylori infection. *Am J Gastroenterol* 2007; **102**: 1808-1825 [PMID: 17608775 DOI: 10.1111/j.1572-0241.2007.01393.x]
7 **Fock KM**, Katelaris P, Sugano K, Ang TL, Hunt R, Talley NJ, Lam SK, Xiao SD, Tan HJ, Wu CY, Jung HC, Hoang BH, Kachintorn U, Goh KL, Chiba T, Rani AA. Second Asia-Pacific Consensus Guidelines for Helicobacter pylori infection. *J Gastroenterol Hepatol* 2009; **24**: 1587-1600 [PMID: 19788600]
8 **Raghunath A**, Hungin AP, Wooff D, Childs S. Prevalence of Helicobacter pylori in patients with gastro-oesophageal reflux disease: systematic review. *BMJ* 2003; **326**: 737 [PMID: 12676842 DOI: 10.1136/bmj.326.7392.737]
9 **Laine L**, Sugg J. Effect of Helicobacter pylori eradication on development of erosive esophagitis and gastroesophageal reflux disease symptoms: a post hoc analysis of eight double blind prospective studies. *Am J Gastroenterol* 2002; **97**: 2992-2997 [PMID: 12492181]
10 **Schwizer W**, Thumshirn M, Dent J, Guldenschuh I, Menne D, Cathomas G, Fried M. Helicobacter pylori and symptomatic relapse of gastro-oesophageal reflux disease: a randomised controlled trial. *Lancet* 2001; **357**: 1738-1742 [PMID: 11403809]
11 **Manes G**, Mosca S, Laccetti M, Lioniello M, Balzano A. Helicobacter pylori infection, pattern of gastritis, and symptoms in erosive and nonerosive gastroesophageal reflux disease. *Scand J Gastroenterol* 1999; **34**: 658-662 [PMID: 10466875]
12 **Malfertheiner P**, MOssner J, Fischbach W, Layer P, Leodolter A, Stolte M, Demleitner K, Fuchs W. Helicobacter pylori eradication is beneficial in the treatment of functional dyspepsia. *Aliment Pharmacol Ther* 2003; **18**: 615-625 [PMID: 12969088]
13 **Bruley Des Varannes S**, Fléjou JF, Colin R, Zaïm M, Meunier A, Bidaut-Mazel C. There are some benefits for eradicating Helicobacter pylori in patients with non-ulcer dyspepsia. *Aliment Pharmacol Ther* 2001; **15**: 1177-1185 [PMID: 11472320]
14 **Yan TL**, Hu QD, Zhang Q, Li YM, Liang TB. National rates of Helicobacter pylori recurrence are significantly and inversely correlated with human development index. *Aliment Pharmacol Ther* 2013; **37**: 963-968 [PMID: 23550618]
15 **Kupcinskas L**, Jonaitis L, Kiudelis G. A 1 year follow-up study of the consequences of Helicobacter pylori eradication in duodenal ulcer patients: unchanged frequency of erosive oesophagitis and decreased prevalence of non-erosive gastro-oesophageal reflux disease. *Eur J Gastroenterol Hepatol* 2004; **16**: 369-374 [PMID: 15028968]
16 **Calvet X**, Ramírez Lázaro MJ, Lehours P, Mégraud F. Diagnosis and epidemiology of Helicobacter pylori infection. *Helicobacter* 2013; **18** Suppl 1: 5-11 [PMID: 24011238]
17 Available from: URL: https: //www.surescreen.com/diagnostics/picture.php?prodid=*H. pylori*T
18 **Jonaitis LV**, Kiudelis G, Kupcinskas L. Evaluation of a novel 14C-urea breath test "Heliprobe" in diagnosis of Helicobacter pylori infection. *Medicina* (Kaunas) 2007; **43**: 32-35 [PMID: 17297281]
19 **Kupcinskas L**, Rasmussen L, Jonaitis L, Kiudelis G, Jørgensen M, Urbonaviciene N, Tamosiunas V, Kupcinskas J, Miciuleviciene J, Kadusevicius E, Berg D, Andersen LP. Evolution of Helicobacter pylori susceptibility to antibiotics during a 10-year period in Lithuania. *APMIS* 2013; **121**: 431-436 [PMID: 23078193]
20 **Jonaitis L**, Kiudelis G, Kupcinskas L. Gastroesophageal reflux disease after Helicobacter pylori eradication in gastric ulcer patients: a one-year follow-up study. *Medicina* (Kaunas) 2008; **44**: 211-215 [PMID: 18413988]
21 **Jonaitis L**, Kiudelis G, Kupcinskas L, Kupcinskas J. Prevalence of Helicobacter pylori among outpatient middle-aged patients in Lithuania and its relation to dyspeptic symptoms. *Helicobacter* 2013; **18**: 104
22 **Jonaitis L**, Kiudelis G, Kupcinskas L. Prevalence of Helicobacter pylori among medical students in Lithuania decreased during last 17 years. *Helicobacter* 2012; **17**: 88
23 **Silva FM**, Navarro-Rodriguez T, Barbuti RC, Mattar R, Hashimoto CL, Eisig JN. Helicobacter pylori reinfection in Brazilian patients with peptic ulcer disease: a 5-year follow-up. *Helicobacter* 2010; **15**: 46-52 [PMID: 20302589 DOI: 10.1111/j.1523-5378.2009.00734]
24 **Pathak CM**, Kaur B, Khanduja KL. 14C-urea breath test is safe for pediatric patients. *Nucl Med Commun* 2010; **31**: 830-835 [PMID: 20864821 DOI: 10.1097/MNM.0b013e32833c3647]
25 **Bentur Y**, Matsui D, Koren G. Safety of 14C-UBT for diagnosis of Helicobacter pylori infection in pregnancy. *Can Fam Physician* 2009; **55**: 479-480 [PMID: 19439698]
26 **Ozdemir E**, Karabacak NI, Degertekin B, Cirak M, Dursun A, Engin D, Unal S, Unlü M. Could the simplified (14)C urea breath test be a new standard in noninvasive diagnosis of Helicobacter pylori infection? *Ann Nucl Med* 2008; **22**: 611-616 [PMID: 18756364 DOI: 10.1007/s12149-008-0168-6]
27 **Oona M**, Rägo T, Maaroos HI. Long-term recurrence rate after treatment of Helicobacter pylori infection in children and adolescents in Estonia. *Scand J Gastroenterol* 2004; **39**: 1186-1191 [PMID: 15742994 DOI: 10.1080/00365520410003461]
28 **McMahon BJ**, Bruce MG, Hennessy TW, Bruden DL, Sacco F, Peters H, Hurlburt DA, Morris JM, Reasonover AL, Dailide G, Berg DE, Parkinson AJ. Reinfection after successful eradication of Helicobacter pylori: a 2-year prospective study in Alaska Natives. *Aliment Pharmacol Ther* 2006; **23**: 1215-1223 [PMID: 16611283 DOI: 10.1111/j.1365-2036.2006.02880]
29 **Kim SY**, Hyun JJ, Jung SW, Koo JS, Yim HJ, Lee SW. Helicobacter pylori recurrence after first- and second-line eradication therapy in Korea: the problem of recrudescence or reinfection. *Helicobacter* 2014; **19**: 202-206 [PMID: 24612156 DOI: 10.1111/hel.12117]
30 **Kim MS**, Kim N, Kim SE, Jo HJ, Shin CM, Lee SH, Park YS, Hwang JH, Kim JW, Jeong SH, Lee DH, Kim JM, Jung HC. Long-term follow-up Helicobacter pylori reinfection rate and its associated factors in Korea. *Helicobacter* 2013; **18**: 135-142 [PMID: 23066652 DOI: 10.1111/hel.12018]
31 **Ryu KH**, Yi SY, Na YJ, Baik SJ, Yoon SJ, Jung HS, Song HJ. Reinfection rate and endoscopic changes after successful eradication of Helicobacter pylori. *World J Gastroenterol* 2010; **16**: 251-255 [PMID: 20066746 DOI: 10.3748/wjg.v16.i2.251]
32 **Zhang YY**, Xia HH, Zhuang ZH, Zhong J. Review article: 'true' re-infection of Helicobacter pylori after successful eradication--worldwide annual rates, risk factors and clinical implications. *Aliment Pharmacol Ther* 2009; **29**: 145-160 [PMID: 18945250 DOI: 10.1111/j.1365-2036.2008.03873]

P- Reviewer: Boyanova L, Biernat MM
S- Editor: Yu J **L- Editor**: Cant MR **E- Editor**: Jiao XK

World Journal of Gastrointestinal Pathophysiology

Submit a Manuscript: http://www.wjgnet.com/esps/
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.4291/wjgp.v7.i1.186

World J Gastrointest Pathophysiol 2016 February 15; 7(1): 186-198
ISSN 2150-5330 (online)

Intra-abdominal pressure: Time ripe to revise management guidelines of acute pancreatitis?

Jiten Jaipuria, Vimal Bhandari, Avneet Singh Chawla, Mohit Singh

Jiten Jaipuria, Vimal Bhandari, Avneet Singh Chawla, Mohit Singh, Department of General Surgery, Vardhman Mahavir Medical College and Safdarjung Hospital, New Delhi 110029, India

Author contributions: Jaipuria J initially conceptualized the idea of paper; Jaipuria J and Bhandari V performed the literature search; all authors read the manuscripts selected in initial search; initial draft was prepared by Jaipuria J which was further modified by intellectual inputs by all the authors; all authors approved the final draft.

Conflict-of-interest statement: All authors have no conflicts of interest or financial ties to disclose.

Data sharing statement: All data pertaining to review was obtained from PubMed, Medline, Scopus and Google search of open access sources. No additional data are available.

Correspondence to: Jiten Jaipuria, MS, MRCS (Edinburgh), FIAGES, Senior Registrar, Department of General Surgery, Vardhman Mahavir Medical College and Safdarjung Hospital, New Delhi 110029, India. jitenjaipuria@yahoo.co.in
Telephone: +91-98-68812182
Fax: +91-11-26163072

Received: June 30, 2015
Peer-review started: July 4, 2015
First decision: July 29, 2015
Revised: September 8, 2015
Accepted: November 3, 2015
Article in press: November 4, 2015
Published online: February 15, 2016

Abstract

AIM: To systematically review evidence on pathophysiology of intra-abdominal pressure (IAP) in acute pancreatitis (AP) with its clinical correlates.

METHODS: Systematic review of available evidence in English literature with relevant medical subject heading terms on PubMed, Medline and Scopus with further search from open access sources on internet as suggested by articles retrieved.

RESULTS: Intra-abdominal hypertension (IAH) is increasingly gaining recognition as a point of specific intervention with potential to alter disease outcome and improve mortality in AP. IAH can be expected in at least 17% of patients presenting with diagnosis of AP to a typical tertiary care hospital (prevalence increasing to 50% in those with severe disease). Abdominal compartment syndrome can be expected in at least 15% patients with severe disease. Recent guidelines on management of AP do not acknowledge utility of surveillance for IAP other than those by Japanese Society of Hepato-Biliary-Pancreatic Surgery. We further outline pathophysiologic mechanisms of IAH; understanding of which advances our knowledge and helps to coherently align common observed variations in management related conundrums (such as fluid therapy, nutrition and antibiotic prophylaxis) with potential to further individualize treatment in AP.

CONCLUSION: We suggest that IAP be given its due place in future practice guidelines and that recommendations be formed with help of a broader panel with inclusion of clinicians experienced in management of IAH.

Key words: Intra-abdominal hypertension; Abdominal compartment syndrome; Pancreatitis; Practice guideline

Core tip: Intra-abdominal hypertension is not merely an epiphenomenon but offers a unique point of specific intervention in acute pancreatitis and there is increasing data to show improved mortality with appropriate management. It is frequent and may be observed in at least 50% patients with severe disease. Moreover it acts as confounder in management related issues of fluid therapy, nutritional support and antibiotic prophylaxis; and understanding its pathophysiology coherently explains many dichotomies which presently lowers internal validity of much available evidence. Incorporating surveillance for intra-abdominal pressure in select subgroup of patients may help better tailor individualized treatment to patients with most severe spectrum of disease. Recommendations by World Society of the Abdominal Compartment Syndrome may be followed by practicing clinician to guide decision making.

Jaipuria J, Bhandari V, Chawla AS, Singh M. Intra-abdominal pressure: Time ripe to revise management guidelines of acute pancreatitis? *World J Gastrointest Pathophysiol* 2016; 7(1): 186-198 Available from: URL: http://www.wjgnet.com/2150-5330/full/v7/i1/186.htm DOI: http://dx.doi.org/10.4291/wjgp.v7.i1.186

INTRODUCTION

Reported data from many countries reveals epidemiology of acute pancreatitis (AP) showing an increasing trend, and it enjoys the crown of being the most frequent gastrointestinal diagnosis leading to hospital admissions in the United States[1]. Despite exciting developments in understanding pathogenesis of AP; none of the molecular targets have proved useful in routine clinical practice, frustrating clinicians to rely much on "clinical" rather than "translational" research in managing patients[2]. Intra-abdominal hypertension/Abdominal Compartment Syndrome (IAH/ACS) is increasingly being recognized as a point of specific intervention in AP with attractive potential to alter disease outcomes[3]. This review focuses on the role and importance of Intra-abdominal pressure (IAP) in AP with a critical perspective, which is now gaining recognition as a vital sign specific for abdomen but lacks mention in clinical guidelines on management of AP except recently those by Japanese Society of Hepato-Biliary-Pancreatic Surgery[4,5].

MATERIALS AND METHODS

In August 2015 electronic database search of PubMed, Medline and Scopus was performed from 1985 till 20th August 2015. Medical Subject Headings terms "AP", "severe AP (SAP)", "necrotizing pancreatitis", "fulminant pancreatitis" were combined with Boolean operator "AND" with studies identified by keywords "IAH", and "ACS". References of retrieved articles were further searched as relevant including open access sources from internet.

Statistical analyses

The available studies (in English literature) were heterogeneous in terms of study populations, study criteria and critical definitions of what constituted SAP, IAH and ACS. It was thus not possible to apply meta analytic methods and a descriptive narration of available evidence is being performed.

RESULTS

Historical perspective and epidemiology-confounding by definitions

The general understanding of pancreatitis in the decade beginning 2000 was dominated by concept of conservative management in initial phase of disease with operative intervention (which should be delayed till later part of disease) reserved for infected pancreatic necrosis[6]. Clinicians broadly classified patients with pancreatitis into groups of mild and severe as proposed by Atlanta classification[7]. Yet they recognized a sub group of patients with fulminant disease course which had rapid onset of multiple organ dysfunctions leading to high mortality rates. Clinical similarities between ACS and such patients with SAP lead to initial study on the role of IAH in AP, which was first published in 2002 from Medical academy of Latvia[3]. Authors emphasized the role of monitoring IAP as a prognostic tool as they found no mortality among patients who had IAP < 25 cm H_2O *vs* 36% mortality in group of patients with IAP > 25 cm H_2O. In the same year another group from United States reported experience with emergency abdominal decompression in three patients with fulminant AP and ACS (in early phase of AP) out of which one survived[8]. They remarked that role of ACS and emergency abdominal decompression in early phase of AP should find mention in prevalent surgical literature of those times.

Meanwhile in 2004 The World Society of the ACS (WSACS) was founded[9]. It performed the commendable job of developing consensus statements and evidence based recommendations which culminated in initial set of guidelines and definitions of IAH/ACS in 2004 which are periodically revisited (latest congress in 2015). Importantly they standardized the methodology of IAP measurement (preferably transvesical method, with maximal instillation of 25 cc saline, in supine position, measured at end expiration, with zeroing done at the level of mid axillary line) and also the cut off and definitions of IAH and ACS. IAH is defined by a sustained or repeated pathologic elevation of IAP $\geqslant$ 12 mmHg with grading as follows: Grade Ⅰ: IAP 12-15 mmHg, Grade Ⅱ: IAP 16-20 mmHg, Grade Ⅲ: IAP 21-25 mmHg, Grade Ⅳ: IAP > 25 mmHg. ACS is defined as a sustained IAP > 20 mmHg [with or without an Abdominal Perfusion Pressure (APP) < 60 mmHg] that

Table 1 Epidemiology of intra-abdominal hypertension and abdominal compartment syndrome in acute pancreatitis as previously reported in literature *n* (%)

Ref.	IAP monitoring	Definition of IAH	Incidence of IAH (among patients with SAP)	Definition of ACS	Incidence of ACS (among patients with SAP)
Pupelis *et al*[3]	Selected	NA	NA	IAP > 25 mmHg	18 (25)
De Waele *et al*[12]	Selected	IAP > 15 mmHg	21 (78)	NA	NA
Pupelis *et al*[13]	Selected	NA	NA	NA	NA
Keskinen *et al*[14]	Selected	IAP > 12 mmHg	31 (84)	IAP > 20 mmHg with new organ dysfunction	18 (49)
Zhang *et al*[15]	Unselected	IAP > 10 cm H_2O (NA)	68 (76)	NA	NA
Rosas *et al*[16]	Unselected (45 patients)	NA	NA	NA	NA
Chen *et al*[17]	Unselected	IAP > 12 mmHg	44 (59)	IAP > 20 mmHg with new organ dysfunction	20 (27)
Al-Bahrani *et al*[18]	Unselected	IAP > 15 mmHg	11 (61)	IAH with organ dysfunction	10 (56)
Dambrauskas *et al*[19]	Unselected	IAP > 12 mmHg	19 (43)	IAP > 20 mmHg with new organ dysfunction	6 (14)
Mentula *et al*[20]	Unselected (26 patients with ACS)	NA	NA	IAP > 20 mmHg with new organ dysfunction	NA
Ke *et al*[21]	Unselected	IAP > 12 mmHg	36 (62)	IAP > 20 mmHg with new organ dysfunction	7 (12)
Bezmarevic *et al*[22]	Unselected	IAP > 12 mmHg	36 (71) (among patients with AP) 27 (97) (among patients with SAP)	IAP > 20 mmHg with new organ dysfunction	6 (12) (among patients with AP) 6 (21) (among patients with SAP)
Boone *et al*[23]	Selected (12 patients undergoing decompressive laparotomy for ACS)	NA	NA	IAP > 20 mmHg with new organ dysfunction	NA
Davis *et al*[24]	Selected	NA	NA	IAP > 20 mmHg with new organ dysfunction	16 (35)
Bhandari *et al*[25]	Unselected	IAP > 12 mmHg	8 (20) (among patients with AP) 8 (50) (among patients with SAP)	IAP > 20 mmHg with new organ dysfunction	3 (7.5) (among patients with AP) 3 (19) (among patients with SAP)
Aitken *et al*[26]	Unselected	IAP > 12 mmHg	36 (17) (among patients with AP)	NA	NA

IAP: Intra-abdominal pressure; IAH: Intra-abdominal hypertension; ACS: Abdominal compartment syndrome; NA: Not available; AP: Acute pancreatitis; SAP: Severe acute pancreatitis.

is associated with new organ dysfunction/failure. APP = mean arterial pressure - IAP[10]. Ke *et al*[11] found IAP was superior to APP as early marker of evolution and predicting complications of AP.

Table 1 summarizes all the studies evaluating the role of IAP in AP[3,12-26]. None of the studies ever enrolled more than 100 patients. Though results from initial studies were luminary for studies to come yet they suffered from drawback of having unselected patients and non-standardized methodology of measuring IAP and defining IAH/ACS. However a general trend towards uniformity of nomenclature could be seen in studies done from 2009 onwards. However in 2012 major changes were proposed in classification of AP and traditional classification of pancreatitis into mild and severe was further widened to include three or four categories[27,28]. Table 2 contrasts the difference in older Atlanta and recent revised Atlanta and determinant based classification of AP while Table 3 details the modified Marshall scoring system that is used in latest classifications to define organ failure[29]. This further complicates meaningful synthesis of information from previous studies to present day context where stage down migration of many cases of severe pancreatitis would occur (many cases classified as severe in older classification may now get classified as moderate).

In light of all above arguments and historical perspective it is very difficult to give an accurate estimate of incidence of IAH/ACS that a clinician using modern classification system of AP is likely to face. However deductive reasoning leads to few obvious conclusions: (1) initial reports of very high incidence of IAH/ACS in patients with SAP needs to be considered with view of selection bias; (2) severe disease in older classification system was a more heterogeneous group (included many cases which would now be classified as moderate) and thus point estimates of IAH/ACS in patients classified as "severe" according to present day classification systems are likely to be higher than the low estimates which later investigators have reported; and (3) the observed incidence of IAH/ACS can further vary due to referral bias of worse cases to tertiary care

Table 2 Comparison of Atlanta, revised Atlanta and determinant based classification system of acute pancreatitis

	Atlanta[7]	Revised atlanta[27]	Determinant based system[28]
Mild	Minimal organ dysfunction and an uneventful recovery; lacks the features of severe acute pancreatitis. Usually normal enhancement of pancreatic parenchyma on contrast-enhanced computed tomography	No organ failure No local or systemic complications	No (peri)pancreatic necrosis and no organ failure[2]
Moderate		Organ failure[2] that resolves within 48 h (transient organ failure) and/or local or systemic complications without persistent organ failure	Sterile (peri)pancreatic necrosis and/or transient organ failure (< 48 h)[2]
Severe	Associated with organ failure[1] and/or local complications such as acute fluid collections, necrosis, abscess or pseudocyst	Persistent organ failure[2] (> 48 h) Single organ failure Multiple organ failure	Infected (peri)pancreatic necrosis or persistent organ failure (> 48 h)[2]
Critical			Infected (peri)pancreatic necrosis and persistent organ failure (> 48 h)[2]

[1]Organ failure and systemic complications defined as - Shock (Systolic blood pressure < 90 mmHg), Pulmonary insufficiency ($PaO_2 \leqslant 60$ mmHg), Renal failure (Creatinine $\geqslant 177$ μmol/L or $\leqslant 2$ mg/dL after rehydration), Gastrointestinal bleeding (500 mL in 24 h), Disseminated intravascular coagulation (Platelets $\leqslant 100000$/mm, fibrinogen < 1.0 g/L and fibrin-split products > 80 μg/L), and Severe metabolic disturbances (Calcium $\leqslant 1.87$ mmol/L or $\leqslant 7.5$ mg/dL); [2]Organ failure defined by modified Marshall scoring (Table 3)[29].

Table 3 Modified Marshall scoring system for organ dysfunction[29]

Organ System	Score				
	0	1	2	3	4
Respiratory (PaO_2/FiO_2)	> 400	301-400	201-300	101-200	$\leqslant 101$
Renal[1]					
(serum creatinine, mmol/L)	$\leqslant 134$	134-169	170-310	311-439	> 439
(serum creatinine, mg/dL)	< 1.4	1.4-1.8	1.9-3.6	3.6-4.9	> 4.9
Cardiovascular (systolic blood pressure, mmHg)[2]	> 90	< 90 and fluid responsive	< 90 and not fluid responsive	< 90, pH < 7.3	< 90, pH < 7.2

[1]A score for patients with pre-existing chronic renal failure depends on the extent of further deterioration of baseline renal function. No formal correction exists for a baseline serum creatinine $\geqslant 134$ μmol/L or $\geqslant 1.4$ mg/dL; [2]Off inotropic support. For non-ventilated patients, the FiO_2 can be estimated from below: (1) Supplemental oxygen (L/min) is room air, the percentage of FiO_2 is 21%; (2) Supplemental oxygen is 2 L/min, 4 L/min, FiO_2 is 25%, 30%, respectively; (3) Supplemental oxygen is 6-8 L/min, FiO_2 is 40%; and (4) Supplemental oxygen is 9-10 L/min, FiO_2 is 50%. A score of 2 or more in any system defines the presence of organ failure.

institutions.

We find data from studies done by Aitken *et al*[26], Bhandari *et al*[25], Bezmarevic *et al*[22] and Dambrauskas *et al*[19] to be useful in this regard as they reported IAP data on all patients with diagnosis of AP visiting their tertiary referral centers. IAH can be expected in at least 17% of patients presenting with diagnosis of AP to a typical tertiary care hospital. Clinicians can expect at least 50% patients with SAP to have IAH. ACS can be expected in at least 15% patients with SAP. No confident estimate can be given for expected incidence of IAH/ACS in patients with moderately severe AP. Similar conclusions have been drawn by other reviewers[30,31].

Pathophysiology and clinical correlates of IAH/ACS in AP

In past decade the central role of zymogen activation due to different reasons as a cause of pancreatic injury has been challenged and modern view accepts both zymogen activation and NF-κB (natural factor kappa beta) activation as parallel players capable of pancreatic injury with aberrant intracellular calcium signaling as the final common pathway[32]. While these mechanisms are playing themselves out in pancreatic acinar cells; the ductal cells may also join the process and enhanced ductal secretions may help wash out the toxins[33]. But they can become overwhelmed and then further contribute to the damage process along with bile. What essentially then ensues is an inflammatory cascade that can propagate to become full blown systemic inflammatory response syndrome (SIRS); which can even lead to multiple organ dysfunction syndrome (MODS) as not only pancreatic acinar cells but inflammatory cells from diverse organs (such as Kupffer cells, peritoneal and alveolar macrophages) become activated during different phases[34]. Peripancreatic adipose tissue upon inflammation can also produce mediators which can facilitate development of SIRS and it can be a more important factor than even waist circumference or BMI in predicting development of SAP[35,36]. There may also be an element of neural mediated inflammation in scheme of events[37]. Initial SIRS is followed by Counter Inflammatory Response Syndrome (CARS) which can contribute to severity

Table 4 Ways in which intra-abdominal hypertension/ abdominal compartment syndrome can be predisposed in patients with acute pancreatitis

Diminished abdominal wall compliance
Prone positioning, head of bed > 30°
High body mass index, central obesity
Acute respiratory failure, especially with elevated intrathoracic pressure
Edema due to excess fluid administered during resuscitation
Increased intra-luminal contents
Gastroparesis
Ileus
Colonic pseudo-obstruction
Increased abdominal contents
Ascites (due to causes such as acute fluid collections, liver dysfunction)
Capillary leak / fluid resuscitation (overload)
Acidosis (pH < 7.2)
Hypotension
Hypothermia (core temperature < 33 ℃)
Coagulopathy (platelets < 55000/mm^3 or prothrombin time > 15 s or partial thromboplastin time > 2 times normal or international standardised ratio > 1.5)
Massive fluid resuscitation (> 5 L/d)
Oliguria
Sepsis

as much as protease activation[38]. CARS leads to suppression of immune system and can facilitate complications such as infection of sterile pancreatic necrosis and nosocomial infections. Certain iatrogenic factors such as timing of resuscitation, quantity of administered fluids and enteric nutrition also modulate this disease process although they are incompletely understood at present[39,40].

Traditional view of gut has been that of an innocent bystander suffering collateral damage but increasingly it is being understood that it (along with abdomen) further contributes in two major inter related ways in the ongoing scheme of events by means of (1) gut barrier dysfunction; and (2) IAH/ACS[41,42]. Gut barrier dysfunction implies damage to intestinal epithelial cells and tight junctions leading to increased intestinal permeability, and pooled meta analyses determined prevalence has been determined to be 59% (95%CI: 48%-70%). It occurs due to ischemia reperfusion injury to gut due to reflex splanchnic vasoconstriction in initial phase which later gets overcome by resuscitation. Resultant increased toxin and enteric bacterial translocation to portal circulation and mesenteric lymph nodes can lead to SIRS, secondary infection, MODS and ultimately death[43,44]. IAH/ACS also contributes to gut barrier dysfunction[42,45].

Further, all studies on IAH/ACS in AP have found it to be variably associated with increased complications and severity of disease (variably in form of higher incidence of organ failure, need for Ⅳ fluids/inotropic support, pancreatic necrosis, extent of pancreatic necrosis, infected pancreatic necrosis, morbidity, hospital stay, and ultimately mortality). There are multiple ways in which IAH/ACS is predisposed in AP (Table 4)[10]. In fact IAH/ACS can contribute to dysfunction in almost any organ[46]. Increased IAP reduces cardiac function with reduced return from Inferior and superior vena cava as well as portal vein while increasing peripheral vascular resistance. It leads to splinting of diaphragm with resultant increased airway pressures and reduced pulmonary capacity; hypercapnia, acidosis and hypoxia can ensue. The splanchnic, mesenteric and hepatic perfusion pressures also decrease which can lead to gut barrier dysfunction as mentioned before as well as hepatic dysfunction potentiating coagulopathy and worsening acidosis. Changes due to abdomino thoracic pressure transmission have been even noted in organs such as orbit and cranium. Renal perfusion pressure decreases due to direct compression effect on renal arteries, veins and kidney parenchyma as well as reduced cardiac output; oliguria and anuria can set in with increasing pressures with simultaneous activation of renin angiotensin system[47]. Indeed, oliguria is one of the early hallmark sign of increased IAP. Recognition of such widespread changes lead WSACS to recognize term "polycompartmental syndrome" ("where two or more anatomical compartments have elevated compartmental pressures") in 2013 consensus definitions[10]. Moreover increases in IAP have been found to positively correlate with reduced transmucosal gastric pH and increased levels of IL-1β, IL-6, TNFα and C-reactive protein in animal models while in human surgical intensive care (ICU) patients it has been found to correlate with IL-10 and adenosine[42,48]. Bodnár *et al*[49] proposes central role of adenosine (released from hypoxemic tissues) in cyclical events of ACS where adenosine induced splanchnic vasodilatation increases IAP and also causes renal arterial dysfunction. Renal dysfunction plays important role propagating other organ dysfunction. They thus propose monitoring adenosine and/or IL-10 in monitoring progression of ACS and results of therapeutic interventions. On a parallel stream of thought pancreatic inflammation is thought to trigger innate immune system which subsequently triggers adaptive immune system. Previously researchers found reduced levels of $CD4^+$ cells in patients with severe disease which also correlated with increased incidence of complications[50,51]. However recently Liu *et al*[52] while revisiting the same concept in SAP found that proportions of $CD4^+$ were significantly lower in patients with ACS in comparison to those with IAH. They further found a $CD4^+$ T cell proportion of 30.3% on day 1 predicted ACS with an area under curve of 0.774, with sensitivity and specificity of 82.5% and 72% respectively.

It is thus easy to understand why IAH/ACS should be considered as a spectrum in continuum, and how it is difficult to discriminate changes due to SIRS of AP from those due to IAH/ACS[31]. But ACS has immediate critical effects on multiple organ systems due to additional physico-mechanical effects which are associated with very high mortality (if left untreated). It may not be unreasonable to argue that few cases of early mortality in AP may be due to untreated ACS[53].

ACS: Epiphenomenon or not? - Are we missing the focus?

Concern is expressed whether IAH/ACS is an epiphenomenon or a driver of events in AP as it has direct bearing on utility of its surveillance and management, and little would be gained if ACS only developed as a terminal phenomenon to a series of morbid events[30,54]. Indeed, almost all clinicians come across critically ill patients with multiple organ dysfunction who have almost all predisposing factors for development of IAH/ACS and little hope for any survival. We cannot reasonably compare clinical picture of acute disease process of pancreatitis with those of chronic and terminally ill patients. Moreover, understanding of pathophysiology from discussed previous evidence proves beyond doubt that ACS is not a silent spectator; so whether IAH/ACS develops as an epiphenomenon or not, it "definitely contributes negatively" to the scheme of events. Two additional points merit attention - almost all studies on IAP in AP conclude that IAH/ACS develops early in the disease course (usually present at admission or within first 2 d). Thus whether IAH/ACS is an epiphenomenon or not can only be judged by carefully timed measurements of IAP before organ failure manifests. Which brings us to the second point of findings in a recent observational study of natural history of IAH in AP where Aitken *et al*[26] found development of IAH/ACS to be sentinel event before progressive organ failure ensued in several patients (an experience with which we also agree). To this we may add that it is practically impossible to detect IAP in a patient prior to when he presents to the hospital; thus natural history of IAP in patients who present with manifest IAH to hospital will remain elusive. Only circumstantial human evidence and experience from animal studies can be used to draw conclusions. A recent animal study by Li *et al*[45] supports the "driver role" of IAH/ACS where they propose early mitigation of IAP as an approach to treat acute necrotizing pancreatitis and hint towards a 6 hour window where appropriately diverted efforts may deliver results. AP was induced in Sprague-Dawley rats and it was observed that IAP showed a continuous upward trend with time with peak in rising rate at 6 h; after which rapid clinical deterioration occurred. Moreover, TNF-α (inflammatory marker), diamine oxidase and D-lactate (markers of gut barrier dysfunction) also peaked at the same time as IAP. When we consider all these arguments in conjunction with evidence of IAH/ACS being associated with higher incidence of complications such pancreatic necrosis, extent of necrosis, presence of infected necrosis, hospital stay and mortality then clinical reasoning only directs attention to surveillance of IAP in appropriate patients and additional efforts to determine optimum management strategies to tackle it.

We find it interesting that the first publication suggesting unique intervention towards ACS in early phase of SAP came from United States in 2002, but latest clinical guidelines (2013) yet do not acknowledge possible unique role of IAP[9,55,56]. We agree that guidelines have to rely on higher order evidence but we believe sufficient evidence exists to merit at least surveillance of IAP in selected subgroup of patients with AP. On an encouraging note Japanese Society Hepato-Biliary-Pancreatic Surgery have been the first to acknowledge the importance of ACS in evolution of AP and recommend sequential measurement of IAP for cases with excessive fluid infusion, high severity, renal and respiratory complications, and fluid accumulation in multiple areas as observed by computed tomography (CT), in recognition of fact that presence of ACS may increase mortality[5]. Lack of support for IAP surveillance from major guidelines is not without consequences and it could be one factor potentiating lack of awareness of IAH/ACS among clinicians treating AP as results from a recent clinical survey indicate less than 30% clinicians being aware of correct definitions of IAH/ACS[57]. It also precluded us from including decompressive laparotomy for ACS in early SAP in our hospital disease management policy in 2009 as regulatory requirements mandated hospital policy to be based on latest accepted national or international clinical guidelines (though we offered it on individual basis with consent and discussion of best available literature)[26]. Such situation may also be faced by other clinicians. This also acts as a barrier to gathering data on the next obvious vexing question.

Does surveillance and intervention for IAH/ACS reduce mortality?

It makes straightforward clinical argument that - "if animal studies and human data show a time period where IAH/ACS occurs before manifest organ failure; and if that time period is of sufficient duration to allow reasonable attempts to correct IAH/ACS; or if IAH/ACS are proximate driver of events which lead to clinical deterioration; implying that they may not be terminal events which occur at the end of morbid clinical incidents" - then, surveillance and intervention for IAH/ACS should be proven to reduce mortality.

The first report in 2002 on abdominal decompression in three patients for ACS in SAP reported one survival[9]. But managing all three patients convinced the authors to recommend consideration for decompressive laparotomy in early phase of SAP; much radical suggestion in contrast to dominant recommendation for conservative management in early phase of disease. A year later, Tao *et al*[58] reported their experience from China where 15 out of 18 patients receiving decompressive laparotomy for ACS survived while 4 out of 5 patients managed conservatively died, and they recommended early diagnosis of ACS for optimum management. On the contrary, in 2005 De Waele *et al*[12] reported universal mortality following decompressive laparotomy for ACS in four patients and cautioned against its routine use. Subsequently a large study by Chen *et al*[17] in 2008 demonstrated obvious amelioration in clinical variables within 24 h after decompression for ACS in patients with SAP and 25% patients survived. They recommended

a low threshold for intervention in patients with AP determined by presence of IAH and early signs of changes in physiologic variables. Further, retrospective study in 26 consecutive cases of decompressive laparotomy for ACS in SAP by Mentula *et al*[20] in 2010 showed 46% mortality (decompressive laparotomy improved renal and respiratory function) which came down to 18% when surgical decompression was carried out within first four days after disease onset. This prompted them to conclude that early decompression reduced mortality. In 2011 Leppäniemi *et al*[59] reported survival in 6 out of 10 patients with a novel minimally invasive endoscopic method of subcutaneous linea alba fasciotomy for decompression of ACS in SAP. However, again in 2012 Bezmarevic *et al*[22] reported mortality in 3 out of 4 patients who underwent decompression of ACS in various time points in disease course; though one patient who did not undergo decompressive laparotomy also did not survive. On the contrary, more recently Boone *et al*[23] in 2013 reported survival in 6 out of 12 consecutive cases with decompressive laparotomy for ACS in SAP with improvement in clinical parameters of multiple organ systems in most cases. In the same year Davis *et al*[24] also reported encouraging results of decompressive laparotomy with survival in 13 out of 16 patients with ACS with no difference in survival whether patients were morbidly obese or not. This stands in stark contrast to prospective observational studies by Bhandari *et al*[25] and Aitken *et al*[26] where all patients with ACS died with conservative approach (avoiding decompressive laparotomy). Recently Jacob *et al*[60] reported their experience from central Australia with early surgical intervention in AP for twin indications of early infected pancreatic necrosis and ACS (not controlled with medical management) over period of 8 years (2005-2012) and reported 0% mortality for SAP in 114 patients.

Could the survival data from decompressive laparotomy for ACS be away from real achievable optimum?

Recognition of clinical heterogeneity of severe pancreatitis and importance of organ failure is one of major advances in our understanding of pancreatitis in the past decade and latest modifications in classification systems of pancreatitis reflect those (apart from better defining complications of AP)[27,28]. Similar paradigms become apparent in ACS: (1) ACS by definition requires development of organ failure but then it further worsens it and induces organ failure in new organs; (2) there may be a reasonable time period between onset of ACS and then further onset of organ failure; (3) there may be heterogeneity in reported outcomes of intervention in ACS because of differences in timing of intervention, as multi organ failure can be irreversible after a point; and (4) there may be other non-modifiable or modifiable but with serious demands on human physiology factors (such as comorbidities, old age) which independently adversely impact survival. All these arguments taken together coherently explain 0% as well as 100% mortality for intervention in ACS. Indeed, almost all the authors reporting outcomes of decompressive laparotomy in ACS except De Waele *et al*[12] and Bezmarevic *et al*[22] recommended early rather than late intervention. These hints towards greater likelihood of better survival than what eyeballing combined results of these studies would indicate. A recent meta analyses on ACS in AP (103 patients) concluded with 49% mortality in patients with ACS (with morbidity upto 90%) *vs* 11% mortality in those without ACS when 84% patients underwent invasive intervention for ACS (which was decompressive laparotomy in majority)[61]. The authors similarly acknowledged that optimum timing and method of intervention for ACS could impact outcomes and recommended further evaluation. In support of human data, animal experiments in controlled environment show coherent conclusive proof that well timed surgical decompression for ACS in SAP reduces mortality compared to delayed decompression[62]. Ke *et al*[62] divided 32 porcine animal models of SAP lasting 24 h into 4 equal groups and induced ACS in three groups. Surgical decompression was carried out in ACS groups at 6, 9 and 12 h respectively and effect on survival was seen. The survival time progressively increased with increasing promptness of timing of decompression and animals in 6-h group had survival times similar to those with SAP without ACS. On a parallel stream of thought Orlando Regional Medical Center (United States) in 2010 reported first large scale prospective clinical data where use of continually revised IAH/ACS management algorithm showed improvement in survival in trauma victims from 50% to 72% in ICU setting in a Level 1 trauma center[63]. Unsurprisingly, with time De Waele *et al*[64,65] changed their position (from exercising caution in recommending decompressive laparotomy) as early as 2008, and now recommend to identify patients early for IAH/ACS during course of disease, and not to hesitate to offer decompressive laparotomy in case non operative methods seem to not help in AP.

Could doing surveillance for IAH/ACS be useful in other ways too?

IAH/ACS is attractive for purpose of prognosis and marker of disease severity for three reasons: (1) It develops early during course of disease; (2) It has been noted to be a proximate event prior to clinical worsening; and (3) It is associated with worse outcomes (may even have a causal role as its coherent fit in clinical events of disease progression and pathophysiology suggests). Unsurprisingly it compares favorably to commonly used markers of disease severity. The pioneering paper with Pupelis *et al*[3] concluded with the final words "Marked increase of the IAP should be considered a serious prognostic sign in patients with severe AP" as they found zero mortality in patients who did not develop IAP > 25 cm H_2O during disease course *vs* 36% mortality in those who did. Subsequently authors reported IAH/ACS to be associated with higher Ranson score[12,17], APACHE 2 score[12-19,24-26], CT severity index[16,25],

Marshall score[17,18,25], Glasgow-Imrie score[19,24,26], SOFA score[13,14,18], Lung injury score[18], multiple organ dysfunction score[19], infected pancreatic necrosis[16,17,25], procalcitonin levels[22], C Reactive Protein levels[22,26], maximal creatinine[14], maximum base deficit[14], lower serum calcium levels[21], lower platelet counts[13], requirement for vasoactive drugs[16], lower enterally provided volume[13], requirement for total parenteral nutrition[16], operative intervention[16,21], and length of ICU stay[12,14,18,21,25]. Definition of ACS by itself implies presence of organ failure and most studies universally conclude ACS to be associated with higher mortality as discussed previously. In fact it compares similarly in terms of sensitivity/specificity for predicting SAP when compared to APACHE 2 Score[25,26], Glasgow Imrie score[26], C reactive protein[26] and procalcitonin levels[22]. Bodnár *et al*[42] recommend presence of IAH/ACS to be a criteria for referral to higher center (a recommendation to which we agree)[25].

Could IAP be a confounder and contribute to heterogeneity in common management related issues in AP?

Confounder is an unobserved exposure that is associated with exposure of interest and is a potential cause for outcome of interest. Failure to control it damages the internal validity of experiment. Similar serious issues occur when population studied under controlled experiment is heterogeneous. The present American College of Gastroenterology as well International Association of Pancreatology/American Pancreatic Association guidelines (which are essentially based on evidence gathered by meta analyses) highlight conflicting evidence on three important areas in management of AP: (1) fluid resuscitation; (2) nutrition; and (3) antibiotics[55,56]. IAH/ACS offers explanation as area of potential heterogeneity as well as confounding, which can coherently align observed outcomes in prevalent pathophysiologic paradigm. This could potentially help plan better individualized treatment for patients with more severe spectrum of disease.

Fluid resuscitation

It is an easy to understand hypothesis that pancreatic hypoperfusion in AP would be worsened by hypotension and experiments in rat models confirm the same[66]. But translational outcome of this evidence falters in humans and contrarily few recent studies indicate better outcomes with non-aggressive fluid resuscitation protocols[67,68]. In fact a recent systematic review concluded with "equipoise" over the question of non-aggressive, *vs* aggressive, *vs* goal directed fluid resuscitation protocol for AP[69]. Most experienced clinicians treating AP would agree that fluid resuscitation is not much problematic with mild AP, and they do not need aggressive fluid management; while also at the same time understanding that that too little fluid is also obviously harmful in AP. We now also better understand that severe pancreatitis according to old Atlanta classification was in fact a heterogeneous group and present classification has set the bar higher for severe disease. This can partially explain incoherent results from previous studies where study cohorts may have included an unrecognized proportion of patients with not as much worse disease but still labelled as severe. We now understand from pathophysiology of ACS that fluid therapy can be tricky, because aggressive fluid therapy may worsen IAH/ACS due to capillary leak and increasing abdominal organ and parietal wall edema while reducing abdominal perfusion pressure; which can have deleterious effects on multiple organs including reduced cardiac index. Thus, it is obvious to see ACS as a confounder here because it may be associated with both aggressive fluid resuscitation as well as poor outcomes. Moreover ACS also adds to clinical heterogeneity among patients with moderate/severe AP. IAH/ACS also adds to confounding in resuscitation protocols which rely on measurement of central venous pressure (CVP) as a goal (used in goal directed fluid therapy protocols derived from sepsis guidelines) as it has been shown that IAP has a "inverted U shaped" relationship with CVP with peak around 15 mmHg[70,71]. This has important therapeutic implications as CVP decreases beyond this point and same CVP value may be attained in two patients with entirely different fluid requirements due to different IAP. Similarly ACS will predispose to reduced urine output due to direct effect on kidneys and thus protocols which rely on urine output have a potential for confounding by IAP. In general, correction factors with regards to IAP have been proposed for CVP (IAP may lead to falsely high CVP due to abdomino thoracic transmission), and usually patients with higher IAP have lower effective circulatory volumes with higher third space losses[46]. Thus initially they paradoxically need more fluids (with close monitoring of IAP) while later if higher grades of IAP/ACS manifests (due to multiple reasons; possibly also including too much fluids if not judiciously administered previously) then immediate measures to reduce IAP (draining excess third space fluid accumulations, decompressing bowel, improving abdominal wall compliance, *etc.*) are required along with possibly more judicious fluid management protocol[72]. Though crystalloids remain the most common used resuscitation fluid, Zhao *et al*[73] report using combinations of crystalloids and colloids (hydroxyl ethyl starch and glutamine) to result in lower incidence of renal dysfunction, MODS and ACS when compared to resuscitation with normal saline alone. However mortality was not different between groups. It is also imperative to understand that renal dysfunction and attendant fluid imbalance due to direct effects of manifest ACS can't usually be reversed by any modification of fluid management protocol and goals of directed therapy would have to consider management of ACS to remain meaningful. Clearly combined hemodynamic and fluid specific indices are better methods to determine fluid requirements and further research with elimination of confounding and heterogeneity is required

to better answer this conundrum.

Nutrition

Enteric feeding is proposed to prevent bacterial overgrowth and reduce bacterial translocation and is one of the ways shown to reduce mortality among patients with AP[74]. Timing of initiation of enteric feeding is critical and benefits seem to be lost if it's delayed beyond 48 h. Although exact patho-physiologic mechanism is unknown but certain issues are highlighted: (1) enteric feeds may increase gut motility due to osmotic effect and thus reduce ileus which may also prevent overgrowth of bacteria and change in micro flora in stagnant bowel; (2) it may prevent gut atrophy (as intestinal epithelial cells may derive direct nutrition from luminal contents) and thus decrease bacterial translocation; and (3) timing of initiation of nutrition may be important as infectious complications can occur very early in AP and current paradigm proposes gut as a source of culprit bacteria. It is easy to see how IAH/ACS fits into above scheme (promotes gut barrier dysfunction and bacterial translocation) and can contribute as a confounder as it may delay initiation of enteric feeds, as patients with IAH/ACS are usually intolerant to it and it may be inadvisable to force feed patients with feeding intolerance[75]. Moreover ACS may itself have been caused by ileus necessitating bowel drainage. Yet it is attractive to align to concept of low volume constant enteric feeds (in comparison to large volume cyclical feeds) in patients with ileus to improve tolerance, thus retaining benefits of enteric nutrition; a concept also endorsed by the American Society for Parenteral and Enteral Nutrition[76,77]. The same concept also coherently aligns with those with rising IAP where low volume constant enteric feeds may theoretically reduce ileus (by promoting gut motility) and prevent IAH (thus promoting splanchnic flow and further aiding gut function). Thus understanding of IAH/ACS explains why initiating early enteric feeds may not necessarily increase IAP in every case, moreover it can be of benefit to those with IAP less than 15 mmHg (even preventing development of IAH), and it may not be appropriate in few cases with manifest ACS who may be intolerant to food where delicate balance of low volume constant enteric feeds/judicious use of parenteral nutrition (in first three days, and as there is no indication for fasting) with early return to enteric feeds may have a place[75]. Thus future trials on enteric nutrition should account for heterogeneity as well as confounding by IAH/ACS.

Antibiotics

There is conflicting data on prophylactic antibiotic use in AP but meta analyses conclude that there is no evidence of benefit from "routine" use[78]. However sample size of RCTs have been rather small and trends (though non-significant) show improvement in mortality and incidence of infections. Clinical heterogeneity reconciles some differences in non-intuitive results of meta analyses since we now understand that severe pancreatitis as defined in older Atlanta classification was not a homogenous entity and included some patients with milder forms of disease with low risk for infections and mortality. Understanding pathophysiology of IAH/ACS further opens up the debate as gut barrier dysfunction and bacterial translocation are inherently explained, and it further contributes to heterogeneity even by present classification system. Overall it's easier to understand with current paradigms (including concepts of IAH/ACS) why "lack of benefit or presence of harm" of prophylactic antibiotics is yet to be proven, and this is not in variance with understanding that infection of pancreatic necrosis may occur much earlier than when it clinically manifests[79]. Since detection of infected pancreatic necrosis can be difficult, and since manifestations of ACS (with inherent organ failure as pre-ordained by definition) are indistinguishable from severe sepsis (and even septic shock), and since there is evidence of harm from delay in instituting antibiotics; position paper by Mentula *et al*[72] for management of IAH/ACS in AP recommend selective prophylactic antibiotic use in patients with organ dysfunction, IAH and other predictors of severe disease till organ dysfunction resolves and there is no evidence of infection. Since IAH/ACS is associated with increased incidence of infected pancreatic necrosis and mortality independently, it should be considered as a contributor to heterogeneity as well as confounding in future studies, and be appropriately analyzed before recommendations are made[16,17,25].

DISCUSSION

The aim of this review was to generate debate on role of IAP and how it may contribute to lowering internal validity in much of available evidence with potential to further individualize treatments to more homogeneous subgroups of patients with AP (a desired goal of management guidelines). Comprehensive coverage of management strategies of IAH/ACS are beyond the scope of this review but are covered well elsewhere[10]. We do not recommend universal IAP surveillance as there is no evidence of advantage of IAP surveillance in patients with milder forms of disease[19,25,80]. But we believe that sufficient evidence exists to selectively offer surveillance for IAH/ACS in a subgroup of patients with AP. We agree with recommendations of Japanese Society Hepato-Biliary-Pancreatic Surgery to recommend IAP surveillance in patients with excessive fluid infusion, high severity, renal and respiratory complications, and fluid accumulation in multiple areas as observed by CT[5]. The current guidelines by WSACS on diagnosis and management of IAH/ACS are also a reference point and offer a roadmap for practicing clinician[10]. SAP is a disease whose management requires close coordination of gastroenterologists, surgeons, intensivists, nursing staff and allied health professionals. Managing open abdomen due to any cause is indeed no small task; but management of open abdomen following surgical intervention has undergone significant changes in past decade.

With newer concepts of early closure, prevention of loss of abdominal domain and negative pressure wound therapy; data on morbidity and mortality following decompressive laparotomy for ACS may stand further revised[81]. Although we have discussed evidence with relevance to AP; much reliably similar and consistent evidence for role and management for IAH can be gleaned from diverse fields of trauma care, burns, sepsis and pediatrics[65]. Overall there is much to expect from upcoming 10 years where we expect to revisit old conclusions in new light of greater homogeneity and less confounding as ACS in AP is a unique entity and incorporating surveillance for IAP in AP offers potential window to further refine diagnostic groups of patients. In the least we expect that IAP be given its due place in future practice guidelines and that recommendations be formed with help of a broader panel with inclusion of clinicians experienced in management of IAH.

CONCLUSION

IAH can be expected in at least 17% of patients presenting with diagnosis of AP to a typical tertiary care hospital. Clinicians can expect at least 50% patients with SAP to have IAH. ACS can be expected in at least 15% patients with SAP. There is increasing data to show that surveillance and management strategies specific to IAH/ACS increases survival in AP. IAH/ACS is useful as a predictive marker for severe disease and prognostic marker for inferior outcomes. Understanding concepts of IAP may help resolve inconsistencies in available literature for management of AP especially in areas relating to fluid management, antibiotic prophylaxis and nutritional management and as such represents an advance in understanding. Future clinical guidelines on AP must include recommendations for surveillance and management of IAH/ACS.

COMMENTS

Background

Towards the end of last decade clinicians increasingly realized the heterogeneity in broad classification of acute pancreatitis (AP) into mild and severe groups and thus recently classification systems were broadened to include more groups. This was one important factor which lowered internal validity of clinical research as patients with intermediate severity disease confounded results from both mild and severe disease groups. Unsurprisingly meta analyses concluded with equipoise on important issues such as antibiotic prophylaxis or fluid resuscitation. Meanwhile increasing evidence has started accumulating about intra-abdominal pressure (IAP) as an important point of specific intervention in AP with potential to reduce mortality.

Research frontiers

The exact epidemiology of IAP remains to be established in new context of new classification systems of AP, but intra-abdominal hypertension (IAH) can be expected in at least 50% patients with severe AP with 15% expected to qualify for intervention specific for abdominal compartment syndrome (ACS). This poses a potential for further refining even recently proposed classification systems as increasing research points towards ACS as a key factor explaining confounding as well as heterogeneity in disease management related issues. ACS (which should be understood as a continuing spectrum of IAH) contributes actively towards multiple organ dysfunction independent of initiating cause and evidence indicates decreased mortality when it is specifically addressed. It complicates issues related to antibiotic prophylaxis, nutritional and fluid management as such patients require intensive individualized management and routine management guidelines do not address its pathophysiology.

Innovations and breakthroughs

This paper is an up to date summary of available evidence about IAP and AP with specific focus on how understanding pathophysiology of IAP helps coherently explain gaps in existing management related issues of AP and why present evidence in AP concludes with equipoise on important aspects. It unambiguously concludes with broad suggestion to include surveillance for IAP in future management related guidelines for AP.

Applications

ACS in AP is a unique entity and incorporating surveillance for IAP in AP offers potential window to further refine diagnostic groups of patients. Precise answers to clinical questions can be obtained if study populations are homogenous and free from confounding potentially aiding future research to conclude with more unambiguous conclusions.

Terminology

IAP is global pressure inside the abdominal cavity and measured preferably by transvesical method, with maximal instillation of 25 cc saline, in supine position, measured at end expiration, with zeroing done at the level of mid axillary line; IAH - defined by a sustained or repeated pathologic elevation of IAP ⩾ 12 mmHg with grading as follows: Grade Ⅰ: IAP 12-15 mmHg, Grade Ⅱ: IAP 16-20 mmHg, Grade Ⅲ: IAP 21-25 mmHg, Grade Ⅳ: IAP > 25 mmHg; ACS - defined as a sustained IAP > 20 mmHg [with or without an abdominal perfusion pressure (APP) < 60 mmHg] that is associated with new organ dysfunction/failure; APP - mean arterial pressure - IAP.

Peer-review

The authors perform an extensive review on the current literature which they provide as evidence for their conclusion. This is an attractive and relevant paper.

REFERENCES

1 **Yadav D**, Lowenfels AB. The epidemiology of pancreatitis and pancreatic cancer. *Gastroenterology* 2013; **144**: 1252-1261 [PMID: 23622135 DOI: 10.1053/j.gastro.2013.01.068]

2 **Sah RP**, Dawra RK, Saluja AK. New insights into the pathogenesis of pancreatitis. *Curr Opin Gastroenterol* 2013; **29**: 523-530 [PMID: 23892538 DOI: 10.1097/MOG.0b013e328363e399]

3 **Pupelis G**, Austrums E, Snippe K, Berzins M. Clinical significance of increased intraabdominal pressure in severe acute pancreatitis. *Acta Chir Belg* 2002; **102**: 71-74 [PMID: 12051093]

4 **Carter BM**, Howard C. A 6th Vital Sign--Potential Use of Nasogastric Tube for Intra-abdominal Pressure Monitoring Method to Detect Feeding Intolerance in Very Low Birth-Weight Preterm Infants (& lt; 1500 g). *Adv Neonatal Care* 2015; **15**: 176-181 [PMID: 26002859 DOI: 10.1097/ANC.0000000000000175]

5 **Yokoe M**, Takada T, Mayumi T, Yoshida M, Isaji S, Wada K, Itoi T, Sata N, Gabata T, Igarashi H, Kataoka K, Hirota M, Kadoya M, Kitamura N, Kimura Y, Kiriyama S, Shirai K, Hattori T, Takeda K, Takeyama Y, Hirota M, Sekimoto M, Shikata S, Arata S, Hirata K. Japanese guidelines for the management of acute pancreatitis: Japanese Guidelines 2015. *J Hepatobiliary Pancreat Sci* 2015; **22**: 405-432 [PMID: 25973947 DOI: 10.1002/jhbp.259]

6 **Uhl W**, Warshaw A, Imrie C, Bassi C, McKay CJ, Lankisch PG, Carter R, Di Magno E, Banks PA, Whitcomb DC, Dervenis C, Ulrich CD, Satake K, Ghaneh P, Hartwig W, Werner J, McEntee G, Neoptolemos JP, Büchler MW. IAP Guidelines for the Surgical Management of Acute Pancreatitis. *Pancreatology* 2002; **2**: 565-573 [PMID: 12435871 DOI: 10.1159/000067684]

7 **Bradley EL**. A clinically based classification system for acute pancreatitis. Summary of the International Symposium on Acute Pancreatitis, Atlanta, Ga, September 11 through 13, 1992. *Arch Surg*

1993; **128**: 586-590 [PMID: 8489394 DOI: 10.1001/archsurg.1993.01420170122019]
8 **Gecelter G**, Fahoum B, Gardezi S, Schein M. Abdominal compartment syndrome in severe acute pancreatitis: an indication for a decompressing laparotomy? *Dig Surg* 2002; **19**: 402-404; discussion 404-405 [PMID: 12435913]
9 **Kirkpatrick AW**, De Waele JJ, De Laet I, De Keulenaer BL, D'Amours S, Björck M, Balogh ZJ, Leppäniemi A, Kaplan M, Chiaka Ejike J, Reintam Blaser A, Sugrue M, Ivatury RR, Malbrain ML. WSACS--The Abdominal Compartment Society. A Society dedicated to the study of the physiology and pathophysiology of the abdominal compartment and its interactions with all organ systems. *Anaesthesiol Intensive Ther* 2015; **47**: 191-194 [PMID: 25973657 DOI: 10.5603/AIT.a2015.0024]
10 **Kirkpatrick AW**, Roberts DJ, De Waele J, Jaeschke R, Malbrain ML, De Keulenaer B, Duchesne J, Bjorck M, Leppaniemi A, Ejike JC, Sugrue M, Cheatham M, Ivatury R, Ball CG, Reintam Blaser A, Regli A, Balogh ZJ, D'Amours S, Debergh D, Kaplan M, Kimball E, Olvera C. Intra-abdominal hypertension and the abdominal compartment syndrome: updated consensus definitions and clinical practice guidelines from the World Society of the Abdominal Compartment Syndrome. *Intensive Care Med* 2013; **39**: 1190-1206 [PMID: 23673399 DOI: 10.1007/s00134-013-2906-z]
11 **Ke L**, Ni HB, Tong ZH, Li WQ, Li N, Li JS. Intra-abdominal pressure and abdominal perfusion pressure: which is a better marker of severity in patients with severe acute pancreatitis. *J Gastrointest Surg* 2011; **15**: 1426-1432 [PMID: 21557012 DOI: 10.1007/s11605-011-1553-3]
12 **De Waele JJ**, Hoste E, Blot SI, Decruyenaere J, Colardyn F. Intra-abdominal hypertension in patients with severe acute pancreatitis. *Crit Care* 2005; **9**: R452-R457 [PMID: 16137360 DOI: 10.1186/cc3422]
13 **Pupelis G**, Plaudis H, Snippe K, Rudakovska M. Increased intra-abdominal pressure: is it of any consequence in severe acute pancreatitis? *HPB* (Oxford) 2006; **8**: 227-232 [PMID: 18333282 DOI: 10.1080/13651820500540956]
14 **Keskinen P**, Leppaniemi A, Pettila V, Piilonen A, Kemppainen E, Hynninen M. Intra-abdominal pressure in severe acute pancreatitis. *World J Emerg Surg* 2007; **2**: 2 [PMID: 17227591 DOI: 10.1186/1749-7922-2-2]
15 **Zhang WF**, Ni YL, Cai L, Li T, Fang XL, Zhang YT. Intra-abdominal pressure monitoring in predicting outcome of patients with severe acute pancreatitis. *Hepatobiliary Pancreat Dis Int* 2007; **6**: 420-423 [PMID: 17690042]
16 **Rosas JM**, Soto SN, Aracil JS, Cladera PR, Borlan RH, Sanchez AV, Ros FB, Posa LG. Intra-abdominal pressure as a marker of severity in acute pancreatitis. *Surgery* 2007; **141**: 173-178 [PMID: 17263972 DOI: 10.1016/j.surg.2006.04.016]
17 **Chen H**, Li F, Sun JB, Jia JG. Abdominal compartment syndrome in patients with severe acute pancreatitis in early stage. *World J Gastroenterol* 2008; **14**: 3541-3548 [PMID: 18567084 DOI: 10.3748/wjg.14.3541]
18 **Al-Bahrani AZ**, Abid GH, Holt A, McCloy RF, Benson J, Eddleston J, Ammori BJ. Clinical relevance of intra-abdominal hypertension in patients with severe acute pancreatitis. *Pancreas* 2008; **36**: 39-43 [PMID: 18192879 DOI: 10.1097/mpa.0b013e318149f5bf]
19 **Dambrauskas Z**, Parseliunas A, Gulbinas A, Pundzius J, Barauskas G. Early recognition of abdominal compartment syndrome in patients with acute pancreatitis. *World J Gastroenterol* 2009; **15**: 717-721 [PMID: 19222096 DOI: 10.3748/wjg.15.717]
20 **Mentula P**, Hienonen P, Kemppainen E, Puolakkainen P, Leppäniemi A. Surgical decompression for abdominal compartment syndrome in severe acute pancreatitis. *Arch Surg* 2010; **145**: 764-769 [PMID: 20713929 DOI: 10.1001/archsurg.2010.132]
21 **Ke L**, Ni HB, Sun JK, Tong ZH, Li WQ, Li N, Li JS. Risk factors and outcome of intra-abdominal hypertension in patients with severe acute pancreatitis. *World J Surg* 2012; **36**: 171-178 [PMID: 21964817 DOI: 10.1007/s00268-011-1295-0]
22 **Bezmarevic M**, Mirkovic D, Soldatovic I, Stamenkovic D, Mitrovic N, Perisic N, Marjanovic I, Mickovic S, Karanikolas M. Correlation between procalcitonin and intra-abdominal pressure and their role in prediction of the severity of acute pancreatitis. *Pancreatology* 2012; **12**: 337-343 [PMID: 22898635 DOI: 10.1016/j.pan.2012.05.007]
23 **Boone B**, Zureikat A, Hughes SJ, Moser AJ, Yadav D, Zeh HJ, Lee KK. Abdominal compartment syndrome is an early, lethal complication of acute pancreatitis. *Am Surg* 2013; **79**: 601-607 [PMID: 23711270]
24 **Davis PJ**, Eltawil KM, Abu-Wasel B, Walsh MJ, Topp T, Molinari M. Effect of obesity and decompressive laparotomy on mortality in acute pancreatitis requiring intensive care unit admission. *World J Surg* 2013; **37**: 318-332 [PMID: 23052814 DOI: 10.1007/s00268-012-1821-8]
25 **Bhandari V**, Jaipuria J, Singh M, Chawla AS. Intra-abdominal pressure in the early phase of severe acute pancreatitis: canary in a coal mine? Results from a rigorous validation protocol. *Gut Liver* 2013; **7**: 731-738 [PMID: 24312716 DOI: 10.5009/gnl.2013.7.6.731]
26 **Aitken EL**, Gough V, Jones A, Macdonald A. Observational study of intra-abdominal pressure monitoring in acute pancreatitis. *Surgery* 2014; **155**: 910-918 [PMID: 24630146 DOI: 10.1016/j.surg.2013.12.028]
27 **Banks PA**, Bollen TL, Dervenis C, Gooszen HG, Johnson CD, Sarr MG, Tsiotos GG, Vege SS. Classification of acute pancreatitis--2012: revision of the Atlanta classification and definitions by international consensus. *Gut* 2013; **62**: 102-111 [PMID: 23100216 DOI: 10.1136/gutjnl-2012-302779]
28 **Dellinger EP**, Forsmark CE, Layer P, Lévy P, Maraví-Poma E, Petrov MS, Shimosegawa T, Siriwardena AK, Uomo G, Whitcomb DC, Windsor JA. Determinant-based classification of acute pancreatitis severity: an international multidisciplinary consultation. *Ann Surg* 2012; **256**: 875-880 [PMID: 22735715 DOI: 10.1097/SLA.0b013e318256f778]
29 **Marshall JC**, Cook DJ, Christou NV, Bernard GR, Sprung CL, Sibbald WJ. Multiple organ dysfunction score: a reliable descriptor of a complex clinical outcome. *Crit Care Med* 1995; **23**: 1638-1652 [PMID: 7587228 DOI: 10.1097/00003246-199510000-00007]
30 **Trikudanathan G**, Vege SS. Current concepts of the role of abdominal compartment syndrome in acute pancreatitis - an opportunity or merely an epiphenomenon. *Pancreatology* 2014; **14**: 238-243 [PMID: 25062870 DOI: 10.1016/j.pan.2014.06.002]
31 **Mifkovic A**, Skultety J, Sykora P, Prochotsky A, Okolicany R. Intra-abdominal hypertension and acute pancreatitis. *Bratisl Lek Listy* 2013; **114**: 166-171 [PMID: 23406186 DOI: 10.4149/bll_2013_036]
32 **Sah RP**, Saluja A. Molecular mechanisms of pancreatic injury. *Curr Opin Gastroenterol* 2011; **27**: 444-451 [PMID: 21844752 DOI: 10.1097/MOG.0b013e328349e346]
33 **Hegyi P**, Rakonczay Z. The role of pancreatic ducts in the pathogenesis of acute pancreatitis. *Pancreatology* 2015; **15**: S13-S17 [PMID: 25921231 DOI: 10.1016/j.pan.2015.03.010]
34 **Gea-Sorlí S**, Closa D. Role of macrophages in the progression of acute pancreatitis. *World J Gastrointest Pharmacol Ther* 2010; **1**: 107-111 [PMID: 21577304 DOI: 10.4292/wjgpt.v1.i5.107]
35 **Franco-Pons N**, Gea-Sorlí S, Closa D. Release of inflammatory mediators by adipose tissue during acute pancreatitis. *J Pathol* 2010; **221**: 175-182 [PMID: 20217859 DOI: 10.1002/path.2691]
36 **Yashima Y**, Isayama H, Tsujino T, Nagano R, Yamamoto K, Mizuno S, Yagioka H, Kawakubo K, Sasaki T, Kogure H, Nakai Y, Hirano K, Sasahira N, Tada M, Kawabe T, Koike K, Omata M. A large volume of visceral adipose tissue leads to severe acute pancreatitis. *J Gastroenterol* 2011; **46**: 1213-1218 [PMID: 21805069 DOI: 10.1007/s00535-011-0430-x]
37 **Vigna SR**, Shahid RA, Liddle RA. Ethanol contributes to neurogenic pancreatitis by activation of TRPV1. *FASEB J* 2014; **28**: 891-896 [PMID: 24221085 DOI: 10.1096/fj.13-236208]
38 **Mayerle J**, Dummer A, Sendler M, Malla SR, van den Brandt C, Teller S, Aghdassi A, Nitsche C, Lerch MM. Differential roles of inflammatory cells in pancreatitis. *J Gastroenterol Hepatol* 2012; **27** Suppl 2: 47-51 [PMID: 22320916 DOI: 10.1111/j.1440-1746.2011.07011.x]
39 **Aggarwal A**, Manrai M, Kochhar R. Fluid resuscitation in acute

pancreatitis. *World J Gastroenterol* 2014; **20**: 18092-18103 [PMID: 25561779 DOI: 10.3748/wjg.v20.i48.18092]
40 **Oláh A**, Romics L. Enteral nutrition in acute pancreatitis: a review of the current evidence. *World J Gastroenterol* 2014; **20**: 16123-16131 [PMID: 25473164 DOI: 10.3748/wjg.v20.i43.16123]
41 **Wu LM**, Sankaran SJ, Plank LD, Windsor JA, Petrov MS. Meta-analysis of gut barrier dysfunction in patients with acute pancreatitis. *Br J Surg* 2014; **101**: 1644-1656 [PMID: 25334028 DOI: 10.1002/bjs.9665]
42 **Bodnár** Z. Intra-Abdominal Hypertension and Abdominal Compartment Syndrome in Critically Ill Surgical Patients (Special Findings in Severe Acute Pancreatitis). InTech. Available from: URL: http://www.researchgate.net/publication221927008_Intrbdominal_Hypertension_and_Abdominal_Compartment_Syndrome_in_Critically_Ill_Surgical_Patients_(Special_Findings_in_Severe_Acute_Pancreatitis)
43 **Liu H**, Li W, Wang X, Li J, Yu W. Early gut mucosal dysfunction in patients with acute pancreatitis. *Pancreas* 2008; **36**: 192-196 [PMID: 18376312 DOI: 10.1097/MPA.0b013e31815a399f]
44 **Capurso G**, Zerboni G, Signoretti M, Valente R, Stigliano S, Piciucchi M, Delle Fave G. Role of the gut barrier in acute pancreatitis. *J Clin Gastroenterol* 2012; **46** Suppl: S46-S51 [PMID: 22955357 DOI: 10.1097/MCG.0b013e3182652096]
45 **Li WD**, Jia L, Ou Y, Huang YX, Jiang SM. Surveillance of intra-abdominal pressure and intestinal barrier function in a rat model of acute necrotizing pancreatitis and its potential early therapeutic window. *PLoS One* 2013; **8**: e78975 [PMID: 24244397 DOI: 10.1371/journal.pone.0078975]
46 **Wauters J**, Wilmer A, Valenza F. Abdomino-thoracic transmission during ACS: facts and figures. *Acta Clin Belg Suppl* 2007; **(1)**: 200-205 [PMID: 17469720 DOI: 10.1179/acb.2007.62.s1.026]
47 **De laet I**, Malbrain ML, Jadoul JL, Rogiers P, Sugrue M. Renal implications of increased intra-abdominal pressure: are the kidneys the canary for abdominal hypertension? *Acta Clin Belg Suppl* 2007; **(1)**: 119-130 [PMID: 17469709]
48 **Ozmen MM**, Zulfikaroglu B, Col C, Cinel I, Isman FK, Cinel L, Besler TH. Effect of increased abdominal pressure on cytokines (IL1 beta, IL6, TNFalpha), C-reactive protein (CRP), free radicals (NO, MDA), and histology. *Surg Laparosc Endosc Percutan Tech* 2009; **19**: 142-147 [PMID: 19390282 DOI: 10.1097/SLE.0b013e31819cdda7]
49 **Bodnár Z**, Keresztes T, Kovács I, Hajdu Z, Boissonneault GA, Sipka S. Increased serum adenosine and interleukin 10 levels as new laboratory markers of increased intra-abdominal pressure. *Langenbecks Arch Surg* 2010; **395**: 969-972 [PMID: 20013289 DOI: 10.1007/s00423-009-0583-8]
50 **Curley PJ**, McMahon MJ, Lancaster F, Banks RE, Barclay GR, Shefta J, Boylston AW, Whicher JT. Reduction in circulating levels of CD4-positive lymphocytes in acute pancreatitis: relationship to endotoxin, interleukin 6 and disease severity. *Br J Surg* 1993; **80**: 1312-1315 [PMID: 7902182 DOI: 10.1002/bjs.1800801031]
51 **Pietruczuk M**, Dabrowska MI, Wereszczynska-Siemiatkowska U, Dabrowski A. Alteration of peripheral blood lymphocyte subsets in acute pancreatitis. *World J Gastroenterol* 2006; **12**: 5344-5351 [PMID: 16981265 DOI: 10.3748/wjg.v12.i33.5344]
52 **Liu Y**, Wang L, Cai Z, Zhao P, Peng C, Zhao L, Wan C. The Decrease of Peripheral Blood CD4+ T Cells Indicates Abdominal Compartment Syndrome in Severe Acute Pancreatitis. *PLoS One* 2015; **10**: e0135768 [PMID: 26287969 DOI: 10.1371/journal.pone.0135768]
53 **Leppäniemi A**, Johansson K, De Waele JJ. Abdominal compartment syndrome and acute pancreatitis. *Acta Clin Belg* 2007; **62** Suppl 1: 131-135 [PMID: 24881709 DOI: 10.1179/acb.2007.62.s1.016]
54 **Miranda CJ**. Intra-abdominal pressure and abdominal perfusion pressure early in severe acute pancreatitis misses the forest for the trees. *J Gastrointest Surg* 2011; **15**: 2300; author reply 2301 [PMID: 21948182 DOI: 10.1007/s11605-011-1701-9]
55 **Tenner S**, Baillie J, DeWitt J, Vege SS. American College of Gastroenterology guideline: management of acute pancreatitis. *Am J Gastroenterol* 2013; **108**: 1400-1415; 1416 [PMID: 23896955 DOI: 10.1038/ajg.2013.218]
56 **Working Group IAP/APA Acute Pancreatitis Guidelines.** IAP/APA evidence-based guidelines for the management of acute pancreatitis. *Pancreatology* 2013; **13**: e1-e15 [PMID: 24054878 DOI: 10.1016/j.pan.2013.07.063]
57 **Wise R,** Roberts DJ, Vandervelden S, Debergh D, De Waele JJ, De Laet I, Kirkpatrick AW, De Keulenaer BL, Malbrain ML. Awareness and knowledge of intra-abdominal hypertension and abdominal compartment syndrome: results of an international survey. *Anaesthesiol Intensive Ther* 2014 Oct 27; Epub ahead of print [PMID: 25251947]
58 **Tao J**, Wang C, Chen L, Yang Z, Xu Y, Xiong J, Zhou F. Diagnosis and management of severe acute pancreatitis complicated with abdominal compartment syndrome. *J Huazhong Univ Sci Technolog Med Sci* 2003; **23**: 399-402 [PMID: 15015646 DOI: 10.1007/BF02829428]
59 **Leppäniemi A**, Hienonen P, Mentula P, Kemppainen E. Subcutaneous linea alba fasciotomy, does it really work? *Am Surg* 2011; **77**: 99-102 [PMID: 21396315]
60 **Jacob AO,** Stewart P, Jacob O. Early surgical intervention in severe acute pancreatitis: Central Australian experience. *ANZ J Surg* 2014 May 30; Epub ahead of print [PMID: 24890051 DOI: 10.1111/ans.12707]
61 **van Brunschot S**, Schut AJ, Bouwense SA, Besselink MG, Bakker OJ, van Goor H, Hofker S, Gooszen HG, Boermeester MA, van Santvoort HC. Abdominal compartment syndrome in acute pancreatitis: a systematic review. *Pancreas* 2014; **43**: 665-674 [PMID: 24921201 DOI: 10.1097/MPA.0000000000000108]
62 **Ke L**, Ni HB, Tong ZH, Li WQ, Li N, Li JS. The importance of timing of decompression in severe acute pancreatitis combined with abdominal compartment syndrome. *J Trauma Acute Care Surg* 2013; **74**: 1060-1066 [PMID: 23511145 DOI: 10.1097/TA.0b013e318283d927]
63 **Cheatham ML**, Safcsak K. Is the evolving management of intra-abdominal hypertension and abdominal compartment syndrome improving survival? *Crit Care Med* 2010; **38**: 402-407 [PMID: 20095067 DOI: 10.1097/CCM.0b013e3181b9e9b1]
64 **De Waele JJ.** Abdominal compartment syndrome in severe acute pancreatitis - When to decompress? *Eur J Trauma Emerg Surg* 2008; **34**: 11-16 [DOI: 10.1007/s00068-008-7170-5]
65 **De Waele JJ**, Ejike JC, Leppäniemi A, De Keulenaer BL, De Laet I, Kirkpatrick AW, Roberts DJ, Kimball E, Ivatury R, Malbrain ML. Intra-abdominal hypertension and abdominal compartment syndrome in pancreatitis, paediatrics, and trauma. *Anaesthesiol Intensive Ther* 2015; **47**: 219-227 [PMID: 25973660 DOI: 10.5603/AIT.a2015.0027]
66 **Kerner T**, Vollmar B, Menger MD, Waldner H, Messmer K. Determinants of pancreatic microcirculation in acute pancreatitis in rats. *J Surg Res* 1996; **62**: 165-171 [PMID: 8632634 DOI: 10.1006/jsre.1996.0190]
67 **Mao EQ**, Fei J, Peng YB, Huang J, Tang YQ, Zhang SD. Rapid hemodilution is associated with increased sepsis and mortality among patients with severe acute pancreatitis. *Chin Med J* (Engl) 2010; **123**: 1639-1644 [PMID: 20819621]
68 **Mao EQ**, Tang YQ, Fei J, Qin S, Wu J, Li L, Min D, Zhang SD. Fluid therapy for severe acute pancreatitis in acute response stage. *Chin Med J* (Engl) 2009; **122**: 169-173 [PMID: 19187641]
69 **Haydock MD**, Mittal A, Wilms HR, Phillips A, Petrov MS, Windsor JA. Fluid therapy in acute pancreatitis: anybody's guess. *Ann Surg* 2013; **257**: 182-188 [PMID: 23207241 DOI: 10.1097/SLA.0b013e31827773ff]
70 **Warndorf MG**, Kurtzman JT, Bartel MJ, Cox M, Mackenzie T, Robinson S, Burchard PR, Gordon SR, Gardner TB. Early fluid resuscitation reduces morbidity among patients with acute pancreatitis. *Clin Gastroenterol Hepatol* 2011; **9**: 705-709 [PMID: 21554987 DOI: 10.1016/j.cgh.2011.03.032]
71 **Yang C**, Yang Z, Chen X, Liu T, Gou S, Chen C, Xiao J, Jin X, He Z, Dong L, Zhang Y, Luo N, Wu H, Wang C. Inverted U-Shaped Relationship between Central Venous Pressure and Intra-Abdominal

Pressure in the Early Phase of Severe Acute Pancreatitis: A Retrospective Study. *PLoS One* 2015; **10**: e0128493 [PMID: 26053865 DOI: 10.1371/journal.pone.0128493]
72 **Mentula P,** Leppäniemi A. Position paper: timely interventions in severe acute pancreatitis are crucial for survival. *World J Emerg Surg* 2014; **9**: 15 [PMID: 24512891 DOI: 10.1186/1749-7922-9-15]
73 **Zhao G**, Zhang JG, Wu HS, Tao J, Qin Q, Deng SC, Liu Y, Liu L, Wang B, Tian K, Li X, Zhu S, Wang CY. Effects of different resuscitation fluid on severe acute pancreatitis. *World J Gastroenterol* 2013; **19**: 2044-2052 [PMID: 23599623 DOI: 10.3748/wjg.v19.i13.2044]
74 **Petrov MS**, Pylypchuk RD, Uchugina AF. A systematic review on the timing of artificial nutrition in acute pancreatitis. *Br J Nutr* 2009; **101**: 787-793 [PMID: 19017421 DOI: 10.1017/s0007114508123443]
75 **Sun JK**, Li WQ, Ke L, Tong ZH, Ni HB, Li G, Zhang LY, Nie Y, Wang XY, Ye XH, Li N, Li JS. Early enteral nutrition prevents intra-abdominal hypertension and reduces the severity of severe acute pancreatitis compared with delayed enteral nutrition: a prospective pilot study. *World J Surg* 2013; **37**: 2053-2060 [PMID: 23674254 DOI: 10.1007/s00268-013-2087-5]
76 **Oláh A**, Romics L. Evidence-based use of enteral nutrition in acute pancreatitis. *Langenbecks Arch Surg* 2010; **395**: 309-316 [PMID: 20309576 DOI: 10.1007/s00423-010-0631-4]
77 **Mirtallo JM**, Forbes A, McClave SA, Jensen GL, Waitzberg DL, Davies AR. International consensus guidelines for nutrition therapy in pancreatitis. *JPEN J Parenter Enteral Nutr* 2012; **36**: 284-291 [PMID: 22457421 DOI: 10.1177/0148607112440823]
78 **Wittau M**, Mayer B, Scheele J, Henne-Bruns D, Dellinger EP, Isenmann R. Systematic review and meta-analysis of antibiotic prophylaxis in severe acute pancreatitis. *Scand J Gastroenterol* 2011; **46**: 261-270 [PMID: 21067283 DOI: 10.3109/00365521.2010.531486]
79 **Gerzof SG**, Banks PA, Robbins AH, Johnson WC, Spechler SJ, Wetzner SM, Snider JM, Langevin RE, Jay ME. Early diagnosis of pancreatic infection by computed tomography-guided aspiration. *Gastroenterology* 1987; **93**: 1315-1320 [PMID: 3678750]
80 **Blaser AR**, Sarapuu S, Tamme K, Starkopf J. Expanded measurements of intra-abdominal pressure do not increase the detection rate of intra-abdominal hypertension: a single-center observational study. *Crit Care Med* 2014; **42**: 378-386 [PMID: 24145841 DOI: 10.1097/CCM.0b013e3182a6459b]
81 **De Waele JJ**, Kaplan M, Sugrue M, Sibaja P, Björck M. How to deal with an open abdomen? *Anaesthesiol Intensive Ther* 2015; **47**: 372-378 [PMID: 25973658 DOI: 10.5603/AIT.a2015.0023]

P- Reviewer: Camara-Lemarroy CR, Cosen-Binker L, Li SD
S- Editor: Tian YL **L- Editor**: A **E- Editor**: Jiao XK

Published by **Baishideng Publishing Group Inc**
8226 Regency Drive, Pleasanton, CA 94588, USA
Telephone: +1-925-223-8242
Fax: +1-925-223-8243
E-mail: bpgoffice@wjgnet.com
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
http://www.wjgnet.com

ISSN 2150-5330 (online)

World Journal of *Gastrointestinal Pathophysiology*

World J Gastrointest Pathophysiol 2016 May 15; 7(2): 199-241

Published by Baishideng Publishing Group Inc

WJGP World Journal of Gastrointestinal Pathophysiology

A peer-reviewed, online, open-access journal of gastrointestinal pathophysiology

Editorial Board

2016-2019

The *World Journal of Gastrointestinal Pathophysiology* Editorial Board consists of 503 members, representing a team of worldwide experts in gastrointestinal pathophysiology. They are from 45 countries, including Argentina (2), Australia (13), Austria (2), Belgium (7), Brazil (9), Brunei Darussalam (1), Canada (21), China (30), Croatia (1), Czech Repoublic (2), Denmark (3), Egypt (1), Estonia (1), Finland (1), France (8), Germany (24), Greece (7), Hungary (5), India (12), Indonesia (1), Iran (2), Ireland (2), Israel (8), Italy (38), Japan (45), Lebanon (3), Malaysia (1), Mexico (2), Netherlands (7), Norway (1), Poland (4), Portugal (1), Romania (1), Russia (1), Singapore (4), South Korea (12), Spain (24), Sweden (11), Switzerland (3), Thailand (2), Turkey (7), Ukraine (1), United Kingdom (10), United States (161) and Venezuela (1).

Zhan-Ju Liu, *Shanghai*
Shi Liu, *Wuhan*
Zhen-Ning Wang, *Shenyang*
Xiao-Hong Wang, *Beijing*
Wei Wei, *Hefei*
Dong-Ping Xie, *Shanghai*
Wen-Xie Xu, *Shanghai*
Xiao Yang, *Beijing*
Hua Yang, *Chongqing*
Wei-Zhen Zhang, *Beijing*
Hua-Chuan Zheng, *Shenyang*
Min-Sheng Zhu, *Nanjing*
Jinxia Zhu, *Beijing*
Da-Ling Zhu, *Harbin*
Yong-Liang Zhu, *Hangzhou*

Croatia

Alen Protic, *Rijeka*

Czech Republic

Pavel Hladik, *Semily*
Martin Vokurka, *Prague*

Denmark

Lars Arendt-Nielsen, *Aalborg*
Frank V Schiodt, *Copenhagen*
Jonas Worsoe, *Aarhus*

Egypt

Mahmoud Aboelneen Khattab, *Minia*

Estonia

Enn Seppet, *Tartu*

Finland

Pauli Antero Puolakkainen, *Turku*

France

Bruno Bonaz, *Grenoble*
Pierre M Dechelotte, *Rouen*
Jean-Paul Lallès, *Saint-Gilles*
Charles-Henri Malbert, *Saint-Gilles*
Thierry Piche, *Nice*
Pascale Plaisancié, *Lyon*
Michelina Plateroti, *Lyon*
Véronique Vitton, *Marseille*

Germany

Hans G Beger, *Ulm*
Carsten Bergmann, *Ingelheim*
Elke Cario, *Essen*
Arno J Dormann, *Koln*
Nikolaus Gassler, *Aachen*
Andreas Geier, *Wuerzburg*
Werner Hartwig, *Heidelberg*
Marion Hewicker-Trautwein, *Hannover*
Jens Hoeppner, *Freiburg*
Tobias Keck, *Freiburg*
Jorg Kleeff, *Munich*
Peter Malfertheiner, *Magdeburg*
Oliver Mann, *Hamburg*
Christoph W Michalski, *Munich*
Andreas K Nussler, *Munich*
Christian Pehl, *Vilsbiburg*
Peter Schemmer, *Heidelberg*
Manuel A Silva, *Penzberg*
Marc Stemmler, *Freiburg*
Frank Tacke, *Aachen*
Sya N Ukena, *Braunschweig*
Brigitte Vollmar, *Rostock*
Thomas M Wex, *Magdeburg*
Margot Zoller, *Heidelberg*

Greece

Stelios F Assimakopoulos, *Patras*
George N Dalekos, *Larissa*
Alkiviadis Efthymiou, *thessaloniki*
Maria Gazouli, *Athens*
Ioannis E Koutroubakis, *Heraklion*
Gerassimos J Mantzaris, *Athens*
George V Papatheodoridis, *Athens*

Hungary

Mária Bagyánszki, *Szeged*
Mihály Boros, *Szeged*
Laszlo Czako, *Szeged*
Pal Miheller, *Budapest*
Zoltan Rakonczay, *Szeged*

India

Anil K Agarwal, *Delhi*
Uday Bandyopadhyay, *Kolkata*
Sriparna Basu, *Varanasi*
Chandra K Chakraborti, *Rourkela*
Nilesh M Dagia, *Mumbai*
Rajeev Garg, *Punjab*
Akhtar Mahmood, *Chandigarh*
Veerareddy P Reddy, *Andhra Pradesh*
Chandra P Sharma, *Thiruvananthapuram*
Shailesh V Shrikhande, *Mumbai*
Virendra Singh, *Chandigarh*
Nicholas J Skill, *Indianapolis*

Indonesia

Laurentius A Lesmana, *Jakarta*

Iran

Gholamreza Roshandel, *Gorgan*
Shahram Shahabi, *Urmia*

Ireland

Billy Bourke, *Dublin*
Stephen J Keely, *Dublin*

Israel

Yosefa Avraham, *Jerusalem*
Yaron Bar-Dayan, *Holon*
Shomron Ben-Horin, *Tel-Hashomer*
Boris Kirshtein, *Beer Sheva*
Stephen Malnick, *Rehovot*
Yaakov Maor, *Tel-Hashomer*
Rifaat Safadi, *Jerusalem*
Nachum Vaisman, *Tel Aviv*

Italy

Rosaria Acquaviva, *Catania*
Alessandro Antonelli, *Pisa*
Giacosa Attilio, *Genova*
Salavtore Auricchio, *Naples*
Guido Basilisco, *Milan*
Antonio Basoli, *Rome*
Massimo Bellini, *Pisa*
Luigi Bonavina, *Milano*
Alfio Brogna, *Catania*
Giuseppe Calamita, *Bari*
Raffaele Capasso, *Naples*
Ignazio Castagliuolo, *Padova*
Francesco Cresi, *Torino*
Rosario Cuomo, *Napoli*
Salvatore Cuzzocrea, *Gazzi*
Mario M D'Elios, *Florence*
Cinzia Domeneghini, *Milan*
Luca Elli, *Milano*
Walter Fries, *Messina*
Eugenio Gaudio, *Rome*
Marco Gobbetti, *Bari*
Fabio Grizzi, *Rozzano*
Enzo Grossi, *Milanese*
Enzo Ierardi, *Foggia*
Pietro Invernizzi, *Monza*
Angelo A Izzo, *Naples*
Anna Kohn, *Rome*
Giovanni Latella, *L'Aquila*
Massimo Marignani, *Rome*
Sergio Morini, *Rome*
Raffaele Pezzilli, *Bologna*
Cristiano Rumio, *Milan*
Giovanni Sarnelli, *Naples*
Edoardo V Savarino, *Padua*
Pierpaolo Sileri, *Roma*
Annamaria Staiano, *Naples*
Giacomo C Sturniolo, *Padua*
Claudio Tiribelli, *Trieste*

Japan

Hirotada Akiho, *Fukuoka*
Akihiro Asakawa, *Kagoshima*
Hisashi Aso, *Sendai*
Yasu-Taka Azuma, *Osaka*
Shotaro Enomoto, *Wakayama*
Mikihiro Fujiya, *Hokkaido*
Takahisa Furuta, *Hamamatsu*
Akira Hokama, *Nishihara*
Ryota Hokari, *Saitama*
Yuichi Hori, *Hyogo*
Masahiro Iizuka, *Akita*
Shoji Ikuo, *Kobe*
Motohiro Imano, *Osaka*

Hajime Isomoto, *Nagasaki*
Tatehiro Kagawa, *Isehara*
Haruki Kitazawa, *Sendai*
Shigeru BH Ko, *Tokyo*
Xiao-Kang Li, *Tokyo*
Noriaki Manabe, *Okayama*
Atsushi Masamune, *Sendai*
Hiroyuki Matsubayashi, *Suntogun*
Kazuyuki Matsushita, *Inohana Chuo-ku*
Reiko Miyazawa, *Gunma*
Kazunari Murakami, *Oita*
Hikaru Nagahara, *Tokyo*
Yuji Naito, *Kyoto*
Atsushi Nakajima, *Yokohama*
Shoji Natsugoe, *Kagoshima City*
Tsutomu Nishida, *Suita*
Koji Nomoto, *Tokyo*
Shouji Shimoyama, *Tokyo*
Goshi Shiota, *Yonago*
Hidekazu Suzuki, *Tokyo*
Hitoshi Takagi, *Gunma*
Toru Takahashi, *Okayama*
Yoshihisa Takahashi, *Tokyo*
Kan Uchiyama, *Chiba*
Yoshiyuki Ueno, *Sendai*
Takato Ueno, *Fukuoka*
Hisayuki Uneyama, *Kwasaki*
Mitsunori Yamakawa, *Yamagata*
Takayuki Yamamoto, *Mie*
Yutaka Yata, *Maebashi-city*
Naohisa Yoshida, *Kyoto*
Hitoshi Yoshiji, *Kashihara*

Lebanon

Costantine F Daher, *Byblos*
Assaad M Soweid, *Beirut*
Julnar Usta, *Beirut*

Malaysia

Andrew CS Boon, *Ipoh*

Mexico

José M de la Roca-Chiapas, *Leon*
Maria-Raquel Huerta-Franco, *Leon*

Netherland

Wouter J de Jonge, *Amsterdam*
Aldo Grefhorst, *Groningen*
Daniel Keszthelyi, *Maastricht*
Cornelis FM Sier, *ZA Leiden*
Pieter J Tanis, *AZ Amsterdam*
Luc JW van der Laan, *Rotterdam*
Sander van der Marel, *Leiden*

Norway

Anne M Bakke, *Oslo*

Poland

Stanistaw A Hac, *Gdansk*
Stanislaw J Kowalski, *Krakow*
Agata Mulak, *Wroclaw*
Napoleon Waszkiewicz, *Choroszcz*

Portugal

Ricardo Marcos, *Porto*

Romania

Mihai Ciocirlan, *Bucharest*

Russia

Ludmila Filaretova, *Petersburg*

Singapore

Madhav Bhatia, *Singapore*
Brian K Goh, *Singapore*
Khek-Yu Ho, *Singapore*
Cliff KS Ong, *Singapore*

South Korea

Jae Hee Cheon, *Seoul*
Myung-Haing Cho, *Seoul*
Ki-Baik Hahm, *Seongnam*
HO Jae Han, *Gwangju*
Chang-Duk Jun, *Gwangju*
Sang Geon Kim, *Seoul*
Hong Joo Kim, *Seoul*
Jin-Kyung Kim, *Gyeongsan-Si*
Won-Jae Lee, *Seoul*
Sung-Joo Park, *Iksan*
Seung Ha Park, *Gangwon Do*
Kwan-Kyu Park, *Daegu*

Spain

Raquel Abalo, *Madrid*
Juan G Abraldes, *Barcelona*
Dario Acuna-Castroviejo, *Armilla*
Agustin Albillos, *Madrid*
Maria-Angeles Aller, *Madrid*
Fernando Azpiroz, *Barcelona*
Ramón Bataller, *Barcelona*
Marco Bustamante-Balén, *Valencia*
Andres Cardenas, *Barcelona*
Joan Claria, *Barcelona*
Pere Clavé, *Barcelona*
Manuel B de Acosta, *Santiago*
Manuel Giner, *Madrid*
Borja Hernandez-Breijo, *Madrid*
Angel I Lanas, *Zaragoza*
Maite T Martin, *Barcelona*
Vicente Martinez, *Bellaterra*
Jose M Mates, *Malaga*
Julio Mayol, *Madrid*
Marcal Pastor-Anglada, *Barcelona*
María E Sáez, *Seville*
Yolanda Sanz, *Burjassot*
Carlos Taxonera, *Madrid*
Maria D Yago, *Granada*

Sweden

Marco Del Chiaro, *Stockholm*
Frida Fak, *Gothenburg*
Gunnar FA Flemstrom, *Uppsala*
Evangelos Kalaitzakis, *Gothenburg*
Kristina Lamas, *Umea*
Bob R Olsson, *Goteborg*
Sara M Regnér, *Malmo*
Peter T Schmidt, *Stockholm*
Xiao-Feng Sun, *Linkoping*
Henrik Thorlacius, *Malmo*
Curt Tysk, *Orebro*

Switzerland

Jyrki J Eloranta, *Zurich*
Remy Meier, *Liestal*
Catherine M Pastor, *Geneva*

Thailand

Thawatchai Akaraviputh, *Bangkok*
Weekitt Kittisupamongkol, *Bangkok*

Turkey

Mehmet Bektas, *Ankara*
Ugur Duman, *Bursa*
Mukaddes Esrefoglu, *Istanbul*
Ahmet Guven, *Ankara*
Muammer Karadeniz, *Bornova-Izmir*
Elvan Ozbek, *Sakarya*
Ilhami Yuksel, *Ankara*

Ukraine

Oksana S Zayavhkivska, *Lviv*

United Kingdom

Geoffrey Burnstock, *London*
Janice E Drew, *Aberdeen*
Girish L Gupte, *Birmingham*
David C Hay, *Edinburgh*
Nusrat Husain, *Manchester*
Michael L Lucas, *Glasgow*
Jamie Murphy, *London*
Vadim Sumbayev, *Chatham Maritime*
Wing-Kin Syn, *Birmingham*
Andrea Varro, *Liverpool*

United States

Sami R Achem, *Florida*
Tauseef Ali, *Oklahoma City*
Shrikant Anant, *Oklahoma*
Mohammed S Anwer, *North Grafton*
Andrew Aronsohn, *Chicago*
Toms Augustin, *Sayre*

WJGP

World Journal of Gastrointestinal Pathophysiology

Contents

Quarterly Volume 7 Number 2 May 15, 2016

REVIEW

MINIREVIEWS

ORIGINAL ARTICLE

Basic Study

World Journal of Gastrointestinal Pathophysiology
Volume 7 Number 2 May 15, 2016

Contents

NAME OF JOURNAL
World Journal of Gastrointestinal Pathophysiology

ISSN
ISSN 2150-5330 (online)

LAUNCH DATE
April 15, 2010

Frequency
Quarterly

Room 903, Building D, Ocean International Center,
No. 62 Dongsihuan Zhonglu, Chaoyang District,
Beijing 100025, China
Telephone: +86-10-85381891
Fax: +86-10-85381893
E-mail: editorialoffice@wjgnet.com
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
http://www.wjgnet.com

PUBLISHER
Baishideng Publishing Group Inc
8226 Regency Drive,
Pleasanton, CA 94588, USA
Telephone: +1-925-223-8242
Fax: +1-925-223-8243
E-mail: bpgoffice@wjgnet.com
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
http://www.wjgnet.com

PUBLICATION DATE
May 15, 2016

World Journal of Gastrointestinal Pathophysiology

Submit a Manuscript: http://www.wjgnet.com/esps/
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.4291/wjgp.v7.i2.199

World J Gastrointest Pathophysiol 2016 May 15; 7(2): 199-210
ISSN 2150-5330 (online)

REVIEW

Differential role of Hedgehog signaling in human pancreatic (patho-) physiology: An up to date review

Eckhard Klieser, Stefan Swierczynski, Christian Mayr, Tarkan Jäger, Johanna Schmidt, Daniel Neureiter, Tobias Kiesslich, Romana Illig

Eckhard Klieser, Johanna Schmidt, Daniel Neureiter, Romana Illig, Institute of Pathology, Paracelsus Medical University, Salzburger Landeskliniken (SALK), 5020 Salzburg, Austria

Stefan Swierczynski, Tarkan Jäger, Department of Surgery, Paracelsus Medical University, Salzburger Landeskliniken (SALK), 5020 Salzburg, Austria

Christian Mayr, Tobias Kiesslich, Department of Internal Medicine I, Paracelsus Medical University, Salzburger Landeskliniken (SALK), 5020 Salzburg, Austria

Christian Mayr, Tobias Kiesslich, Laboratory for Tumour Biology and Experimental Therapies (TREAT), Institute of Physiology and Pathophysiology, Paracelsus Medical University, 5020 Salzburg, Austria

Author contributions: Mayr C and Kiesslich T designed the figures; Neureiter D and Illig R outlined and designed the manuscript; all authors contributed individual chapters of the manuscript.

Conflict-of-interest statement: The authors declared no conflict of interest.

Correspondence to: Daniel Neureiter, MA, Associate Professor, Institute of Pathology, Paracelsus Medical University, Salzburger Landeskliniken (SALK), Müllner Haupstrasse 48, 5020 Salzburg, Austria. d.neureiter@salk.at
Telephone: +43-662-44824701

Received: July 27, 2015
Peer-review started: July 30, 2015
First decision: September 28, 2015
Revised: October 21, 2015
Accepted: March 7, 2016
Article in press: March 9, 2016
Published online: May 15, 2016

Abstract

Since the discovery of the Hedgehog (Hh) pathway in drosophila melanogaster, our knowledge of the role of Hh in embryonic development, inflammation, and cancerogenesis in humans has dramatically increased over the last decades. This is the case especially concerning the pancreas, however, real therapeutic breakthroughs are missing until now. In general, Hh signaling is essential for pancreatic organogenesis, development, and tissue maturation. In the case of acute pancreatitis, Hh has a protective role, whereas in chronic pancreatitis, Hh interacts with pancreatic stellate cells, leading to destructive parenchym fibrosis and atrophy, as well as to irregular tissue remodeling with potency of initiating cancerogenesis. *In vitro* and *in situ* analysis of Hh in pancreatic cancer revealed that the Hh pathway participates in the development of pancreatic precursor lesions and ductal adenocarcinoma including critical interactions with the tumor microenvironment. The application of specific inhibitors of components of the Hh pathway is currently subject of ongoing clinical trials (phases 1 and 2). Furthermore, a combination of Hh pathway inhibitors and established chemotherapeutic drugs could also represent a promising therapeutic approach. In this review, we give a structured survey of the role of the Hh pathway in pancreatic development, pancreatitis, pancreatic carcinogenesis and pancreatic cancer as well as an overview of current clinical trials concerning Hh pathway inhibitors and pancreas cancer.

Key words: Pancreatic cancer; Hedgehog; Pancreatitis; Pancreas; Development

Core tip: The Hedgehog (Hh) pathway is a ligand-dependent and evolutionary conserved cellular signaling mechanism with various physiologic (development) and pathogenetic functions (especially carcinogenesis). Concerted Hh signaling is essential for human pancreatic development and homeostasis of the gastrointestinal tract. Aberrant expression within the Hh signaling pathway results in malformations like annular pancreas. The Janus aspect of Hh in pancreatitis is reflected by the protective role of Hh in acute pancreatitis *vs* the disease-progressive function of Hh in chronic pancreatitis (CP), whereby CP is linked to pancreatic cancerogenesis *via* pancreatic intraepithelial neoplasia (PanIn). Starting with PanIn and ending up at metastatic disease, Hh pathway is expressed in ductal pancreatic cancer thereby influencing and being paracrine influenced by the tumor microenvironment.

Klieser E, Swierczynski S, Mayr C, Jäger T, Schmidt J, Neureiter D, Kiesslich T, Illig R. Differential role of Hedgehog signaling in human pancreatic (patho-) physiology: An up to date review. *World J Gastrointest Pathophysiol* 2016; 7(2): 199-210 Available from: URL: http://www.wjgnet.com/2150-5330/full/v7/i2/199.htm DOI: http://dx.doi.org/10.4291/wjgp.v7.i2.199

INTRODUCTION

Hedgehog (*Hh*) genes were originally identified in drosophila melanogaster as regulators of body patterning during embryonic development[1]. Today it is known that the Hh pathway plays a central role in diverse biological processes in mammals, such as embryonic development, cell proliferation, differentiation, tissue repair and maintenance of stem cell status in the adult[2].

In general, activation of the Hh pathway relies on the binding of a secreted ligand to its receptor. Three ligand homologues are known in mammals: Desert hedgehog (*Dhh*), Indian hedgehog (*Ihh*) and Sonic hedgehog (*Shh*). The ligands are produced as precursors and are secreted after extensive modifications to bind to their membrane bound receptor, called Patched. In mammals, two homologues exist, Patched1 (Ptch1) and Patched2 (Ptch2). After signal transduction *via* the co-receptor Smoothened (Smo), the executing transcription factors of the Hh pathway are the Gli proteins, of which three homologues are known in mammals: Gli1, Gli2 and Gli3[3]. Using a simplified model, the canonical Hh signaling can be described as follows[2,4]: In the absence of a Hh ligand, Ptch inactivates Smo - probably by preventing its localization into the primary cilium, a cell organelle that is thought to be essential for proper Hh signaling[5,6]. As a consequence, the Gli proteins are processed in such a way that they act as transcriptional repressors of the Hh target genes. However, upon binding of the Hh ligand to the receptor Ptch, inactivation of Smo is ended, allowing Smo to translocate to the primary cilium and initiate a cascade of events that ultimately lead to the conversion of Gli factors into their active form. The latter then shuttle into the nucleus and enable transcription of Hh target genes, including components of the pathway itself, such as *Ptch* and *Gli1,* indicating a built-in feedback loop within the Hh signaling cascade[2]. In addition to the "classical and canonical" Hh signaling described above, also non-canonical (Gli-independent), non-classical (ligand-independent) and aberrant Hh signaling (driven by activating mutations) have been identified at different stages of carcinogenesis (Figure 1)[7].

The pancreas is a fundamental organ of the digestive system with specialized endocrine and exocrine functions. The acinar cells within the exocrine pancreatic compartment produce and secrete numerous digestive enzymes into the duodenum. In the endocrine compartment, specialized cells produce hormones and directly release them into the blood stream - most importantly to control and regulate the blood glucose concentration. It is known from previous studies that physiologic Hh pathway signaling is crucial for correct development of the pancreas[8,9]. With this review, we give an overview of the current understanding of the role of Hh signaling in pancreatic development, cell differentiation and functional specialization. In a second part, the pathomechanistic implications of deregulated Hh signaling are discussed for the clinically most important pancreatic pathologies.

Hh SIGNALING IN HUMAN PANCREATIC DEVELOPMENT

Development of the pancreas

Pancreatic development is based on: (1) The fusion of two evaginations of the foregut to one single organ; and (2) endodermal growth by dichotomy branching. According the classical Carnegie stages[10,11], in stage 13, the dorsal pancreatic bud arises at first as a thickening of the endodermal tube, which proliferates, into the dorsal mesogastrium. In close, in stage 14, the ventral pancreatic bud evaginates to the liver primordium. As a result of differential growth of the duodenum, which rotates 90 degrees clockwise and becomes "C"-shaped, the ventral pancreatic bud comes to lie below and behind the dorsal pancreatic bud in stage 15. Until stage 17, both pancreatic buds have fused: The ventral pancreatic bud forms the posterior part of the head and the posterior part of the uncinated process, whereas the rest of the pancreas is formed by the dorsal pancreatic bud (the anterior part of the head, the body and the tail). Failure of the ventral pancreatic bud to migrate will result in an annular pancreas with consequent duodenal stenosis[12]. The main pancreatic duct (of Wirsung) is formed by the fusion of the distal part of the dorsal pancreatic duct and the entire ventral pancreatic duct and enters the duodenum combined with the bile duct at

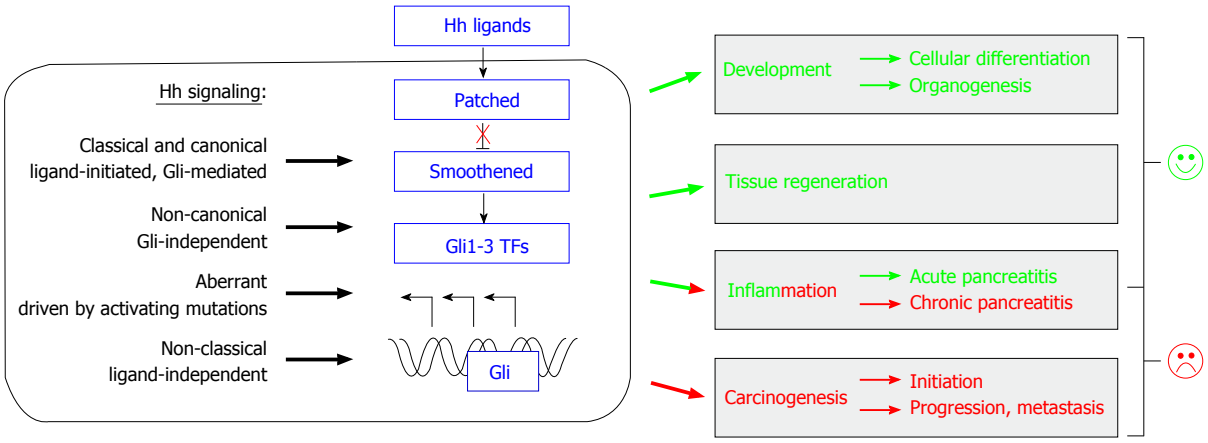


Figure 1 Overview of the Hedgehog signaling cascade and different activation modes (for details see ref. [7]). As described in detail in chapters Ⅱ to Ⅳ, the Hh pathway exerts positive and negative (labeled with green and red color, respectively) functions during development, regeneration, inflammation and cancerogenesis in the pancreas. Hh: Hedgehog; TF: Transcription factors.

the major papilla. Until the postnatal period, the proximal portion of the dorsal pancreatic duct either obliterates or persists as an accessory duct (of Santorini), entering the duodenum at the minor papilla (10% adults), so-called pancreatic divisum.

Cellular development of the pancreas

Differentiation and early specification of pancreatic endoderm is induced by fibroblast growth factor 2 and activin [a transforming growth factor beta (TGF-β) family member], both produced by the notochord and endothelium of the dorsal aorta. Both repress the expression of the transcription factor *Shh* locally in the gut endoderm, destined to form the dorsal pancreatic bud. Endoderm lying caudally to the pancreatic region does not respond to those signals[13]. The ventral bud is induced by upregulation of the pancreatic and duodenal homeobox 1 *(PDX1)* gene from the splanchnic mesoderm.

From 10th to 15th week, the primitive endodermal ductal epithelium provides the stem cell population for all the secretory cells, which are initially located in the duct walls or in the buds, from which they arise. Islet differentiation proceeds in two phases[13]: Phase Ⅰ (9th-15th week) is characterized by the proliferation of polyhormonal cells, whereas the differentiation of monohormonal cells is seen from week 16 onwards, referred to as phase Ⅱ. Later, these endocrine cells accumulate in pancreatic islets (of Langerhans) and scatter throughout the pancreas, starting with insulin and amylin secretion by β-cells approximately at the 5th month until neonatal period. The dorsal bud gives rise mostly to α-cells, which produce glucagon; however, most of the pancreatic polypeptide producing γ-cells develop from the ventral bud. After week 30, somatostatin-producing δ-cells are seen. The remaining primitive duct cells will either differentiate into definitive duct cells with microvilli and cilia or into acinar cells in which zymogen granules or acinar cell markers can be detected at weeks 12-16[13].

Correct ductal branching pattern and formation of acinar structures is determined by pancreatic mesenchyme which gives rise to connective tissue between the ducts resulting in pancreatic proliferation and maintaining the relative proportions of acinar, α-and β-cells. Additionally, it provides cell lines for smooth muscle within the pancreatic tissue, and angiogenic mesenchyme produces blood and lymphatic vessels.

Molecular regulation of pancreatic development by Hh signaling

Pancreas development is regulated by the activation/inactivation of Hh signaling members, which are ex-/repressed either within pancreatic tissue (*e.g., Ihh*) or in adjacent tissue (*e.g., Shh*)[14]. Initial absence of *Shh* signaling is required for regular pancreatic development, because ectopic expression of *Shh* leads to transformation of pancreatic mesoderm into intestinal mesenchyme in mice[15]. In single mutant mice (*i.e.*, *Shh*$^{-/-}$ or *Ihh*$^{-/-}$), gastrointestinal defects of the developing endoderm like annular pancreas or other malformations have been reported, suggesting similarities to human gut malformations[8,16].

It was shown that the graded response to Hh-signaling controls regular pancreatic development in mice, in which Hh signaling occurs at low levels during early organogenesis to ensure the correct establishment of organ boundaries and tissue architecture, and is up-regulated at later developmental stages to promote proliferation and maturation of the tissue[9,17-19]. Nielsen *et al*[20] confirmed the suggested concerted Hh signaling also in human pancreatic organogenesis: In early pancreatic development (7.5 wk), Gli3 was highly expressed in developing pancreatic ducts - while Smo and Gli2 were absent. In contrast, Smo and Gli2 were highly expressed between weeks 14 to 18, whereas the expression of Gli3 was reduced.

PDX1 (a pancreatic-promoting transcription factor; syn.: Insulin promotor factor 1) is also expressed in the preduodenal endoderm - including the sites of dorsal and ventral pancreatic bud formation. Total absence of the

pancreas is observed in homozygous *PDX1* mutant mice that suggest that *PDX1* is necessary for the formation of the pancreas and may be essential in the differentiation of pancreatic precursor cells[21,22]. Although all of the involved downstream effectors of human pancreas development have not been determined in detail yet, it appears that expression of the paired homeobox genes *PAX4* and *PAX6* specifies the endocrine cell lineage: Cells expressing both become β-, δ-, and γ-cells; whereas those expressing only *PAX6* become α-cells.

Hh IN PANCREATITIS

The cellular and molecular processes in acute pancreatitis (AP) and chronic pancreatitis (CP) were intensively elucidated in the last years providing valuable detailed insights which could be important in the next years for a further therapeutical approach in this partially lethal disease entity (reviewed in detail in[23-25]). In short, in the phase of AP, the major cellular key players are neutrophils, monocytes and macrophages which interact by building and secretion of cytokines and inflammatory mediators, mainly tumor necrosis factor α, interleukin (IL) 1β and 6, and monocyte chemotactic protein. In the phase of CP, pancreatic stellate cells (PSC), tissue infiltrating myeloid cells, and particularly macrophages are coming to the fore by induced and increased progressive fibrosing of the pancreas tissue, being mediated mainly by nuclear factor (NF)-κB[26,27]. Finally, the crosstalk of the mentioned cells is linked to T-subsets (CD-8$^+$/central memory cells as well as T-regulator cells) which are involved in the pathogenesis of CP[28,29]. Additionally, CP is commonly regarded as a relevant risk factor for ductal pancreatic cancer (DPC) by irregular ductal changes leading to acino-ductal metaplasia and pancreatic intraepithelial neoplasia (PanIn)[30,31].

Focusing on the linkage between the Hh pathway and AP as well CP, experimental investigations demonstrated that the members of the Hh pathway could be detected in different amounts in AP and CP, whereby the definitive functional role of Hh in AP and CP seems to be very different. Additionally, in the process of CP forward to DPC an irregular expression pattern of the Hh members are observed compared to the normal and structured embryonic development of the pancreas[9,32,33].

AP

Compared to CP, the role of Hh in AP has been dealt with only in few studies. Summarizing these data, activation of the Hh signaling is linked to injury and repair using the cerulean-mediated model, whereby the unequivocal conclusion of the available experimental data is that the Hh has protective function in AP. In 2008, Fendrich *et al*[33] presented a functional analysis of the Hh pathway in AP using pharmacologic and genetic techniques (like Ptch1-LacZ reporter mice and two different *Cre-driven* pancreas-specific depletion mice models of Smo) demonstrating that Hh is essentially involved in effective regeneration of the exocrine pancreas. By this approach, *Shh*, *Ihh*, and Gli1 are increasingly expressed in caerulein treated mice, whereby the pharmacologic and genetic inhibition lead to persistence of PDX1 expressing metaplastic intermediates and impaired tissue repair. Additionally, the group of Zhou *et al*[34] used a Cerulein-induced AP model in mice to show elegantly that: (1) *Shh*, not *Ihh* or *Dhh*, is involved in this model; (2) *Shh*-inhibition aggravates the AP; and (3) the anti-inflammatory autocrine effect of *Shh* is mediated by IL-10. A recent experimental study from 2014 showed that Gli1, the downstream member of the Hh cascade, could essentially influence the inflammatory reaction in the circumstances of remodeling processes of the pancreas. Based on genetic analysis of deletion of a single allele of Gli1, the authors postulated that the canonical Hh pathway, respectively the transcription factor Gli1, is essential for pancreatic recovery in inflammatory processes *via* Gli1 targeted cytokines, including IL-6, murine homolog of IL8, monocyte chemoattractant protein-1, and Macrophage colony-stimulating factor M-csf, leading to pancreatic tumorgenesis *via* improper stromal remodeling and persistence of the inflammatory infiltrate[35].

Chronic pancreatitis

Empiric studies in humans with CP demonstrated a heterogeneous upregulated expression of *Ihh*, its receptors Ptch and Hedgehog-Interacting Protein, and Smo in different histological distribution and cellular localization of human tissue with CP using Northern blotting, immunohistochemistry and Western-blotting[32,36,37]. Interestingly, the members of the Hh pathway were localized mainly in the islet cells, whereas the Hh signaling members were present in degenerated acinar and tubular complexes of CP[36,37]. In addition, Kayed *et al*[37] could show that the inhibition of the Hh pathway *via* Cyclopamin led to growth inhibition of TAKA-1 pancreatic ductal cells through cell cycle arrest *in vitro*.

Based on cDNA microarrays, Bhanot *et al*[31] could support the findings, that the Hh pathway is altered in microdissected ectatic ducts of CP whereby dysregulation of Hh could enhance the probability of DPC *via* duct ectasia, acino-ductal metaplasia or intraepithelial neoplasia as reviewed by Bahnot *et al*[31] in 2008.

As mentioned above, PSCs are essentially involved in the pathogenesis of the CP, whereby the main question is, how the Hh pathway regulates the activation of these PSCs.

The experimental analysis of the group of Shinozaki *et al*[38] revealed that *Ihh* has no evident effect on expression of collagen-1 or alpha-smooth muscle actin or on proliferation of PSCs, but *Ihh* modulates the migration potency by changing the amount of membrane-type 1 matrix metalloproteinase and its localization on the plasma membrane leading to a pro-migration status of PSCs. Although the *Ihh* effects are mediated by Gli1, experimental overexpression of Gli1 using an adenovirus-mediated or RNA interference techniques revealed a negatively regulation by Gli1 to *Ihh* effects *in vitro*.

But the question remains: Why is Hh pathway up-

Table 1 Summary of the role of Hedgehog signaling in pancreatitis, indicating the protective role in acute pancreatitis *vs* disease-progressive function in chronic pancreatitis as well the possible association to pancreatic cancerogenesis[32-38,42,43]

	Acute pancreatitis	Chronic pancreatitis
Pathogenetic effect of Hh	Protective	Progressive
Detected members of Hh	↑ *Shh* (*Ihh*, *Dhh*), Gli1	↑ *Ihh* (*Shh*), Ptc, Hip, SMO, Gli1, Gli2
Interactive cells (auto-and paracrine effects)	Acinar/ductal cells with; acute inflammatory cells	Acinar/ductal cells with; PSC
(Immune) mediators of inflammation	IL-10, IL-6, mIL-8, Mcp-1, and M-csf (Csf1)	MT1-MMP, MMP9, TGF-β1, smooth muscle actin, fibronectin 1, type Ⅰ collagen
Association to cancerogenesis	No	Yes, possibly *via* ADM and PanIn

Hh: Hedgehog; *Shh*: Sonic Hh; *Ihh*: Indian Hh; *Dhh*: Desert hedgehog; IL: Interleukin; mIL-8: Murine homolog of IL8; Mcp-1: Monocyte chemoattractant protein-1; M-csf: Macrophage colony-stimulating factor; Ptc: Patched; Hip: Hh-interacting protein; SMO: Smoothened; MT1-MMP: Membrane-type 1 matrix metalloproteinase; MMP-9: Matrix metallopeptidase 9; TGF-β: Transforming growth factor beta; ADM: Acino-ductal metaplasia; PanIn: Pancreatic intraductal neoplasia; PSC: Pancreatic stellate cells.

regulated within the fibrogenic process of CP? Based on *in vitro* and *in situ* studies with xenografts as well as in humans with pancreatitis, it is postulated that para- and partially autocrine activation of stromal cells by Hh ligands from epithelial components and vice versa are responsible[39-41]. The experimental data of Jung *et al*[42] are based on transgenic phenotypes in zebrafish with over-expression of either *Ihh* or *Shh* along with green fluorescence protein. Consecutive analysis of these transgenic phenotypes using quantitative and qualitative investigations of mRNA and protein levels including PCR, *in situ* hybridization, and immunohistochemistry revealed that myofibroblasts and ductal cells are activated and proliferate which is triggered by paracrine Hh signaling in a restricted expression of Ptc1, Smo and Gli1/2. Additionally, Hh ligands could induce matrix metallopeptidase 9 and TGF-β1 in this animal model[42].

Recent investigations by Tsang *et al*[43] could support the published findings of pro-fibrinogenic effects of Hh in CP by using an *in vivo* model. The application of Rhein, a natural anthraquinone derivative, reduces the activation of PSCs in mice with experimental induced CP. The morphological effect of Rhein in reduced pancreatic fibrosis was paralleled by reduced molecular expression of fibrogenic markers including alpha-smooth muscle actin, fibronectin 1, type Ⅰ collagen as well as the members of the Hh pathway *Shh* and Gli1.

Interestingly, the promoting fibrotic effect of Hh signaling is not only existent in pancreas, but also could be observed in other organs like lung, bile duct and liver implicating a tissue independent overriding principle of the Hh pathway in this pathogenesis[44-46].

CP and pancreatic carcinogenesis

Since chronic recurrent inflammation has been linked to carcinogenesis, especially in pancreas, some findings of Hh in AP/CP and pancreatic carcinogenesis are presented in the following for supporting this already emphasized linkage[47,48]. First of all, Hh modulates the axis between inflammation and cancerogenesis *via* activation and production of cytokines by human peripheral $CD4^+$ T cells[49]. Furthermore, experimental studies of Hh in AP and CP revealed morphological changes like ductal metaplasia promoted by *Shh*, which are *per se* no pre-tumorous conditions[33,50]. Nevertheless, during progression of CP, morphological changes of the ductal pancreatic tissue like papillary lesions with nuclear atypia resulting in PanIn lesions could be observed which have a high association to aberrant Hh expression and pancreatic cancer[31,50].

In conclusion (summarized in Table 1), members of the Hh pathway have protective properties in case of AP, whereby the face of Hh changes to a progressive and disease-promoting function in CP. Especially in CP, the negative effects of Hh on tissue remodeling and repair favored the possibility of cancerogenesis *via* de- and trans-differentiation[51-54].

Hh IN PANCREATIC CANCER: FROM *IN VITRO* TO *IN SITU*

In vitro: Findings in cell culture experiments and xenografts

Hh signaling in the normal pancreas and in pancreatic ductal adenocarcinoma is exclusively paracrine with expression of *Shh* (tumor cell and stroma signal circle as shown in Figure 2)[55]. The silencing of Smo in pancreatic cancer epithelium in mice showed no altered tumor spread or development, so the Hh signaling does not occur in an autocrine way[55].

In paracrine signaling, the Hh ligand sends signals directly to the stroma and provides a selective tumor growth advantage. This was established through a pancreatic cancer model where Hh signal was needed for overall tumor growth while the particular tumor cells themselves did not respond to Hh ligand[56].

The existence of cancer stem cells (CSC) in different tumors, including pancreatic cancer, offers an explanation why some therapy assessments are ineffective[57,58]. Therefore, a good knowledge base for new therapies, which target pancreatic CSCs, is very important. The Hh signaling pathway plays a vital role in pancreatic and embryonic development; autocrine or paracrine secreted *Shh* activates a signal transduction cascade that includes other Hh members like Ptch and Smo, which then activates the canonical Hh pathway through Gli.

This leads to transcription of multiple targets like

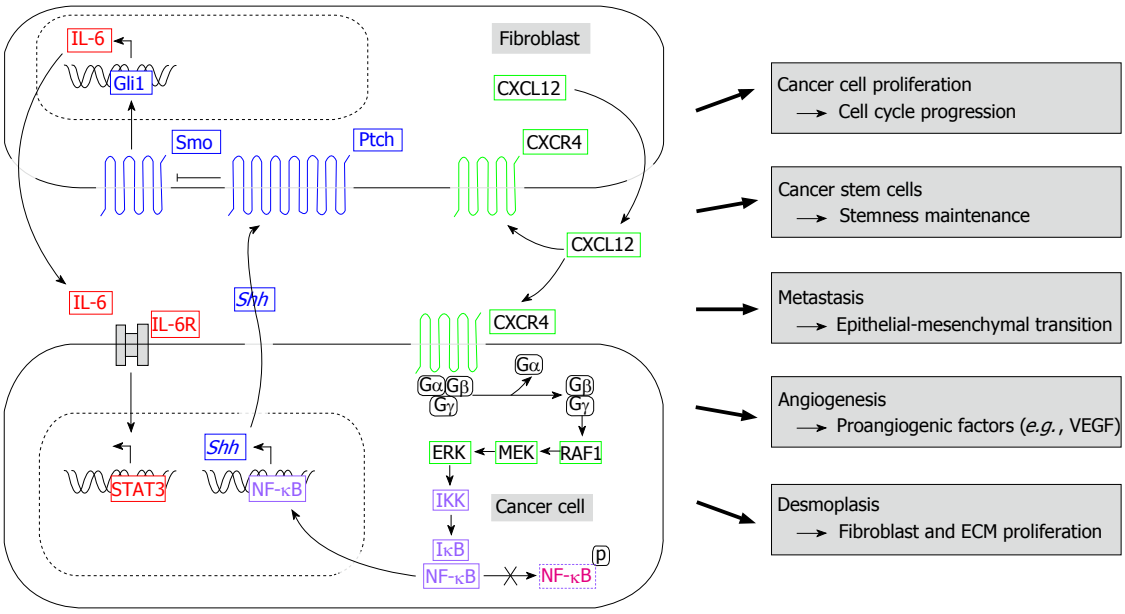


Figure 2 Illustrated summary of Hedgehog pathway and its effetcs at different stages in the formation and progression of ductal pancreatic carcinoma. Stromal cells secrete CXCL12 that binds to its receptor CXCR4 of ductal pancreatic cancer (DPC) cells (paracrine) and of stromal cells themselves (autocrine) resulting in *Shh* expression. *Shh*, secreted by DPC cells, binds in a paracrine manner Smo on stromal cells of the tumor microenvironment ending up in IL-6 secretion, which is known to regulate the progression of precursor lesions and tumor formation. For details see chapter Ⅳ. Based on[63,77,85]. CXCL12/CXCR4: CXC-motif-chemokine 12/CXC chemokine receptor type 4; ECM: Extracellular matrix; ERK: Extracellular signal-regulated kinases; Hh: Hedgehog; IκB: Inhibitor of kappa B; IKK: Inhibitor of nuclear factor kappa-B kinase; IL-6: Interleukin-6; MEK: Mitogen-activated protein kinase; NF-κB: nuclear factor-κB; Ptch: Patched; RAF1: V-Raf-1 murine leukemia viral oncogene homolog 1; *Shh*: Sonic Hh; Smo: Smoothened; STAT3: Signal transducer and activator of transcription 3; VEGF: Vascular endothelial growth factor.

Nanog, Cyclin D1, *Ptch*, Gli1 and Gli2. Activation of *Shh* signaling seems to precede the transformation of pancreatic tissue stem cells to cancerous stem cells. This was shown in mice, which were treated with sulforaphane to inhibit the growth of these stem cells. Sulforaphane is a natural compound found in cruciferous vegetables like broccoli that as an inhibitor acts on various receptors and pathways with anti-cancerous properties like apoptosis induction and cell proliferation[59]. This experimental study showed that human pancreatic stem cells need the activity of the Hh-Gli pathway for proliferation, survival, self-renewal and tumorigenicity[60].

In 2002, Chen *et al*[61] modulated mammalian embryonic pancreas development *in vitro* using cyclopamine treated pancreatic explants. A recombinant form of *Shh* was added to pancreatic buds to activate the Hh signaling pathway. The fluorescently labeled epithelium of the pancreatic explants underwent extensive growth and branching when treated by cyclopamine, which indicates that Hh inhibition did not block branching in the epithelium[61].

Walter *et al*[62] isolated pancreatic fibroblasts from benign and malignant primary pancreatic resection specimens by immunohistochemistry marker selection through vimentin. Together with two fibroblast cell lines, SC2 and SC3 (from non-neoplastic pancreas), the cancer-associated fibroblasts (CAF) were characterized for Hh activity. The fibroblast cell lines and the isolated CAFs where treated with *Shh* ligands to observe any expression changes on Gli mRNA. As a result they detected overexpression of Smo in pancreatic CAFs, which could transduce the *Shh* signal followed by Gli1 activation. The Hh pathway has been identified as activated in cancer associated stromal fibroblasts in mouse models of pancreatic cancer. CAFs can actively transduce the Hh signal to induce Gli expression. CAFs expressing Smo respond to exogenous Hh ligand, whereas control fibroblasts lacking Smo expression are unresponsive to Hh ligand, and downregulation of Smo in CAFs inhibits transduction of the Hh signal[62].

In human tumor xenografts, expression of *Shh* by tumor cells correlated with increased expression of GLi1 and Ptch1 in the stromal compartment. Pathway inhibition affected only stromal *Gli1* and *Ptch1* expression and resulted in decreased tumor growth exclusively in Hh ligand-expressing tumors[63].

Tian *et al*[64] demonstrated that the expression of an oncogenic allele of Smo (SmoM2) in mouse pancreas neither activated Hh signaling in epithelial cells nor promoted their neoplastic transformation. In murine pancreatic cancer models as well as in human pancreatic cancer specimens, activation of the Hh pathway was observed only in stromal cells surrounding Hh ligand-expressing tumor cells[64].

In-situ: Findings in human specimen of pancreas

Tumors of the pancreas can develop either from ductal, neuroendocrine or acinar cell populations. Due to a lack of information about the role of the Hh signaling pathway in acinar and neuroendocrine tumors of the pancreas, the following paragraphs will concentrate on DPC.

Among all cancers, DPC has one of the worst pro-

gnoses among all cancers with an overall 5-year survival rate of less than 5%[65]. Chemo- and radiotherapy are largely ineffective; furthermore, metastatic spread frequently occurs even after complete surgical resection[66]. The Hh pathway is one highly promising signaling transduction pathway for a better understanding of the origin of DPC.

Expression of Hh pathway members is usually not present in healthy adult pancreatic tissue[67]. In 2008, a global sequencing analysis revealed that the Hh pathway is one of the core signaling pathways that undergoes somatic alterations in nearly all pancreatic cancers[68]. Kayed *et al*[37] showed an aberrant activity of the Hh pathway in chronic pancreatitis and pancreatic cancer. Later on, it was recognized that *Shh* expression enhances the proliferation of pancreatic duct epithelial cells[69] and is not only up-regulated in the setting of pancreatic injury, but also in noninvasive precursor lesions of DPC: (1) PanIn; and (2) intraductal papillary mucinous neoplasia (IPMN) starting with rising expression values up to Hh pathway persistence in metastatic state[67,70,71]. Additionally, it was stated that up-regulation of the *Shh* ligand is sufficient to misdirect the pancreatic ductal epithelium towards a gastrointestinal metaplastic phenotype, which explains the involvement in IPMN formation[50,63].

However, dysfunction, or rather re-activation of the Hh pathway is not the only reason for the development of PanIn and DPC. Lauth *et al*[72] described a synergistic molecular crosstalk between Hh pathway and activated V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (K-RAS) signaling pathway[72]. Over 90% of patients suffering from DPC showed a *K-RAS* mutation, thus identifying the K-RAS pathway as another key mediator of pancreatic carcinogenesis[68,73]. Patients with a K-RAS mutation developed PanIn; and an additional P53 loss of function leads to subsequent development of the lesion towards DPC[74]. According to various studies, the crosstalk between Hh and K-RAS takes place *via* the RAF/MEK/MAPK pathway[75,76].

In 2013, Mills *et al*[77] were able to identify Gli1 as an effector of K-RAS at early stages of pancreatic carcinogenesis. They showed in a mouse model that loss of Gli1 impairs K-RAS-induced carcinogenesis. Although the mice still developed PanIn, the incidence of PanIn decreased and as a result, no mice suffered from DPC[77].

In recent studies, another central role in pre-neoplastic lesions of the pancreas is awarded to the signal transducer and activator of transcription 3 (STAT3) and its upstream cytokine IL-6. It is supposed that STAT3 activation is involved in driving early changes in the microenvironment promoting PanIn formation in the presence of oncogenic K-RAS[78,79]. Mills *et al*[77] stated that Gli1 also acts on CAF by paracrine regulation of the IL-6/STAT3 pathway in stromal cells of the tumor microenvironment (TME) and, thus, regulating the progression of precursor lesions and tumor formation (Figure 2).

DPC pathogenesis is characterized by a desmoplastic reaction to invading tumor cells, including a dense extracellular matrix that was recently shown to be the result of epithelial to mesenchymal transition (EMT)[80,81]. The epithelial-mesenchymal interaction, especially in the paracrine model of the Hh pathway, plays a distinctive role in different carcinoma entities as well as in DPC. Deregulated Hh pathway in PanIn and DPC leads to the secretion of Hh ligands *Shh* and *Ihh*, followed by a paracrine activation of CAFs in the surrounding stroma leading to expansion and desmoplasia[40,82,83]. In detail, neoplastic epithelium secretes *Shh*, which binds to the cognate Ptch-receptor on stromal cells, followed by desmoplastic stromal expansion and microenvironment remodeling. Moreover, supporting the paracrine action model of Hh pathway in DPC, Yauch *et al*[83] showed that treatment with Hh pathway antagonist results in downregulation of *Hh* target genes only in the tumor stroma but not in the epithelial cancer cell. In the same way, Smo expression decreases in mesenchymal cells in the pancreas resulting in Hh pathway activation. However, Lee *et al*[80] described that Hh pathway activity controls the balance between epithelial and stromal elements: Pathway activation causes stromal hyperplasia and reduced epithelial growth, whereas Hh inhibition causes accelerated growth of epithelial elements and suppression of desmoplasia.

It is suggested that the TME and extensive desmoplasia are partly responsible for chemoresistance in DPC by creating a "fence" around the tumor cells, which protects them against therapeutic compounds[84]. Therefore, tearing down this barrier could be a promising strategy to improve therapeutic approaches. Singh *et al*[85] already showed that inhibition of Hh pathway depleted tumor-associated stromal tissue.

There are many other different tumor specific characteristics that are influenced by the interrelation of Hh pathway and the TME. Bailey *et al*[86] identified paracrine *Shh*-mediated fibroblasts within the TME as source of Hypoxia-inducible factor 1 alpha (HIF-1α), which is known to be a regulator of angiogenesis and metastasis in cancer. Another example is the CXC-motif-chemokine 12/CXC chemokine receptor type 4 (CXCL12/CXCR4) pathway, which is on the one hand critical for normal cellular processes, but on the other hand contributes to metastasis, growth, survival and stem cell characteristics of cancer cells[87-89]. CXCL12, the sole ligand for CXCR4, is produced by tumor-associated stromal cells, is increased in DPC; and after binding to its receptor CXCR4, leads to activation of extracellular signal-regulated kinases resulting in release and nuclear translocation of NF-κB, which then directly binds the *Shh* promotor[85,90,91]. In summary, Hh pathway acts in a predominantly paracrine manner, thereby influencing and being influenced by the TME (for an overview of Hh-dependent interactions between tumor and stroma cells in DPC, Figure 2).

CSC, also called tumor initiating cells are suggested to be responsible for cancer initiation, progression and chemo-resistance in several malignancies including DPC[92]. The transcription factors Nanog, octamer-binding transcription factor 4 and BMI1 Proto-Oncogene, Polycomb Ring Finger (BMI-1) are essential for the

Table 2 Clinical trials of Hedgehog inhibitors for pancreatic cancer (https://clinicaltrials.gov/)

Drug	Combination	Phase	Status	Trial ID
GDC-0449	Gemcitabine	0	N	NCT01713218
		1/2	A	NCT01195415
		2	A	NCT01064622
	Erlotinib, gemcitabine	1	A	NCT00878163
	Gemcitabine, nab-paclitaxel	2	A	NCT01088815
LDE-225	Gemcitabine, nab-paclitaxel	1/2	R	NCT01431794
		1/2	R	NCT02358161
	BKM120	1	C	NCT01576666
IPI-926	Gemcitabine	1/2	C	NCT01130142

A: Active, not recruiting; C: Completed; N: Not yet recruiting; R: Recruiting.

"stemness", including characteristics like self-renewal of CSC[93-95]. The Hh pathway is implicated in the maintenance of pancreatic CSCs: For example, Li *et al*[96] stated that *Shh* expression was 46-fold greater in pancreatic CSCs ($CD24^+/CD44^+/ESA^+$) as in other DPC cells ($CD24^-/CD44^-/ESA^-$). Additionally, *Gli1* is known to up-regulate genes that are crucial for many properties for stemness of CSC - like *Nanog* and *BMI-1*[97-99].

Recapitulating this chapter, Hh pathway plays an important role in DPC, beginning from PanIn precursors to progressed metastatic disease. Hh signaling cross talks with a variety of other signaling pathways, like K-RAS, requires the interaction with the EMT in particular *via* paracrine pathway stimulation in order to contribute to the development of DPC (Figure 1).

Hh-BASED CLINICAL TRIALS FOR PANCREATIC CANCER

At present, clinical trials using Hh inhibitors enroll patients with pancreatic malignancies including advanced, metastatic, recurrent or resectable pancreatic cancer. Currently, no trials are listed within the United States National Institutes of Health database (www.clinicaltrials.gov) which target pancreatitis or other pancreatic non-neoplastic conditions. As summarized in Table 2, most trials in the phase 1 or 2 setting use GDC-0449 (vismodegib) which is a small molecular weight inhibitor of Smo[100] thereby interfering with Hh signaling at the plasma membrane level similarly to cyclopamine, a naturally occurring Smo antagonist[101]. Other Hh-targeting drugs in current clinical trials on pancreatic cancer are the Smo-inhibitors LDE-225 (Sonidegib)[102] and IPI-926 (Saridegib)[103].

For the latter, a preclinical study on pancreatic cancer in mice demonstrated that IPI-926 depletes tumor-associated stromal tissue and facilitated the delivery and increased the intratumoral concentration of gemcitabine[84]. In line with these results, all currently ongoing clinical trials combine selective Hh antagonists with established chemotherapies (gemcitabine, paclitaxel) or other targeted drugs (erlotinib epidermal growth factor receptor inhibitor) or BKM120 (Phosphatidylinositol-4,5-bisphosphate 3-kinase inhibitor) to investigate possible therapeutic benefits of these drug combinations. Taken together, current clinical studies employ inhibitors of the Smo co-receptor in combination with established chemotherapeutic drugs. Novel experimental inhibitors targeting the Hh pathway at the level of the transcriptional regulation (*e.g.*, Gant-61, Gant-58) have not yet entered the stage of clinical evaluation[104].

CONCLUSION

Besides its physiologic functions in human pancreatic development, the Hh pathway is activated in numerous pathological conditions, including carcinogenesis. However, the data on its functional aspects currently available draw a more nuanced picture. Progression from pancreatic cancer precursors lesions (PanIn) to DPC and metastatic disease is strongly influenced by a paracrine Hh signal modulating the interaction between DPC cells and CAFs. This Hh driven signaling predominantly includes the IL-6/STAT3 and the CXCL12/CXCR4 pathways resulting in disease progression by invasion, angiogenesis, metastasis formation and chemoresistance as well as gaining of stem cell like characteristics. Therefore, therapeutic targeting of the Hh pathway may provide new therapeutic approaches to improved disease control and prognosis for both, chronic pancreatitis and pancreatic carcinogenesis.

REFERENCES

1 **Nüsslein-Volhard C**, Wieschaus E. Mutations affecting segment number and polarity in Drosophila. *Nature* 1980; **287**: 795-801 [PMID: 6776413 DOI: 10.1038/287795a0]
2 **Teglund S**, Toftgård R. Hedgehog beyond medulloblastoma and basal cell carcinoma. *Biochim Biophys Acta* 2010; **1805**: 181-208 [PMID: 20085802 DOI: 10.1016/j.bbcan.2010.01.003]
3 **Lees C**, Howie S, Sartor RB, Satsangi J. The hedgehog signalling pathway in the gastrointestinal tract: implications for development, homeostasis, and disease. *Gastroenterology* 2005; **129**: 1696-1710 [PMID: 16285967 DOI: 10.1053/j.gastro.2005.05.010]
4 **Heretsch P**, Tzagkaroulaki L, Giannis A. Modulators of the hedgehog signaling pathway. *Bioorg Med Chem* 2010; **18**: 6613-6624 [PMID: 20708941 DOI: 10.1016/j.bmc.2010.07.038]
5 **Huangfu D**, Liu A, Rakeman AS, Murcia NS, Niswander L, Anderson KV. Hedgehog signalling in the mouse requires intraflagellar transport proteins. *Nature* 2003; **426**: 83-87 [PMID: 14603322 DOI: 10.1038/nature02061]
6 **May SR**, Ashique AM, Karlen M, Wang B, Shen Y, Zarbalis K, Reiter J, Ericson J, Peterson AS. Loss of the retrograde motor for IFT disrupts localization of Smo to cilia and prevents the expression of both activator and repressor functions of Gli. *Dev Biol* 2005; **287**: 378-389 [PMID: 16229832 DOI: 10.1016/j.ydbio.2005.08.050]
7 **Shevde LA**, Samant RS. Nonclassical hedgehog-GLI signaling and its clinical implications. *Int J Cancer* 2014; **135**: 1-6 [PMID: 23929208 DOI: 10.1002/ijc.28424]
8 **Hebrok M**. Hedgehog signaling in pancreas development. *Mech Dev* 2003; **120**: 45-57 [PMID: 12490295 DOI: 10.1016/S0925-4773(02)00331-3]
9 **Lau J**, Kawahira H, Hebrok M. Hedgehog signaling in pancreas development and disease. *Cell Mol Life Sci* 2006; **63**: 642-652 [PMID: 16465449 DOI: 10.1007/s00018-005-5357-z]
10 **Hill MA**. Early human development. *Clin Obstet Gynecol* 2007; **50**: 2-9 [PMID: 17304021 DOI: 10.1097/GRF.0b013e31802f119d]
11 **Yi H**, Xue L, Guo MX, Ma J, Zeng Y, Wang W, Cai JY, Hu HM, Shu HB, Shi YB, Li WX. Gene expression atlas for human embryo-

genesis. *FASEB J* 2010; **24**: 3341-3350 [PMID: 20430792 DOI: 10.1096/fj.10-158782]
12 **Etienne D**, John A, Menias CO, Ward R, Tubbs RS, Loukas M. Annular pancreas: a review of its molecular embryology, genetic basis and clinical considerations. *Ann Anat* 2012; **194**: 422-428 [PMID: 22694842 DOI: 10.1016/j.aanat.2012.04.006]
13 **Howard ER**, Stringer MD, Colombani PM. Surgery of the Liver, Bile Ducts and Pancreas in Children. 2nd ed. London: Arnold Publishers; 2002: 239-246
14 **Hebrok M**, Kim SK, St Jacques B, McMahon AP, Melton DA. Regulation of pancreas development by hedgehog signaling. *Development* 2000; **127**: 4905-4913 [PMID: 11044404]
15 **Apelqvist A**, Ahlgren U, Edlund H. Sonic hedgehog directs specialised mesoderm differentiation in the intestine and pancreas. *Curr Biol* 1997; **7**: 801-804 [PMID: 9368764 DOI: 10.1016/S0960-9822(06)00340-X]
16 **Ramalho-Santos M**, Melton DA, McMahon AP. Hedgehog signals regulate multiple aspects of gastrointestinal development. *Development* 2000; **127**: 2763-2772 [PMID: 10821773]
17 **Kawahira H**, Scheel DW, Smith SB, German MS, Hebrok M. Hedgehog signaling regulates expansion of pancreatic epithelial cells. *Dev Biol* 2005; **280**: 111-121 [PMID: 15766752 DOI: 10.1016/j.ydbio.2005.01.008]
18 **Cano DA**, Hebrok M, Zenker M. Pancreatic development and disease. *Gastroenterology* 2007; **132**: 745-762 [PMID: 17258745 DOI: 10.1053/j.gastro.2006.12.054]
19 **van den Brink GR**. Hedgehog signaling in development and homeostasis of the gastrointestinal tract. *Physiol Rev* 2007; **87**: 1343-1375 [PMID: 17928586 DOI: 10.1152/physrev.00054.2006]
20 **Nielsen SK**, Møllgård K, Clement CA, Veland IR, Awan A, Yoder BK, Novak I, Christensen ST. Characterization of primary cilia and Hedgehog signaling during development of the human pancreas and in human pancreatic duct cancer cell lines. *Dev Dyn* 2008; **237**: 2039-2052 [PMID: 18629868 DOI: 10.1002/dvdy.21610]
21 **Jonsson J**, Carlsson L, Edlund T, Edlund H. Insulin-promoter-factor 1 is required for pancreas development in mice. *Nature* 1994; **371**: 606-609 [PMID: 7935793 DOI: 10.1038/371606a0]
22 **Offield MF**, Jetton TL, Labosky PA, Ray M, Stein RW, Magnuson MA, Hogan BL, Wright CV. PDX-1 is required for pancreatic outgrowth and differentiation of the rostral duodenum. *Development* 1996; **122**: 983-995 [PMID: 8631275]
23 **Thrower E**, Husain S, Gorelick F. Molecular basis for pancreatitis. *Curr Opin Gastroenterol* 2008; **24**: 580-585 [PMID: 19122498 DOI: 10.1097/MOG.0b013e32830b10e6]
24 **Zheng L**, Xue J, Jaffee EM, Habtezion A. Role of immune cells and immune-based therapies in pancreatitis and pancreatic ductal adenocarcinoma. *Gastroenterology* 2013; **144**: 1230-1240 [PMID: 23622132 DOI: 10.1053/j.gastro.2012.12.042]
25 **Gukovsky I**, Li N, Todoric J, Gukovskaya A, Karin M. Inflammation, autophagy, and obesity: common features in the pathogenesis of pancreatitis and pancreatic cancer. *Gastroenterology* 2013; **144**: 1199-209.e4 [PMID: 23622129 DOI: 10.1053/j.gastro.2013.02.007]
26 **Omary MB**, Lugea A, Lowe AW, Pandol SJ. The pancreatic stellate cell: a star on the rise in pancreatic diseases. *J Clin Invest* 2007; **117**: 50-59 [PMID: 17200706 DOI: 10.1172/JCI30082]
27 **Treiber M**, Neuhöfer P, Anetsberger E, Einwächter H, Lesina M, Rickmann M, Liang S, Kehl T, Nakhai H, Schmid RM, Algül H. Myeloid, but not pancreatic, RelA/p65 is required for fibrosis in a mouse model of chronic pancreatitis. *Gastroenterology* 2011; **141**: 1473-1485 [PMID: 21763242 DOI: 10.1053/j.gastro.2011.06.087]
28 **Schmitz-Winnenthal H**, Pietsch DH, Schimmack S, Bonertz A, Udonta F, Ge Y, Galindo L, Specht S, Volk C, Zgraggen K, Koch M, Büchler MW, Weitz J, Beckhove P. Chronic pancreatitis is associated with disease-specific regulatory T-cell responses. *Gastroenterology* 2010; **138**: 1178-1188 [PMID: 19931255 DOI: 10.1053/j.gastro.2009.11.011]
29 **Grundsten M**, Liu GZ, Permert J, Hjelmstrom P, Tsai JA. Increased central memory T cells in patients with chronic pancreatitis. *Pancreatology* 2005; **5**: 177-182 [PMID: 15849488 DOI: 10.1159/000085269]
30 **Becker AE**, Hernandez YG, Frucht H, Lucas AL. Pancreatic ductal adenocarcinoma: risk factors, screening, and early detection. *World J Gastroenterol* 2014; **20**: 11182-11198 [PMID: 25170203 DOI: 10.3748/wjg.v20.i32.11182]
31 **Bhanot UK**, Möller P. Mechanisms of parenchymal injury and signaling pathways in ectatic ducts of chronic pancreatitis: implications for pancreatic carcinogenesis. *Lab Invest* 2009; **89**: 489-497 [PMID: 19308045 DOI: 10.1038/labinvest.2009.19]
32 **Kayed H**, Kleeff J, Osman T, Keleg S, Büchler MW, Friess H. Hedgehog signaling in the normal and diseased pancreas. *Pancreas* 2006; **32**: 119-129 [PMID: 16552330 DOI: 10.1097/01.mpa.0000202937.55460.0c]
33 **Fendrich V**, Esni F, Garay MV, Feldmann G, Habbe N, Jensen JN, Dor Y, Stoffers D, Jensen J, Leach SD, Maitra A. Hedgehog signaling is required for effective regeneration of exocrine pancreas. *Gastroenterology* 2008; **135**: 621-631 [PMID: 18515092 DOI: 10.1053/j.gastro.2008.04.011]
34 **Zhou X**, Liu Z, Jang F, Xiang C, Li Y, He Y. Autocrine Sonic hedgehog attenuates inflammation in cerulein-induced acute pancreatitis in mice via upregulation of IL-10. *PLoS One* 2012; **7**: e44121 [PMID: 22956998 DOI: 10.1371/journal.pone.0044121]
35 **Mathew E**, Collins MA, Fernandez-Barrena MG, Holtz AM, Yan W, Hogan JO, Tata Z, Allen BL, Fernandez-Zapico ME, di Magliano MP. The transcription factor GLI1 modulates the inflammatory response during pancreatic tissue remodeling. *J Biol Chem* 2014; **289**: 27727-27743 [PMID: 25104358 DOI: 10.1074/jbc.M114.556563]
36 **Kayed H**, Kleeff J, Esposito I, Giese T, Keleg S, Giese N, Büchler MW, Friess H. Localization of the human hedgehog-interacting protein (Hip) in the normal and diseased pancreas. *Mol Carcinog* 2005; **42**: 183-192 [PMID: 15754313 DOI: 10.1002/mc.20088]
37 **Kayed H**, Kleeff J, Keleg S, Büchler MW, Friess H. Distribution of Indian hedgehog and its receptors patched and smoothened in human chronic pancreatitis. *J Endocrinol* 2003; **178**: 467-478 [PMID: 12967338]
38 **Shinozaki S**, Ohnishi H, Hama K, Kita H, Yamamoto H, Osawa H, Sato K, Tamada K, Mashima H, Sugano K. Indian hedgehog promotes the migration of rat activated pancreatic stellate cells by increasing membrane type-1 matrix metalloproteinase on the plasma membrane. *J Cell Physiol* 2008; **216**: 38-46 [PMID: 18286538 DOI: 10.1002/jcp.21372]
39 **Kolterud A**, Grosse AS, Zacharias WJ, Walton KD, Kretovich KE, Madison BB, Waghray M, Ferris JE, Hu C, Merchant JL, Dlugosz AA, Kottmann AH, Gumucio DL. Paracrine Hedgehog signaling in stomach and intestine: new roles for hedgehog in gastrointestinal patterning. *Gastroenterology* 2009; **137**: 618-628 [PMID: 19445942 DOI: 10.1053/j.gastro.2009.05.002]
40 **Bailey JM**, Swanson BJ, Hamada T, Eggers JP, Singh PK, Caffery T, Ouellette MM, Hollingsworth MA. Sonic hedgehog promotes desmoplasia in pancreatic cancer. *Clin Cancer Res* 2008; **14**: 5995-6004 [PMID: 18829478 DOI: 10.1158/1078-0432.CCR-08-0291]
41 **Wicking C**, Smyth I, Bale A. The hedgehog signalling pathway in tumorigenesis and development. *Oncogene* 1999; **18**: 7844-7851 [PMID: 10630637 DOI: 10.1038/sj.onc.1203282]
42 **Jung IH**, Jung DE, Park YN, Song SY, Park SW. Aberrant Hedgehog ligands induce progressive pancreatic fibrosis by paracrine activation of myofibroblasts and ductular cells in transgenic zebrafish. *PLoS One* 2011; **6**: e27941 [PMID: 22164219 DOI: 10.1371/journal.pone.0027941]
43 **Tsang SW**, Zhang H, Lin C, Xiao H, Wong M, Shang H, Yang ZJ, Lu A, Yung KK, Bian Z. Rhein, a natural anthraquinone derivative, attenuates the activation of pancreatic stellate cells and ameliorates pancreatic fibrosis in mice with experimental chronic pancreatitis. *PLoS One* 2013; **8**: e82201 [PMID: 24312641 DOI: 10.1371/journal.pone.0082201]
44 **Lin N**, Tang Z, Deng M, Zhong Y, Lin J, Yang X, Xiang P, Xu R. Hedgehog-mediated paracrine interaction between hepatic stellate cells and marrow-derived mesenchymal stem cells. *Biochem Biophys Res Commun* 2008; **372**: 260-265 [PMID: 18486597 DOI:

10.1016/j.bbrc.2008.05.029]
45 **Omenetti A**, Porrello A, Jung Y, Yang L, Popov Y, Choi SS, Witek RP, Alpini G, Venter J, Vandongen HM, Syn WK, Baroni GS, Benedetti A, Schuppan D, Diehl AM. Hedgehog signaling regulates epithelial-mesenchymal transition during biliary fibrosis in rodents and humans. *J Clin Invest* 2008; **118**: 3331-3342 [PMID: 18802480 DOI: 10.1172/JCI35875]
46 **Stewart GA**, Hoyne GF, Ahmad SA, Jarman E, Wallace WA, Harrison DJ, Haslett C, Lamb JR, Howie SE. Expression of the developmental Sonic hedgehog (Shh) signalling pathway is up-regulated in chronic lung fibrosis and the Shh receptor patched 1 is present in circulating T lymphocytes. *J Pathol* 2003; **199**: 488-495 [PMID: 12635140 DOI: 10.1002/path.1295]
47 **Blaylock RL**. Cancer microenvironment, inflammation and cancer stem cells: A hypothesis for a paradigm change and new targets in cancer control. *Surg Neurol Int* 2015; **6**: 92 [PMID: 26097771 DOI: 10.4103/2152-7806.157890]
48 **Ling SB**, Feng TT, Jia KQ, Tian Y, Li Y. Inflammation to cancer: The molecular biology in the pancreas (Review). *Oncol Lett* 2014; **7**: 1747-1754 [PMID: 24932227 DOI: 10.3892/ol.2014.2003]
49 **Stewart GA**, Lowrey JA, Wakelin SJ, Fitch PM, Lindey S, Dallman MJ, Lamb JR, Howie SE. Sonic hedgehog signaling modulates activation of and cytokine production by human peripheral CD4+ T cells. *J Immunol* 2002; **169**: 5451-5457 [PMID: 12421920]
50 **Strobel O**, Rosow DE, Rakhlin EY, Lauwers GY, Trainor AG, Alsina J, Fernández-Del Castillo C, Warshaw AL, Thayer SP. Pancreatic duct glands are distinct ductal compartments that react to chronic injury and mediate Shh-induced metaplasia. *Gastroenterology* 2010; **138**: 1166-1177 [PMID: 20026066 DOI: 10.1053/j.gastro.2009.12.005]
51 **Neureiter D**, Zopf S, Dimmler A, Stintzing S, Hahn EG, Kirchner T, Herold C, Ocker M. Different capabilities of morphological pattern formation and its association with the expression of differentiation markers in a xenograft model of human pancreatic cancer cell lines. *Pancreatology* 2005; **5**: 387-397 [PMID: 15980667 DOI: 10.1159/000086539]
52 **Quint K**, Stintzing S, Alinger B, Hauser-Kronberger C, Dietze O, Gahr S, Hahn EG, Ocker M, Neureiter D. The expression pattern of PDX-1, SHH, Patched and Gli-1 is associated with pathological and clinical features in human pancreatic cancer. *Pancreatology* 2009; **9**: 116-126 [PMID: 19077462 DOI: 10.1159/000178882]
53 **DI Fazio P**, Montalbano R, Quint K, Alinger B, Kemmerling R, Kiesslich T, Ocker M, Neureiter D. The pan-deacetylase inhibitor panobinostat modulates the expression of epithelial-mesenchymal transition markers in hepatocellular carcinoma models. *Oncol Lett* 2013; **5**: 127-134 [PMID: 23255907 DOI: 10.3892/ol.2012.951]
54 **Quint K**, Tonigold M, Di Fazio P, Montalbano R, Lingelbach S, Rückert F, Alinger B, Ocker M, Neureiter D. Pancreatic cancer cells surviving gemcitabine treatment express markers of stem cell differentiation and epithelial-mesenchymal transition. *Int J Oncol* 2012; **41**: 2093-2102 [PMID: 23026911 DOI: 10.3892/ijo.2012.1648]
55 **Honselmann KC**, Pross M, Jung CM, Wellner UF, Deichmann S, Keck T, Bausch D. Regulation mechanisms of the hedgehog pathway in pancreatic cancer: a review. *JOP* 2015; **16**: 25-32 [PMID: 25640779 DOI: 10.6092/1590-8577/2894]
56 **Marini KD**, Payne BJ, Watkins DN, Martelotto LG. Mechanisms of Hedgehog signalling in cancer. *Growth Factors* 2011; **29**: 221-234 [PMID: 21875383 DOI: 10.3109/08977194.2011.610756]
57 **Allegra A**, Alonci A, Penna G, Innao V, Gerace D, Rotondo F, Musolino C. The cancer stem cell hypothesis: a guide to potential molecular targets. *Cancer Invest* 2014; **32**: 470-495 [PMID: 25254602 DOI: 10.3109/07357907.2014.958231]
58 **O'Connor ML**, Xiang D, Shigdar S, Macdonald J, Li Y, Wang T, Pu C, Wang Z, Qiao L, Duan W. Cancer stem cells: A contentious hypothesis now moving forward. *Cancer Lett* 2014; **344**: 180-187 [PMID: 24333726 DOI: 10.1016/j.canlet.2013.11.012]
59 **Chinembiri TN**, du Plessis LH, Gerber M, Hamman JH, du Plessis J. Review of natural compounds for potential skin cancer treatment. *Molecules* 2014; **19**: 11679-11721 [PMID: 25102117 DOI: 10.3390/molecules190811679]
60 **Li SH**, Fu J, Watkins DN, Srivastava RK, Shankar S. Sulforaphane regulates self-renewal of pancreatic cancer stem cells through the modulation of Sonic hedgehog-GLI pathway. *Mol Cell Biochem* 2013; **373**: 217-227 [PMID: 23129257 DOI: 10.1007/s11010-012-1493-6]
61 **Chen JK**, Taipale J, Cooper MK, Beachy PA. Inhibition of Hedgehog signaling by direct binding of cyclopamine to Smoothened. *Genes Dev* 2002; **16**: 2743-2748 [PMID: 12414725 DOI: 10.1101/gad.1025302]
62 **Walter K**, Omura N, Hong SM, Griffith M, Vincent A, Borges M, Goggins M. Overexpression of smoothened activates the sonic hedgehog signaling pathway in pancreatic cancer-associated fibroblasts. *Clin Cancer Res* 2010; **16**: 1781-1789 [PMID: 20215540 DOI: 10.1158/1078-0432.CCR-09-1913]
63 **Rosow DE**, Liss AS, Strobel O, Fritz S, Bausch D, Valsangkar NP, Alsina J, Kulemann B, Park JK, Yamaguchi J, LaFemina J, Thayer SP. Sonic Hedgehog in pancreatic cancer: from bench to bedside, then back to the bench. *Surgery* 2012; **152**: S19-S32 [PMID: 22770959 DOI: 10.1016/j.surg.2012.05.030]
64 **Tian H**, Callahan CA, DuPree KJ, Darbonne WC, Ahn CP, Scales SJ, de Sauvage FJ. Hedgehog signaling is restricted to the stromal compartment during pancreatic carcinogenesis. *Proc Natl Acad Sci USA* 2009; **106**: 4254-4259 [PMID: 19246386 DOI: 10.1073/pnas.0813203106]
65 **Jemal A**, Siegel R, Xu J, Ward E. Cancer statistics, 2010. *CA Cancer J Clin* 2010; **60**: 277-300 [PMID: 20610543 DOI: 10.3322/caac.20073]
66 **Li J**, Wientjes MG, Au JL. Pancreatic cancer: pathobiology, treatment options, and drug delivery. *AAPS J* 2010; **12**: 223-232 [PMID: 20198462 DOI: 10.1208/s12248-010-9181-5]
67 **Thayer SP**, di Magliano MP, Heiser PW, Nielsen CM, Roberts DJ, Lauwers GY, Qi YP, Gysin S, Fernández-del Castillo C, Yajnik V, Antoniu B, McMahon M, Warshaw AL, Hebrok M. Hedgehog is an early and late mediator of pancreatic cancer tumorigenesis. *Nature* 2003; **425**: 851-856 [PMID: 14520413 DOI: 10.1038/nature02009]
68 **Jones S**, Zhang X, Parsons DW, Lin JC, Leary RJ, Angenendt P, Mankoo P, Carter H, Kamiyama H, Jimeno A, Hong SM, Fu B, Lin MT, Calhoun ES, Kamiyama M, Walter K, Nikolskaya T, Nikolsky Y, Hartigan J, Smith DR, Hidalgo M, Leach SD, Klein AP, Jaffee EM, Goggins M, Maitra A, Iacobuzio-Donahue C, Eshleman JR, Kern SE, Hruban RH, Karchin R, Papadopoulos N, Parmigiani G, Vogelstein B, Velculescu VE, Kinzler KW. Core signaling pathways in human pancreatic cancers revealed by global genomic analyses. *Science* 2008; **321**: 1801-1806 [PMID: 18772397 DOI: 10.1126/science.1164368]
69 **Morton JP**, Mongeau ME, Klimstra DS, Morris JP, Lee YC, Kawaguchi Y, Wright CV, Hebrok M, Lewis BC. Sonic hedgehog acts at multiple stages during pancreatic tumorigenesis. *Proc Natl Acad Sci USA* 2007; **104**: 5103-5108 [PMID: 17372229 DOI: 10.1073/pnas.0701158104]
70 **Hingorani SR**, Wang L, Multani AS, Combs C, Deramaudt TB, Hruban RH, Rustgi AK, Chang S, Tuveson DA. Trp53R172H and KrasG12D cooperate to promote chromosomal instability and widely metastatic pancreatic ductal adenocarcinoma in mice. *Cancer Cell* 2005; **7**: 469-483 [PMID: 15894267 DOI: 10.1016/j.ccr.2005.04.023]
71 **Feldmann G**, Dhara S, Fendrich V, Bedja D, Beaty R, Mullendore M, Karikari C, Alvarez H, Iacobuzio-Donahue C, Jimeno A, Gabrielson KL, Matsui W, Maitra A. Blockade of hedgehog signaling inhibits pancreatic cancer invasion and metastases: a new paradigm for combination therapy in solid cancers. *Cancer Res* 2007; **67**: 2187-2196 [PMID: 17332349 DOI: 10.1158/0008-5472.CAN-06-3281]
72 **Lauth M**, Bergström A, Shimokawa T, Tostar U, Jin Q, Fendrich V, Guerra C, Barbacid M, Toftgård R. DYRK1B-dependent autocrine-to-paracrine shift of Hedgehog signaling by mutant RAS. *Nat Struct Mol Biol* 2010; **17**: 718-725 [PMID: 20512148 DOI: 10.1038/nsmb.1833]
73 **Hingorani SR**, Petricoin EF, Maitra A, Rajapakse V, King C, Jacobetz MA, Ross S, Conrads TP, Veenstra TD, Hitt BA,

Kawaguchi Y, Johann D, Liotta LA, Crawford HC, Putt ME, Jacks T, Wright CV, Hruban RH, Lowy AM, Tuveson DA. Preinvasive and invasive ductal pancreatic cancer and its early detection in the mouse. *Cancer Cell* 2003; **4**: 437-450 [PMID: 14706336 DOI: 10.1016/S1535-6108(03)00309-X]

74 **Collins MA**, Bednar F, Zhang Y, Brisset JC, Galbán S, Galbán CJ, Rakshit S, Flannagan KS, Adsay NV, Pasca di Magliano M. Oncogenic Kras is required for both the initiation and maintenance of pancreatic cancer in mice. *J Clin Invest* 2012; **122**: 639-653 [PMID: 22232209 DOI: 10.1172/JCI59227]

75 **Merchant A**, Joseph G, Wang Q, Brennan S, Matsui W. Gli1 regulates the proliferation and differentiation of HSCs and myeloid progenitors. *Blood* 2010; **115**: 2391-2396 [PMID: 20107231 DOI: 10.1182/blood-2009-09-241703]

76 **Ji Z**, Mei FC, Xie J, Cheng X. Oncogenic KRAS activates hedgehog signaling pathway in pancreatic cancer cells. *J Biol Chem* 2007; **282**: 14048-14055 [PMID: 17353198 DOI: 10.1074/jbc.M611089200]

77 **Mills LD**, Zhang Y, Marler RJ, Herreros-Villanueva M, Zhang L, Almada LL, Couch F, Wetmore C, Pasca di Magliano M, Fernandez-Zapico ME. Loss of the transcription factor GLI1 identifies a signaling network in the tumor microenvironment mediating KRAS oncogene-induced transformation. *J Biol Chem* 2013; **288**: 11786-11794 [PMID: 23482563 DOI: 10.1074/jbc.M112.438846]

78 **Fukuda A**, Wang SC, Morris JP, Folias AE, Liou A, Kim GE, Akira S, Boucher KM, Firpo MA, Mulvihill SJ, Hebrok M. Stat3 and MMP7 contribute to pancreatic ductal adenocarcinoma initiation and progression. *Cancer Cell* 2011; **19**: 441-455 [PMID: 21481787 DOI: 10.1016/j.ccr.2011.03.002]

79 **Lesina M**, Kurkowski MU, Ludes K, Rose-John S, Treiber M, Klöppel G, Yoshimura A, Reindl W, Sipos B, Akira S, Schmid RM, Algül H. Stat3/Socs3 activation by IL-6 transsignaling promotes progression of pancreatic intraepithelial neoplasia and development of pancreatic cancer. *Cancer Cell* 2011; **19**: 456-469 [PMID: 21481788 DOI: 10.1016/j.ccr.2011.03.009]

80 **Lee JJ**, Perera RM, Wang H, Wu DC, Liu XS, Han S, Fitamant J, Jones PD, Ghanta KS, Kawano S, Nagle JM, Deshpande V, Boucher Y, Kato T, Chen JK, Willmann JK, Bardeesy N, Beachy PA. Stromal response to Hedgehog signaling restrains pancreatic cancer progression. *Proc Natl Acad Sci USA* 2014; **111**: E3091-E3100 [PMID: 25024225 DOI: 10.1073/pnas.1411679111]

81 **Rhim AD**, Mirek ET, Aiello NM, Maitra A, Bailey JM, McAllister F, Reichert M, Beatty GL, Rustgi AK, Vonderheide RH, Leach SD, Stanger BZ. EMT and dissemination precede pancreatic tumor formation. *Cell* 2012; **148**: 349-361 [PMID: 22265420 DOI: 10.1016/j.cell.2011.11.025]

82 **Nakashima H**, Nakamura M, Yamaguchi H, Yamanaka N, Akiyoshi T, Koga K, Yamaguchi K, Tsuneyoshi M, Tanaka M, Katano M. Nuclear factor-kappaB contributes to hedgehog signaling pathway activation through sonic hedgehog induction in pancreatic cancer. *Cancer Res* 2006; **66**: 7041-7049 [PMID: 16849549 DOI: 10.1158/0008-5472.CAN-05-4588]

83 **Yauch RL**, Gould SE, Scales SJ, Tang T, Tian H, Ahn CP, Marshall D, Fu L, Januario T, Kallop D, Nannini-Pepe M, Kotkow K, Marsters JC, Rubin LL, de Sauvage FJ. A paracrine requirement for hedgehog signalling in cancer. *Nature* 2008; **455**: 406-410 [PMID: 18754008 DOI: 10.1038/nature07275]

84 **Olive KP**, Jacobetz MA, Davidson CJ, Gopinathan A, McIntyre D, Honess D, Madhu B, Goldgraben MA, Caldwell ME, Allard D, Frese KK, Denicola G, Feig C, Combs C, Winter SP, Ireland-Zecchini H, Reichelt S, Howat WJ, Chang A, Dhara M, Wang L, Rückert F, Grützmann R, Pilarsky C, Izeradjene K, Hingorani SR, Huang P, Davies SE, Plunkett W, Egorin M, Hruban RH, Whitebread N, McGovern K, Adams J, Iacobuzio-Donahue C, Griffiths J, Tuveson DA. Inhibition of Hedgehog signaling enhances delivery of chemotherapy in a mouse model of pancreatic cancer. *Science* 2009; **324**: 1457-1461 [PMID: 19460966 DOI: 10.1126/science.1171362]

85 **Singh AP**, Arora S, Bhardwaj A, Srivastava SK, Kadakia MP, Wang B, Grizzle WE, Owen LB, Singh S. CXCL12/CXCR4 protein signaling axis induces sonic hedgehog expression in pancreatic cancer cells via extracellular regulated kinase- and Akt kinase-mediated activation of nuclear factor κB: implications for bidirectional tumor-stromal interactions. *J Biol Chem* 2012; **287**: 39115-39124 [PMID: 22995914 DOI: 10.1074/jbc.M112.409581]

86 **Bailey JM**, Mohr AM, Hollingsworth MA. Sonic hedgehog paracrine signaling regulates metastasis and lymphangiogenesis in pancreatic cancer. *Oncogene* 2009; **28**: 3513-3525 [PMID: 19633682 DOI: 10.1038/onc.2009.220]

87 **Sun X**, Cheng G, Hao M, Zheng J, Zhou X, Zhang J, Taichman RS, Pienta KJ, Wang J. CXCL12/CXCR4/CXCR7 chemokine axis and cancer progression. *Cancer Metastasis Rev* 2010; **29**: 709-722 [PMID: 20839032 DOI: 10.1007/s10555-010-9256-x]

88 **Hermann PC**, Huber SL, Herrler T, Aicher A, Ellwart JW, Guba M, Bruns CJ, Heeschen C. Distinct populations of cancer stem cells determine tumor growth and metastatic activity in human pancreatic cancer. *Cell Stem Cell* 2007; **1**: 313-323 [PMID: 18371365 DOI: 10.1016/j.stem.2007.06.002]

89 **Mayr C**, Neureiter D, Pichler M, Berr F, Wagner A, Kiesslich T, Namberger K. Cytotoxic effects of chemokine receptor 4 inhibition by AMD3100 in biliary tract cancer cells: Potential drug synergism with gemcitabine. *Mol Med Rep* 2015; **12**: 2247-2252 [PMID: 25846744 DOI: 10.3892/mmr.2015.3589]

90 **Maréchal R**, Demetter P, Nagy N, Berton A, Decaestecker C, Polus M, Closset J, Devière J, Salmon I, Van Laethem JL. High expression of CXCR4 may predict poor survival in resected pancreatic adenocarcinoma. *Br J Cancer* 2009; **100**: 1444-1451 [PMID: 19352387 DOI: 10.1038/sj.bjc.6605020]

91 **Matsuo Y**, Ochi N, Sawai H, Yasuda A, Takahashi H, Funahashi H, Takeyama H, Tong Z, Guha S. CXCL8/IL-8 and CXCL12/SDF-1alpha co-operatively promote invasiveness and angiogenesis in pancreatic cancer. *Int J Cancer* 2009; **124**: 853-861 [PMID: 19035451 DOI: 10.1002/ijc.24040]

92 **Lee CJ**, Li C, Simeone DM. Human pancreatic cancer stem cells: implications for how we treat pancreatic cancer. *Transl Oncol* 2008; **1**: 14-18 [PMID: 18607507]

93 **Kashyap V**, Rezende NC, Scotland KB, Shaffer SM, Persson JL, Gudas LJ, Mongan NP. Regulation of stem cell pluripotency and differentiation involves a mutual regulatory circuit of the NANOG, OCT4, and SOX2 pluripotency transcription factors with polycomb repressive complexes and stem cell microRNAs. *Stem Cells Dev* 2009; **18**: 1093-1108 [PMID: 19480567 DOI: 10.1089/scd.2009.0113]

94 **Mueller MT**, Hermann PC, Witthauer J, Rubio-Viqueira B, Leicht SF, Huber S, Ellwart JW, Mustafa M, Bartenstein P, D'Haese JG, Schoenberg MH, Berger F, Jauch KW, Hidalgo M, Heeschen C. Combined targeted treatment to eliminate tumorigenic cancer stem cells in human pancreatic cancer. *Gastroenterology* 2009; **137**: 1102-1113 [PMID: 19501590 DOI: 10.1053/j.gastro.2009.05.053]

95 **Mayr C**, Neureiter D, Wagner A, Pichler M, Kiesslich T. The role of polycomb repressive complexes in biliary tract cancer. *Expert Opin Ther Targets* 2015; **19**: 363-375 [PMID: 25424424 DOI: 10.1517/14728222.2014.986460]

96 **Li C**, Heidt DG, Dalerba P, Burant CF, Zhang L, Adsay V, Wicha M, Clarke MF, Simeone DM. Identification of pancreatic cancer stem cells. *Cancer Res* 2007; **67**: 1030-1037 [PMID: 17283135 DOI: 10.1158/0008-5472.CAN-06-2030]

97 **Inaguma S**, Riku M, Hashimoto M, Murakami H, Saga S, Ikeda H, Kasai K. GLI1 interferes with the DNA mismatch repair system in pancreatic cancer through BHLHE41-mediated suppression of MLH1. *Cancer Res* 2013; **73**: 7313-7323 [PMID: 24165159 DOI: 10.1158/0008-5472.CAN-13-2008]

98 **Wang X**, Venugopal C, Manoranjan B, McFarlane N, O'Farrell E, Nolte S, Gunnarsson T, Hollenberg R, Kwiecien J, Northcott P, Taylor MD, Hawkins C, Singh SK. Sonic hedgehog regulates Bmi1 in human medulloblastoma brain tumor-initiating cells. *Oncogene* 2012; **31**: 187-199 [PMID: 21685941 DOI: 10.1038/onc.2011.232]

99 **Po A**, Ferretti E, Miele E, De Smaele E, Paganelli A, Canettieri G, Coni S, Di Marcotullio L, Biffoni M, Massimi L, Di Rocco

C, Screpanti I, Gulino A. Hedgehog controls neural stem cells through p53-independent regulation of Nanog. *EMBO J* 2010; **29**: 2646-2658 [PMID: 20581804 DOI: 10.1038/emboj.2010.131]

100 **Robarge KD**, Brunton SA, Castanedo GM, Cui Y, Dina MS, Goldsmith R, Gould SE, Guichert O, Gunzner JL, Halladay J, Jia W, Khojasteh C, Koehler MF, Kotkow K, La H, Lalonde RL, Lau K, Lee L, Marshall D, Marsters JC, Murray LJ, Qian C, Rubin LL, Salphati L, Stanley MS, Stibbard JH, Sutherlin DP, Ubhayaker S, Wang S, Wong S, Xie M. GDC-0449-a potent inhibitor of the hedgehog pathway. *Bioorg Med Chem Lett* 2009; **19**: 5576-5581 [PMID: 19716296 DOI: 10.1016/j.bmcl.2009.08.049]

101 **Cooper MK**, Porter JA, Young KE, Beachy PA. Teratogen-mediated inhibition of target tissue response to Shh signaling. *Science* 1998; **280**: 1603-1607 [PMID: 9616123 DOI: 10.1126/science.280.5369.1603]

102 **Pan S**, Wu X, Jiang J, Gao W, Wan Y, Cheng D, Han D, Liu J, Englund NP, Wang Y, Peukert S, Miller-Moslin K, Yuan J, Guo R, Matsumoto M, Vattay A, Jiang Y, Tsao J, Sun F, Pferdekamper AC, Dodd S, Tuntland T, Maniara W, Kelleher JF, Yao YM, Warmuth M, Williams J, Dorsch M. Discovery of NVP-LDE225, a Potent and Selective Smoothened Antagonist. *ACS Med Chem Lett* 2010; **1**: 130-134 [PMID: 24900187 DOI: 10.1021/ml1000307]

103 **Tremblay MR**, Lescarbeau A, Grogan MJ, Tan E, Lin G, Austad BC, Yu LC, Behnke ML, Nair SJ, Hagel M, White K, Conley J, Manna JD, Alvarez-Diez TM, Hoyt J, Woodward CN, Sydor JR, Pink M, MacDougall J, Campbell MJ, Cushing J, Ferguson J, Curtis MS, McGovern K, Read MA, Palombella VJ, Adams J, Castro AC. Discovery of a potent and orally active hedgehog pathway antagonist (IPI-926). *J Med Chem* 2009; **52**: 4400-4418 [PMID: 19522463 DOI: 10.1021/jm900305z]

104 **Lauth M**, Bergstrom A, Shimokawa T, Toftgard R. Inhibition of GLI-mediated transcription and tumor cell growth by small-molecule antagonists. *Proc Natl Acad Sci USA* 2007; **104**: 8455-8460 [PMID: 17494766 DOI: 10.1073/pnas.0609699104]

P- Reviewer: Boros M, Morini S **S- Editor**: Kong JX
L- Editor: A **E- Editor**: Li D

World Journal of Gastrointestinal Pathophysiology

Submit a Manuscript: http://www.wjgnet.com/esps/
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.4291/wjgp.v7.i2.211

World J Gastrointest Pathophysiol 2016 May 15; 7(2): 211-217
ISSN 2150-5330 (online)

REVIEW

Insulin resistance in development and progression of nonalcoholic fatty liver disease

Shahinul Alam, Golam Mustafa, Mahabubul Alam, Nooruddin Ahmad

Shahinul Alam, Golam Mustafa, Mahabubul Alam, Nooruddin Ahmad, Department of Hepatology, Bangabandhu Sheikh Mujib Medical University, Dhaka 1000, Bangladesh

Author contributions: All authors contributed equally to this paper in the conception and design of the study, literature review and analysis, drafting, critical revisions and editing; all authors approved the final version of the manuscript.

Conflict-of-interest statement: The authors have no potential conflicts of interest.

Correspondence to: Shahinul Alam, Associate Professor, Department of Hepatology, Bangabandhu Sheikh Mujib Medical University, Shahbag, Dhaka 1000, Bangladesh. shahinul67@yahoo.com
Telephone: +88-019-73007173

Received: August 31, 2015
Peer-review started: September 5, 2015
First decision: November 30, 2015
Revised: January 3, 2016
Accepted: March 7, 2016
Article in press: March 9, 2016
Published online: May 15, 2016

Abstract

Although insulin resistance (IR) is strongly associated with nonalcoholic fatty liver disease (NAFLD), the association of IR and NAFLD is not universal and correlation between IR and severity of NAFLD is still controversial. In this review, we summarize recent evidence that partially dissociates insulin resistance from NAFLD. It has also been reported that single-nucleotide polymorphisms in the diacylglycerol acyltransferase gene, rather than IR, account for the variability in liver fat content. Polymorphisms of the patatin-like phospholipase 3 gene have also been reported to be associated with NAFLD without metabolic syndrome, which suggests that genetic conditions that promote the development of fatty changes in the liver may occur independently of IR. Moreover, environmental factors such as nutrition and physical activity as well as small intestinal bacterial overgrowth have been linked to the pathogenesis of NAFLD, although some of the data are conflicting. Therefore, findings from both genetically engineered animal models and humans with genetic conditions, as well as recent studies that have explored the role of environmental factors, have confirmed the view that NAFLD is a polygenic disease process caused by both genetic and environmental factors. Therefore, IR is not the sole predictor of the pathogenesis of NAFLD.

Key words: Nonalcoholic fatty liver disease; Insulin resistance; Metabolic syndrome; Diabetes; Nonalcoholic steatohepatitis

Core tip: Insulin resistance is considered as the major contributor for the development and progression of nonalcoholic fatty liver disease (NAFLD). However, recent evidence that has shown that non-obese individuals from developing countries are also affected by NAFLD, thus the conventional paradigm of NAFLD as the "hepatic manifestation of metabolic syndrome" has become outdated. Recent studies have highlighted novel pathophysiological mechanisms for the development and progression of NAFLD. Insulin resistance contributes to the disease process, but it is evident that environmental and genetic factors also contribute for development of necroinflammation and subsequent

progression to fibrosis. This review provides a summary of current knowledge of the pathogenesis of NAFLD and discusses factors that dissociate insulin resistance from NAFLD.

Alam S, Mustafa G, Alam M, Ahmad N. Insulin resistance in development and progression of nonalcoholic fatty liver disease. *World J Gastrointest Pathophysiol* 2016; 7(2): 211-217 Available from: URL: http://www.wjgnet.com/2150-5330/full/v7/i2/211.htm DOI: http://dx.doi.org/10.4291/wjgp.v7.i2.211

INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is an emerging public health problem[1] due to increasing prevalence in developed and developing countries. NAFLD is the second leading cause of chronic liver diseases after hepatitis C in Western countries and affects individuals of all age groups[2]. NAFLD includes a wide spectrum of conditions that range from a simple steatosis to nonalcoholic steatohepatitis (NASH) which may further progress to cirrhosis and its complications, in absence of alcohol consumption; or a low daily consumption of alcohol (< 30 g/d for men, < 20 g/d for women)[3-5]. NAFLD has been linked to insulin resistance (IR) and other components of metabolic syndrome such as diabetes mellitus, central abdominal obesity and dyslipidemia[6]. Patients with NAFLD are at an increased risk for all-cause mortality, including liver-related deaths and non-liver-related deaths such as death due to cardiovascular disease and diabetes[7].

Recent evidence that has shown that non-obese individuals from developing countries are also affected by NAFLD; thus, the conventional paradigm of NAFLD as the "hepatic manifestation of metabolic syndrome" has become outdated[8]. Recent studies have highlighted novel pathophysiological mechanisms in the development and progression of NAFLD. IR contributes to the disease process, but it is evident that environmental and genetic factors also have the contribution in the development of necroinflammation and subsequent fibrosis. The dogma of a sequential progression of simple steatosis to NASH to cirrhosis in NAFLD is currently under scrutiny.

The pathogenesis of NAFLD is now conceptualized as a complex and multifaceted process that requires further understanding. This review provides a summary of our current understanding of these processes, particularly the evidence that IR is not the lone predictor for NAFLD, but rather, the disease is multifactorial and may be caused by the involvement of genetic and environmental factors.

RESEARCH

We searched MEDLINE, EMBASE, and PubMed using the MeSH terms "insulin resistance", "nonalcoholic fatty liver disease", and "nonalcoholic steatohepatitis". The reference lists of the articles selected for inclusion were also reviewed for additional relevant papers. The search was limited to studies that were reported in the English language and that were published between 1995 and March 2015. Articles that are specifically related to the epidemiology, diagnosis and current treatment strategies for NAFLD and NASH are summarized.

Burden of NAFLD

The reported prevalence of NAFLD from Western countries is 20%-30%, from Asian countries is approximately 15%[9-11]. In normal-weight individuals without any known metabolic risk factors, the prevalence of NAFLD is reported to be approximately 16%. However, the prevalence is much higher among high-risk groups such as diabetics (60%), patients with hyperlipidemia (90%) and obese patients undergoing bariatric surgery (91%)[9-13]. Only 20% of patients under the age of 20 have NAFLD, but among patients aged 60 and above, the prevalence is more than 40%[14]. This findings further strengthened in another study where older age is identified as an independent risk factor for disease progression from simple steatosis to NASH and for the development of fibrosis and cirrhosis[15]. Hamabe *et al*[16] showed that smoking is an independent risk factor for NAFLD. A few studies have also reported ethnic variation in the prevalence of NAFLD, but these reports present contrasting data[17,18]. The risk of mortality is higher in NASH and advanced fibrosis compared with simple steatosis[19]. The progression to advanced fibrosis has been shown to be associated with the patient's age and the degree of inflammation[20]. In a long-term longitudanal study of 129 patients with NAFLD, Ekstedt *et al*[19] explored that mortality was not increased in patients with simple steatosis but was increased in NASH patient. Although the mortality was primarily due to cardiovascular disease, liver-related deaths were more common in patients with NASH-related cirrhosis[21].

Pathogenesis

Traditional concept: The two-hit hypothesis: Day *et al*[22] first proposed the current concept of the "two-hit hypothesis in NAFLD" in 1998 (Figure 1). The first hit is primarily as a result of IR, increased dietary intake and enhanced hepatic lipogenesis there is accumulation of free fatty acids (FFAs) and triglycerides (TGs) in hepatocytes[22]. The second hits is a combination of oxidative stress, lipid peroxidation, mitochondrial dysfunction and the release of inflammatory mediators, which leads to progressive liver injury which constitute steatohepatitis and fibrosis[22]. The activation of proinflammatory pathways and toll-like receptors merge at the junction of two main intracellular signaling pathways known as nuclear factor-κB (NF-κB) and c-Jun N-terminal kinase (JNK)[23,24]. NF-κB activation has been reported in NASH and can lead to increased transcription of many proinflammatory genes, whereas JNK activation causes IR *via* the direct phosphorylation and degradation of insulin receptor substrate 1 (IRS1); this in turn reduces the intracellular signaling pathway activity downstream of the insulin receptor[23]. Lipid peroxidation can promote

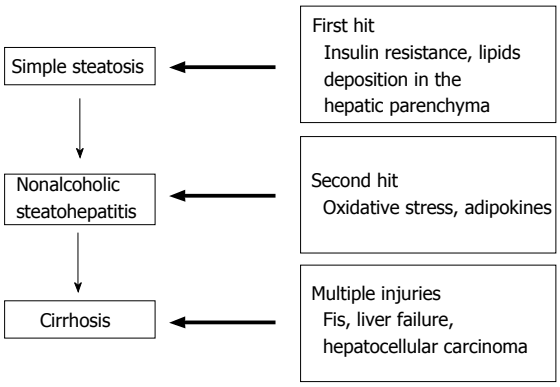


Figure 1 Two-hit hypothesis of nonalcoholic fatty liver disease (traditional view).

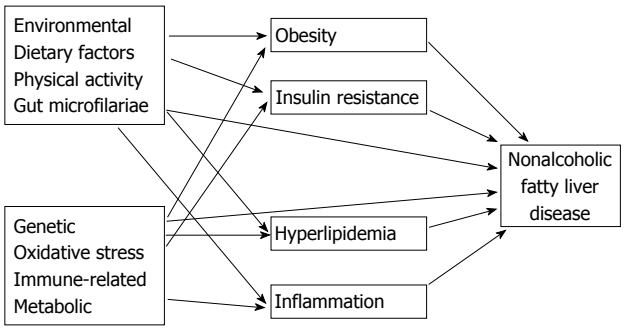


Figure 2 Interplay among environmental and genetic factors in the development of nonalcoholic fatty liver disease.

the proliferation of stellate cells, which contributes to fibrogenesis[25]. Reactive oxygen species induce the release of cytokines from hepatocytes, which leads to the initiation of various immune-mediated mechanisms that contribute to further liver cell injury. The combination of hyperinsulinemia, hepatic iron and lipid peroxidation induces oxidative stress[17], which can cause mitochondrial dysfunction in NASH and can contribute to TG accumulation and eventually to cell necrosis[11].

Multiple-hit pathogenesis

Accumulations of knowledge in recent years have challenged the traditional "two-hit" pathogenesis. Knowledge of interaction between insulin resistance, adipokines, adipose tissue inflammation and other less recognized pathogenic factors has been argued that multiple hits from adipose tissue and the gut occur at the same time and promote liver inflammation (Figure 2). This process suggests that cellular inflammation and insulin resistance occur concurrently[26,27]. Progression of NAFLD to NASH is explained by subsequent "two-hit" theory. In the "multiple-hit" model[28,29] hepatic steatosis may represent an epiphenomenon of several distinct injurious mechanisms including IR rather than a true "first hit"[30]. Hyperinsulinemia, results in increased hepatic *de novo* lipogenesis and increased adipose tissue lipolysis; leads to an increased efflux of free fatty acids to the liver[31,32]. After the initial development of steatosis, the liver becomes extremely vulnerable. Multipe series of pathogenic and injurious factors including oxidative damage, activation of transforming growth factor-beta pathway, dysregulation of multiple adipokines and apoptpsis and activation of hepatic stellate cell may lead to hepatocyte injury and finally to the progression from simple steatosis to NASH and fibrosis[33]. So multiple factors ineract in the complicated ways for development and progression of steatosis, NASH and fibrosis[14,34,35].

Distinct-hit hypothesis

A more recent model has proposed that the development of simple steatosis and NASH follows distinct pathways. The activation of these pathways is a complex process and is not only the result of a simple hepatic insult. Many other factors promote the activation of the pathways that lead to the development of steatosis and NASH[34]. The most important factors include genetic factors, the activation the hedgehog pathway and hepatic progenitor cells[36].

Role of IR in NAFLD

Studies have demonstrated that NAFLD is associated with higher IR compared with controls, even after the exclusion of overweight and obese subjects, and that IR increases with increasing degrees of steatosis[37-40]. IR in NAFLD is predominantly peripheral and occurs in the skeletal muscle and adipose tissue. Peripheral IR in the skeletal muscle causes reduced glucose uptake, which leads to hyperglycemia. In adipose tissue, IR impairs the anti-lipolytic action of insulin, which leads to an increased release of FFA. Elevated plasma concentrations of insulin, glucose, and fatty acids then impair the β-oxidation of fatty acids by negative feedback and promote the uptake of hepatic fatty acids and triglycerides, *de novo* lipid synthesis (*via* SREBP) (sterol-regulatory element-binding protein) and the expression of C/enhancer-binding protein (CCAAT/EBP). Insulin resistance also increases the amount of intra-hepatocytic fatty acids *via* an increase in glycolysis and a decrease in apolipoprotein B-100, which blocks the export of VLDL. The development of IR in NAFLD is most likely related to the imbalance between pro-insulin (adiponectin) and anti-insulin (TNFα) cytokines, specially, those secreted by adipose tissue. Alterations in several molecules, including FFAs, TNFα, membrane glycoprotein PC-1, and leptin, interfere with the insulin signaling pathway. FFAs are both the result and cause of IR. Excess FFAs cause hepatic IR *via* the down regulation of IRS1 signaling and by the activation of the inhibitor kappa B kinase (IKK-B)/NF-κB pathway. Patients with NAFLD have increased insulin resistance not only in muscle but also in liver and adipose tissue[41], and this reduced insulin sensitivity plays a major role in the pathogenesis of NAFLD. This IR, increases peripheral lipolysis in adipose tissue that leads to increase in the delivery of FFAs to the liver and *de novo* lipogenesis[17,35]. In addition, lipid overload in pancreatic-B cells leads to dysregulated insulin secretion and changes in the expression of peroxisome proliferator-activated receptor(PPAR)-α, glucokinase, the glucose

transporter-2, pre-pro-insulin and pancreatic duodenal homeobox-1, which can lead to IR as a result of FFA-induced B-cell apoptosis[12]. It has been suggested that IR in the liver is sufficient to produce dyslipidemia and increase the risk of atherosclerosis[42]. However, current evidences are not sufficient to demonstrate a consistent association between any particular type of adipokine and the histological severity of NAFLD[43].

IR IS THE CAUSE OR A CONSEQUENCE?

Although the development and progression of NAFLD is strongly associted with metabolic syndrome and IR, several studies have evidenced that all obese and diabetic dividuals dont have NAFLD. There are also evideces that NAFLD can occur in nonobese, as well as persons without metabolic syndrome[44]. Therefore, it could be hypothesized that factors other than IR could be the determinant of the development and severity of NAFLD. Familial lustering[45,46] and in the ethnic variation in the prevalence of NAFLD strengthen the initial concept[17]. Single-nucleotide polymorphisms in the adiponectin, interleukin-6, *TNFα* and *apoE* genes has been studied[47-49]. Multiethnic genome-wide association study with NAFLD revealed that the patatin-like phospholipase domain containing protein 3 (also known as adiponutrin) gene is strongly associated with hepatic TG content[50]. Allelic variants of the patatin-like phospholipase domain containing protein 3 (*PNPLA3*) genes have been found to be correlated with amounts of hepatic fat in Hispanics and African-Americans, and to be associted with prevalence of NAFLD. *PNPLA3* has also been independently identified in a separate population-based genome-wide study that influences the alanine aminotransferase (ALT) livel[51]. Environmental factors like; sedentary life styles, excess food intake, constituents of food and intestinal bacterial overgrowth have evidences to contribute in the pathogenesis of NAFLD. Obesity resulting from excess food intake and lack of exercise has been proven to contribute to the progression of fibrosis in patients with NAFLD[19]. An increased consumption of meat, soft drinks, saturated fat and cholesterol and a low consumption of fish and polyunsaturated fat (PUFA) were found to be associated with NAFLD[52-55]. Dietary supplementation with PUFA has been demonstrated in randomized control trial to be benifical in regression of fatty liver and reduction of ALT compared to dietery advice alone[56,57]. On the other hand highcarbohydrate and lowfat diets are associated with more progressive disease[58,59]. Conversely, studies in mice[60] and non-human primates[61], exposure to a maternal high-fat diet associated with development and progression of NAFLD in the offspring. Small intestinal bacterial overgrowth increases gut permeability, which leads to portal endotoxemia and increased numbers of circulating inflammatory cytokines, both of which have crucial role in the progression of NAFLD to NASH[62]. Several studies have reported an association between small intestinal bacterial overgrowth and the progression of NAFLD[63-65]. Dietary supplimentatation of probiotics and treatment with antibiotics resulted in benificial effects in NAFLD, which has furter sterngthen the concept[65].

FROM SIMPLE STEATOSIS TO NASH

Linear progression vs different entity

Although simple steatosis and NASH are currently classified as two histological subtypes of NAFLD, the two conditions are likely distinct from both a histological and a pathophysiological standpoint[34]. The American Association for the Study of Liver Diseases has recently suggested the classification of patients within the NAFLD spectrum into two main categories: NASH and "not steatohepatitis, with steatosis" ("simple steatosis")[66]. Differentiation is on histological variation where NASH is defined by the findings of lobular inflammation, portal inflammation, cellular ballooning, and fibrosis. In contrast, "not steatohepatitis, with steatosis" is characterized by simple fat infiltration with minimal/no inflammation[66]. NASH is a progressive disease, may progress to cirrhosis upto 9%-20% over a period of 5-10 years[67-69]. Vernon *et al*[9] explored that, only NASH is progressive and associated with the development of cirrhosis and hepatocellular carcinoma. In contrast, "simple steatosis" tends to be stable over time[69]. Though there is recent study of progression of steatosis to NASH and also there is progression to fibrosis[70], In agreement with these findings, Musso *et al*[71] in a meta-analysis concluded that a minority of patients with pure fatty liver will progress to NASH and only NASH seems to be associated with an increased risk of progressive liver disease[71]. Along these lines, a community based study of NAFLD outcomes has shown that no patients with simple steatosis died during a 7.6-year follow-up, whereas 35% of patients with NASH died during[69]. All these results established that NASH and "not steatohepatitis, with steatosis" are two distinct entities rather than a real progression of histological changes that can progress over time. For this reason, simple steatosis and NASH should be considered as a separate disease entity that develops along a distinct pathogenic pathway with multiple hits. The conceptualisation of these pathophysiological mechanisms would not only improve our biological understanding of NAFLD but may also allow clinicians to interven the patogenesis more accurately in future.

TREATMENT OF NAFLD

Considering that IR is a primary factor in the pathogenesis of NAFLD, several insulin sensitizers have been used in different settings. Table 1 summarizes a few of these trials. According to these trials, none of these drugs was effective, and thus further studies are warranted to identify their role. Notably, metformin was shown to improve liver injury, but this medication, which is typically used in the treatment of type 2 diabetes, could not prevent fibrosis in patients with steatosis[72]. Additionally, glitazones, which are PPARγ agonists, were found to be efficient in the management

Table 1 Insulin-sensitizing agents and anti-diabetic drug trials for halting nonalcoholic fatty liver disease progression

Insulin-sensitizing agent	Results of the study	Relevance to NAFLD	Ref.
Metformin	Improvements in liver histology and ALT levels in 30% of patients with NASH	Appears to be beneficial for NAFLD patients but not for non-obese patients with early-stage NAFLD	Loomba *et al*[72]
Pioglitazone	Improvement in the biochemical and histological features of NASH	Could be used as a treatment for NAFLD	Promrat *et al*[73]
Pioglitazone	Improvement in insulin resistance but not in hepatic fibrosis and ALT levels	Not adapted to treat NAFLD	Sanyal *et al*[74]

NASH: Nonalcoholic steatohepatitis; NAFLD: Nonalcoholic fatty liver disease; ATL: Alanine aminotransferase.

of NAFLD *via* a notable decrease in liver fibrosis[73]. In contrast, another study revealed that pioglitazone does not promote beneficial effects with respect to liver fibrosis, but it diminished inflammation and steatosis[74]. Therefore, further studies are required to elucidate these contradictory results. Additionally, salsalate, a potential anti-diabetic drug that is currently under development, has been shown to improve glycemia in diabetic patients through a downregulation of the proinflammatory IKKβ/NFκB pathway[75]. Additionally, this agent likely improves NAFLD through an induction of adiponectin[76].

CONCLUSION

Hepatic steatosis is recognized to be the consequence of a complex interplay among diet, environment and liver and adipose tissues, although a comprehensive understanding of pathogenesis of NAFLD has not yet been complete. Therefore, NAFLD is currently perceived as multifactorial pathogenic disease with both genetic and environmental factors. Genome-wide association studies have identified specific genetic associations that are involved in NAFLD. From a therapeutic point of view, pathogenic-based interventions aimed at the reversal of NAFLD are likely to be a rational approach to the prevention and treatment of hepatic IR, metabolic syndrome and related complications. Further studies are required to explore the relationship among adiponutrin mutations, steatosis and IR. A better understanding of the different factors involved in the pathophysiology of NAFLD will open the opportunity to intervene its progression in future.

REFERENCES

1 **Zafrani ES**. Non-alcoholic fatty liver disease: an emerging pathological spectrum. *Virchows Arch* 2004; **444**: 3-12 [PMID: 14685853 DOI: 10.1007/s00428-003-0943-7]

2 **Bellentani S**, Scaglioni F, Marino M, Bedogni G. Epidemiology of non-alcoholic fatty liver disease. *Dig Dis* 2010; **28**: 155-161 [PMID: 20460905 DOI: 10.1159/000282080]

3 **Younossi ZM**, Stepanova M, Rafiq N, Makhlouf H, Younoszai Z, Agrawal R, Goodman Z. Pathologic criteria for nonalcoholic steatohepatitis: interprotocol agreement and ability to predict liver-related mortality. *Hepatology* 2011; **53**: 1874-1882 [PMID: 21360720 DOI: 10.1002/hep.24268]

4 **Matteoni CA**, Younossi ZM, Gramlich T, Boparai N, Liu YC, McCullough AJ. Nonalcoholic fatty liver disease: a spectrum of clinical and pathological severity. *Gastroenterology* 1999; **116**: 1413-1419 [PMID: 10348825 DOI: 10.1016/S0016-5085(99)70506-8]

5 **Scaglioni F**, Ciccia S, Marino M, Bedogni G, Bellentani S. ASH and NASH. *Dig Dis* 2011; **29**: 202-210 [PMID: 21734385 DOI: 10.1159/000323886]

6 **Chang E**, Park CY, Park SW. Role of thiazolidinediones, insulin sensitizers, in non-alcoholic fatty liver disease. *J Diabetes Investig* 2013; **4**: 517-524 [PMID: 24843703 DOI: 10.1111/jdi.12107]

7 **Armstrong MJ**, Adams LA, Canbay A, Syn WK. Extrahepatic complications of nonalcoholic fatty liver disease. *Hepatology* 2014; **59**: 1174-1197 [PMID: 24002776 DOI: 10.1002/hep.26717]

8 **Das K**, Das K, Mukherjee PS, Ghosh A, Ghosh S, Mridha AR, Dhibar T, Bhattacharya B, Bhattacharya D, Manna B, Dhali GK, Santra A, Chowdhury A. Nonobese population in a developing country has a high prevalence of nonalcoholic fatty liver and significant liver disease. *Hepatology* 2010; **51**: 1593-1602 [PMID: 20222092 DOI: 10.1002/hep.23567]

9 **Vernon G**, Baranova A, Younossi ZM. Systematic review: the epidemiology and natural history of non-alcoholic fatty liver disease and non-alcoholic steatohepatitis in adults. *Aliment Pharmacol Ther* 2011; **34**: 274-285 [PMID: 21623852 DOI: 10.1111/j.1365-2036.2011.04724.x]

10 **Wanless IR**, Lentz JS. Fatty liver hepatitis (steatohepatitis) and obesity: an autopsy study with analysis of risk factors. *Hepatology* 1990; **12**: 1106-1110 [PMID: 2227807 DOI: 10.1002/hep.1840120505]

11 **Williamson RM**, Price JF, Glancy S, Perry E, Nee LD, Hayes PC, Frier BM, Van Look LA, Johnston GI, Reynolds RM, Strachan MW. Prevalence of and risk factors for hepatic steatosis and nonalcoholic Fatty liver disease in people with type 2 diabetes: the Edinburgh Type 2 Diabetes Study. *Diabetes Care* 2011; **34**: 1139-1144 [PMID: 21478462 DOI: 10.2337/dc10-2229]

12 **Machado M**, Marques-Vidal P, Cortez-Pinto H. Hepatic histology in obese patients undergoing bariatric surgery. *J Hepatol* 2006; **45**: 600-606 [PMID: 16899321 DOI: 10.1016/j.jhep.2006.06.013]

13 **Gaggini M**, Morelli M, Buzzigoli E, DeFronzo RA, Bugianesi E, Gastaldelli A. Non-alcoholic fatty liver disease (NAFLD) and its connection with insulin resistance, dyslipidemia, atherosclerosis and coronary heart disease. *Nutrients* 2013; **5**: 1544-1560 [PMID: 23666091 DOI: 10.3390/nu5051544]

14 **Targher G**, Marra F, Marchesini G. Increased risk of cardiovascular disease in non-alcoholic fatty liver disease: causal effect or epiphenomenon? *Diabetologia* 2008; **51**: 1947-1953 [PMID: 18762907 DOI: 10.1007/s00125-008-1135-4]

15 **Attar BM**, Van Thiel DH. Current concepts and management approaches in nonalcoholic fatty liver disease. *ScientificWorldJournal* 2013; **2013**: 481893 [PMID: 23576902 DOI: 10.1155/2013/481893]

16 **Hamabe A**, Uto H, Imamura Y, Kusano K, Mawatari S, Kumagai K, Kure T, Tamai T, Moriuchi A, Sakiyama T, Oketani M, Ido A, Tsubouchi H. Impact of cigarette smoking on onset of nonalcoholic fatty liver disease over a 10-year period. *J Gastroenterol* 2011; **46**: 769-778 [PMID: 21302121 DOI: 10.1007/s00535-011-0376-z]

17 **Browning JD**, Szczepaniak LS, Dobbins R, Nuremberg P, Horton JD, Cohen JC, Grundy SM, Hobbs HH. Prevalence of hepatic steatosis in an urban population in the United States: impact of

ethnicity. *Hepatology* 2004; **40**: 1387-1395 [PMID: 15565570 DOI: 10.1002/hep.20466]
18 **Bambha K**, Belt P, Abraham M, Wilson LA, Pabst M, Ferrell L, Unalp-Arida A, Bass N. Ethnicity and nonalcoholic fatty liver disease. *Hepatology* 2012; **55**: 769-780 [PMID: 21987488 DOI: 10.1002/hep.24726]
19 **Ekstedt M**, Franzén LE, Mathiesen UL, Thorelius L, Holmqvist M, Bodemar G, Kechagias S. Long-term follow-up of patients with NAFLD and elevated liver enzymes. *Hepatology* 2006; **44**: 865-873 [PMID: 17006923 DOI: 10.1002/hep.21327]
20 **Argo CK**, Northup PG, Al-Osaimi AM, Caldwell SH. Systematic review of risk factors for fibrosis progression in non-alcoholic steatohepatitis. *J Hepatol* 2009; **51**: 371-379 [PMID: 19501928 DOI: 10.1016/j.jhep.2009.03.019]
21 **Perazzo H**, Munteanu M, Ngo Y, Lebray P, Seurat N, Rutka F, Couteau M, Jacqueminet S, Giral P, Monneret D, Imbert-Bismut F, Ratziu V, Hartemann-Huertier A, Housset C, Poynard T. Prognostic value of liver fibrosis and steatosis biomarkers in type-2 diabetes and dyslipidaemia. *Aliment Pharmacol Ther* 2014; **40**: 1081-1093 [PMID: 25186086 DOI: 10.1111/apt.12946]
22 **Day CP**, James OF. Steatohepatitis: a tale of two "hits"? *Gastroenterology* 1998; **114**: 842-845 [PMID: 9547102 DOI: 10.1016/S0016-5085(98)70599-2]
23 **Hirosumi J**, Tuncman G, Chang L, Görgün CZ, Uysal KT, Maeda K, Karin M, Hotamisligil GS. A central role for JNK in obesity and insulin resistance. *Nature* 2002; **420**: 333-336 [PMID: 12447443 DOI: 10.1038/nature01137]
24 **Cai D**, Yuan M, Frantz DF, Melendez PA, Hansen L, Lee J, Shoelson SE. Local and systemic insulin resistance resulting from hepatic activation of IKK-beta and NF-kappaB. *Nat Med* 2005; **11**: 183-190 [PMID: 15685173 DOI: 10.1038/nm1166]
25 **Ikura Y**, Ohsawa M, Suekane T, Fukushima H, Itabe H, Jomura H, Nishiguchi S, Inoue T, Naruko T, Ehara S, Kawada N, Arakawa T, Ueda M. Localization of oxidized phosphatidylcholine in nonalcoholic fatty liver disease: impact on disease progression. *Hepatology* 2006; **43**: 506-514 [PMID: 16496325 DOI: 10.1002/hep.21070]
26 **Kim CH**, Younossi ZM. Nonalcoholic fatty liver disease: a manifestation of the metabolic syndrome. *Cleve Clin J Med* 2008; **75**: 721-728 [PMID: 18939388 DOI: 10.3949/ccjm.75.10.721]
27 **Asrih M**, Jornayvaz FR. Metabolic syndrome and nonalcoholic fatty liver disease: Is insulin resistance the link? *Mol Cell Endocrinol* 2015; **418** Pt 1: 55-65 [PMID: 25724480 DOI: 10.1016/j.mce.2015.02.018]
28 **Tilg H**, Moschen AR. Evolution of inflammation in nonalcoholic fatty liver disease: the multiple parallel hits hypothesis. *Hepatology* 2010; **52**: 1836-1846 [PMID: 21038418 DOI: 10.1002/hep.24001]
29 **Wree A**, Broderick L, Canbay A, Hoffman HM, Feldstein AE. From NAFLD to NASH to cirrhosis-new insights into disease mechanisms. *Nat Rev Gastroenterol Hepatol* 2013; **10**: 627-636 [PMID: 23958599 DOI: 10.1038/nrgastro.2013.149]
30 **Cortez-Pinto H**, de Moura MC, Day CP. Non-alcoholic steatohepatitis: from cell biology to clinical practice. *J Hepatol* 2006; **44**: 197-208 [PMID: 16274837 DOI: 10.1016/j.jhep.2005.09.002]
31 **Mehta K**, Van Thiel DH, Shah N, Mobarhan S. Nonalcoholic fatty liver disease: pathogenesis and the role of antioxidants. *Nutr Rev* 2002; **60**: 289-293 [PMID: 12296456 DOI: 10.1301/00296640232 0387224]
32 **Paradis V**, Perlemuter G, Bonvoust F, Dargere D, Parfait B, Vidaud M, Conti M, Huet S, Ba N, Buffet C, Bedossa P. High glucose and hyperinsulinemia stimulate connective tissue growth factor expression: a potential mechanism involved in progression to fibrosis in nonalcoholic steatohepatitis. *Hepatology* 2001; **34**: 738-744 [PMID: 11584370 DOI: 10.1053/jhep.2001.28055]
33 **Harrison SA**, Kadakia S, Lang KA, Schenker S. Nonalcoholic steatohepatitis: what we know in the new millennium. *Am J Gastroenterol* 2002; **97**: 2714-2724 [PMID: 12425538 DOI: 10.1016/s0002-9270(02)05486-2]
34 **Yilmaz Y**. Review article: is non-alcoholic fatty liver disease a spectrum, or are steatosis and non-alcoholic steatohepatitis distinct conditions? *Aliment Pharmacol Ther* 2012; **36**: 815-823 [PMID: 22966992 DOI: 10.1111/apt.12046]
35 **Brea A**, Puzo J. Non-alcoholic fatty liver disease and cardiovascular risk. *Int J Cardiol* 2013; **167**: 1109-1117 [PMID: 23141876 DOI: 10.1016/j.ijcard.2012.09.085]
36 **Day CP**. Non-alcoholic steatohepatitis (NASH): where are we now and where are we going? *Gut* 2002; **50**: 585-588 [PMID: 11950797 DOI: 10.1136/gut.50.5.585]
37 **Sanyal AJ**, Campbell-Sargent C, Mirshahi F, Rizzo WB, Contos MJ, Sterling RK, Luketic VA, Shiffman ML, Clore JN. Nonalcoholic steatohepatitis: association of insulin resistance and mitochondrial abnormalities. *Gastroenterology* 2001; **120**: 1183-1192 [PMID: 11266382 DOI: 10.1053/gast.2001.23256]
38 **Yki-Järvinen H**. Liver fat in the pathogenesis of insulin resistance and type 2 diabetes. *Dig Dis* 2010; **28**: 203-209 [PMID: 20460912 DOI: 10.1159/000282087]
39 **Fabbrini E**, Magkos F, Mohammed BS, Pietka T, Abumrad NA, Patterson BW, Okunade A, Klein S. Intrahepatic fat, not visceral fat, is linked with metabolic complications of obesity. *Proc Natl Acad Sci USA* 2009; **106**: 15430-15435 [PMID: 19706383 DOI: 10.1073/pnas.0904944106]
40 **Bugianesi E**, Gastaldelli A, Vanni E, Gambino R, Cassader M, Baldi S, Ponti V, Pagano G, Ferrannini E, Rizzetto M. Insulin resistance in non-diabetic patients with non-alcoholic fatty liver disease: sites and mechanisms. *Diabetologia* 2005; **48**: 634-642 [PMID: 15747110 DOI: 10.1007/s00125-005-1682-x]
41 **Marchesini G**, Brizi M, Morselli-Labate AM, Bianchi G, Bugianesi E, McCullough AJ, Forlani G, Melchionda N. Association of nonalcoholic fatty liver disease with insulin resistance. *Am J Med* 1999; **107**: 450-455 [PMID: 10569299 DOI: 10.1016/S0002-9343(99)00271-5]
42 **Brown MS**, Goldstein JL. Selective versus total insulin resistance: a pathogenic paradox. *Cell Metab* 2008; **7**: 95-96 [PMID: 18249166 DOI: 10.1016/j.cmet.2007.12.009]
43 **Farrell GC**, George J, Hall PdlM, McCullough AJ. Fatty liver disease: NASH and related disorders. Malden: John Wiley & Sons; 2008
44 **Bacon BR**, Farahvash MJ, Janney CG, Neuschwander-Tetri BA. Nonalcoholic steatohepatitis: an expanded clinical entity. *Gastroenterology* 1994; **107**: 1103-1109 [PMID: 7523217]
45 **Struben VM**, Hespenheide EE, Caldwell SH. Nonalcoholic steatohepatitis and cryptogenic cirrhosis within kindreds. *Am J Med* 2000; **108**: 9-13 [PMID: 11059435 DOI: 10.1016/S0002-9343(99)00315-0]
46 **Willner IR**, Waters B, Patil SR, Reuben A, Morelli J, Riely CA. Ninety patients with nonalcoholic steatohepatitis: insulin resistance, familial tendency, and severity of disease. *Am J Gastroenterol* 2001; **96**: 2957-2961 [PMID: 11693332 DOI: 10.1111/j.1572-0241.2001.04667.x]
47 **Musso G**, Gambino R, De Michieli F, Durazzo M, Pagano G, Cassader M. Adiponectin gene polymorphisms modulate acute adiponectin response to dietary fat: Possible pathogenetic role in NASH. *Hepatology* 2008; **47**: 1167-1177 [PMID: 18311774 DOI: 10.1002/hep.22142]
48 **Tokushige K**, Hashimoto E, Noto H, Yatsuji S, Taniai M, Torii N, Shiratori K. Influence of adiponectin gene polymorphisms in Japanese patients with non-alcoholic fatty liver disease. *J Gastroenterol* 2009; **44**: 976-982 [PMID: 19484180 DOI: 10.1007/s00535-009-0085-z]
49 **Sazci A**, Akpinar G, Aygun C, Ergul E, Senturk O, Hulagu S. Association of apolipoprotein E polymorphisms in patients with non-alcoholic steatohepatitis. *Dig Dis Sci* 2008; **53**: 3218-3224 [PMID: 18465245 DOI: 10.1007/s10620-008-0271-5]
50 **Romeo S**, Kozlitina J, Xing C, Pertsemlidis A, Cox D, Pennacchio LA, Boerwinkle E, Cohen JC, Hobbs HH. Genetic variation in PNPLA3 confers susceptibility to nonalcoholic fatty liver disease. *Nat Genet* 2008; **40**: 1461-1465 [PMID: 18820647 DOI: 10.1038/ng.257]
51 **Yuan X**, Waterworth D, Perry JR, Lim N, Song K, Chambers JC, Zhang W, Vollenweider P, Stirnadel H, Johnson T, Bergmann S, Beckmann ND, Li Y, Ferrucci L, Melzer D, Hernandez D,

Singleton A, Scott J, Elliott P, Waeber G, Cardon L, Frayling TM, Kooner JS, Mooser V. Population-based genome-wide association studies reveal six loci influencing plasma levels of liver enzymes. *Am J Hum Genet* 2008; **83**: 520-528 [PMID: 18940312 DOI: 10.1016/j.ajhg.2008.09.012]

52 **Musso G**, Gambino R, De Michieli F, Cassader M, Rizzetto M, Durazzo M, Fagà E, Silli B, Pagano G. Dietary habits and their relations to insulin resistance and postprandial lipemia in nonalcoholic steatohepatitis. *Hepatology* 2003; **37**: 909-916 [PMID: 12668986 DOI: 10.1053/jhep.2003.50132]

53 **Toshimitsu K**, Matsuura B, Ohkubo I, Niiya T, Furukawa S, Hiasa Y, Kawamura M, Ebihara K, Onji M. Dietary habits and nutrient intake in non-alcoholic steatohepatitis. *Nutrition* 2007; **23**: 46-52 [PMID: 17140767 DOI: 10.1016/j.nut.2006.09.004]

54 **Zelber-Sagi S**, Nitzan-Kaluski D, Goldsmith R, Webb M, Blendis L, Halpern Z, Oren R. Long term nutritional intake and the risk for non-alcoholic fatty liver disease (NAFLD): a population based study. *J Hepatol* 2007; **47**: 711-717 [PMID: 17850914 DOI: 10.1016/j.jhep.2007.06.020]

55 **Kim CH**, Kallman JB, Bai C, Pawloski L, Gewa C, Arsalla A, Sabatella ME, Younossi ZM. Nutritional assessments of patients with non-alcoholic fatty liver disease. *Obes Surg* 2010; **20**: 154-160 [PMID: 18560947 DOI: 10.1007/s11695-008-9549-0]

56 **Spadaro L**, Magliocco O, Spampinato D, Piro S, Oliveri C, Alagona C, Papa G, Rabuazzo AM, Purrello F. Effects of n-3 polyunsaturated fatty acids in subjects with nonalcoholic fatty liver disease. *Dig Liver Dis* 2008; **40**: 194-199 [PMID: 18054848 DOI: 10.1016/j.dld.2007.10.003]

57 **Zhu FS**, Liu S, Chen XM, Huang ZG, Zhang DW. Effects of n-3 polyunsaturated fatty acids from seal oils on nonalcoholic fatty liver disease associated with hyperlipidemia. *World J Gastroenterol* 2008; **14**: 6395-6400 [PMID: 19009658 DOI: 10.3748/wjg.14.6395]

58 **Solga S**, Alkhuraishe AR, Clark JM, Torbenson M, Greenwald A, Diehl AM, Magnuson T. Dietary composition and nonalcoholic fatty liver disease. *Dig Dis Sci* 2004; **49**: 1578-1583 [PMID: 15573908]

59 **Kang H**, Greenson JK, Omo JT, Chao C, Peterman D, Anderson L, Foess-Wood L, Sherbondy MA, Conjeevaram HS. Metabolic syndrome is associated with greater histologic severity, higher carbohydrate, and lower fat diet in patients with NAFLD. *Am J Gastroenterol* 2006; **101**: 2247-2253 [PMID: 17032189 DOI: 10.1111/j.1572-0241.2006.00719.x]

60 **Bruce KD**, Cagampang FR, Argenton M, Zhang J, Ethirajan PL, Burdge GC, Bateman AC, Clough GF, Poston L, Hanson MA, McConnell JM, Byrne CD. Maternal high-fat feeding primes steatohepatitis in adult mice offspring, involving mitochondrial dysfunction and altered lipogenesis gene expression. *Hepatology* 2009; **50**: 1796-1808 [PMID: 19816994 DOI: 10.1002/hep.23205]

61 **McCurdy CE**, Bishop JM, Williams SM, Grayson BE, Smith MS, Friedman JE, Grove KL. Maternal high-fat diet triggers lipotoxicity in the fetal livers of nonhuman primates. *J Clin Invest* 2009; **119**: 323-335 [PMID: 19147984 DOI: 10.1172/jci32661]

62 **Brun P**, Castagliuolo I, Di Leo V, Buda A, Pinzani M, Palù G, Martines D. Increased intestinal permeability in obese mice: new evidence in the pathogenesis of nonalcoholic steatohepatitis. *Am J Physiol Gastrointest Liver Physiol* 2007; **292**: G518-G525 [PMID: 17023554]

63 **Wigg AJ**, Roberts-Thomson IC, Dymock RB, McCarthy PJ, Grose RH, Cummins AG. The role of small intestinal bacterial overgrowth, intestinal permeability, endotoxaemia, and tumour necrosis factor alpha in the pathogenesis of non-alcoholic steatohepatitis. *Gut* 2001; **48**: 206-211 [PMID: 11156641 DOI: 10.1136/gut.48.2.206]

64 **Sabaté JM**, Jouët P, Harnois F, Mechler C, Msika S, Grossin M, Coffin B. High prevalence of small intestinal bacterial overgrowth in patients with morbid obesity: a contributor to severe hepatic steatosis. *Obes Surg* 2008; **18**: 371-377 [PMID: 18286348 DOI: 10.1007/s11695-007-9398-2]

65 **Sajjad A**, Mottershead M, Syn WK, Jones R, Smith S, Nwokolo CU. Ciprofloxacin suppresses bacterial overgrowth, increases fasting insulin but does not correct low acylated ghrelin concentration in non-alcoholic steatohepatitis. *Aliment Pharmacol Ther* 2005; **22**: 291-299 [PMID: 16097995 DOI: 10.1111/j.1365-2036.2005.02562.x]

66 **Sanyal AJ**, Brunt EM, Kleiner DE, Kowdley KV, Chalasani N, Lavine JE, Ratziu V, McCullough A. Endpoints and clinical trial design for nonalcoholic steatohepatitis. *Hepatology* 2011; **54**: 344-353 [PMID: 21520200 DOI: 10.1002/hep.24376]

67 **Harrison SA**, Torgerson S, Hayashi PH. The natural history of nonalcoholic fatty liver disease: a clinical histopathological study. *Am J Gastroenterol* 2003; **98**: 2042-2047 [PMID: 14499785 DOI: 10.1111/j.1572-0241.2003.07659.x]

68 **Ong JP**, Younossi ZM. Nonalcoholic fatty liver disease (NAFLD)--two decades later: are we smarter about its natural history? *Am J Gastroenterol* 2003; **98**: 1915-1917 [PMID: 14499766 DOI: 10.1111/j.1572-0241.2003.07667.x]

69 **Adams LA**, Lymp JF, St Sauver J, Sanderson SO, Lindor KD, Feldstein A, Angulo P. The natural history of nonalcoholic fatty liver disease: a population-based cohort study. *Gastroenterology* 2005; **129**: 113-121 [PMID: 16012941 DOI: 10.1053/j.gastro.2005.04.014]

70 **McPherson S**, Hardy T, Henderson E, Burt AD, Day CP, Anstee QM. Evidence of NAFLD progression from steatosis to fibrosing-steatohepatitis using paired biopsies: implications for prognosis and clinical management. *J Hepatol* 2015; **62**: 1148-1155 [PMID: 25477264 DOI: 10.1016/j.jhep.2014.11.034]

71 **Musso G**, Gambino R, Cassader M, Pagano G. Meta-analysis: natural history of non-alcoholic fatty liver disease (NAFLD) and diagnostic accuracy of non-invasive tests for liver disease severity. *Ann Med* 2011; **43**: 617-649 [PMID: 21039302 DOI: 10.3109/07853890.2010.518623]

72 **Loomba R**, Lutchman G, Kleiner DE, Ricks M, Feld JJ, Borg BB, Modi A, Nagabhyru P, Sumner AE, Liang TJ, Hoofnagle JH. Clinical trial: pilot study of metformin for the treatment of non-alcoholic steatohepatitis. *Aliment Pharmacol Ther* 2009; **29**: 172-182 [PMID: 18945255 DOI: 10.1111/j.1365-2036.2008.03869.x]

73 **Promrat K**, Lutchman G, Uwaifo GI, Freedman RJ, Soza A, Heller T, Doo E, Ghany M, Premkumar A, Park Y, Liang TJ, Yanovski JA, Kleiner DE, Hoofnagle JH. A pilot study of pioglitazone treatment for nonalcoholic steatohepatitis. *Hepatology* 2004; **39**: 188-196 [PMID: 14752837 DOI: 10.1002/hep.20012]

74 **Sanyal AJ**, Chalasani N, Kowdley KV, McCullough A, Diehl AM, Bass NM, Neuschwander-Tetri BA, Lavine JE, Tonascia J, Unalp A, Van Natta M, Clark J, Brunt EM, Kleiner DE, Hoofnagle JH, Robuck PR. Pioglitazone, vitamin E, or placebo for nonalcoholic steatohepatitis. *N Engl J Med* 2010; **362**: 1675-1685 [PMID: 20427778 DOI: 10.1056/NEJMoa0907929]

75 **Goldfine AB**, Silver R, Aldhahi W, Cai D, Tatro E, Lee J, Shoelson SE. Use of salsalate to target inflammation in the treatment of insulin resistance and type 2 diabetes. *Clin Transl Sci* 2008; **1**: 36-43 [PMID: 19337387]

76 **Jung TW**, Choi HY, Lee SY, Hong HC, Yang SJ, Yoo HJ, Youn B-S, Baik SH, Choi KM. Salsalate and Adiponectin Improve Palmitate-Induced Insulin Resistance via Inhibition of Selenoprotein P through the AMPK-FOXO1α Pathway. *PLoS One* 2013; **8**: e66529 [PMID: 23825542 DOI: 10.1371/journal.pone.0066529]

P- Reviewer: Hwu CM, Ikura Y **S- Editor**: Qiu S
L- Editor: A **E- Editor**: Li D

World Journal of Gastrointestinal Pathophysiology

Submit a Manuscript: http://www.wjgnet.com/esps/
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.4291/wjgp.v7.i2.218

World J Gastrointest Pathophysiol 2016 May 15; 7(2): 218-222
ISSN 2150-5330 (online)

Clinical impacts of mesothelin expression in gastrointestinal carcinomas

Takahiro Einama, Futoshi Kawamata, Hirofumi Kamachi, Hiroshi Nishihara, Shigenori Homma, Fumihiko Matsuzawa, Tatsuzo Mizukami, Yuji Konishi, Munenori Tahara, Toshiya Kamiyama, Okio Hino, Akinobu Taketomi, Satoru Todo

Takahiro Einama, Futoshi Kawamata, Hirofumi Kamachi, Shigenori Homma, Fumihiko Matsuzawa, Tatsuzo Mizukami, Yuji Konishi, Munenori Tahara, Toshiya Kamiyama, Akinobu Taketomi, Department of General Surgery, Hokkaido University Graduate School of Medicine, Sapporo, Hokkaido 060-8638, Japan

Hiroshi Nishihara, Department of Translational Pathology, Hokkaido University Graduate School of Medicine, Sapporo, Hokkaido 060-8638, Japan

Okio Hino, Department of Pathology and Oncology, Juntendo University School of Medicine, Tokyo 113-8421, Japan

Satoru Todo, Research institute of St. Mary's Hospital, Kurume 830-8558, Japan

Author contributions: All authors equally contributed to this paper regarding its conception, literature review, editing and approval of the final version.

Conflict-of-interest statement: No potential conflicts of interest.

Correspondence to: Takahiro Einama, MD, PhD, Department of General Surgery, Hokkaido University Graduate School of Medicine, Kita-Ku, Kita 14, Nishi 7, Sapporo, Hokkaido 060-8638, Japan. titiuehahaue@hotmail.com
Telephone: +81-11-7065927
Fax: +81-11-7177515

Received: September 29, 2015
Peer-review started: October 2, 2015
First decision: November 4, 2015
Revised: December 8, 2015
Accepted: January 27, 2016
Article in press: January 29, 2016
Published online: May 15, 2016

Abstract

Mesothelin, C-ERC/mesothelin is a 40-kDa cell surface glycoprotein that is normally present on normal mesothelial cells lining the pleura, peritoneum, and pericardium. Moreover, mesothelin has been shown to be overexpressed in several human cancers, including virtually all mesothelioma and pancreatic cancer, approximately 70% of ovarian cancer and extra bile duct cancer, and 50% of lung adenocarcinomas and gastric cancer. The full-length human mesothelin gene encodes the primary product, a 71-kDa precursor protein. The 71-kDa mesothelin precursor is cleaved into two products, 40-kDa C-terminal fragment that remains membrane-bound *via* glycosylphosphatidylinositol anchor, and a 31-kDa N-terminal fragment, megakaryocyte potentiating factor, which is secreted into the blood. The biological functions of mesothelin remain largely unknown. However, results of recent studies have suggested that the mesothelin may play a role of cell proliferation and migration. In pancreatic cancer, mesothelin expression was immunohistochemically observed in all cases, but absent in normal pancreas and in chronic pancreatitis. Furthermore, the expression of mesothelin was correlated with an poorer patient outcome in several human cancers. The limited mesothelin expression in normal tissues and high expression in many cancers makes it an attractive candidate for cancer therapy. The present review discusses the expression and function of mesothelin in cancer cells and the utility of mesothelin as

a target of cancer therapy.

Key words: Mesothelin; Luminal membrane expression; Cytoplasmic expression; Tumor marker; Cancer therapy

Core tip: Mesothelin is a 40-kDa cell surface glycoprotein expressed on normal mesothelial cells lining the pleura, pericardium, and peritoneum. Moreover, mesothelin has been shown to be overexpressed in several cancer types. Recent studies have suggested that the overexpression of mesothelin increases cell proliferation and migration. Furthermore, the expression of mesothelin was related to an unfavourable patient outcome in several human cancers. The limited mesothelin expression in normal tissues and high expression in many cancers makes it an attractive candidate for cancer therapy.

Einama T, Kawamata F, Kamachi H, Nishihara H, Homma S, Matsuzawa F, Mizukami T, Konishi Y, Tahara M, Kamiyama T, Hino O, Taketomi A, Todo S. Clinical impacts of mesothelin expression in gastrointestinal carcinomas. *World J Gastrointest Pathophysiol* 2016; 7(2): 218-222 Available from: URL: http://www.wjgnet.com/2150-5330/full/v7/i2/218.htm DOI: http://dx.doi.org/10.4291/wjgp.v7.i2.218

INTRODUCTION

Mesothelin is a 40-kDa cell surface glycoprotein that is normally present on normal mesothelial cells lining the pleura, peritoneum, and pericardium[1,2]. Moreover, mesothelin has been shown to be overexpressed in several human cancers, including virtually all mesothelioma and pancreatic cancer, approximately 70% of ovarian cancer and extra bile duct cancer, and 50% of lung adenocarcinomas and gastric cancer[3-6] (Table 1). The full-length human mesothelin gene (Full-ERC/mesothelin) encodes a 71-kDa precursor protein. The 71-kDa mesothelin precursor is cleaved into two products, 40-kDa C-terminal fragment (C-ERC/mesothelin) that remains membrane-bound *via* glycosylphosphatidylinositol anchor[7], and a 31-kDa N-terminal fragment (N-ERC/mesothelin, megakaryocyte potentiating factor), which is secreted into the blood (Figure 1)[1]. The function of mesothelin in cancer is still unclear. However, results of recent studies have suggested that the mesothelin may play a role of tumor progression *in vitro*[8-11] and *in vivo*[11,12].

MESOTHELIN EXPRESSION IN GASTROINTESTINAL CANCERS

Co-expression of mesothelin and CA125 correlates with unfavorable patient outcome in pancreatic ductal adenocarcinoma

Mesothelin could play a role of the binding to CA125[13-15]. Mesothelin and CA125 binding may be important in the peritoneal spread[13,15]. In ovarian cancer, advanced clinical stage and/or high histological grade patients showed mesothelin expression and CA125 expressions[15]. Our group showed that the co-expression of mesothelin and CA125 group was a higher histological grade and a higher level of blood vessel permeation and correlated with recurrence rate and poor patient outcome in pancreatic ductal adenocarcinoma[16]. These findings suggest that the co-expression of mesothelin and CA125 may lead to tumor development, metastasis, and a poorer patient prognosis.

Luminal membrane expression of mesothelin is a prominent poor prognostic factor

The expression of mesothelin was related to an unfavorable patient outcome in pancreatic ductal adenocarcinoma[16,17]. Our group investigated mesothelin expression in gastric cancers by using immunohistochemistry, especially focusing on the localization of mesothelin, *i.e.,* "luminal membrane-positive" and/or "cytoplasm-positive" (Figure 2)[18].

The overall survival revealed that the "luminal membrane-positive" group showed a significantly poorer outcome compared to the "luminal membrane-negative" group. On the other hand, the "mesothelin-positive" group and the "cytoplasmic-positive" group were not correlated with overall survival in the gastric cancer patients.

Intraductal papillary mucinous neoplasm (IPMN) of the pancreas has a histological spectrum ranging from benign adenoma to invasive cancer. We performed an immunohistochemical analysis of mesotheelin expression in IPMN. Mesothelin was absent in all of the normal pancreatic tissues. But, mesothelin was expressed in both adenoma and carcinoma cells. Most of mesothelin expressed adenoma cells exhibited slight "cytoplasmic-positive", and the "luminal membrane-positive" group has a tendency of poor prognosis and high recurrence rate[19].

Based on these results, the "luminal membrane-positive" of mesothelin is a useful prognostic factor, implying that membrane-localized mesothelin might have the significant function of the aggressive behavior in the cancer cells.

THE ROLE OF MESOTHELIN EXPRESSION IN TUMOR BIOLOGY

Our study generated the novel finding, the potential role of the "luminal membrane-positive" mesothelin in the malignant behavior of tumor cells[18-21]. The human mesothelin gene encodes a 71-kDa precursor protein (Full-ERC/mesothelin). This precursor protein is cleaved by furin-like proteases into a 31-kDa N-terminal secreted form (N-ERC/mesothelin) and a C-terminal fragment, 40-kDa mesothelin (C-ERC/mesothelin)[1,7,22]. The 5B2 anti-mesothelin antibody, which we used in our studies,

Table 1 Mesothelin expression in human cancer detected by immunohistochemistry

Tumour	Mesothelin expressions (%)	Comments	Ref.
Pancreatic cancer	86-100	Co-expression of mesothelin and CA125 group associated with a poorer patient prognosis	[6,16,17]
Gastric cancer	29-59	Luminal membrane expression is one of the poor prognostic factors	[6,18,27,35]
Extrahepatic bile duct cancer	72-100	Luminal membrane expression or cytoplasmic expression of mesothelin could be a reliable prognostic factor	[6,21]
Colorectal cancer	28-58	Luminal membrane expression was associated with lymphatic invasion	[6,20]
Intraductal papillary mucinous neoplasm	57	Luminal membrane expression was correlated with the histological classification of the tumor and the recurrence rate	[19]

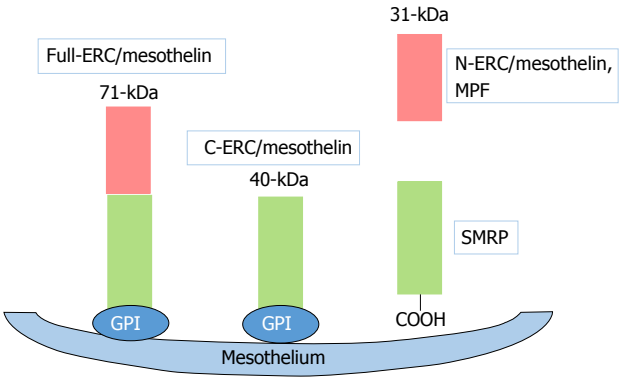


Figure 1 Schematics showing the maturation of mesothelin protein. The primary product of the full-ERC/mesothelin gene is a 71-kDa precursor protein. This protein is physiologically cleaved, releasing a 31-kDa fragment, N-ERC/mesothelin, into the blood. MPF: Megakaryocyte potentiating factor; SMRP: Soluble mesothelin-related peptide; GPI: Glycosylphosphatidylinositol.

can detect the 71-kDa precursor protein (Full-ERC/mesothelin) and the 40-kDa C-terminal fragment (C-ERC/mesothelin), but not the 30-kDa N-terminal fragment (N-ERC/mesothelin). Based on the specificity of this antibody, the "luminal membrane-positive" mesothelin observed in our study might have indicated the existence of 40-kDa mesothelin (C-ERC/mesothelin) membrane-bound form, while the "cytoplasmic-positive" mesothelin might have indicated the presence of the the 71-kDa precursor protein (Full-ERC/mesothelin). To demonstrate the mechanism of the membranous localization of mesothelin, we enforced the expression of Full-, C-, and N-ERC/mesothelin in human colorectal cancer (CRC) cell lines[20]. The 7E7 antibody, which recognizes the 30-kDa N-terminal fragment (N-ERC/mesothelin), revealed the diffuse cytoplasmic expression of Full- and N-ERC/mesothelin in Full-WiDr and N-WiDr. In contrast, the 22A31 antibody, which recognizes 40-kDa mesothelin (C-ERC/mesothelin), demonstrated a dot-like expression of Full- and C-ERC/mesothelin in Full-WiDr and C-WiDr. Moreover, some of the dot-like spots along with the cellular membrane were merged with actin, showing yellow signals. According to these results, we confirmed the membranous expressions of C-ERC/mesothelin in CRC cell lines.

To demonstrate the biological role of Full-, C-, and N-ERC/mesothelin in the lymphatic invasion of CRC, we performed an *in vitro* lymphatic invasion assay. C-ERC/mesothelin, the 40-kDa membrane-localized fragment, promoted the lymphatic invasion by increasing cell adhesion to lymphatic endothelial cells.

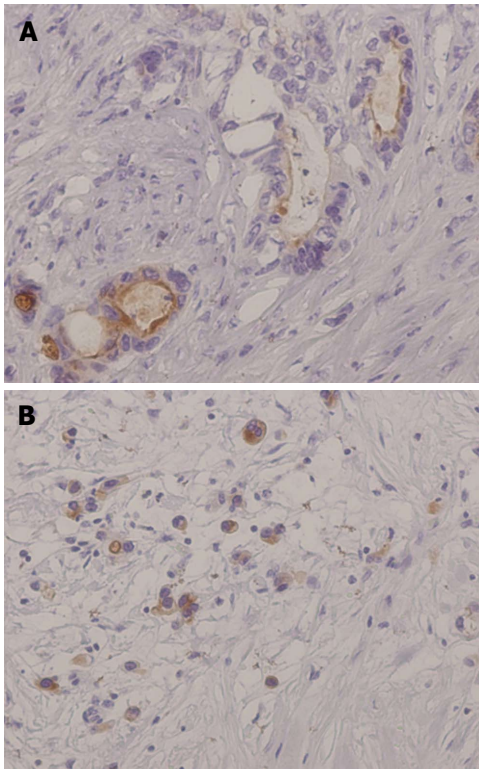


Figure 2 The expression of mesothelin in gastric cancer. A: Luminal membrane expression. The entire circumference of the cell membrane was stained; B: "Cytoplasmic expression" with granular cytoplasmic staining in cancer cells.

THE PATHWAYS OF MESOTHELIN INVOLVED IN CANCER

Recent studies reported that mesothelin is not only associated with increased cell proliferation and the migration of pancreatic cancer cells *in vitro*[11,23], but also contributes to tumor progression *in vivo*[11]. Mesothelin protects cancer cells from paclitaxel-induced apoptosis through both the concomitant activation of PI3K/Akt and MAPK/ERK pathways[24]. Overexpression of mesothelin in pancreatic cancer cells leads to constitutive activation of signal transducer and activator of transcription 3, which results in enhanced expression of cyclin E and cyclin

E/cyclin-dependent kinase 2 complex formation as well as increased G1-S transition[23]. Mesothelin expression correlated closely with interleukin (IL)-6 in human pancreatic cancer specimens and cell lines. Cancer cell with forced mesothelin expression grow faster than control cells by producing higher quantities of IL-6[9,10].

BLOOD TEST FOR MESOTHELIN

Several ELISAs have been developed to measure the levels of soluble mesothelin-related peptide (SMRP) and megakaryocyte potentiating factor (MPF, N-ERC/mesothelin). The soluble form of mesothelin is likely due to an abnormal splicing event resulting in a frameshift mutation and premature termination at amino acid 600 deleting the amino acids at the COOH terminus that are responsible for its association with the cell membrane. The full-length human mesothelin gene encodes the primary product, a 71-kDa precursor protein. It can be physiologically cleaved by some furin-like proteases into a 40-kDa C-terminal fragment that remains membrane-bound, and a 31-kDa N-terminal fragment, which is secreted into the blood. The C-terminal 40-kDa fragment is referred to as mesothelin. In contrast, the N-terminal 31-kDa fragment is a secreted protein identified as MPF. SMRP has proven to be a promising cancer biomarker in the sera of patients with tumors of mesothelial origin[25,26]. MPF has been reported to be expressed in gastrointestinal cancers[27,28].

Wu *et al*[29] revealed that SMRP performs better than CA125 as a tumor marker for epithelial ovarian cancer, it increases only in malignant patients and not in benign patients or healthy volunteers. Furthermore, the sensitivity is enhanced when combined with CA125. Hassan *et al*[30] identified a positive correlation with the tumor burden and SMRP levels, as a marker for monitoring the response to treatment o malignant mesothelioma.

MESOTHELIN TARGET IMMUNOTHERAPY

Because of the high expression of mesothelin in many malignancies and its limited expression in normal tissues, mesothelin has been suggested as an attractive target for immunotherapy. Several therapeutic agents that target mesothelin have been developed and some are being evaluated in preclinical and clinical studies. SS1P is an immunotoxin being clinically tested as a systemic agent in solid tumor patients. Two phase Ⅰ trials of single-agent SS1P have been performed[31,32]. The majority of patients developed antidrug antibodies by the end of their first cycle, resulting in non-therapeutic drug levels if any additional cycles were given. MORAb-009 (amatuximab) is a chimeric antibody. A phase Ⅰ clinical trial of MORAb-009 for mesothelioma, pancreatic cancer, and ovarian cancer patients has been completed[33]. Eleven of 24 subjects had stable disease. Phase Ⅱ studies of MORAb-009 in different mesothelin-expressing cancers are ongoing. The mesothelin tumor vaccine in clinical development is CRS-207. The safety of this vaccine was established in a phase Ⅰ clinical trial of patients with mesothelin-expressing refractory cancers[34].

CONCLUSION

Mesothelin is an attractive antigen that is expressed in several gastrointestinal cancers. Recent studies have revealed oncogenic functions of mesothelin in cancer proliferation and invasion and drug resistance. Also, soluble mesothelin could be useful as a tumor marker. The limited mesothelin expression in normal tissues and high expression in many cancers makes it an attractive candidate for cancer therapy.

REFERENCES

1 **Chang K**, Pastan I. Molecular cloning of mesothelin, a differentiation antigen present on mesothelium, mesotheliomas, and ovarian cancers. *Proc Natl Acad Sci USA* 1996; **93**: 136-140 [PMID: 8552591 DOI: 10.1073/pnas.93.1.136]

2 **Chang K**, Pastan I, Willingham MC. Isolation and characterization of a monoclonal antibody, K1, reactive with ovarian cancers and normal mesothelium. *Int J Cancer* 1992; **50**: 373-381 [PMID: 1735605 DOI: 10.1002/ijc.2910500308]

3 **Argani P**, Iacobuzio-Donahue C, Ryu B, Rosty C, Goggins M, Wilentz RE, Murugesan SR, Leach SD, Jaffee E, Yeo CJ, Cameron JL, Kern SE, Hruban RH. Mesothelin is overexpressed in the vast majority of ductal adenocarcinomas of the pancreas: identification of a new pancreatic cancer marker by serial analysis of gene expression (SAGE). *Clin Cancer Res* 2001; **7**: 3862-3868 [PMID: 11751476]

4 **Hassan R**, Kreitman RJ, Pastan I, Willingham MC. Localization of mesothelin in epithelial ovarian cancer. *Appl Immunohistochem Mol Morphol* 2005; **13**: 243-247 [PMID: 16082249 DOI: 10.1097/01.pai.00000141545.36485.d6]

5 **Ordóñez NG**. Value of mesothelin immunostaining in the diagnosis of mesothelioma. *Mod Pathol* 2003; **16**: 192-197 [PMID: 12640097 DOI: 10.1097/01.MP.0000056981.16578.C3]

6 **Ordóñez NG**. Application of mesothelin immunostaining in tumor diagnosis. *Am J Surg Pathol* 2003; **27**: 1418-1428 [PMID: 14576474 DOI: 10.1097/00000478-200311000-00003]

7 **Hassan R**, Bera T, Pastan I. Mesothelin: a new target for immunotherapy. *Clin Cancer Res* 2004; **10**: 3937-3942 [PMID: 15217923 DOI: 10.1158/1078-0432.CCR-03-0801]

8 **Wang K**, Bodempudi V, Liu Z, Borrego-Diaz E, Yamoutpoor F, Meyer A, Woo RA, Pan W, Dudek AZ, Olyaee MS, Esfandyari T, Farassati F. Inhibition of mesothelin as a novel strategy for targeting cancer cells. *PLoS One* 2012; **7**: e33214 [PMID: 22485139 DOI: 10.1371/journal.pone.0033214]

9 **Bharadwaj U**, Marin-Muller C, Li M, Chen C, Yao Q. Mesothelin overexpression promotes autocrine IL-6/sIL-6R trans-signaling to stimulate pancreatic cancer cell proliferation. *Carcinogenesis* 2011; **32**: 1013-1024 [PMID: 21515913 DOI: 10.1093/carcin/bgr075]

10 **Bharadwaj U**, Marin-Muller C, Li M, Chen C, Yao Q. Mesothelin confers pancreatic cancer cell resistance to TNF-α-induced apoptosis through Akt/PI3K/NF-κB activation and IL-6/Mcl-1 overexpression. *Mol Cancer* 2011; **10**: 106 [PMID: 21880146 DOI: 10.1186/1476-4598-10-106]

11 **Li M**, Bharadwaj U, Zhang R, Zhang S, Mu H, Fisher WE, Brunicardi FC, Chen C, Yao Q. Mesothelin is a malignant factor and therapeutic vaccine target for pancreatic cancer. *Mol Cancer Ther* 2008; **7**: 286-296 [PMID: 18281514 DOI: 10.1158/1535-7163.MCT-07-0483]

12 **Servais EL**, Colovos C, Rodriguez L, Bograd AJ, Nitadori J, Sima C, Rusch VW, Sadelain M, Adusumilli PS. Mesothelin overexpression promotes mesothelioma cell invasion and MMP-9 secretion in an orthotopic mouse model and in epithelioid pleural mesothelioma

patients. *Clin Cancer Res* 2012; **18**: 2478-2489 [PMID: 22371455 DOI: 10.1158/1078-0432.CCR-11-2614]
13 **Gubbels JA**, Belisle J, Onda M, Rancourt C, Migneault M, Ho M, Bera TK, Connor J, Sathyanarayana BK, Lee B, Pastan I, Patankar MS. Mesothelin-MUC16 binding is a high affinity, N-glycan dependent interaction that facilitates peritoneal metastasis of ovarian tumors. *Mol Cancer* 2006; **5**: 50 [PMID: 17067392 DOI: 10.1186/1476-4598-5-50]
14 **Kaneko O**, Gong L, Zhang J, Hansen JK, Hassan R, Lee B, Ho M. A binding domain on mesothelin for CA125/MUC16. *J Biol Chem* 2009; **284**: 3739-3749 [PMID: 19075018 DOI: 10.1074/jbc.M806776200]
15 **Rump A**, Morikawa Y, Tanaka M, Minami S, Umesaki N, Takeuchi M, Miyajima A. Binding of ovarian cancer antigen CA125/MUC16 to mesothelin mediates cell adhesion. *J Biol Chem* 2004; **279**: 9190-9198 [PMID: 14676194 DOI: 10.1074/jbc.M312372200]
16 **Einama T**, Kamachi H, Nishihara H, Homma S, Kanno H, Takahashi K, Sasaki A, Tahara M, Okada K, Muraoka S, Kamiyama T, Matsuno Y, Ozaki M, Todo S. Co-expression of mesothelin and CA125 correlates with unfavorable patient outcome in pancreatic ductal adenocarcinoma. *Pancreas* 2011; **40**: 1276-1282 [PMID: 21775916 DOI: 10.1097/MPA.0b013e318221bed8]
17 **Shimizu A**, Hirono S, Tani M, Kawai M, Okada K, Miyazawa M, Kitahata Y, Nakamura Y, Noda T, Yokoyama S, Yamaue H. Coexpression of MUC16 and mesothelin is related to the invasion process in pancreatic ductal adenocarcinoma. *Cancer Sci* 2012; **103**: 739-746 [PMID: 22320398 DOI: 10.1111/j.1349-7006.2012.02214.x]
18 **Einama T**, Homma S, Kamachi H, Kawamata F, Takahashi K, Takahashi N, Taniguchi M, Kamiyama T, Furukawa H, Matsuno Y, Tanaka S, Nishihara H, Taketomi A, Todo S. Luminal membrane expression of mesothelin is a prominent poor prognostic factor for gastric cancer. *Br J Cancer* 2012; **107**: 137-142 [PMID: 22644300 DOI: 10.1038/bjc.2012.235]
19 **Einama T**, Kamachi H, Nishihara H, Homma S, Kanno H, Ishikawa M, Kawamata F, Konishi Y, Sato M, Tahara M, Okada K, Muraoka S, Kamiyama T, Taketomi A, Matsuno Y, Furukawa H, Todo S. Importance of luminal membrane mesothelin expression in intraductal papillary mucinous neoplasms. *Oncol Lett* 2015; **9**: 1583-1589 [PMID: 25789005 DOI: 10.3892/ol.2015.2969]
20 **Kawamata F**, Homma S, Kamachi H, Einama T, Kato Y, Tsuda M, Tanaka S, Maeda M, Kajino K, Hino O, Takahashi N, Kamiyama T, Nishihara H, Taketomi A, Todo S. C-ERC/mesothelin provokes lymphatic invasion of colorectal adenocarcinoma. *J Gastroenterol* 2014; **49**: 81-92 [PMID: 23512344 DOI: 10.1007/s00535-013-0773-6]
21 **Kawamata F**, Kamachi H, Einama T, Homma S, Tahara M, Miyazaki M, Tanaka S, Kamiyama T, Nishihara H, Taketomi A, Todo S. Intracellular localization of mesothelin predicts patient prognosis of extrahepatic bile duct cancer. *Int J Oncol* 2012; **41**: 2109-2118 [PMID: 23064529 DOI: 10.3892/ijo.2012.1662]
22 **Cheng WF**, Huang CY, Chang MC, Hu YH, Chiang YC, Chen YL, Hsieh CY, Chen CA. High mesothelin correlates with chemoresistance and poor survival in epithelial ovarian carcinoma. *Br J Cancer* 2009; **100**: 1144-1153 [PMID: 19293794 DOI: 10.1038/sj.bjc.6604964]
23 **Bharadwaj U**, Li M, Chen C, Yao Q. Mesothelin-induced pancreatic cancer cell proliferation involves alteration of cyclin E via activation of signal transducer and activator of transcription protein 3. *Mol Cancer Res* 2008; **6**: 1755-1765 [PMID: 19010822 DOI: 10.1158/1541-7786.MCR-08-0095]
24 **Chang MC**, Chen CA, Hsieh CY, Lee CN, Su YN, Hu YH, Cheng WF. Mesothelin inhibits paclitaxel-induced apoptosis through the PI3K pathway. *Biochem J* 2009; **424**: 449-458 [PMID: 19747165 DOI: 10.1042/BJ20082196]
25 **Robinson BW**, Creaney J, Lake R, Nowak A, Musk AW, de Klerk N, Winzell P, Hellstrom KE, Hellstrom I. Mesothelin-family proteins and diagnosis of mesothelioma. *Lancet* 2003; **362**: 1612-1616 [PMID: 14630441 DOI: 10.1016/S0140-6736(03)14794-0]
26 **Scholler N**, Fu N, Yang Y, Ye Z, Goodman GE, Hellström KE, Hellström I. Soluble member(s) of the mesothelin/megakaryocyte potentiating factor family are detectable in sera from patients with ovarian carcinoma. *Proc Natl Acad Sci USA* 1999; **96**: 11531-11536 [PMID: 10500211 DOI: 10.1073/pnas.96.20.11531]
27 **Ito T**, Kajino K, Abe M, Sato K, Maekawa H, Sakurada M, Orita H, Wada R, Kajiyama Y, Hino O. ERC/mesothelin is expressed in human gastric cancer tissues and cell lines. *Oncol Rep* 2014; **31**: 27-33 [PMID: 24146039 DOI: 10.3892/or.2013.2803]
28 **Inami K**, Kajino K, Abe M, Hagiwara Y, Maeda M, Suyama M, Watanabe S, Hino O. Secretion of N-ERC/mesothelin and expression of C-ERC/mesothelin in human pancreatic ductal carcinoma. *Oncol Rep* 2008; **20**: 1375-1380 [PMID: 19020717]
29 **Wu X**, Li D, Liu L, Liu B, Liang H, Yang B. Serum soluble mesothelin-related peptide (SMRP): a potential diagnostic and monitoring marker for epithelial ovarian cancer. *Arch Gynecol Obstet* 2014; **289**: 1309-1314 [PMID: 24370956 DOI: 10.1007/s00404-013-3128-x]
30 **Hassan R**, Remaley AT, Sampson ML, Zhang J, Cox DD, Pingpank J, Alexander R, Willingham M, Pastan I, Onda M. Detection and quantitation of serum mesothelin, a tumor marker for patients with mesothelioma and ovarian cancer. *Clin Cancer Res* 2006; **12**: 447-453 [PMID: 16428485 DOI: 10.1158/1078-0432.CCR-05-1477]
31 **Hassan R**, Broaddus VC, Wilson S, Liewehr DJ, Zhang J. Anti-mesothelin immunotoxin SS1P in combination with gemcitabine results in increased activity against mesothelin-expressing tumor xenografts. *Clin Cancer Res* 2007; **13**: 7166-7171 [PMID: 18056197 DOI: 10.1158/1078-0432.CCR-07-1592]
32 **Kreitman RJ**, Hassan R, Fitzgerald DJ, Pastan I. Phase I trial of continuous infusion anti-mesothelin recombinant immunotoxin SS1P. *Clin Cancer Res* 2009; **15**: 5274-5279 [PMID: 19671873 DOI: 10.1158/1078-0432.CCR-09-0062]
33 **Hassan R**, Cohen SJ, Phillips M, Pastan I, Sharon E, Kelly RJ, Schweizer C, Weil S, Laheru D. Phase I clinical trial of the chimeric anti-mesothelin monoclonal antibody MORAb-009 in patients with mesothelin-expressing cancers. *Clin Cancer Res* 2010; **16**: 6132-6138 [PMID: 21037025 DOI: 10.1158/1078-0432.CCR-10-2275]
34 **Le DT**, Brockstedt DG, Nir-Paz R, Hampl J, Mathur S, Nemunaitis J, Sterman DH, Hassan R, Lutz E, Moyer B, Giedlin M, Louis JL, Sugar EA, Pons A, Cox AL, Levine J, Murphy AL, Illei P, Dubensky TW, Eiden JE, Jaffee EM, Laheru DA. A live-attenuated Listeria vaccine (ANZ-100) and a live-attenuated Listeria vaccine expressing mesothelin (CRS-207) for advanced cancers: phase I studies of safety and immune induction. *Clin Cancer Res* 2012; **18**: 858-868 [PMID: 22147941 DOI: 10.1158/1078-0432.CCR-11-2121]
35 **Baba K**, Ishigami S, Arigami T, Uenosono Y, Okumura H, Matsumoto M, Kurahara H, Uchikado Y, Kita Y, Kijima Y, Kitazono M, Shinchi H, Ueno S, Natsugoe S. Mesothelin expression correlates with prolonged patient survival in gastric cancer. *J Surg Oncol* 2012; **105**: 195-199 [PMID: 21780126 DOI: 10.1002/jso.22024]

P- Reviewer: Munoz M, Zhu YL
S- Editor: Qiu S **L- Editor**: A **E- Editor**: Li D

World Journal of Gastrointestinal Pathophysiology

Submit a Manuscript: http://www.wjgnet.com/esps/
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.4291/wjgp.v7.i2.223

World J Gastrointest Pathophysiol 2016 May 15; 7(2): 223-234
ISSN 2150-5330 (online)

ORIGINAL ARTICLE

Basic Study

Sieving characteristics of cytokine- and peroxide-induced epithelial barrier leak: Inhibition by berberine

Katherine M DiGuilio, Christina M Mercogliano, Jillian Born, Brendan Ferraro, Julie To, Brittany Mixson, Allison Smith, Mary Carmen Valenzano, James M Mullin

Katherine M DiGuilio, Christina M Mercogliano, Brittany Mixson, Allison Smith, Mary Carmen Valenzano, James M Mullin, Lankenau Institute for Medical Research, Lankenau Medical Center, Wynnewood, PA 19096, United States

Jillian Born, Brendan Ferraro, Julie To, Departments of Biology and Biomedical Engineering, Drexel University, Philadelphia, PA 19104, United States

Author contributions: DiGuilio KM, Mercogliano CM, Born J, Ferraro B, To J, Mixson B, Smith A, Valenzano MC conducted experiments described in this study; DiGuilio KM and Mullin JM wrote the initial draft of the paper and analyzed the data; Mullin JM suggested experimental directions.

Institutional review board statement: No human subjects were used in this study.

Institutional animal care and use committee statement: No animals were used in this study.

Conflict-of-interest statement: None of the authors of this manuscript have any conflicts of interest, financial or non-financial to declare.

Data sharing statement: Additional data will be shared upon request concerning the action of other micronutrients on cytokine and peroxide-induced leak across gastrointestinal cell layers.

Correspondence to: James M Mullin, PhD, AGAF, Professor, Lankenau Institute for Medical Research, Lankenau Medical Center, 100 Lancaster Avenue, Wynnewood, PA 19096, United States. mullinj@mlhs.org

Telephone: +1-484-4762708
Fax: +1-484-4762205

Received: December 30, 2015
Peer-review started: January 2, 2016
First decision: February 2, 2016
Revised: March 1, 2016
Accepted: March 17, 2016
Article in press: March 18, 2016
Published online: May 15, 2016

Abstract

AIM: To study whether the inflammatory bowel disease (IBD) colon which exhibits varying severity and cytokine levels across its mucosa create varying types of transepithelial leak.

METHODS: We examined the effects of tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ), interleukin-1-β (IL1β) and hydrogen peroxide (H_2O_2) - singly and in combinations - on barrier function of CACO-2 cell layers. Our focus was on the type (not simply the magnitude) of transepithelial leak generated by these agents as measured by transepithelial electrical resistance (TER) and transepithelial flux of ^{14}C-D-mannitol, ^{3}H-Lactulose and ^{14}C-Polyethylene glycol as radiolabeled probe molecules. The isoquinoline alkaloid, berberine, was then examined for its ability to reduce specific types of transepithelial leak.

RESULTS: Exposure to TNF-α alone (200 ng/mL; 48 h) induced a 50% decrease in TER, *i.e.*, increased leak of Na^+ and Cl^- - with only a marginal but statistically significant increase in transepithelial leak of ^{14}C-mannitol (J_m). Exposure to TNF-α + IFN-γ (200 ng/mL; 48 h) + IL1β (50 ng/mL; 48 h) did not increase the TER change (from TNF-α alone), but there was now a 100% increase in

J_m. There however was no increase in transepithelial leak of two larger probe molecules, ^{3}H-lactulose and ^{14}C-polyethylene glycol (PEG). However, exposure to TNF-α + IFN-γ + IL1β followed by a 5 h exposure to 2 mmol/L H_2O_2 resulted in a 500% increase in ^{14}C-PEG leak as well as leak to the luminal mitogen, epidermal growth factor.

CONCLUSION: This model of graded transepithelial leak is useful in evaluating therapeutic agents reducing IBD morbidity by reducing barrier leak to various luminal substances.

Key words: Intestine; Crohn's disease; Tight junction; Ulcerative colitis; CACO-2; Berberine; Micronutrient; Cytokine

Core tip: A cell culture model of graded transepithelial leak can be very valuable in evaluating the various types and magnitudes of leak that can exhibit across the inflammatory bowel disease mucosa. This graded leak can be achieved through various combinations of proinflammatory cytokines and peroxide. Berberine provides an example of a micronutrient that can be more effective against one type of induced leak than another.

DiGuilio KM, Mercogliano CM, Born J, Ferraro B, To J, Mixson B, Smith A, Valenzano MC, Mullin JM. Sieving characteristics of cytokine- and peroxide-induced epithelial barrier leak: Inhibition by berberine. *World J Gastrointest Pathophysiol* 2016; 7(2): 223-234 Available from: URL: http://www.wjgnet.com/2150-5330/full/v7/i2/223.htm DOI: http://dx.doi.org/10.4291/wjgp.v7.i2.223

INTRODUCTION

The idiopathic inflammatory bowel diseases (IBD), the major types being Crohn's disease (CD) and ulcerative colitis (UC), are autoimmune diseases affecting the gastrointestinal tract and causing chronic intestinal inflammation. CD and UC have similar key characteristics, perhaps the most important being an observed compromise of epithelial barrier function. The crucial role of the mucosal layer of the gastrointestinal tract is to actively separate gut luminal contents from the underlying interstitium. The epithelial cell layer that lines the gastrointestinal (GI) tract functions as a selectively permeable barrier. A major component of this barrier is the tight junctional (TJ) protein complex, which prevents free diffusion along the paracellular pathway. In the case of IBD, the integrity of the TJ barrier is compromised in part as a result of the inflammatory response increasing local and systemic pro-inflammatory cytokine [tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ), interleukin-1-β (IL1β)] production. Luminal antigens now able to traverse the "leaky" barrier exacerbate the inflammatory response in the mucosa and submucosa, which in turn further worsens the integrity of the TJ complex and the cell layer overall[1-6]. It is debated whether intestinal leak is causal or simply a result of the disease. A study by Hollander *et al*[7] found a two-fold increase in permeability to ^{14}C-polyethylene glycol (PEG)-400 of CD patients and their healthy relatives as compared to normal controls, suggesting intestinal barrier leak may precede clinical intestinal inflammation and be an etiologic factor in IBD. However the overall involvement of compromised barrier function in IBD is not debated.

TNF-α is a key proinflammatory cytokine involved in intestinal inflammation in IBD[6]. Experimentally it has been shown to increase epithelial TJ permeability in several cell types including the human intestinal epithelial monolayers: CACO-2, T84, and HT29/B6[1,8-11]. In CACO-2, TNF-α decreased transepithelial electrical resistance in both a dose- and time- dependent manner[1,9,10]. A decrease in transepithelial electrical resistance (TER) was shown after 24 h incubation with 100 ng/mL TNF-α which reached a maximum at 48 h, and was sustained for up to 8 d after TNF-α removal[10]. TNF-α reduction of TER was associated with an increase in mannitol (182 MW) as well as inulin (5000 MW) permeability[9]. The exact characteristics of the TNF-α effect on barrier function vary across different cell lines. In the renal epithelium model, LLC-PK1, TNF-α produces a rapidly reversible reduction of TER within 2 h and is accompanied by increased permeability to molecules as large as PEG (4000 MW)[11]. TNF-α was also capable of producing a pronounced effect on HT29/B6 cell layers, reducing TER by 81% of the control[12]. The mechanism by which TNF-α generates leak in CACO-2 cell layers has been shown not to be simply a direct result of apoptosis, but rather attributed to TNF-α's ability to activate NF-κB, induce myosin light chain kinase (MLCK) protein expression and activity, and engender TJ leak[9].

The proinflammatory cytokine IFN-γ has also been reported to increase TJ permeability across T84, HT29/B6, and CACO-2 cell layers[1,13]. T84 cells treated basal-laterally with IFN-γ for 24 h showed a decrease in TER that continued for five days after exposure[13]. Watson *et al*[14] (2005) demonstrated that in T84 cell layers, IFN-γ caused a greater increase in permeability to larger-sized than to smaller-sized molecules, proposing that IFN-γ selectively activates specific permeation pathways within the TJ. In several studies, using both HT29/B6 and CACO-2 cell layers, TNF-α has been used in combination with IFN-γ to produce a synergistic effect on TER[11,15]. In the CACO-2 model, IFN-γ (10 ng/mL) and TNF-α (2.5 ng/mL) individually did not have an effect on TER or paracellular flux of 3 kD dextran. However, when the cell layers were first primed with IFN-γ for 24 h followed by treatment with TNF-α for 8 h, the cells exhibited both a significant decrease in TER and an increase in 3 kD dextran flux[15].

Increased levels of IL1β in IBD patients have also

been associated with increased intestinal inflammation[16]. In CACO-2 cell layers, IL1β caused a drop in TER that was maximal after 48 h treatment. This decrease in TER was accompanied by approximately a 20-fold increase in paracellular permeability to inulin. IL1β was also shown to affect TJ proteins, inducing a decrease in occludin protein expression and an increase in claudin-1 expression. The CACO-2 TJ permeability increase as a result of IL1β exposure involved NF-κB activation and *MLCK* gene regulation, not induction of apoptosis. Studies by Al-Sadi *et al*[17,18] suggest a role for p38-kinase dependent activation of the nuclear transcription factor, activating transcription factor-2.

Increased production of mucosal-damaging oxygen radicals by white blood cells has been shown in IBD[19]. Decreased nutritional intake of a variety of antioxidants in IBD patients can lead to an imbalance that causes an additional increase in reactive oxygen species levels resulting in exacerbated oxidative stress of the inflamed intestinal tissue[20,21]. Furthermore, Strus *et al*[22] (2009) demonstrated that hydrogen peroxide-producing bacteria, present in samples from IBD patients, may be another contributing factor behind increased hydrogen peroxide in the IBD mucosa. In the literature, oxidative stress of CACO-2 cells induced by treatment with H_2O_2 causes increased paracellular permeability as evidenced by a decrease in TER, as well as increases in both mannitol and inulin flux. Hydrogen peroxide is capable of disrupting the TJ through a specific mechanism that involves protein tyrosine phosphorylation[23-25].

The objectives of the following study were to: (1) Observe the effects of the proinflammatory cytokines, TNF-α, IFN-γ, IL1β, and H_2O_2, alone and in combination, on CACO-2 barrier function in order to create an *in vitro* model of graded leak that can reflect the clinical situation in IBD at different sites along the intestinal mucosa; (2) determine if this leak allows for barrier breakdown to biologically active proteins such as epidermal growth factor (EGF); and (3) determine if a previously described nutraceutical capable of barrier protection can in fact reduce barrier compromise under these extreme conditions.

MATERIALS AND METHODS

Cell culture

The CACO-2 cell culture, an epithelial cell line derived from human colon adenocarcinoma[26], was used between passages 52 and 64. Upon confluence, cells were passaged on a weekly basis by trypsinization [0.25% trypsin, 2.2 mmol/L EDTA (Corning Cellgro, Manassas, VA)] and were seeded at 7.5 × 10^5 cells/Falcon 75-cm^2 culture flask with 25 mL of Dulbecco's Modified Minimum Essential Medium (Corning Cellgro) supplemented with 2 mmol/L L-Glutamine, 1% non essential amino acids, 1 mmol/L Sodium Pyruvate (all culture medium additives, Corning Cellgro) and 10% defined fetal bovine serum (HyClone, Logan, UT). Cultures were incubated at 37 ℃ in 95% air-5% CO_2 atmosphere.

Treatment with cytokines and/or hydrogen peroxide

Human recombinant proteins TNF-α, IL1β and IFN-γ were obtained from Life Technologies (Frederick, MD). For individual exposures and combinations of cytokine treatment, 200 ng/mL TNF-α, 50 ng/mL IL1β, and between 100 and 200 ng/mL IFN-γ were applied (in complete medium) to both the apical and basal-lateral compartments for 48 h. This combination of three cytokines is referred to in this manuscript as "cytomix" for purposes of brevity. Media was first filter sterilized with a 0.2 μm disc filter unit (Corning). Seven- and twenty-one day post-confluent CACO-2 cell layers were used in our studies, as barrier function at these days is highly similar in CACO-2 monolayers, and we did not observe a difference between their responses to cytokines. For hydrogen peroxide (Sigma Life Science, St. Louis, MO) exposure, with or without a prior 48 h incubation with cytokines, the CACO-2 cells were treated both apically and basal-laterally with 2 mmol/L hydrogen peroxide in Dulbecco's Phosphate Buffered Saline containing calcium and magnesium (Corning Cellgro) supplemented with 5 mmol/L glucose for 5 h. The dosages of cytokines and hydrogen peroxide were fixed in a given experiment; however, over the course of experiments, concentration and exposure time were reduced, yet conditions remained capable of achieving the maximum effect, as we had been using saturating levels of cytokines in a receptor-mediated response.

Transepithelial electrophysiology and permeability

Cells were seeded into sterile Millipore Millicell polycarbonate (PCF) permeable supports (30 mm diameter with 0.4 μm pore size) on day 0 at a seeding density of 5 × 10^5 cells/insert. Four sterile Millicell PCF inserts were placed into a 100 mm petri dish. On day 1, all cell layers were refed (2 mL apical/15 mL basal-lateral) with control medium containing penicillin (50 U/mL) and streptomycin (50 mcg/mL), followed by refeedings every 2-3 d until exposure. Depending on the specific exposure combination, cells were fed medium supplemented with the appropriate cytokines for 48 h treatment, followed in certain experiments with 5 h of peroxide exposure, then followed by transepithelial electrophysiological measurements and radiotracer flux studies with 0.1 mmol/L, 0.1 μCi/mL ^{14}C-D-mannitol (PerkinElmer, Boston, MA), 0.1 mmol/L, 0.25 μCi/mL ^{3}H-Lactulose (American Radiolabeled Chemicals, Inc., St Louis, MO) and/or 0.1 mmol/L, 0.3 μCi/mL ^{14}C-Polyethylene glycol (PerkinElmer, Waltham, MA).

On the day of transepithelial experiments (for cells treated with cytokines only), the cell layers were refed with fresh control medium and allowed to incubate at 37 ℃ for 1 to 1.5 h prior to electrophysiological readings. Potential difference, TER, and short-circuit current (I_{sc}) were measured using 1 s, 40 μA direct current pulses, with TER calculated using Ohm's law. As soon as electrical measurements were completed, the basal-lateral medium was aspirated and replaced with

15 mL of medium containing the appropriate radioisotope and incubated at 37 ℃. Triplicate basal-lateral medium samples were taken for liquid scintillation counting (LSC) for specific activity (cpm/micromole) determination. Duplicate samples were taken from the apical medium at 60 and 120 min for LSC to determine radioisotope flux rates. The media lost due to sampling from the apical compartment were replaced with fresh medium of the same sample volume. The flux rate (in cpm/min per square centimeter and pmol/min per square centimetre) was calculated for the radioisotope diffusing across the cell layer. In experiments that included exposure to hydrogen peroxide, the cell layers were rinsed in saline before being refed with saline, with or without hydrogen peroxide. After five hours of incubation, the basal-lateral saline was aspirated and replaced with 15 mL of saline containing 0.1 mmol/L, 0.3 μCi/mL ^{14}C-polyethylene glycol. Triplicate basal-lateral samples and duplicate apical samples were taken at 75 min, and the flux rate was calculated as before.

Paracellular flux of ^{125}I-EGF

CACO-2 cell layers, treated as described above prior to exposure, were refed in control medium or medium containing 50 ng/mL TNF-α, 100 ng/mL IFN-γ, and 50 ng/mL IL1β in the apical and basal-lateral compartments for 48 h. On the day of experimental measurements, the cell layers were exposed to control saline or saline containing 1 mmol/L hydrogen peroxide for 3 h. The apical saline was then replaced with medium containing 0.5 μCi/mL, 10 mmol/L ^{125}I-EGF and the basal-lateral saline was replaced with control medium. After a 2 h incubation period, apical and basal-lateral samples were taken for LSC to determine EGF flux rates. Basal-lateral medium was also sampled for column (G-25) chromatography analysis. Total ^{125}I-EGF flux rates (as cpm/min per square centimetre) were adjusted based upon the percent of intact ^{125}I-EGF in the basal-lateral compartment.

Pretreatment with berberine chloride prior to cytokine exposure

Seven-day post-confluent CACO-2 cell layers were refed in control medium or medium containing 100 μmol/L berberine chloride in the apical and basal-lateral compartments. A berberine chloride (Sigma-Aldrich) stock solution (2.7 mmol/L) was prepared in deionized distilled water, but was made each day at the time of use. Following a 24 h berberine pretreatment, the appropriate cell layers were continued in control medium or berberine medium and additionally exposed to either no cytokines, TNF-α, or cytomix for 48 h prior to transepithelial electrophysiology and permeability measurements. For studies that included hydrogen peroxide exposure, on the day of the experiment the cell layers were treated for 5 h with control saline or saline containing 1 mmol/L hydrogen peroxide ± berberine.

Statistical analysis

For electrophysiology and radiotracer flux studies, cytokine- and/or hydrogen peroxide-exposed cell samples were compared against appropriate matched controls within the same experiment. All data are expressed as the mean ± standard error of the mean with the number of replicates provided for each set of studies. Differences between means are evaluated by two-sided Student's *t* tests for two groups or by one-way ANOVA followed by Tukey's *post hoc* testing where multiple conditions existed.

RESULTS

Exposure to TNF-α

Treatment for 48 h of 7-d post-confluent CACO-2 monolayers with apical and basal-lateral 200 ng/mL TNF-α resulted in a 50% decrease in TER (Figure 1A). The reduction in TER was associated with only a marginal statistically significant increase in transepithelial leak of ^{14}C-D-mannitol (Figure 1B). Unlike the consistent decrease in TER, this increase in mannitol flux did not always achieve statistical significance within each individual experiment, as exemplified by the lack of statistical significance in Figure 2A.

Exposure to TNF-α and IFN-γ

Forty-eight hours combined exposure to 200 ng/mL TNF-α and 100-200 ng/mL IFN-γ (apical and basal-lateral) also caused a significant decrease in TER. Interestingly, this 35% reduction of TER was consistently less than that produced by TNF-α alone (50%) (Figure 2B). A simultaneous slight increase in mannitol flux (J_m) was again observed (Figure 2A), although not quite achieving statistical significance. Exposure to IFN-γ alone reduced TER by only 20% and did not have a significant effect on J_m.

Exposure to TNF-α, IFN-γ, and IL1β

Exposure of 21-d post-confluent CACO-2 cell layers to TNF-α, IFN-γ and 50 ng/mL IL1β (cytomix) on both cell surfaces led to a similar decrease of TER as seen with the combination of TNF-α and IFN-γ (approximately 30%-35% decrease) (Figure 3A). IL1β alone did not generate leak any greater than TNF-α or IFN-γ achieved individually (data not shown). Upon adding IL1β to the mixture of TNF-α and IFN-γ, there was now however a dramatic and consistent 100% increase in J_m (Figure 3B). Further investigation into the impact of cytomix on paracellular permeability showed that the leak pathway produced did not however allow for an increase in flux of the larger probe molecules, ^{3}H-lactulose (MW 342) or ^{14}C-PEG (MW 4000) (Figure 4).

Exposure to cytomix and hydrogen peroxide

Treatment of both 7- and 21-d post-confluent CACO-2 cell layers with cytomix for 48 h followed by 5 h exposure to 2 mmol/L H_2O_2 (apical and basal-lateral) induced on average a 500% increase in PEG transepithelial leak. H_2O_2 alone caused only a 35% increase in PEG leak (Figure 5). In the PEG flux studies, column (G-25)

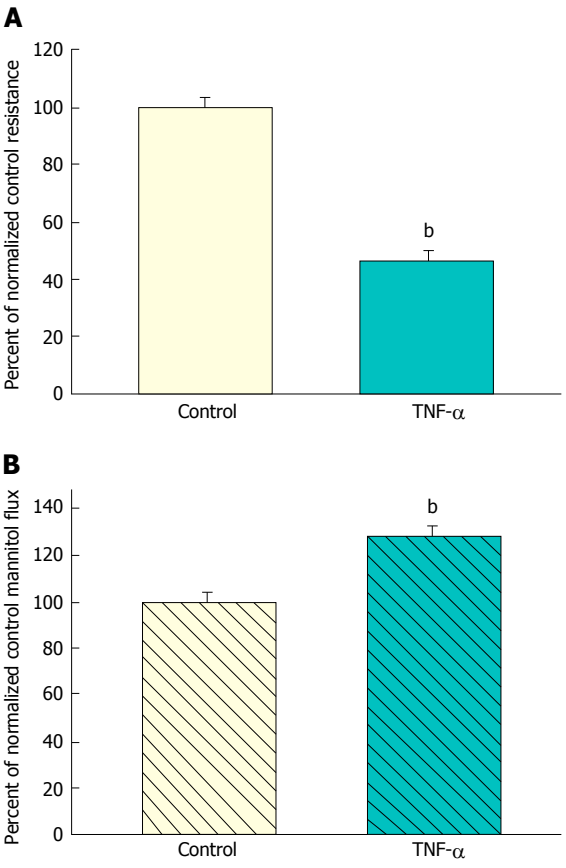


Figure 1 The effect of tumor necrosis factor-α on CACO-2 transepithelial electrical resistance and transepithelial flux of ^{14}C-D-mannitol. A: Seven-day post-confluent CACO-2 cell layers on Millipore polycarbonate filters were refed in control medium or medium containing 200 ng/mL tumor necrosis factor-α (TNF-α) (apical and basal-lateral compartments) 48 h prior to electrical measurements. Data shown represent the mean ± SE of 16 cell layers per condition. Data represent the percent of control resistance normalized across 3 experiments; B: After electrical measurements, radiotracer flux studies with 0.1 mmol/L, 0.1 μCi/mL 14C-D-mannitol were performed on CACO-2 cell layers, as described in Materials and Methods. Data represent the percent of control flux rate normalized across 4 experiments, and is expressed as the mean ± SE of 20 cell layers per condition. $^{b}P < 0.001$ *vs* control (Student's *t* test, one-tailed).

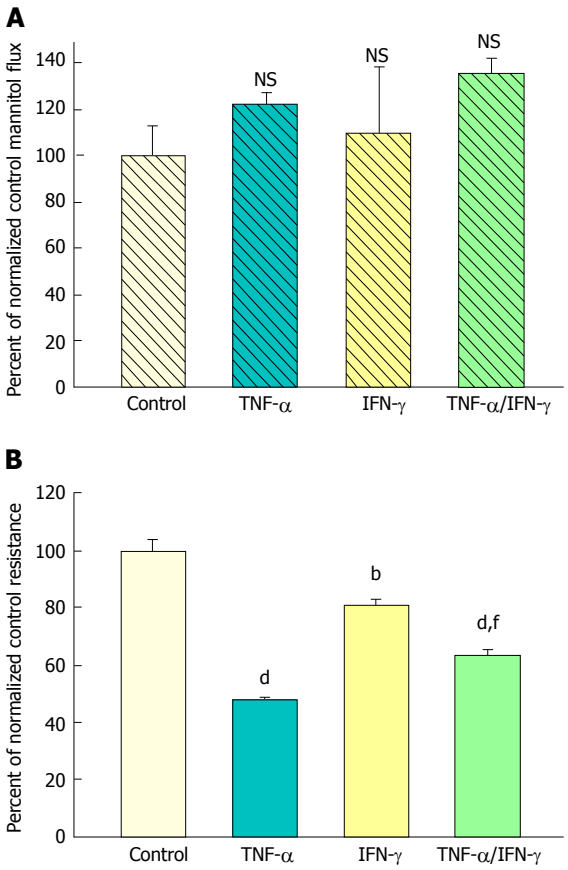


Figure 2 The effect of tumor necrosis factor-α and interferon-γ on CACO-2 transepithelial electrical resistance and transepithelial flux of ^{14}C-D-mannitol. A: Radiotracer flux studies were conducted as described in Figure 1 with the treatment conditions listed above. Data represent the percent of control flux rate, and is expressed as the mean ± SE of 4 cell layers per condition. NS indicates non significance *vs* control; B: CACO-2 cell layers were cultured and treated as described in Figure 1, using the following conditions: Control medium; medium containing 200 ng/mL tumor necrosis factor-α (TNF-α); medium containing 200 ng/mL Interferon-γ (IFN-γ); or medium containing a combination of 200 ng/mL TNF-α and 200 ng/mL IFN-γ. Data shown represent the mean ± SE of 4 cell layers per condition, with data expressed as the percent of control resistance. $^{b}P < 0.01$ *vs* control; $^{d}P < 0.001$ *vs* control; $^{f}P < 0.01$ *vs* TNF-α alone (one-way ANOVA followed by Tukey's *post hoc* testing).

chromatography was used to verify leak of a 4000 MW species of PEG (data not shown). After the 5 h H_2O_2 exposure, the combination of cytomix and H_2O_2 resulted in an 80%-90% decrease of TER (data not shown).

Hematoxylin and eosin-stained cross sections of CACO-2 cell layers were used to evaluate the histological effects of the various cytokine/H_2O_2 exposure regimens. As shown in Figure 6, cytomix alone produced no observable morphological changes in cross sections of the epithelial cell layer. Exposure to 2 mmol/L H_2O_2 resulted in increased blebbing of membranes from the apical surface of occasional cells. Exposure to both cytomix and peroxide induced not only blebbing of apical membranes in occasional cells but also frequent apoptotic nuclei and rare, though occasional, sites of cell detachment.

Transepithelial leak of EGF

CACO-2 cell layers exposed for 48 h to 50 ng/mL TNF-α, 100 ng/mL IFN-γ, and 50 ng/mL IL1β followed by a 3 h 1 mmol/L H_2O_2 treatment manifested a transepithelial leak pathway that allowed for not only a leak to 4000 MW PEG, but also an over 30-fold increase in ^{125}I-EGF permeation. This EGF flux was performed in an apical to basal-lateral direction to mimic the diffusion gradient for EGF that would exist *in vivo* (Table 1). In these EGF studies, the leak of ^{125}I isotope across the cell layer was analyzed by gel filtration chromatography to determine the amount of transepithelial isotope diffusion that corresponded solely with the compound of interest, 6100 MW EGF. Transepithelial leak of actual 6100 MW EGF - and not simply ^{125}I-EGF degradation products - was thus verified.

Evaluation of berberine as a potential therapeutic agent using the graded transepithelial leak model

A major benefit of this *in vitro* model of graded epithelial barrier leak is the capability of evaluating a great number

Table 1 The effect of tumor necrosis factor-α + interferon-γ + interleukin-1β and hydrogen peroxide on transepithelial flux of ^{14}C-polyethylene glycol and ^{125}I-epidermal growth factor

	^{14}C-PEG flux		^{125}I-EGF flux	
	cpm/min per square centimeter	pmol/min per square centimeter	cpm/min per square centimeter	fmol/min per square centimeter
Control	3.74 ± 0.07	7.54 ± 0.20	43.7 ± 2.0	0.034 ± 0.005
Cytomix	3.40 ± 0.12	6.87 ± 0.27	30.3 ± 1.4	0.015 ± 0.001
Cytomix/H_2O_2	9.80 ± 0.28[a]	20.97 ± 0.64[a]	192.0 ± 4.0[c]	1.21 ± 0.029[c]

[a]$P < 0.05$ *vs* control; [c]$P < 0.05$ *vs* Cytomix-only condition (one-way ANOVA followed by Tukey's *post hoc* testing). CACO-2 cell layers on Millipore polycarbonate filters were refed in control medium or medium containing the combination of 50 ng/mL tumor necrosis factor-α, 100 ng/mL interferon-γ, and 50 ng/mL interleukin-1β (apical and basal-lateral compartments) for 48 h. On the day of radiotracer flux studies, the cell layers were exposed to control saline or saline containing 1 mmol/L hydrogen peroxide for 3 h. These studies were performed using 0.1 mmol/L, 0.025 μCi/mL ^{14}C-polyethylene glycol (MW 4000) and 10 nmol/L, 0.5 μCi/mL ^{125}I-EGF (MW 6100), as described in Materials and Methods. Total ^{125}I flux rates (as cpm/min per square centimeter) were adjusted, based upon the percent of intact ^{125}I-EGF in the basal-lateral compartment (using column chromatography), and expressed finally as fmol/min per square centimeter. Similar gel chromatography analyses were performed for ^{14}C-PEG experiments, but here all isotopes that diffused across the epithelial cell layer was found to be 4000 MW PEG. Data shown represent the mean standard error for an $n = 8$ in all cases. PEG: Polyethylene glycol; EGF: Epidermal growth factor.

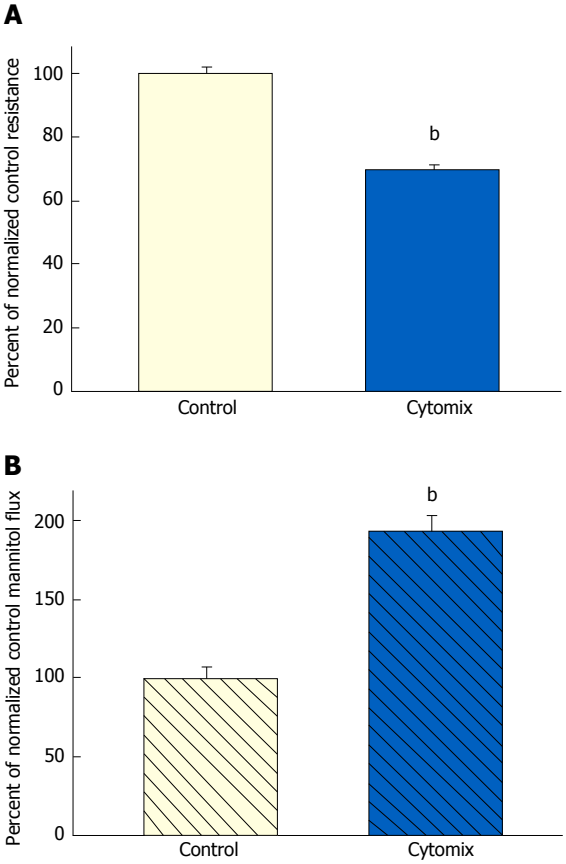


Figure 3 The effect of tumor necrosis factor-α + interferon-γ + interleukin-1β on CACO-2 transepithelial electrical resistance and transepithelial flux of ^{14}C-D-mannitol. A: Twenty-one day post-confluent CACO-2 cell layers cultured and treated as described in Figure 1, were refed in control medium or medium containing the combination of 200 ng/mL tumor necrosis factor-α, 150 ng/mL interferon-γ, and 50 ng/mL interleukin-1β. Data shown represent the mean ± SE of 16 cell layers per condition. Data represent the percent of control resistance normalized across 4 experiments; B: Radiotracer flux studies were conducted as described in Figure 1, with the same conditions listed above for panel A. Data represent the percent of control flux rate normalized across 2 experiments, and is expressed as the mean ± SE of 8 cell layers per condition. [b]$P < 0.001$ *vs* control (Student's *t* test, one-tailed).

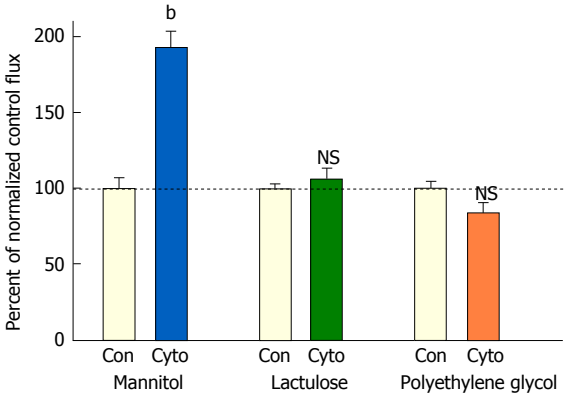


Figure 4 The effect of tumor necrosis factor-α + interferon-γ + interleukin-1β on transepithelial flux of ^{14}C-D-mannitol, ^{3}H-lactulose, and ^{14}C-polyethylene glycol across CACO-2 cell layers. Twenty-one day post-confluent CACO-2 cell layers on Millipore PCF filters were refed in control medium or medium containing the combination of 200 ng/mL tumor necrosis factor-α, 150 ng/mL interferon-γ, and 50 ng/mL interleukin-1β (apical and basal-lateral compartments) 48 h prior to radiotracer flux studies. These studies were performed using 0.1 mmol/L, 0.1 μCi/mL ^{14}C-D-mannitol; 0.1 mmol/L, 0.25 μCi/mL ^{3}H-lactulose; and 0.1 mmol/L, 0.3 μCi/mL ^{14}C-polyethylene glycol as described in Materials and Methods. Data represent the percent of control flux rate normalized across 2 experiments, and is expressed as the mean ± SE of 8 cell layers per condition for the mannitol flux and 4 cell layers per condition for both the lactulose and polyethylene glycol fluxes. NS indicates non significance. [b]$P < 0.001$ *vs* control (Student's *t* test, one-tailed).

of potentially efficacious micronutrients - or combinations thereof - for reducing each type of leak that may occur across the surface of the IBD mucosa. In this study, 100 μmol/L berberine chloride pretreatment and simultaneous exposure was evaluated for its effects on the ability of TNF-α and cytomix to impair CACO-2 barrier function. Berberine treatment not only increased basal TER, but also reduced both the TNF-α- and cytomix-induced decrease in TER (Figure 7A). Additionally, berberine reduced the TNF-α- and cytomix-induced increase in J_m (Figure 7B). Berberine also effectively - and significantly - reduced the macromolecule leak resulting from cell layer exposure to cytomix and H_2O_2 (Figure 8A). Berberine likewise reduced both the H_2O_2- and cytomix + H_2O_2-induced decrease in resistance (Figure 8B).

DISCUSSION

Colon mucosa in active IBD is rarely homogeneously inflamed; rather, there is typically much heterogeneity

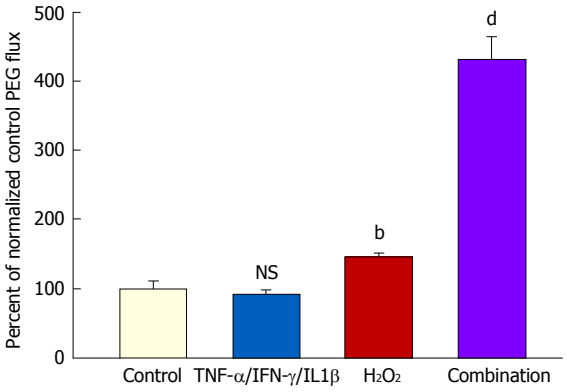


Figure 5 The effect of tumor necrosis factor-α + interferon-γ + interleukin-1β plus hydrogen peroxide on transepithelial flux of ^{14}C-polyethylene glycol. Seven-day and 21-d post-confluent CACO-2 cell layers on Millipore PCF filters were refed in control medium or medium containing the combination of 200 ng/mL TNF-α, 200 ng/mL IFN-γ, and 50 ng/mL IL1β (apical and basal-lateral compartments) 48 h prior to radiotracer flux studies. On the day of the experiment, the cell layers were treated for 5 h with control saline or saline containing 2 mmol/L H_2O_2. Paracellular permeability was assessed using 0.1 mmol/L, 0.3 μCi/mL ^{14}C-polyethylene glycol as described in materials and methods. Data represent the percent of control flux rate normalized across 2 experiments, and is expressed as the mean ± SE for 8 cell layers per condition. NS indicates non significance *vs* control. $^{b}P < 0.01$ *vs* control; $^{d}P < 0.001$ *vs* control (one-way ANOVA followed by Tukey's *post hoc* testing). IFN-γ: Interferon-γ; IL1β: Interleukin-1β; TNF-α: Tumor necrosis factor-α.

across the apical surface. This can range from grossly normal, non-inflamed tissue, to normal-appearing tissue but with histological evidence of inflammation (seen, *e.g.*, in white blood cell infiltration in stained tissue sections), to tissue that appears obviously inflamed grossly. In addition, there can be granulomas, pseudopolyps and even micro-ulceration areas that are denuded of epithelium[27-29]. It is well known that inflammation can lead to increased epithelial barrier leak through elevated cytokine levels and other mechanisms[30]. Such grossly observed heterogeneity reflects itself in different degrees of barrier compromise. This variability in barrier compromise can manifest itself not only in the quantitative magnitude of leak, but also in the types of solutes able to leak across the epithelial barrier. As described in Mullin *et al*[31] (1997) and Watson *et al*[14] (2005), induction of transepithelial paracellular leak by different agents can result in leak to only small molecules or it can extend to paracellular permeation of macromolecules.

A decrease in TER typically signifies (in "low resistance" epithelial tissues like ileum or colon or CACO-2 cell layers) increased paracellular conductance or diffusion of Na^+ and Cl^- ions. It implies nothing about potential leak to larger (or uncharged) molecules. And as shown in Figure 1, such was the situation that we observed when CACO-2 cell layers were treated with TNF-α - a significant decrease in TER with only a marginal (and variable) increase in leak to D-mannitol [and no significant leak to the disaccharide lactulose (MW 342) (data not shown)]. This implies that paracellular pathways to Na^+ and Cl^- ions were increased by TNF-α treatment of the cell layers, but the paracellular pathways that would allow D-mannitol (MW 182) (or larger molecules) to pass, were hardly affected.

However, when cell layers were also treated with IL1β and IFN-γ as well as TNF-α, a sizable increase (100%) in D-mannitol leak was combined with the TER decrease. Obviously, paracellular leak pathways that would admit D-mannitol, were now being induced. This signifies a different type of paracellular pathway that would likely allow for transepithelial paracellular leak of monosaccharides and perhaps neutral amino acids as well as inorganic salts. This could have an effect on the efficiency of gastrointestinal nutrient absorption, as well as ATP consumption by gastrointestinal mucosa. But it is difficult to see how this type of leak would induce inflammation in GI mucosa, since luminal molecules (antigens) capable of eliciting an inflammatory response are typically much larger. It is noteworthy that in our study, even with the increased leak of D-mannitol, leak to the larger probe molecules, lactulose and PEG (4000 MW), was unaffected (Table 2). This not only implies distinct paracellular leak pathways for these different molecules, but also shows that induction of paracellular leak can be a staged, graded phenomenon. Only when exposure of cell layers to cytokines was combined with subsequent treatment with hydrogen peroxide (a situation that reflects certain inflamed tissue in IBD) was a paracellular leak to large molecules observed.

Transepithelial leak can be both a manifestation of morbidity as well as a driver of morbidity, depending upon the nature of the molecules that are leaking. If leak is induced only to Na^+ and Cl^- ions (as we observed with TNF-α treatment of CACO-2 cell layers), a situation exists that may not generate serious morbidity. Induced leak to D-mannitol along with decreased TER, however, has implications for the physiological efficiency of nutrient absorption that may then have metabolic/bioenergetic implications for the organism. However, induced leak to molecules larger than D-mannitol, as we observed only for combined treatment with cytokines and peroxide, is the situation most problematic in IBD, because now there can be leak of peptides/proteins present in the GI lumen, into the interstitial fluid compartment, where activation of inflammatory cascades is possible. Substances normally sequestered in the GI lumen such as bacterial toxins and antigens, could - at least on a basis of size - now leak across the epithelial barrier into the interstitial compartment under the epithelium. Bacterial toxins such as *Clostridium perfringens* enterotoxin (CPE) - which is active from only the abluminal compartment[32] - as well as simple lipopolysaccharide endotoxin, might begin to permeate and either simply raise an immune response (and more cytokine production) in the interstitium or further damage the epithelial barrier directly (in the case of CPE) and compound the barrier compromise even further.

A protein not often considered in the paracellular leak scenarios out of the GI lumen and across a compromised GI barrier is the potent mitogenic growth factor, EGF. EGF

Table 2 Summary of the magnitude of effect of combinations of cytokines and hydrogen peroxide on transepithelial electrical resistance, mannitol leak, lactulose leak and polyethylene glycol leak

	Transepithelial electrical resistance decrease	Mannitol leak increase	Lactulose leak increase	Polyethylene glycol leak increase
TNF	++	[1]	[0]	ND
TNF/IFN	+	[1]	[0]	ND
TNF/IFN/IL1β	+	++	[0]	[0]
TNF/IFN/IL1β + hydrogen peroxide	++++	++++	++++	++++

CACO-2 cell layers having been exposed to the above combinations of cytokines and hydrogen peroxide were evaluated for type (salts, mannitol, lactulose, PEG) and magnitude of leak produced. All treatments increased leakage of salts as seen by a decrease in TER, but large molecule leak was seen only in the presence of peroxide alone or both cytokines and hydrogen peroxide, never in the presence of cytokines alone. [1]Indicates that the observed effect did not routinely achieve statistical significance; [0]Indicates that an effect was measured but no significant change was observed. ND: Not determined; IFN-γ: Interferon-γ; IL1β: Interleukin-1β; TNF-α: Tumor necrosis factor-α; PEG: Polyethylene glycol.

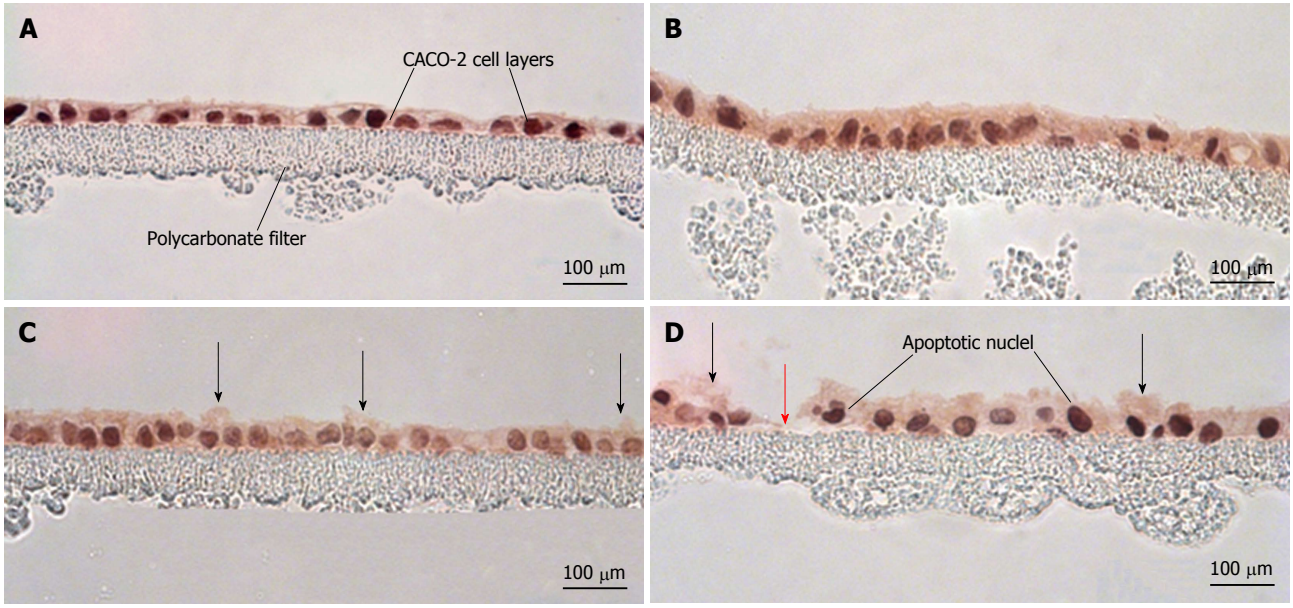


Figure 6 Morphological effects of cytokines and hydrogen peroxide on CACO-2 cell layers. CACO-2 cell layers were cultured at confluent density onto Millipore polycarbonate filters. Seven day post-seeding, the cell layers were refed with control medium or medium containing the combination of 200 ng/mL tumor necrosis factor-α, 200 ng/mL interferon-γ, and 50 ng/mL interleukin-1β ("cytomix") (apical and basal-lateral compartments) for 48 h, followed by exposure to saline or saline containing 2 mmol/L H_2O_2 for 5 h. Cell layers were then fixed in formalin and stained with hematoxylin and eosin. A: CACO-2 cell layers exposed to control medium and control saline; B: CACO-2 cell layers exposed to cytomix medium and control saline; C: CACO-2 cell layers exposed to control medium and saline containing hydrogen peroxide; D: CACO-2 cell layers exposed to cytomix medium and saline containing hydrogen peroxide. In C and D, black arrows indicate instances of cytoplasmic blebbing. The red arrow points at a gap in the epithelial barrier arising from cell death and detachment.

(MW 6100) exists in the GI lumen at concentrations over a thousand fold greater than that in the bloodstream, as a result of EGF synthesis and vectorial secretion by salivary glands and Brunner's glands[33,34]. This EGF is typically biologically inactive however because its receptors are found on the abluminal side of GI epithelia and on interstitial fibroblasts - not on the apical surface of epithelia[35]. However, we show (Table 1) that combined treatment with cytokines and peroxide allows for EGF leak out of the luminal compartment - a situation predicted from the increased leak of PEG (Figure 5). This leakage of luminally situated EGF (down a very steep concentration gradient) into the interstitium may be a major contributing cause for the increased risk of neoplasia in UC, because one could now be putting colonic epithelial cells (whose DNA may be compromised by increased free radical generation) under a near constant replication stimulus.

The greatest utility of the *in vitro* model of graded transepithelial leak being presented here may derive however from the recent research surrounding improvement/recovery of epithelial barrier function by a diverse - and growing - array of natural (*e.g.*, micronutrients) and synthetic enhancers of TJ barrier function. There have been numerous recent reviews and publications focused on the ability of certain micronutrients like zinc, berberine, quercetin, butyrate, indole, *etc*., to modify TJ protein composition and in the process yield a TJ - and epithelial barrier - that is less leaky[36-39]. The list of naturally occurring compounds with this capability being reported in the biomedical literature is expanding year by year. One must consider not only the action of these agents in isolation, but also contend with the possibility that certain combinations of these agents can

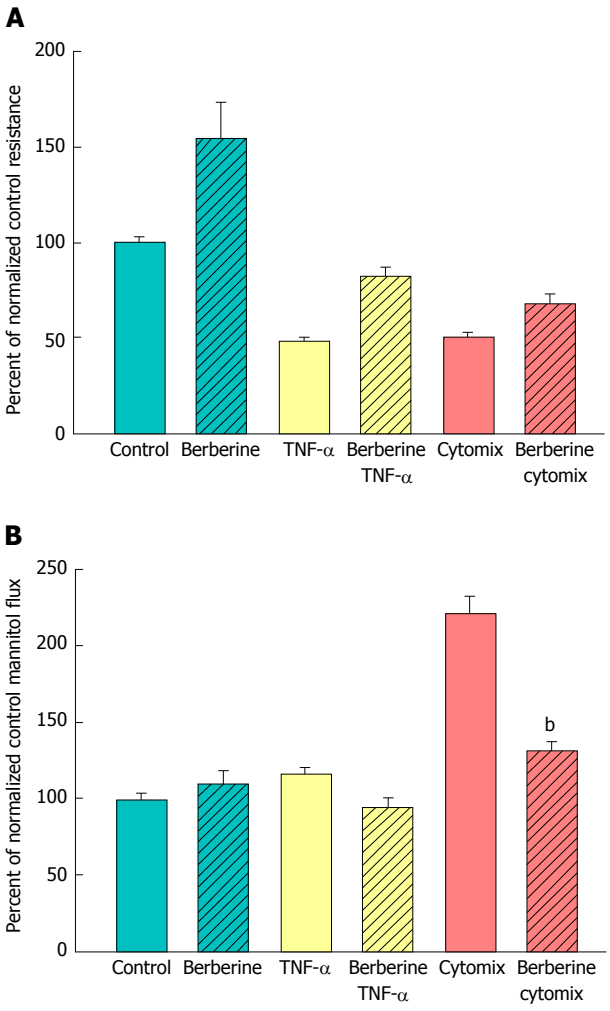


Figure 7 The effect of berberine on cytokine-induced leak of CACO-2 cell layers. A: Seven-day post-confluent CACO-2 cell layers on Millipore polycarbonate filters were refed in control medium or medium containing 100 μmol/L berberine. After 24 h treatment with berberine alone, the cell layers were given either control or berberine medium in addition to being exposed to no cytokines, TNF-α alone, or cytomix (apical and basal-lateral compartments) for 48 h prior to electrical measurements. Data shown represent the mean ± SE of 8 cell layers per condition. Data represent the percent of control resistance normalized across experiments; B: After electrical measurements, the same CACO-2 cell layers represented in A were used to perform radiotracer flux studies with 0.1 mmol/L, 0.1 μCi/mL ^{14}C-D-mannitol, as described in Materials and Methods. Data represent the percent of control flux rate normalized across experiments and is expressed as the mean ± SE of 8 cell layers per condition. $^{b}P < 0.001$ *vs* cytomix alone (one-way ANOVA followed by Tukey's *post hoc* testing). TNF-α: Tumor necrosis factor-α.

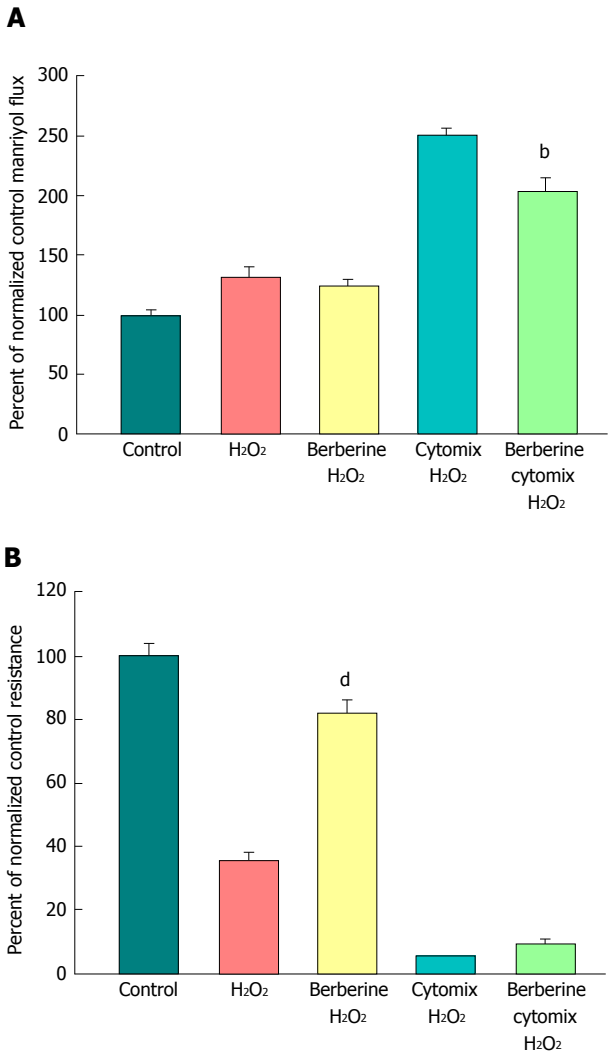


Figure 8 The effect of berberine on cytokine- and peroxide-induced leak of CACO-2 cell layers. A: After electrical measurements, the same CACO-2 cell layers represented in B were used to perform radiotracer flux studies with 0.1 mmol/L, 0.025 μCi/mL ^{14}C-PEG. Data represent the percent of control flux rate, and is expressed as the mean ± SE of 4 cell layers per condition (3 cell layers for the condition of cytomix + peroxide, due to removal of one outlier data point, as determined by a 90% confidence level in the Dixon's Q test); B: Seven-day post-confluent CACO-2 cell layers on Millipore polycarbonate filters were refed in control medium or medium containing 100 μmol/L berberine. After 24 h treatment with berberine alone, the cell layers were given either control or berberine medium, in addition to being exposed to either cytomix (50 ng/mL tumor necrosis factor-α, 100 ng/mL interferon-γ, 50 ng/mL interleukin-1β) or no cytokines (apical and basal-lateral compartments) for 48 h. On the day of the experiment, the cell layers were treated for 5 h with control saline or saline containing 1 mmol/L hydrogen peroxide ± berberine. Data shown represent the mean ± SE of 4 cell layers per condition, with data expressed as the percent of control resistance. $^{b}P < 0.01$ *vs* cytomix + H_2O_2; $^{d}P < 0.001$ *vs* H_2O_2 alone (one-way ANOVA followed by Tukey's *post hoc* testing). Experiment was repeated with similar results.

display even greater efficacy than single agents alone in remodeling and enhancing TJs[40]. In short, it is a situation that demands the testing of a large number of agents and permutations of agents. Moreover, one needs to test not only for improvement of basal barrier function, but also for ability to offset the action of proinflammatory proteins and molecules that compromise barrier function. And as we described above, those agents can induce different states of leak, further complicating the testing situation. An *in vitro* system like what we describe here is ideal for the testing of a large number of different enhancers of barrier function in a range of distinct leak states. This is particularly needed because agents that successfully enhance barrier function in one permeability state of a barrier may or may not be effective in yet another permeability state. As an example, the effectiveness of berberine in reducing leak was tested in this *in vitro* system and found to not only enhance basal CACO-2 barrier integrity but also to reduce the proinflammtory cytokine - induced compromise in epithelial barrier function (Figure 7). It is the first demonstrated effectiveness of berberine in the context of selective

leak induction by treating epithelial cell layers with combinations of cytokines + peroxide. Berberine's effectiveness in attenuating not only cytokine-induced leak to small molecules but also the transepithelial leak to larger molecules that is seen with cytokines + peroxide, portends potential clinical therapeutic value for IBD.

In our studies, berberine was presented to both cell surfaces simultaneously, as has also been done in earlier studies with berberine and CACO-2 cell layers, studies also showing berberine effectiveness in reducing cytokine-induced barrier disruption[41]. Future studies by our group will evaluate potential sidedness aspects to berberine's effectiveness in this model. Basal-lateral effectiveness of berberine has been shown pointedly by Taylor *et al*[42,43] in colon tissue studies. *In vivo* studies where berberine has been proven effective when given orally[44] may suggest an action from the apical surface, but it is equally possible that berberine is diffusing across damaged epithelial mucosa and engaging the cell from the basal-lateral surface. This is in fact suggested in studies where mucosal barrier damage is modeled through the use of cytochalasin-D treatment[43], and studies showing poor intestinal absorption of berberine into the bloodstream[45].

In conclusion, the expanding array of compounds that are effective in reducing leak across epithelial barriers, both basal as well as induced leak, may be the source of an entirely new class of therapeutics in IBD, therapeutics that could work in complementarity to agents that reduce inflammatory response directly, such as the anti-TNF-α drugs or the salicylates. A need exists for a cell culture model system that could allow large numbers of such compounds (and their combinations) to be tested *in vitro* before animal model and human studies are undertaken.

COMMENTS

Background

In inflammation and inflammatory diseases impacting epithelial cell layers, there are many molecular agents assaulting the epithelial barrier. Pro-inflammatory cytokines such as tumor necrosis factor-α, interferon-γ and interleukin-1β, and the chemical hydrogen peroxide, are four very common entities in the inflammatory microenvironment that impact an epithelium. In this study, the authors considered that each agent and combinations of agents may have unique effects in terms of the barrier leak that they produce. The authors asked the question of whether these varying effects would confer leakiness to different sizes of molecules. The authors then asked if an agent capable of improving barrier integrity and resisting leak would confer its benefits on specific types of leak.

Research frontiers

It has been well established by numerous research groups that the above three cytokines and hydrogen peroxide all possess ability to induce transepithelial leak, but the nature of the leak produced by the different agents-and especially their combinations-has not been clearly delineated.

Innovations and breakthroughs

In a barrier-related disease such as inflammatory bowel disease (IBD), transepithelial leakage of small molecules (salts, water, sugars, amino acids) will have different medical implications than leak of macromolecules such as protein growth factors, food antigens, and bacterial toxins and antigens. In the testing of various agents capable of barrier protection it is important to note what types of leak are reduced by the agent under study. Berberine, for instance, is shown here to be able to redress the most severe form of leak, namely the leak to macromolecules produced by the combination of cytokines and peroxide.

Applications

Certain aspects of IBD could be alleviated specifically by shutting down or reducing the unregulated leak of molecules across the inflammation-damaged mucosal lining. This manuscript highlights the issue that specific therapeutic agents will be uniquely able to target certain types of leak.

Terminology

Transepithelial leak is not a monolithic entity. It can result from altered tight junctions giving rise to greater leak to small molecules. Or it can result from full disappearance of tight junctions, giving rise to unrestricted paracellular leak, as can happen in epithelial-to-mesenchymanl transition. Or it can result from the disappearance of whole cells (by death and/or detachment), which gives rise to similarly unrestricted leak, as in tight junction disappearance, but here requiring very different mechanisms (including cell replication and motility) to close the leak. All forms are likely in play in IBD, and each-by being regulated differently-can be affected by unique agents capable of restoring aspects of barrier function.

Peer-review

This paper indicated hyper permeability of colon cancer cell layer by cytokine and inhibition by berberine. The results are interesting and the manuscript is well written.

REFERENCES

1 **Al-Sadi R**, Boivin M, Ma T. Mechanism of cytokine modulation of epithelial tight junction barrier. *Front Biosci* (Landmark Ed) 2009; **14**: 2765-2778 [PMID: 19273235 DOI: 10.2741/3413]

2 **Bruewer M**, Samarin S, Nusrat A. Inflammatory bowel disease and the apical junctional complex. *Ann N Y Acad Sci* 2006; **1072**: 242-252 [PMID: 17057204 DOI: 10.1196/annals.1326.017]

3 **Nusrat A**, Turner JR, Madara JL. Molecular physiology and pathophysiology of tight junctions. IV. Regulation of tight junctions by extracellular stimuli: nutrients, cytokines, and immune cells. *Am J Physiol Gastrointest Liver Physiol* 2000; **279**: G851-G857 [PMID: 11052980]

4 **Shen L**, Turner JR. Role of epithelial cells in initiation and propagation of intestinal inflammation. Eliminating the static: tight junction dynamics exposed. *Am J Physiol Gastrointest Liver Physiol* 2006; **290**: G577-G582 [PMID: 16537969 DOI: 10.1152/ajpgi.00439.2005]

5 **Thoreson R**, Cullen JJ. Pathophysiology of inflammatory bowel disease: an overview. *Surg Clin North Am* 2007; **87**: 575-585 [PMID: 17560413 DOI: 10.1016/j.suc.2007.03.001]

6 **Fakhoury M**, Negrulj R, Mooranian A, Al-Salami H. Inflammatory bowel disease: clinical aspects and treatments. *J Inflamm Res* 2014; **7**: 113-120 [PMID: 25075198 DOI: 10.2147/JIR.S65979]

7 **Hollander D**, Vadheim CM, Brettholz E, Petersen GM, Delahunty T, Rotter JI. Increased intestinal permeability in patients with Crohn's disease and their relatives. A possible etiologic factor. *Ann Intern Med* 1986; **105**: 883-885 [PMID: 3777713 DOI: 10.7326/0003-4819-105-6-883]

8 **Fish SM**, Proujansky R, Reenstra WW. Synergistic effects of interferon gamma and tumour necrosis factor alpha on T84 cell function. *Gut* 1999; **45**: 191-198 [PMID: 10403730 DOI: 10.1136/gut.45.2.191]

9 **Ma TY**, Iwamoto GK, Hoa NT, Akotia V, Pedram A, Boivin MA, Said HM. TNF-alpha-induced increase in intestinal epithelial tight junction permeability requires NF-kappa B activation. *Am J Physiol Gastrointest Liver Physiol* 2004; **286**: G367-G376 [PMID: 14766535 DOI: 10.1152/ajpgi.00173.2003]

10 **Marano CW**, Lewis SA, Garulacan LA, Soler AP, Mullin JM. Tumor necrosis factor-alpha increases sodium and chloride conductance across the tight junction of CACO-2 BBE, a human intestinal epithelial cell line. *J Membr Biol* 1998; **161**: 263-274 [PMID: 9493132 DOI: 10.1007/s002329900333]

11 **Mullin JM**, Laughlin KV, Marano CW, Russo LM, Soler AP. Modulation of tumor necrosis factor-induced increase in renal (LLC-PK1) transepithelial permeability. *Am J Physiol* 1992; **263**: F915-F924 [PMID: 1279987]

12 **Schmitz H**, Fromm M, Bentzel CJ, Scholz P, Detjen K, Mankertz J, Bode H, Epple HJ, Riecken EO, Schulzke JD. Tumor necrosis factor-alpha (TNFalpha) regulates the epithelial barrier in the human intestinal cell line HT-29/B6. *J Cell Sci* 1999; **112** (Pt 1): 137-146 [PMID: 9841910]

13 **Adams RB**, Planchon SM, Roche JK. IFN-gamma modulation of epithelial barrier function. Time course, reversibility, and site of cytokine binding. *J Immunol* 1993; **150**: 2356-2363 [PMID: 8450217]

14 **Watson CJ**, Hoare CJ, Garrod DR, Carlson GL, Warhurst G. Interferon-gamma selectively increases epithelial permeability to large molecules by activating different populations of paracellular pores. *J Cell Sci* 2005; **118**: 5221-5230 [PMID: 16249235 DOI: 10.1242/jcs.02630]

15 **Wang F**, Graham WV, Wang Y, Witkowski ED, Schwarz BT, Turner JR. Interferon-gamma and tumor necrosis factor-alpha synergize to induce intestinal epithelial barrier dysfunction by up-regulating myosin light chain kinase expression. *Am J Pathol* 2005; **166**: 409-419 [PMID: 15681825 DOI: 10.1016/S0002-9440(10)62264-X]

16 **Reinecker HC**, Steffen M, Doehn C, Petersen J, Pflüger I, Voss A, Raedler A. Proinflammatory cytokines in intestinal mucosa. *Immunol Res* 1991; **10**: 247-248 [PMID: 1955748 DOI: 10.1007/BF02919700]

17 **Al-Sadi R**, Guo S, Ye D, Dokladny K, Alhmoud T, Ereifej L, Said HM, Ma TY. Mechanism of IL-1β modulation of intestinal epithelial barrier involves p38 kinase and activating transcription factor-2 activation. *J Immunol* 2013; **190**: 6596-6606 [PMID: 23656735 DOI: 10.4049/jimmunol.1201876]

18 **Al-Sadi RM**, Ma TY. IL-1beta causes an increase in intestinal epithelial tight junction permeability. *J Immunol* 2007; **178**: 4641-4649 [PMID: 17372023 DOI: 10.4049/jimmunol.178.7.4641]

19 **Rugtveit J**, Haraldsen G, Høgåsen AK, Bakka A, Brandtzaeg P, Scott H. Respiratory burst of intestinal macrophages in inflammatory bowel disease is mainly caused by CD14+L1+ monocyte derived cells. *Gut* 1995; **37**: 367-373 [PMID: 7590432 DOI: 10.1136/gut.37.3.367]

20 **Kruidenier L**, Kuiper I, Lamers CB, Verspaget HW. Intestinal oxidative damage in inflammatory bowel disease: semi-quantification, localization, and association with mucosal antioxidants. *J Pathol* 2003; **201**: 28-36 [PMID: 12950014 DOI: 10.1002/path.1409]

21 **Alzoghaibi MA**. Concepts of oxidative stress and antioxidant defense in Crohn's disease. *World J Gastroenterol* 2013; **19**: 6540-6547 [PMID: 24151379 DOI: 10.3748/wjg.v19.i39.6540]

22 **Strus M**, Gosiewski T, Fyderek K, Wedrychowicz A, Kowalska-Duplaga K, Kochan P, Adamski P, Heczko PB. A role of hydrogen peroxide producing commensal bacteria present in colon of adolescents with inflammatory bowel disease in perpetuation of the inflammatory process. *J Physiol Pharmacol* 2009; **60** Suppl 6: 49-54 [PMID: 20224151]

23 **Basuroy S**, Seth A, Elias B, Naren AP, Rao R. MAPK interacts with occludin and mediates EGF-induced prevention of tight junction disruption by hydrogen peroxide. *Biochem J* 2006; **393**: 69-77 [PMID: 16134968 DOI: 10.1042/BJ20050959]

24 **Rao RK**, Baker RD, Baker SS, Gupta A, Holycross M. Oxidant-induced disruption of intestinal epithelial barrier function: role of protein tyrosine phosphorylation. *Am J Physiol* 1997; **273**: G812-G823 [PMID: 9357822]

25 **Rao RK**, Li L, Baker RD, Baker SS, Gupta A. Glutathione oxidation and PTPase inhibition by hydrogen peroxide in Caco-2 cell monolayer. *Am J Physiol Gastrointest Liver Physiol* 2000; **279**: G332-G340 [PMID: 10915642]

26 **Grasset E**, Pinto M, Dussaulx E, Zweibaum A, Desjeux JF. Epithelial properties of human colonic carcinoma cell line Caco-2: electrical parameters. *Am J Physiol* 1984; **247**: C260-C267 [PMID: 6476109]

27 **Feldman M**, Sleisenger MH, Friedman LS, Brandt LJ. Sleisenger & Fordtran's Gastrointestinal and Liver Disease. Philadelphia, PA: Saunders/Elsevier, 2010; **2**: 1948-1986

28 **Fenoglio-Preiser CM**. Gastrointestinal Pathology: An Atlas and Text. Philadelphia, PA: Wolters Kluwer Health/Lippincott Williams & Wilkins, 2008; 610-647

29 **Geboes K**. Histopathology of Crohn's Disease and Ulcerative Colitis. In: Satsangi J, Sutherland LR, ed. Inflammatory Bowel Diseases, London, England: Churchill Livingstone, 2003: 257-261

30 **Fries W**, Belvedere A, Vetrano S. Sealing the broken barrier in IBD: intestinal permeability, epithelial cells and junctions. *Curr Drug Targets* 2013; **14**: 1460-1470 [PMID: 24060148 DOI: 10.2174/13894501131412001 1]

31 **Mullin JM**, Marano CW, Laughlin KV, Nuciglio M, Stevenson BR, Soler P. Different size limitations for increased transepithelial paracellular solute flux across phorbol ester and tumor necrosis factor-treated epithelial cell sheets. *J Cell Physiol* 1997; **171**: 226-233 [PMID: 9130471 DOI: 10.1002/(SICI)1097-4652(199705)171:2<226::AID-JCP14>3.0.CO;2-B]

32 **Sonoda N**, Furuse M, Sasaki H, Yonemura S, Katahira J, Horiguchi Y, Tsukita S. Clostridium perfringens enterotoxin fragment removes specific claudins from tight junction strands: Evidence for direct involvement of claudins in tight junction barrier. *J Cell Biol* 1999; **147**: 195-204 [PMID: 10508866 DOI: 10.1083/jcb.147.1.195]

33 **Heitz PU**, Kasper M, van Noorden S, Polak JM, Gregory H, Pearse AG. Immunohistochemical localisation of urogastrone to human duodenal and submandibular glands. *Gut* 1978; **19**: 408-413 [PMID: 350728 DOI: 10.1136/gut.19.5.408]

34 **Playford RJ**, Wright NA. Why is epidermal growth factor present in the gut lumen? *Gut* 1996; **38**: 303-305 [PMID: 8675078 DOI: 10.1136/gut.38.3.303]

35 **Scheving LA**, Shiurba RA, Nguyen TD, Gray GM. Epidermal growth factor receptor of the intestinal enterocyte. Localization to laterobasal but not brush border membrane. *J Biol Chem* 1989; **264**: 1735-1741 [PMID: 2912982]

36 **Amasheh M**, Andres S, Amasheh S, Fromm M, Schulzke JD. Barrier effects of nutritional factors. *Ann N Y Acad Sci* 2009; **1165**: 267-273 [PMID: 19538315 DOI: 10.1111/j.1749-6632.2009.04063.x]

37 **Gu L**, Li N, Li Q, Zhang Q, Wang C, Zhu W, Li J. The effect of berberine in vitro on tight junctions in human Caco-2 intestinal epithelial cells. *Fitoterapia* 2009; **80**: 241-248 [PMID: 19243699 DOI: 10.1016/j.fitote.2009.02.005]

38 **Bansal T**, Alaniz RC, Wood TK, Jayaraman A. The bacterial signal indole increases epithelial-cell tight-junction resistance and attenuates indicators of inflammation. *Proc Natl Acad Sci USA* 2010; **107**: 228-233 [PMID: 19966295 DOI: 10.1073/pnas.0906112107]

39 **Wang X**, Valenzano MC, Mercado JM, Zurbach EP, Mullin JM. Zinc supplementation modifies tight junctions and alters barrier function of CACO-2 human intestinal epithelial layers. *Dig Dis Sci* 2013; **58**: 77-87 [PMID: 22903217 DOI: 10.1007/s10620-012-2328-8]

40 **Mercado J**, Valenzano MC, Jeffers C, Sedlak J, Cugliari MK, Papanikolaou E, Clouse J, Miao J, Wertan NE, Mullin JM. Enhancement of tight junctional barrier function by micronutrients: compound-specific effects on permeability and claudin composition. *PLoS One* 2013; **8**: e78775 [PMID: 24236048 DOI: 10.1371/journal.pone.0078775]

41 **Li N**, Gu L, Qu L, Gong J, Li Q, Zhu W, Li J. Berberine attenuates pro-inflammatory cytokine-induced tight junction disruption in an in vitro model of intestinal epithelial cells. *Eur J Pharm Sci* 2010; **40**: 1-8 [PMID: 20149867 DOI: 10.1016/j.ejps.2010.02.001]

42 **Taylor CT**, Baird AW. Berberine inhibition of electrogenic ion transport in rat colon. *Br J Pharmacol* 1995; **116**: 2667-2672 [PMID: 8590987 DOI: 10.1111/j.1476-5381.1995.tb17224.x]

43 **Taylor CT**, Winter DC, Skelly MM, O'Donoghue DP, O'Sullivan GC, Harvey BJ, Baird AW. Berberine inhibits ion transport in human colonic epithelia. *Eur J Pharmacol* 1999; **368**: 111-118 [PMID: 10096776 DOI: 10.1016/S0014-2999(99)00023-0]

44 **Chen C**, Yu Z, Li Y, Fichna J, Storr M. Effects of berberine in the gastrointestinal tract - a review of actions and therapeutic im-

plications. *Am J Chin Med* 2014; **42**: 1053-1070 [PMID: 25183302 DOI: 10.1142/S0192415X14500669]

45 **Pan GY**, Wang GJ, Liu XD, Fawcett JP, Xie YY. The involvement of P-glycoprotein in berberine absorption. *Pharmacol Toxicol* 2002; **91**: 193-197 [PMID: 12530470 DOI: 10.1034/j.1600-0773.2002.t01-1-910403.x]

P- Reviewer: Can G, Suzuki H, Touil-Boukoffa C
S- Editor: Ji FF **L- Editor**: A **E- Editor**: Li D

World Journal of Gastrointestinal Pathophysiology

Submit a Manuscript: http://www.wjgnet.com/esps/
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.4291/wjgp.v7.i2.235

World J Gastrointest Pathophysiol 2016 May 15; 7(2): 235-241
ISSN 2150-5330 (online)

ORIGINAL ARTICLE

Basic Study

Visualization of sphingolipids and phospholipids in the fundic gland mucosa of human stomach using imaging mass spectrometry

Nobuya Kurabe, Hisaki Igarashi, Ippei Ohnishi, Shogo Tajima, Yusuke Inoue, Yoshihiko Takahashi, Mitsutoshi Setou, Haruhiko Sugimura

Nobuya Kurabe, Hisaki Igarashi, Ippei Ohnishi, Yusuke Inoue, Yoshihiko Takahashi, Haruhiko Sugimura, Department of Tumor Pathology, Hamamatsu University School of Medicine, Shizuoka 431-3192, Japan

Ippei Ohnishi, Division of Pathology, Iwata City Hospital, Shizuoka 438-8550, Japan

Shogo Tajima, Department of Pathology, the University of Tokyo, Tokyo 113-0033, Japan

Yusuke Inoue, Second Division, Department of Internal Medicine, Hamamatsu University School of Medicine, Shizuoka 431-3192, Japan

Mitsutoshi Setou, Department of Cell Biology and Anatomy, Hamamatsu University School of Medicine, Shizuoka 431-3192, Japan

Author contributions: Kurabe N, Igarashi H, Ohnishi I, Tajima S, Inoue Y, Setou M and Sugimura H substantially contributed to the conception and design of the study and acquisition, analysis, and interpretation of data; all authors drafted the manuscript, made critical revisions related to the intellectual content of the manuscript, and approved the final version of the manuscript to be published.

Institutional review board statement: All the gastric tissue specimens were residual tissues from surgical procedures. The patients gave informed consent for research use, provided that the study was conducted anonymously.

Conflict-of-interest statement: To the best of our knowledge, no conflict of interest exists.

Data sharing statement: No additional data are available.

Correspondence to: Haruhiko Sugimura, MD, PhD, Professor, Department of Tumor Pathology, Hamamatsu University School of Medicine, 1-20-1 Handayama, Higashi-Ku, Hamamatsu, Shizuoka 431-3192, Japan. hsugimur@hama-med.ac.jp
Telephone: +81-53-4352220
Fax: +81-53-4352225

Received: December 10, 2015
Peer-review started: December 11, 2015
First decision: January 13, 2016
Revised: January 21, 2016
Accepted: March 1, 2016
Article in press: March 2, 2016
Published online: May 15, 2016

Abstract

AIM: To analyze the lipid distribution in gastric mucosae.

METHODS: Imaging mass spectrometry (MS) is a useful tool to survey the distribution of biomolecules in surgical specimens. Here we used the imaging MS apparatus named iMScope to identify the dominant molecules present in the human gastric mucosa near the fundic glands. Five gastric specimens were subjected to iMScope analysis. These specimens were also analyzed by immunohistochemistry using MUC5AC, H(+)-K(+)-ATPaseβ Claudin18 antibodies.

RESULTS: Three major molecules with *m/z* 725.5, 780.5, and 782.5 detected in the gastric mucosa were identified as sphingomyelin (SM) (d18:1/16:0), phosphatidylcholine

(PC) (16:0/18:2), and PC (16:0/18:1), respectively, through MS/MS analyses. Using immunohistological staining, SM (d18:1/16:0) signals were mainly co-localized with the foveolar epithelium marker MUC5AC. In contrast, PC (16:0/18:2) signals were observed in the region testing positive for the fundic gland marker H(+)-K(+)-ATPaseβ. PC (16:0/18:1) signals were uniformly distributed throughout the mucosa.

CONCLUSION: Our basic data will contribute to the studies of lipid species in physical and pathological conditions of the human stomach.

Key words: Imaging mass spectrometry; iMScope; Sphingomyelin; Phosphatidylcholine; Gastric mucosa

Core tip: Imaging mass spectrometry (MS) is a useful tool to survey the distribution of biomolecules in surgical specimens. Here we used the imaging MS apparatus named iMScope to identify the dominant molecules present in the human gastric mucosa near the fundic glands. Three major molecules with *m/z* 725.5, 780.5, and 782.5 detected in the gastric mucosa were identified as sphingomyelin (d18:1/16:0), phosphatidylcholine (PC) (16:0/18:2), and PC (16:0/18:1), respectively.

Kurabe N, Igarashi H, Ohnishi I, Tajima S, Inoue Y, Takahashi Y, Setou M, Sugimura H. Visualization of sphingolipids and phospholipids in the fundic gland mucosa of human stomach using imaging mass spectrometry. *World J Gastrointest Pathophysiol* 2016; 7(2): 235-241 Available from: URL: http://www.wjgnet.com/2150-5330/full/v7/i2/235.htm DOI: http://dx.doi.org/10.4291/wjgp.v7.i2.235

INTRODUCTION

The wall of the stomach is composed of mucosa, submucosa, muscularis propria, and subserosa[1]. Except for the mucosa and proper glands, the structures of these layers are the same throughout the gastrointestinal tract. The mucosa of the stomach contains two structurally different layers: A superficial layer with foveolae and a deep layer with coiled glands. The lamina propria exists beneath the foveolar epithelium and harbors the proper gastric glands. The gastric mucosa possesses the ability to protect itself from numerous internal and external stimuli. Various intrinsic factors and systems, such as acid, mucus, bicarbonate, prostaglandins, biotin, blood flow, and the self-renewal of the epithelium as well as extrinsic infections, contribute to this defense mechanism. Loss of gastric mucosa causes gastric ulceration, erosion, or gastritis.

Imaging mass spectrometry (MS) is a recently developed modality that combines microscopy and MS[2-6]. Using this technique, the spatial distribution and molecular profiling of the analytes can be assessed simultaneously in a non-targeted manner. In fact, some lipids and proteins can be identified solely through imaging MS[7-9]. Because antibodies against lipids are difficult to generate, imaging MS is the most suitable option for the study of the lipid "metabolome". Shimadzu Co. (Shimadzu, Kyoto, Japan) has developed a novel application for imaging MS named iMScope[10]. Because of its higher resolution compared with other imaging MS apparatuses, it enables us to visualize the localization of many lipids at one time. Using iMScope, we have already demonstrated the exact spatial distribution of lung surfactant and also discovered a specific phosphatidylcholine that is a potential biomarker in colorectal cancer tissue[11,12].

In this study, to investigate the molecular profile of human gastric mucosa in detail, iMScope was used to analyze the lipid distribution in the human gastric mucosa near the fundic glands. We identified, for the first time, the exact localization of lipids, including phospholipids and sphingolipid, in the human gastric mucosa near the fundic glands.

MATERIALS AND METHODS

Sample preparation

Five gastric samples were retrieved from the archives of Hamamatsu University Hospital. Non-disease portions (fundic gland area) of gastric tissues obtained from gastric surgical specimens were snap-frozen in liquid nitrogen and stored at -80 ℃. The tissue blocks were put in the cryostat (CM1950; Leica, Microsystems, Wetzlar, Germany) at -20 ℃ for 30 min. The tissue blocks were then sectioned to a thickness of 8 μm at -20 ℃. Then, the tissue sections were subjected to hematoxylin and eosin (HE) staining. The adjacent sections were mounted on indium-tin-oxide (ITO)-coated glass slides (Bruker Daltonics, Billerica, MA, United States) for imaging MS and on MAS coated glass slides for immunohistochemistry. The tissue sections on the ITO-coated glass slides were then kept at room temperature. Next, 2,5-dihydroxybenzoic acid (DHB; Bruker Daltonics) was deposited on the sections using a deposition apparatus[11].

Imaging MS and MS/MS analysis

An iMScope (Shimadzu) instrument, which consists of an atmospheric pressure matrix-assisted laser desorption/ionization system equipped with a quadrupole ion trap-time of flight analyzer, was used to obtain the imaging MS data[10]. The sample was scanned with a focused laser (a diode-pumped 355-nm Nd:YAG laser) to acquire the mass spectrum of each spot with a laser shot number of 200 per pixel and a 1000 Hz frequency. The reflection mode was applied to each measurement. The mass range was set to *m/z* 700-900 with a scan pitch of 7.5 μm (for 20 × magnification) or a 20 μm (for 2.5 × magnification) pixel size. The BioMap software (freeware: www.maldi-msi.org) graphical interface was used to visualize the ion images[13].

Table 1 Summary of averaged peak intensities in gastric mucosae

m/z	Averaged intensity
723.4	5.02 ± 1.75
725.5	29.09 ± 13.48
741.4	8.24 ± 5.30
756.5	7.11 ± 2.70
772.4	5.99 ± 3.03
780.5	22.16 ± 23.27
781.5	10.69 ± 9.95
782.5	22.55 ± 16.04
796.5	12.69 ± 8.95
798.5	14.90 ± 9.58
804.5	8.09 ± 4.87
806.5	6.55 ± 3.02
808.5	9.61 ± 6.41
820.5	5.92 ± 2.40
824.5	8.26 ± 4.54

For each spectrum, baseline subtraction, smoothing, normalization to the total ion current, and recalibration were conducted using ClinProTools 2.2 software (Bruker Daltonics)[12]. The total ion currents were the sum of all spectrum intensities. The spectra processing parameters were as follows: Baseline correction [Top Hat algorithm (minimal baseline width set to 10%), resolution (500 ppm), and smoothing (Savitzky Golay, 5 cycles with a 2 *m/z* width)]. Recalibration was performed to reduce mass shifts. Peak picking was also performed based on the overall average spectrum for the whole mass range (signal to noise threshold of 5). The treated data was the average spectrum of input data sets. MS/MS analyses were performed to assign the molecular species using QSTAR Elite (Applied Biosystems, Foster City, CA, United States)[12]. The MS/MS spectral data were then verified using the LIPID MAPS database (http://lipidmaps.org).

Immunohistochemistry

Tissue preparation and immunohistochemical procedures were performed as previously described[14,15]. The 5 μm-thick sections were treated with 0.3% hydrogen peroxide to inactivate endogenous peroxidase activity. To identify the structure of the gastric mucosa, antibodies against MUC5AC (1:50, clone CLH2; Novocastra Laboratories, United Kingdom), claudin-18 (1:200, clone 5G7F2; proteintech, IL, United States), and H(+)-K(+)-ATPaseβ (1:1600, clone 2G11; Abcam, United Kingdom) were used to indicate the foveolar epithelium, fundic glands and foveolar epithelium, and fundic glands, respectively. For antigen retrieval, the slides were heated at 96 ℃ for 30 min in Tris-HCl-EDTA (TE) buffer (pH 9.0), followed by incubation at room temperature for 30 min. The sections were then incubated with a peroxidase-conjugated secondary antibody (Histofine Simple Stain MAX PO; Nichirei, Japan) at room temperature for 30 min. Next, the sections were treated with diaminobenzidine (DAB) substrate-chromogen solution (DAKO Cytomation; Carpinteria, CA, United States), followed by counterstaining with 0.1% hematoxylin. Images of these sections were obtained using Keyence BZ-9000 (Keyence, Tokyo, Japan). The stained sections were histologically evaluated by experienced pathologists[11,12].

RESULTS

We used the imaging MS modality called iMScope to analyze the spatial distribution of lipids in the gastric mucosae from five individuals. The gastric mucosal region (Figure 1A; inset) was subjected to imaging MS analysis. Table 1 presents the list of ions obtained in the five gastric mucosae using imaging MS analysis. A representative mass spectrum obtained from the gastric mucosa near the fundic gland is shown in Figure 1B. Three major peaks were observed (*m/z* 725.5, 780.5, and 782.5) among these ions. The most intense peak was the ion at *m/z* 725.5. We subsequently used BioMap software to image the spatial distribution of these ions. Figure 1C presents the region of interest (ROI) of the gastric mucosa used to perform imaging MS. The strong signals from these ions were observed in the mucosal region of the gastric wall (Figure 1D for *m/z* 725.5, E for *m/z* 780.5, and F for *m/z* 782.5).

Identification of gastric mucosa specific lipids

MS/MS analyses were performed to assign these ion species. Figure 2A presents the MS/MS spectrum obtained for the ion at *m/z* 725.5. This spectral pattern was identical to the one previously reported by Sudano *et al*[16]. Thus, this ion was shown to be sphingomyelin (SM) [SM (d18:1/16:0) + Na]$^+$. The ions at *m/z* 780.5 and 782.5 were identified as phosphatidylcholoine (PC) [PC (16:0/18:2) + Na]$^+$ and [PC (16:0/18:1) + Na]$^+$, respectively, because of the neutral losses of 59 Da and 183 Da (Figure 2D and G).

Detailed spatial distribution of the identified lipids

To specify the spatial distribution patterns of these lipids more precisely, we compared the ion images with the staining of three gastric mucosal markers. Figure 2B, E, and H are the low-power field ion images of these lipids, and Figure 2C, F, and I are the immunohistological staining patterns of the gastric mucosal markers MUC5AC[17], H(+)-K(+)-ATPaseβ[18] and claudin18[19], respectively. MUC5AC staining was specific for the surface region of the mucosa. H(+)-K(+)-ATPaseβ is a fundic gland marker. Claudin18 is expressed throughout the mucosa. The ion at *m/z* 725.5 was present in the surface of the gastric mucosa, which corresponded to the area of MUC5AC staining. The ion at *m/z* 780.5 was highly expressed in the bottom of the mucosa, which contains the fundic glands. The ion at *m/z* 782.5 was uniformly spread in the mucosa, similar to the area of claudin18 staining.

DISCUSSION

Lipids are important functional molecules in the human body. Phospholipids, which are constituents of plasma membrane, have recently been recognized to have important roles in cellular systems. For example, PC

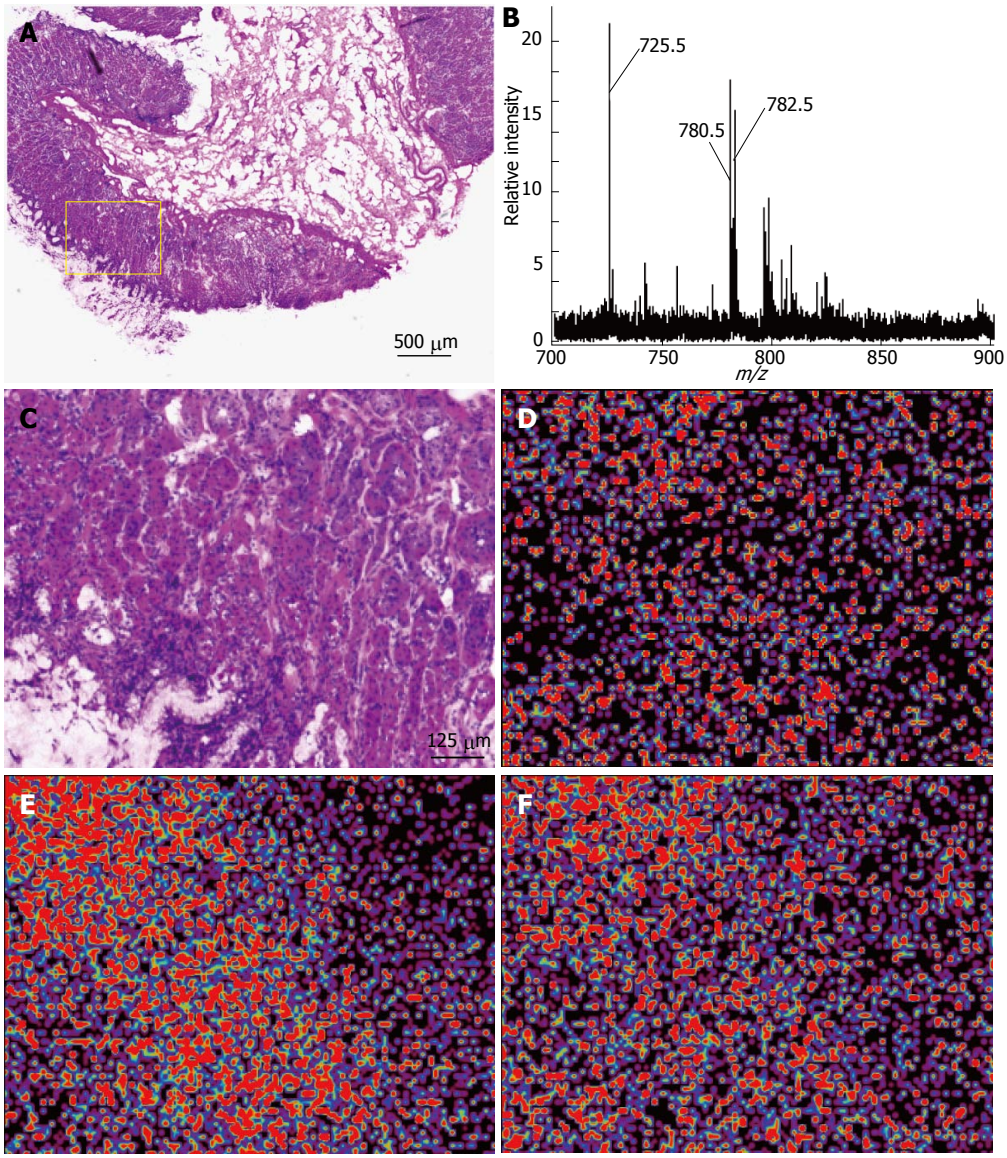


Figure 1 Imaging mass spectrometry analysis of a gastric mucosa. A: HE staining of the gastric mucosa. Inset, ROI of the imaging analysis; B: Averaged spectra obtained from five gastric mucosae; C: Magnified view of the ROI represented in the inset of (A), HE; D: The ion at *m/z* 725.5; E: The ion at *m/z* 780.5; F: The ion at *m/z* 782.5 were imaged using BioMap.

(16:0/16:0) plays an important role as a surfactant in the reduction of surface tension in the lung[20,21]. PC (16:0/18:1) has been shown to be a physiological PPARα ligand, regulating lipid metabolism and glucose homeostasis[22]. Moreover, PC (16:0/20:4) and PC (16:0/18:2) are crucial for the inactivation of Akt kinase[23]. Sphingolipids are also involved in cellular functions such as the cell cycle, apoptosis, senescence, and inflammation[24-26]. In this study, we identified three highly expressed lipid molecules, SM (d18:1/16:0), PC (16:0/18:2) and PC (16:0/18:1), in gastric mucosae (Figures 1 and 2). SM (d18:1/16:0) was mainly localized to the foveolar epithelium of the gastric mucosa (Figure 2B and C). The foveolar epithelium secretes mucus and bicarbonate ions to prevent the damaging effects by pepsin and acid. Because SM molecules are mainly distributed in the plasma membrane, they may cooperate with mucus and bicarbonate ions to protect the mucosal surface. PC (16:0/18:2) co-localized with the fundic gland marker H(+)-K(+)-ATPaseβ. Intriguingly, this observation may be related to the knowledge that Akt phosphorylation is suppressed in fundic glands under ordinary conditions (Figure 2E and F). Considering that Akt phosphorylation may increase the risk of various cancers, including gastric cancer[27,28], the presence of PC (16:0/18:2) may be involved in the sustainability of the gastric mucosa, including the prevention of malignant transformation of gastric mucosae. The role of PC (16:0/18:1) in the gastric mucosa is unknown. This PC species is an endogenous PPARα ligand, leading to the activation of target genes such as *Acox1* and *Cpt1a*; this pathway lowers triglycerides and raises HDL. However, PPARα itself is not expressed in the stomach[29]; it is abundant in the liver. Thus PC (16:0/18:1) in gastric mucosae may have a function other than as a PPARα ligand (Figure 2H and I).

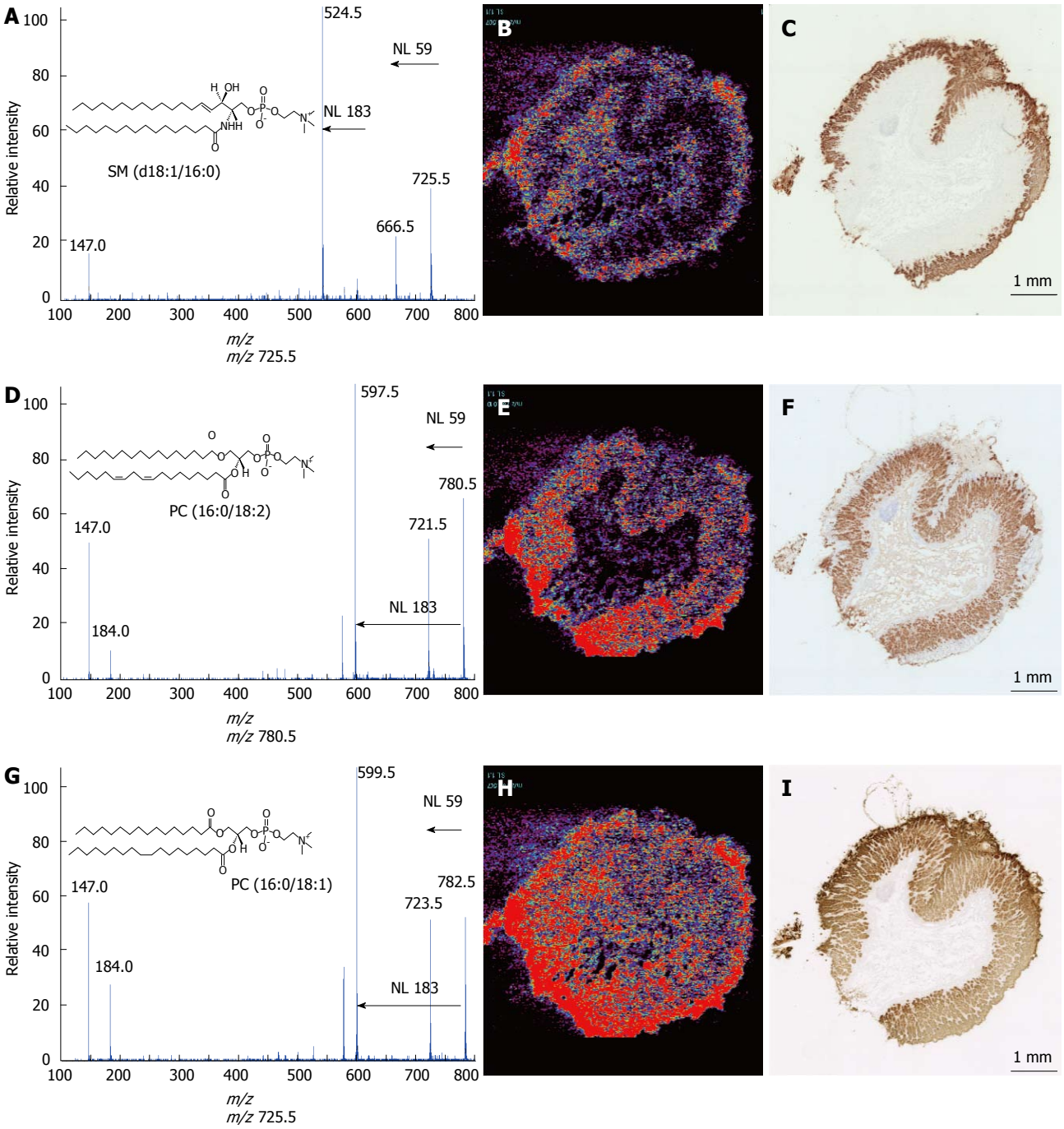


Figure 2 Ion assignment of *m/z* 725.5, 780.5, and 782.5 and immunohistochemical analyses of the gastric mucosae. MS/MS analyses were performed to identify the ions at *m/z* 725.5 (A), 780.5 (D), and 782.5 (G). The ion images of *m/z* 725.5 (B), 780.5 (E), and 782.5 (H) are shown using BioMap software. Immunohistochemical analyses were performed using the antibodies against MUC5AC (C), H(+)-K(+)-ATPaseβ (F), and claudin18 (I) in the adjacent specimens used in the imaging MS analyses. Scale bar, 1 mm. MS: Mass spectrometry.

In conclusion, this study, for the first time, clarified the lipids localized in the human gastric mucosa near the fundic glands. Because we have just reached this level of the modality, in terms of resolution and the ability to identify molecules, the information available on human tissue is currently limited. Our results will be the basis for further investigations of phosphatidylcholine and sphingomyelin species in physical and pathological conditions of the human stomach and will help the precise understanding of the nature of lipid function in the stomach.

COMMENTS

Background

Because antibodies against lipids are difficult to generate, more innovative methodologies are needed in lipid research field to analyze human disease. The authors developed the imaging mass spectrometry (MS) apparatus "iMScope"

to visualize the lipid distribution in the pathological specimen and applied this technique to the measurement of gastric mucosae.

Research frontires

iMScope can irradiate using a thinner laser than other imaging MS modalities, which enables the finest ion image of lipids in the world.

Innovations and breakthroughs

To the best of the authors' knowledge, this is the first time that lipid images of gastric mucosae were obtained.

Applications

Because the authors showed functional lipid images in gastric mucosae, these lipid distributions may reflect the significant role of lipids in the homeostasis of gastric mucosae.

Terminology

Imaging MS is a novel technique that enables us to visualize many biomolecules at one time. The apparatus of imaging MS is composed of a microscope and a mass spectrometer. In the microscopic part, the authors can determine the region of interest (ROI) within the specimen sample and then scan this ROI with the laser. Ions from the evaporated vapors are transferred to the mass spectrometric part, where their mass spectra are obtained. The scanned data are then visualized along a two-dimensional axis.

Peer-review

This report combines the imaging MS with immunohistochemistry to show the lipid spatial distribution on gastric mucosae. Imaging MS is shown to be a useful tool to survey the distribution of biomolecules in the pathological samples. This report firstly applied the iMSope to locate the lipids including both phospholipids and sphingolipid in gastric mucosa, which is helpful to better understand the lipid's function in stomach.

REFERENCES

1 **David AO**. Stomach. In: Histology for Pathologists. 4th ed. Mills SE, editor. Philadelphia: Lippincott Williams Wilkins, 2012: 633-646

2 **McDonnell LA**, Heeren RM. Imaging mass spectrometry. *Mass Spectrom Rev* 2007; **26**: 606-643 [PMID: 17471576 DOI: 10.1002/mas.20124]

3 **Chaurand P**, Sanders ME, Jensen RA, Caprioli RM. Proteomics in diagnostic pathology: profiling and imaging proteins directly in tissue sections. *Am J Pathol* 2004; **165**: 1057-1068 [PMID: 15466373 DOI: 10.1016/S0002-9440(10)63367-6]

4 **Cornett DS**, Reyzer ML, Chaurand P, Caprioli RM. MALDI imaging mass spectrometry: molecular snapshots of biochemical systems. *Nat Methods* 2007; **4**: 828-833 [PMID: 17901873 DOI: 10.1038/nmeth1094]

5 **Seeley EH**, Caprioli RM. Molecular imaging of proteins in tissues by mass spectrometry. *Proc Natl Acad Sci USA* 2008; **105**: 18126-18131 [PMID: 18776051 DOI: 10.1073/pnas.0801374105]

6 **Kakimoto Y**, Tsuruyama T, Yamamoto T, Furuta M, Kotani H, Ozeki M, Yoshizawa A, Haga H, Tamaki K. Novel in situ pretreatment method for significantly enhancing the signal in MALDI-TOF MS of formalin-fixed paraffin-embedded tissue sections. *PLoS One* 2012; **7**: e41607 [PMID: 22899997 DOI: 10.1371/journal.pone.0041607PONE-D-12-07910]

7 **Cazares LH**, Troyer D, Mendrinos S, Lance RA, Nyalwidhe JO, Beydoun HA, Clements MA, Drake RR, Semmes OJ. Imaging mass spectrometry of a specific fragment of mitogen-activated protein kinase/extracellular signal-regulated kinase kinase kinase 2 discriminates cancer from uninvolved prostate tissue. *Clin Cancer Res* 2009; **15**: 5541-5551 [PMID: 19690195 DOI: 10.1158/1078-0432.CCR-08-2892]

8 **Morita Y**, Ikegami K, Goto-Inoue N, Hayasaka T, Zaima N, Tanaka H, Uehara T, Setoguchi T, Sakaguchi T, Igarashi H, Sugimura H, Setou M, Konno H. Imaging mass spectrometry of gastric carcinoma in formalin-fixed paraffin-embedded tissue microarray. *Cancer Sci* 2010; **101**: 267-273 [PMID: 19961487 DOI: 10.1111/j.1349-7006.2009.01384.x]

9 **Liu Y**, Chen Y, Momin A, Shaner R, Wang E, Bowen NJ, Matyunina LV, Walker LD, McDonald JF, Sullards MC, Merrill AH. Elevation of sulfatides in ovarian cancer: an integrated transcriptomic and lipidomic analysis including tissue-imaging mass spectrometry. *Mol Cancer* 2010; **9**: 186 [PMID: 20624317 DOI: 10.1186/1476-4598-9-186]

10 **Harada T**, Yuba-Kubo A, Sugiura Y, Zaima N, Hayasaka T, Goto-Inoue N, Wakui M, Suematsu M, Takeshita K, Ogawa K, Yoshida Y, Setou M. Visualization of volatile substances in different organelles with an atmospheric-pressure mass microscope. *Anal Chem* 2009; **81**: 9153-9157 [PMID: 19788281 DOI: 10.1021/ac901872n]

11 **Kurabe N**, Hayasaka T, Igarashi H, Mori H, Sekihara K, Tao H, Yamada H, Kahyo T, Onishi I, Tsukui H, Kawase A, Matsuura S, Inoue Y, Shinmura K, Funai K, Setou M, Sugimura H. Visualization of phosphatidylcholine (16: 0/16: 0) in type II alveolar epithelial cells in the human lung using imaging mass spectrometry. *Pathol Int* 2013; **63**: 195-200 [PMID: 23692419 DOI: 10.1111/pin.12050]

12 **Kurabe N**, Hayasaka T, Ogawa M, Masaki N, Ide Y, Waki M, Nakamura T, Kurachi K, Kahyo T, Shinmura K, Midorikawa Y, Sugiyama Y, Setou M, Sugimura H. Accumulated phosphatidylcholine (16: 0/16: 1) in human colorectal cancer; possible involvement of LPCAT4. *Cancer Sci* 2013; **104**: 1295-1302 [PMID: 23815430 DOI: 10.1111/cas.12221]

13 **Shimma S**, Sugiura Y, Hayasaka T, Zaima N, Matsumoto M, Setou M. Mass imaging and identification of biomolecules with MALDI-QIT-TOF-based system. *Anal Chem* 2008; **80**: 878-885 [PMID: 18166020 DOI: 10.1021/ac071301v]

14 **Sugimura H**, Mori H, Nagura K, Kiyose S, Tao H, Isozaki M, Igarashi H, Shinmura K, Hasegawa A, Kitayama Y, Tanioka F. Fluorescence in situ hybridization analysis with a tissue microarray: 'FISH and chips' analysis of pathology archives. *Pathol Int* 2010; **60**: 543-550 [PMID: 20618731 DOI: 10.1111/j.1440-1827.2010.02561.x]

15 **Igarashi H**, Sugimura H, Maruyama K, Kitayama Y, Ohta I, Suzuki M, Tanaka M, Dobashi Y, Kino I. Alteration of immunoreactivity by hydrated autoclaving, microwave treatment, and simple heating of paraffin-embedded tissue sections. *APMIS* 1994; **102**: 295-307 [PMID: 7516673]

16 **Sudano MJ**, Santos VG, Tata A, Ferreira CR, Paschoal DM, Machado R, Buratini J, Eberlin MN, Landim-Alvarenga FD. Phosphatidylcholine and sphingomyelin profiles vary in Bos taurus indicus and Bos taurus taurus in vitro- and in vivo-produced blastocysts. *Biol Reprod* 2012; **87**: 130 [PMID: 23053436 DOI: 10.1095/biolreprod.112.102897]

17 **Reis CA**, David L, Nielsen PA, Clausen H, Mirgorodskaya K, Roepstorff P, Sobrinho-Simões M. Immunohistochemical study of MUC5AC expression in human gastric carcinomas using a novel monoclonal antibody. *Int J Cancer* 1997; **74**: 112-121 [PMID: 9036879]

18 **Chow DC**, Forte JG. Characterization of the beta-subunit of the H(+)-K(+)-ATPase using an inhibitory monoclonal antibody. *Am J Physiol* 1993; **265**: C1562-C1570 [PMID: 8279517]

19 **Niimi T**, Nagashima K, Ward JM, Minoo P, Zimonjic DB, Popescu NC, Kimura S. claudin-18, a novel downstream target gene for the T/EBP/NKX2.1 homeodomain transcription factor, encodes lung- and stomach-specific isoforms through alternative splicing. *Mol Cell Biol* 2001; **21**: 7380-7390 [PMID: 11585919 DOI: 10.1128/MCB.21.21.7380-7390.2001]

20 **Bernhard W**, Haagsman HP, Tschernig T, Poets CF, Postle AD, van Eijk ME, von der Hardt H. Conductive airway surfactant: surface-tension function, biochemical composition, and possible alveolar origin. *Am J Respir Cell Mol Biol* 1997; **17**: 41-50 [PMID: 9224208 DOI: 10.1165/ajrcmb.17.1.2594]

21 **Lang CJ**, Postle AD, Orgeig S, Possmayer F, Bernhard W, Panda AK, Jürgens KD, Milsom WK, Nag K, Daniels CB. Dipalmitoylphosphatidylcholine is not the major surfactant phospholipid species in all mammals. *Am J Physiol Regul Integr Comp Physiol* 2005; **289**: R1426-R1439 [PMID: 16037124 DOI: 10.1152/ajpregu.00496.2004]

22 **Chakravarthy MV**, Lodhi IJ, Yin L, Malapaka RR, Xu HE, Turk J, Semenkovich CF. Identification of a physiologically relevant endogenous ligand for PPARalpha in liver. *Cell* 2009; **138**: 476-488 [PMID: 19646743 DOI: 10.1016/j.cell.2009.05.036]
23 **Koeberle A**, Shindou H, Koeberle SC, Laufer SA, Shimizu T, Werz O. Arachidonoyl-phosphatidylcholine oscillates during the cell cycle and counteracts proliferation by suppressing Akt membrane binding. *Proc Natl Acad Sci USA* 2013; **110**: 2546-2551 [PMID: 23359699 DOI: 10.1073/pnas.1216182110]
24 **Smith ER**, Merrill AH, Obeid LM, Hannun YA. Effects of sphingosine and other sphingolipids on protein kinase C. *Methods Enzymol* 2000; **312**: 361-373 [PMID: 11070884]
25 **Venable ME**, Lee JY, Smyth MJ, Bielawska A, Obeid LM. Role of ceramide in cellular senescence. *J Biol Chem* 1995; **270**: 30701-30708 [PMID: 8530509]
26 **Hla T**. Physiological and pathological actions of sphingosine 1-phosphate. *Semin Cell Dev Biol* 2004; **15**: 513-520 [PMID: 15271296 DOI: 10.1016/j.semcdb.2004.05.002]
27 **Cinti C**, Vindigni C, Zamparelli A, La Sala D, Epistolato MC, Marrelli D, Cevenini G, Tosi P. Activated Akt as an indicator of prognosis in gastric cancer. *Virchows Arch* 2008; **453**: 449-455 [PMID: 18841391 DOI: 10.1007/s00428-008-0676-8]
28 **Murakami D**, Tsujitani S, Osaki T, Saito H, Katano K, Tatebe S, Ikeguchi M. Expression of phosphorylated Akt (pAkt) in gastric carcinoma predicts prognosis and efficacy of chemotherapy. *Gastric Cancer* 2007; **10**: 45-51 [PMID: 17334718 DOI: 10.1007/s10120-006-0410-7]
29 **Braissant O**, Foufelle F, Scotto C, Dauça M, Wahli W. Differential expression of peroxisome proliferator-activated receptors (PPARs): tissue distribution of PPAR-alpha, -beta, and -gamma in the adult rat. *Endocrinology* 1996; **137**: 354-366 [PMID: 8536636 DOI: 10.1210/endo.137.1.8536636]

P- Reviewer: Liu DL **S- Editor**: Qi Y **L- Editor**: A
E- Editor: Lu YJ

Published by **Baishideng Publishing Group Inc**
8226 Regency Drive, Pleasanton, CA 94588, USA
Telephone: +1-925-223-8242
Fax: +1-925-223-8243
E-mail: bpgoffice@wjgnet.com
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
http://www.wjgnet.com

ISSN 2150-5330 (online)

World Journal of *Gastrointestinal Pathophysiology*

World J Gastrointest Pathophysiol 2016 August 15; 7(3): 242-299

Published by Baishideng Publishing Group Inc

WJGP World Journal of Gastrointestinal Pathophysiology

A peer-reviewed, online, open-access journal of gastrointestinal pathophysiology

Editorial Board

2016-2019

The *World Journal of Gastrointestinal Pathophysiology* Editorial Board consists of 503 members, representing a team of worldwide experts in gastrointestinal pathophysiology. They are from 45 countries, including Argentina (2), Australia (13), Austria (2), Belgium (7), Brazil (9), Brunei Darussalam (1), Canada (21), China (30), Croatia (1), Czech Repoublic (2), Denmark (3), Egypt (1), Estonia (1), Finland (1), France (8), Germany (24), Greece (7), Hungary (5), India (12), Indonesia (1), Iran (2), Ireland (2), Israel (8), Italy (38), Japan (45), Lebanon (3), Malaysia (1), Mexico (2), Netherlands (7), Norway (1), Poland (4), Portugal (1), Romania (1), Russia (1), Singapore (4), South Korea (12), Spain (24), Sweden (11), Switzerland (3), Thailand (2), Turkey (7), Ukraine (1), United Kingdom (10), United States (161) and Venezuela (1).

Zhan-Ju Liu, *Shanghai*
Shi Liu, *Wuhan*
Zhen-Ning Wang, *Shenyang*
Xiao-Hong Wang, *Beijing*
Wei Wei, *Hefei*
Dong-Ping Xie, *Shanghai*
Wen-Xie Xu, *Shanghai*
Xiao Yang, *Beijing*
Hua Yang, *Chongqing*
Wei-Zhen Zhang, *Beijing*
Hua-Chuan Zheng, *Shenyang*
Min-Sheng Zhu, *Nanjing*
Jinxia Zhu, *Beijing*
Da-Ling Zhu, *Harbin*
Yong-Liang Zhu, *Hangzhou*

Croatia

Alen Protic, *Rijeka*

Czech Republic

Pavel Hladik, *Semily*
Martin Vokurka, *Prague*

Denmark

Lars Arendt-Nielsen, *Aalborg*
Frank V Schiodt, *Copenhagen*
Jonas Worsoe, *Aarhus*

Egypt

Mahmoud Aboelneen Khattab, *Minia*

Estonia

Enn Seppet, *Tartu*

Finland

Pauli Antero Puolakkainen, *Turku*

France

Bruno Bonaz, *Grenoble*
Pierre M Dechelotte, *Rouen*
Jean-Paul Lallès, *Saint-Gilles*
Charles-Henri Malbert, *Saint-Gilles*
Thierry Piche, *Nice*
Pascale Plaisancié, *Lyon*
Michelina Plateroti, *Lyon*
Véronique Vitton, *Marseille*

Germany

Hans G Beger, *Ulm*
Carsten Bergmann, *Ingelheim*
Elke Cario, *Essen*
Arno J Dormann, *Koln*
Nikolaus Gassler, *Aachen*
Andreas Geier, *Wuerzburg*
Werner Hartwig, *Heidelberg*
Marion Hewicker-Trautwein, *Hannover*
Jens Hoeppner, *Freiburg*
Tobias Keck, *Freiburg*
Jorg Kleeff, *Munich*
Peter Malfertheiner, *Magdeburg*
Oliver Mann, *Hamburg*
Christoph W Michalski, *Munich*
Andreas K Nussler, *Munich*
Christian Pehl, *Vilsbiburg*
Peter Schemmer, *Heidelberg*
Manuel A Silva, *Penzberg*
Marc Stemmler, *Freiburg*
Frank Tacke, *Aachen*
Sya N Ukena, *Braunschweig*
Brigitte Vollmar, *Rostock*
Thomas M Wex, *Magdeburg*
Margot Zoller, *Heidelberg*

Greece

Stelios F Assimakopoulos, *Patras*
George N Dalekos, *Larissa*
Alkiviadis Efthymiou, *thessaloniki*
Maria Gazouli, *Athens*
Ioannis E Koutroubakis, *Heraklion*
Gerassimos J Mantzaris, *Athens*
George V Papatheodoridis, *Athens*

Hungary

Mária Bagyánszki, *Szeged*
Mihály Boros, *Szeged*
Laszlo Czako, *Szeged*
Pal Miheller, *Budapest*
Zoltan Rakonczay, *Szeged*

India

Anil K Agarwal, *Delhi*
Uday Bandyopadhyay, *Kolkata*
Sriparna Basu, *Varanasi*
Chandra K Chakraborti, *Rourkela*
Nilesh M Dagia, *Mumbai*
Rajeev Garg, *Punjab*
Akhtar Mahmood, *Chandigarh*
Veerareddy P Reddy, *Andhra Pradesh*
Chandra P Sharma, *Thiruvananthapuram*
Shailesh V Shrikhande, *Mumbai*
Virendra Singh, *Chandigarh*
Nicholas J Skill, *Indianapolis*

Indonesia

Laurentius A Lesmana, *Jakarta*

Iran

Gholamreza Roshandel, *Gorgan*
Shahram Shahabi, *Urmia*

Ireland

Billy Bourke, *Dublin*
Stephen J Keely, *Dublin*

Israel

Yosefa Avraham, *Jerusalem*
Yaron Bar-Dayan, *Holon*
Shomron Ben-Horin, *Tel-Hashomer*
Boris Kirshtein, *Beer Sheva*
Stephen Malnick, *Rehovot*
Yaakov Maor, *Tel-Hashomer*
Rifaat Safadi, *Jerusalem*
Nachum Vaisman, *Tel Aviv*

Italy

Rosaria Acquaviva, *Catania*
Alessandro Antonelli, *Pisa*
Giacosa Attilio, *Genova*
Salavtore Auricchio, *Naples*
Guido Basilisco, *Milan*
Antonio Basoli, *Rome*
Massimo Bellini, *Pisa*
Luigi Bonavina, *Milano*
Alfio Brogna, *Catania*
Giuseppe Calamita, *Bari*
Raffaele Capasso, *Naples*
Ignazio Castagliuolo, *Padova*
Francesco Cresi, *Torino*
Rosario Cuomo, *Napoli*
Salvatore Cuzzocrea, *Gazzi*
Mario M D'Elios, *Florence*
Cinzia Domeneghini, *Milan*
Luca Elli, *Milano*
Walter Fries, *Messina*
Eugenio Gaudio, *Rome*
Marco Gobbetti, *Bari*
Fabio Grizzi, *Rozzano*
Enzo Grossi, *Milanese*
Enzo Ierardi, *Foggia*
Pietro Invernizzi, *Monza*
Angelo A Izzo, *Naples*
Anna Kohn, *Rome*
Giovanni Latella, *L'Aquila*
Massimo Marignani, *Rome*
Sergio Morini, *Rome*
Raffaele Pezzilli, *Bologna*
Cristiano Rumio, *Milan*
Giovanni Sarnelli, *Naples*
Edoardo V Savarino, *Padua*
Pierpaolo Sileri, *Roma*
Annamaria Staiano, *Naples*
Giacomo C Sturniolo, *Padua*
Claudio Tiribelli, *Trieste*

Japan

Hirotada Akiho, *Fukuoka*
Akihiro Asakawa, *Kagoshima*
Hisashi Aso, *Sendai*
Yasu-Taka Azuma, *Osaka*
Shotaro Enomoto, *Wakayama*
Mikihiro Fujiya, *Hokkaido*
Takahisa Furuta, *Hamamatsu*
Akira Hokama, *Nishihara*
Ryota Hokari, *Saitama*
Yuichi Hori, *Hyogo*
Masahiro Iizuka, *Akita*
Shoji Ikuo, *Kobe*
Motohiro Imano, *Osaka*

Hajime Isomoto, *Nagasaki*
Tatehiro Kagawa, *Isehara*
Haruki Kitazawa, *Sendai*
Shigeru BH Ko, *Tokyo*
Xiao-Kang Li, *Tokyo*
Noriaki Manabe, *Okayama*
Atsushi Masamune, *Sendai*
Hiroyuki Matsubayashi, *Suntogun*
Kazuyuki Matsushita, *Inohana Chuo-ku*
Reiko Miyazawa, *Gunma*
Kazunari Murakami, *Oita*
Hikaru Nagahara, *Tokyo*
Yuji Naito, *Kyoto*
Atsushi Nakajima, *Yokohama*
Shoji Natsugoe, *Kagoshima City*
Tsutomu Nishida, *Suita*
Koji Nomoto, *Tokyo*
Shouji Shimoyama, *Tokyo*
Goshi Shiota, *Yonago*
Hidekazu Suzuki, *Tokyo*
Hitoshi Takagi, *Gunma*
Toru Takahashi, *Okayama*
Yoshihisa Takahashi, *Tokyo*
Kan Uchiyama, *Chiba*
Yoshiyuki Ueno, *Sendai*
Takato Ueno, *Fukuoka*
Hisayuki Uneyama, *Kwasaki*
Mitsunori Yamakawa, *Yamagata*
Takayuki Yamamoto, *Mie*
Yutaka Yata, *Maebashi-city*
Naohisa Yoshida, *Kyoto*
Hitoshi Yoshiji, *Kashihara*

Lebanon

Costantine F Daher, *Byblos*
Assaad M Soweid, *Beirut*
Julnar Usta, *Beirut*

Malaysia

Andrew CS Boon, *Ipoh*

Mexico

José M de la Roca-Chiapas, *Leon*
Maria-Raquel Huerta-Franco, *Leon*

Netherland

Wouter J de Jonge, *Amsterdam*
Aldo Grefhorst, *Groningen*
Daniel Keszthelyi, *Maastricht*
Cornelis FM Sier, *ZA Leiden*
Pieter J Tanis, *AZ Amsterdam*
Luc JW van der Laan, *Rotterdam*
Sander van der Marel, *Leiden*

Norway

Anne M Bakke, *Oslo*

Poland

Stanistaw A Hac, *Gdansk*
Stanislaw J Kowalski, *Krakow*
Agata Mulak, *Wroclaw*
Napoleon Waszkiewicz, *Choroszcz*

Portugal

Ricardo Marcos, *Porto*

Romania

Mihai Ciocirlan, *Bucharest*

Russia

Ludmila Filaretova, *Petersburg*

Singapore

Madhav Bhatia, *Singapore*
Brian K Goh, *Singapore*
Khek-Yu Ho, *Singapore*
Cliff KS Ong, *Singapore*

South Korea

Jae Hee Cheon, *Seoul*
Myung-Haing Cho, *Seoul*
Ki-Baik Hahm, *Seongnam*
HO Jae Han, *Gwangju*
Chang-Duk Jun, *Gwangju*
Sang Geon Kim, *Seoul*
Hong Joo Kim, *Seoul*
Jin-Kyung Kim, *Gyeongsan-Si*
Won-Jae Lee, *Seoul*
Sung-Joo Park, *Iksan*
Seung Ha Park, *Gangwon Do*
Kwan-Kyu Park, *Daegu*

Spain

Raquel Abalo, *Madrid*
Juan G Abraldes, *Barcelona*
Dario Acuna-Castroviejo, *Armilla*
Agustin Albillos, *Madrid*
Maria-Angeles Aller, *Madrid*
Fernando Azpiroz, *Barcelona*
Ramón Bataller, *Barcelona*
Marco Bustamante-Balén, *Valencia*
Andres Cardenas, *Barcelona*
Joan Claria, *Barcelona*
Pere Clavé, *Barcelona*
Manuel B de Acosta, *Santiago*
Manuel Giner, *Madrid*
Borja Hernandez-Breijo, *Madrid*
Angel I Lanas, *Zaragoza*
Maite T Martin, *Barcelona*
Vicente Martinez, *Bellaterra*
Jose M Mates, *Malaga*
Julio Mayol, *Madrid*
Marcal Pastor-Anglada, *Barcelona*
María E Sáez, *Seville*
Yolanda Sanz, *Burjassot*
Carlos Taxonera, *Madrid*
Maria D Yago, *Granada*

Sweden

Marco Del Chiaro, *Stockholm*
Frida Fak, *Gothenburg*
Gunnar FA Flemstrom, *Uppsala*
Evangelos Kalaitzakis, *Gothenburg*
Kristina Lamas, *Umea*
Bob R Olsson, *Goteborg*
Sara M Regnér, *Malmo*
Peter T Schmidt, *Stockholm*
Xiao-Feng Sun, *Linkoping*
Henrik Thorlacius, *Malmo*
Curt Tysk, *Orebro*

Switzerland

Jyrki J Eloranta, *Zurich*
Remy Meier, *Liestal*
Catherine M Pastor, *Geneva*

Thailand

Thawatchai Akaraviputh, *Bangkok*
Weekitt Kittisupamongkol, *Bangkok*

Turkey

Mehmet Bektas, *Ankara*
Ugur Duman, *Bursa*
Mukaddes Esrefoglu, *Istanbul*
Ahmet Guven, *Ankara*
Muammer Karadeniz, *Bornova-Izmir*
Elvan Ozbek, *Sakarya*
Ilhami Yuksel, *Ankara*

Ukraine

Oksana S Zayavhkivska, *Lviv*

United Kingdom

Geoffrey Burnstock, *London*
Janice E Drew, *Aberdeen*
Girish L Gupte, *Birmingham*
David C Hay, *Edinburgh*
Nusrat Husain, *Manchester*
Michael L Lucas, *Glasgow*
Jamie Murphy, *London*
Vadim Sumbayev, *Chatham Maritime*
Wing-Kin Syn, *Birmingham*
Andrea Varro, *Liverpool*

United States

Sami R Achem, *Florida*
Tauseef Ali, *Oklahoma City*
Shrikant Anant, *Oklahoma*
Mohammed S Anwer, *North Grafton*
Andrew Aronsohn, *Chicago*
Toms Augustin, *Sayre*

WJGP

World Journal of Gastrointestinal Pathophysiology

Contents

Quarterly Volume 7 Number 3 August 15, 2016

Contents

World Journal of Gastrointestinal Pathophysiology
Volume 7 Number 3 August 15, 2016

NAME OF JOURNAL
World Journal of Gastrointestinal Pathophysiology

ISSN
ISSN 2150-5330 (online)

LAUNCH DATE
April 15, 2010

Frequency
Quarterly

EDITOR-IN-CHIEF
Thomas Y Ma, MD, PhD, Professor, Chief, Division of Gastroenterology and Hepatology, University of New Mexico, MSC10 5550, 1 UNM, Albuquerque, NM 87131, United States

EDITORIAL OFFICE
Jin-Lei Wang, Director
Xiu-Xia Song, Vice Director
World Journal of Gastrointestinal Pathophysiology

Room 903, Building D, Ocean International Center,
No. 62 Dongsihuan Zhonglu, Chaoyang District,
Beijing 100025, China
Telephone: +86-10-85381891
Fax: +86-10-85381893
E-mail: editorialoffice@wjgnet.com
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
http://www.wjgnet.com

PUBLISHER
Baishideng Publishing Group Inc
8226 Regency Drive,
Pleasanton, CA 94588, USA
Telephone: +1-925-223-8242
Fax: +1-925-223-8243
E-mail: bpgoffice@wjgnet.com
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
http://www.wjgnet.com

PUBLICATION DATE
August 15, 2016

INSTRUCTIONS TO AUTHORS
http://www.wjgnet.com/bpg/gerinfo/204

ONLINE SUBMISSION
http://www.wjgnet.com/esps/

World Journal of Gastrointestinal Pathophysiology

Submit a Manuscript: http://www.wjgnet.com/esps/
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.4291/wjgp.v7.i3.242

World J Gastrointest Pathophysiol 2016 August 15; 7(3): 242-255
ISSN 2150-5330 (online)

REVIEW

Immunobiology of hepatocarcinogenesis: Ways to go or almost there?

Pavan Patel, Steven E Schutzer, Nikolaos Pyrsopoulos

Pavan Patel, Steven E Schutzer, Department of Medicine, Rutgers-New Jersey Medical School, Newark, NJ 07101-1709, United States

Nikolaos Pyrsopoulos, Division of Gastroenterology and Hepatology, Rutgers-New Jersey Medical School, Newark, NJ 07101-1709, United States

Author contributions: Patel P wrote the paper; Schutzer SE and Pyrsopoulos N contributed to the manuscript by providing revision and oversight of its writing.

Conflict-of-interest statement: Nikolaos Pyrsopoulos, MD: Advisory board for GILEAD, BMS, ABBVIE research for ABBVIE.

Manuscript source: Invited manuscript

Correspondence to: Nikolaos Pyrsopoulos, Chief, Division of Gastroenterology and Hepatology, Rutgers-New Jersey Medical School, 185 South Orange Avenue, MSB H538, Newark, NJ 07101-1709, United States. pyrsopni@njms.rutgers.edu
Telephone: +1-973-9725252
Fax: +1-973-9723144

Received: April 26, 2016
Peer-review started: April 28, 2016
First decision: June 16, 2016
Revised: July 1, 2016
Accepted: July 20, 2016
Article in press: July 22, 2016
Published online: August 15, 2016

Abstract

Hepatocellular carcinoma is on the rise and occurs in the setting of chronic liver disease and cirrhosis. Though treatment modalities are available, mortality from this cancer remains high. Medical therapy with the utilization of biologic compounds such as the Food and Drug Administration approved sorafenib might be the only option that can increase survival. Immunotherapy, with modern pharmacologic developments, is a new frontier in cancer therapy and therefore the immunobiology of hepatocarcinogenesis is under investigation. This review will discuss current concepts of immunobiology in hepatocarcinogenesis along with current treatment modalities employing immunotherapy. The tumor microenvironment along with a variety of immune cells coexists and interplays to lead to tumorigenesis. Tumor infiltrating lymphocytes including $CD8^+$ T cells, $CD4^+$ T cells along with regulatory T cells, tumor associated macrophages, tumor associated neutrophils, myeloid derived suppressor cells, and natural killer cells interact to actively provide anti-tumor or pro-tumor effects. Furthermore, oncogenic pathways such as Raf/mitogen-activated protein kinase/extracellular-signal-regulated kinase pathway, phosphatidyl-3-kinase/AKT/mammalian target or rapamycin, Wnt/β-catenin, nuclear factor-κB and signal transducers and activators of transcription 3 may lead to activation and proliferation of tumor cells and are also considered cornerstones in tumorigenesis. Immunotherapy directed at this complex milieu of cells has been showned to be successful in cancer treatment. The use of vaccines, adoptive cell therapy and immune checkpoint inhibitor modulation are current options for therapy. Further translational research will shed light to concepts such as anti-tumor immunity which can add another alternative in the therapeutic armamentarium.

Key words: Hepatocarcinogenesis; Adoptive cell therapy; Tumorigenesis; Hepatocellular carcinoma; Immunology;

Tumorigenesis; Immunotherapy; Immunobiology; Immune checkpoint inhibitors

Core tip: Hepatocellular carcinoma is on the rise and is associated with high mortality. Cancer immunology is an expanding field with promise. This review will summarize the current concepts in the immunobiology of hepatocarcinogenesis including the interplay of a variety of immune cells involved in anti-tumor and pro-tumor effects. Oncogenic pathways currently known to effect hepatocarcinogenesis will also be discussed. Finally, currently tested and developed treatment modalities employing immunotherapy will be discussed with an outlook on future therapies.

Patel P, Schutzer SE, Pyrsopoulos N. Immunobiology of hepatocarcinogenesis: Ways to go or almost there? *World J Gastrointest Pathophysiol* 2016; 7(3): 242-255 Available from: URL: http://www.wjgnet.com/2150-5330/full/v7/i3/242.htm DOI: http://dx.doi.org/10.4291/wjgp.v7.i3.242

INTRODUCTION

Hepatocellular carcinoma (HCC) is a tumor of the hepatocytes that often occurs in the setting of chronic liver disease and cirrhosis[1]. In men, it is the fifth most commonly diagnosed cancer worldwide and the second leading cause of cancer mortality in the world with rising incidence[2,3]. The incidence of HCC varies throughout the world. The rates of liver cancer are highest in both East and South-East Asia as well as Northern and Western Africa[2]. The higher incidence of HCC in these areas could reflect the elevated prevalence of chronic hepatitis B virus (HBV) infection[4]. In contrast, in North America, Europe, and Japan, infection with hepatitis C virus (HCV) and use of alcohol are the main risk factors[5]. HBV and HCV account for one-third of infection-related cancer cases such as gastric, liver and cervical. Most of these cancers were liver in origin[6,7]. These chronic infections induce inflammation and over time can lead to liver fibrosis and subsequently cirrhosis. Cirrhosis is the formation of dysplastic nodules that predispose patients to hepatocarcinogenesis. It has been reported that cirrhosis is a major factor in hepatocarcinogenesis in patients with HCV. Though for hepatitis B and apparently for non-alcoholic steatohepatitis (NASH), cirrhosis is not a prerequisite[8].

The diagnosis of HCC is based on strict screening and surveillance protocols published by various liver societies such as the American Association for the Study of Liver Diseases (AASLD), European Association for the Study of the Liver (EASL), and the Asian Pacific Association for the Study of the Liver (APASL)[9-11]. If these practices are not followed patients typically present at the late stages of the disease and the therapeutic options available are rather limited. Grading and prognosis criteria such as the Barcelona Clinic Liver Cancer (BCLC) staging system help determine appropriate treatment options[12]. Resection and transplantation are cornerstones for curative management. Locoregional therapies including percutaneous ablation, radiofrequency ablation (RFA), trans-arterial chemo-embolization (TACE) and radiation therapy are available for those patients who aren't suitable for resection or transplantation[13,14].

HCC diagnosis has progressed but it remains a major cause of cancer mortality with median survival beyond 5 years only if using better selection criteria and optimum treatment delivery[15]. Drug-based therapies that target tumor signaling pathways are being developed. Of these, sorafenib, a multi-targeted tyrosine kinase inhibitor is the only drug that has been Food and Drug Administration (FDA) approved that prolongs survival in patients with HCC[16].

Immunotherapy is an evolving frontier in cancer therapy and further research into the immunobiology of HCC might help develop targeted novel therapies in anticipation of improving mortality.

TUMOR MICROENVIRONMENT

Tumors acquire mutations in oncogenes and tumor suppressor genes which help promote tumorigenesis[17]. However, it is now becoming evident that the nonmalignant cells in the microenvironment of the tumor can aid and provide support to its malignant expression as well[17]. This tumor microenvironment is essential in cancer development and behavior. Chronic inflammation and a variety of host components including stromal cells, angiogenesis, and the inflammatory infiltrate produce an environment favorable for tumor growth[18-21].

Both the local and systemic environment are essential in tumorigenesis as they are involved in the production of a persistent inflammatory response to a myriad of stimuli[22]. This persistent inflammation might be induced by a chronic infection with viruses such as hepatitis B and C in the liver and fatty infiltration. This inflammation has been associated with cancer initiation[23].

Chronic viral hepatitis induces changes in the liver that can lead to hepatocarcinogenesis and ultimately HCC[24]. Chronic HCV for example can induce changes in lipid metabolism and gene expression and HBV is believed to alter the transcription of several genes that may lead to the development of HCC[25,26]. Chronic inflammation alone can lead to induction of hepatocarcinogenesis even if active viral hepatitis is not detected[27].

A variety of immune cells coexist and interplay in a complex cascade of pathways that ultimately lead to tumor carcinogenesis and proliferation. Below we discuss a few of those cells and their role in the formation of cancer and in particular hepatocarcinogenesis.

TUMOR INFILTRATING LYMPHOCYTES

Tumor infiltrating lymphocytes (TIL) are a class of cells

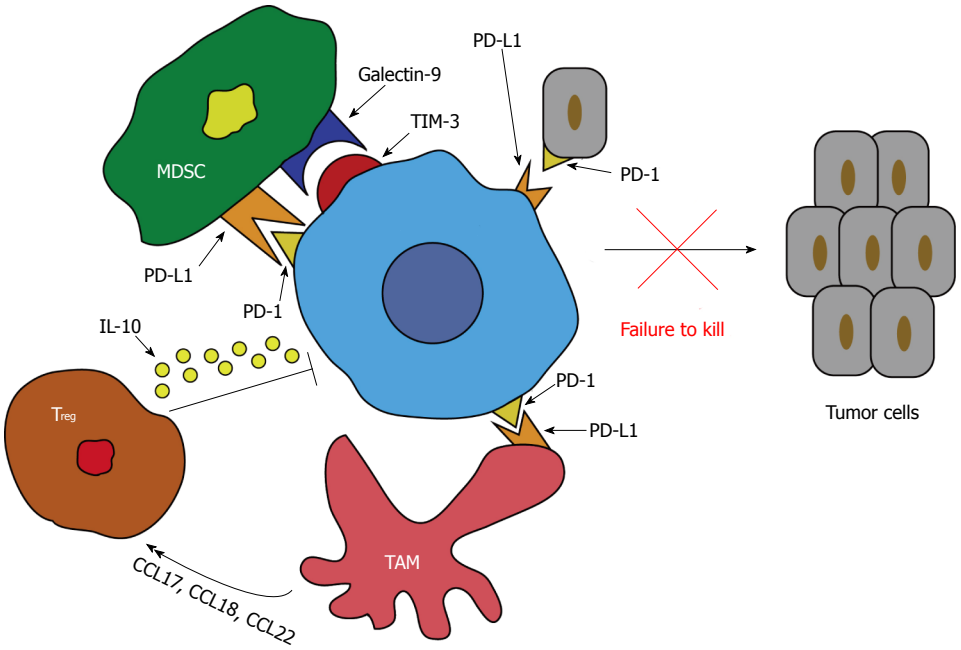


Figure 1 Mechanisms leading to CD8$^+$ T cell suppression. Failure of CD8$^+$ T cells to kill tumor cells involves signals from multiple cells including MDSC, Treg, and TAMs. The interaction of PD-L1 with PD-1 on the CD8$^+$ T cell causes suppression and decrease in its effector function leading to decreased tumor cell death. Furthermore, the Galectin-9 and TIM-3 interaction on MDSC's and IL-10 secretion by Treg cause a similar effect. PD-1: Programmed death 1; IL: Interleukine; TAM: Tumor associated macrophages; MDSC: Myeloid derived suppressor cells; TIM-3: T-cell immunoglobulin and mucin-domain containing-3; T_{reg}: Regulatory T cells.

that shape the tumor microenvironment and therefore effect carcinogenesis. Most cancers that express a high amount of CD8$^+$ T cells usually portend a better prognosis[28,29] as opposed to tumors with increased expression of protumor cells such as regulatory T cells (T_{reg}) which are usually associated with a worse prognosis[30-32]. Therefore, the balance between these counter-regulatory TILs is crucial to the determination of antitumor response. TILs interact with a variety of tumor-associated antigens (TAAs) which are responsible for producing an immune response. A variety of TAAs are found in HCC. The major ones include oncofetal antigens such as α-fetoprotein (AFP) and glypican-3 (GPC-3) along with cancer antigens such as melanoma-associated antigen 1 (MAGEA), synovial sarcoma X breakpoint 2 (SSX2), NY-ESO-1, and human telomerase-reverse transcriptase (hTERT)[33]. The breakdown in response to the above TAAs is responsible for hepatocarcinogenesis. The following cells are responsible for an effective anti-tumor response but complex interplay amongst a variety of molecules can lead to pro-tumoral effects.

CD8$^+$ T cells

CD8$^+$ T cells are integral to antitumor immunity *via* direct antigen-specific cytotoxic targeting of tumors. Most tumors or their antigens are ingested by the host antigen presenting cell and are processed to produce peptides. These peptides are then displayed bound to class I MHC molecules in order to be recognized by CD8$^+$ T cells[34]. Studies have shown that an increased number of CD8$^+$ T cells infiltrating cancer tissue is connected to a favorable prognosis in ovarian[29] and colorectal cancers[35].

In HCC, a similar association has been found with tumor penetration of predominantly CD8$^+$ T cells[36]. These patients have a lower recurrence of cancer, better recurrence-free survival after liver resection and better overall prognosis[37,38]. These T cells contributed to an inflammatory microenvironment that significantly improved patient survival and therefore served an anti-tumoral role in HCC. One mechanism of the cytotoxic effect on tumor cells was described in mice models of HCC in which IL-12 mediated activation of CD8$^+$ T cells caused IFN-γ production and apoptosis of hepatoma cells[39].

Recent work by Flecken *et al*[40] has further elucidated CD8$^+$ T cells that respond to specific TAAs in HCC that were mentioned earlier. Important findings from the study show that TAA-specific CD8$^+$ T cell activity was detectable in more than 50% of HCC patients and was seen with even early stage disease. Furthermore, the presence of these TAA-specific CD8$^+$ T cell responses was associated with an improved progression-free survival, once again confirming that the cytotoxic activity of these cells is important to anti-tumor immunity. Lastly, responses to multiple TAA's showed a trend toward better progression-free survival, though a study with a larger cohort may be necessary to confirm this finding[40].

In contrast, dysfunction of CD8$^+$ T cells in patients with HCC has also been seen[41]. Programmed death 1 (PD-1) is a co-inhibitory molecule that is seen on activated T and B cells and is a pivotal molecule for T cell activity[42]. The ligand for PD-1 (PD-L1) is expressed on a variety of tumor cells and is responsible for delivering a signal for inhibition to PD-1 expressing T cells leading to suppression of the cytotoxic T cell response[43]. This inhibition leads to apoptosis and unresponsiveness of these T cells[44]. Studies have shown that the interaction of PD-1 and PD-L1 negatively regulate T cell function in tumors and ultimately may affect the aggressiveness of the tumor (Figure 1). In a cohort of HCC patients, it was demonstrated that there was a significant increase in peripheral and intratumor PD-1 expression on CD8$^+$ T cells. The tumor cells were also rich in PD-L1 expression and therefore predicted a poorer outcome and early

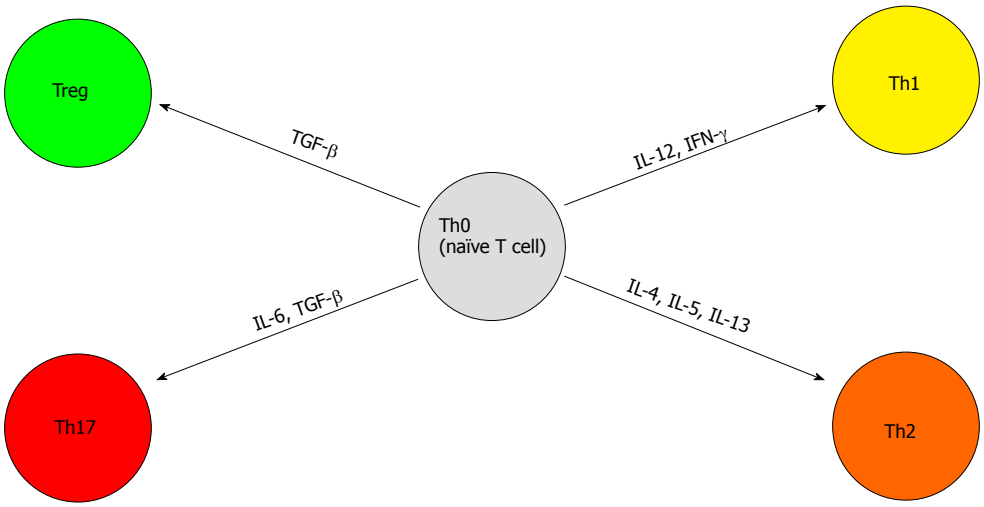


Figure 2 Differentiation of naïve T cells. The Th0 T cell can differentiate into a variety of $CD4^+$ cells based on stimulatory signals as seen above. TGF-β: Transforming growth factor beta; IFN-γ: Interferon gamma; IL: Interleukine; Treg: Regulatory T cells.

recurrence of HCC after liver resection due to the promotion of $CD8^+$ T cell apoptosis[45].

$CD4^+$ T cells

Better known as helper T cells, $CD4^+$ T cells can differentiate to subsets of cells *via* the expression of a variety of cytokines and transcription factors, namely T_H1, T_H2, T_H17 and T_{reg} cells[46].

T_H1 is responsible for antitumor response and differentiates from naive $CD4^+$ T cells *via* signaling from IL-12 and IFN-γ (mentioned above). IFN-γ exerts its antiviral, proinflammatory, and antitumor effects on cells by regulation of numerous genes and *via* specific interaction with the IFN-γ receptor on cell membranes[47]. This receptor has been shown to up-regulated in acute and chronic liver disease but diminished in cases of HCC[48]. Therefore, down-regulation or loss of the IFN-γ receptor on the surface of HCC cells may be another escape mechanism of host immune surveillance ultimately leading to HCC progression and metastasis[49].

T_H2 polarization, on the other hand, has been shown to promote tumor formation and progression[50]. This process is coordinated by IL-4 signaling through STAT-6. These polarized cells secrete IL-4, IL-5 and IL-13 and are associated with potent humoral immunity[50]. This polarization of $CD4^+$ T cells to T_H2 blocks the differentiation towards T_H1 and therefore inhibits the antitumor response. These cells have also been shown to suppress the $CD8^+$ T cell responses mentioned above[51].

T_H17 differentiation is induced by the presence of IL-6 and transforming growth factor-β (TGF-β). The presence of these cytokines leads to expression of the transcription factor ROR-γt *via* activation of the signal transducers and activators of transcription 3 (STAT-3) signaling pathway[52]. These T_H17 cells have potent pro-inflammatory properties *via* the secretion of IL-17[53,54]. In mouse models, IL-17 has been found to promote tumor growth by amplifying angiogenesis and increasing the intra-tumoral burden of phagocytes[55,56]. T_H17 cells have been seen in a myriad of cancers including HCC and have been shown to promote HCC growth[57,58] *via* the activation of the STAT-3 signaling pathway[59]. Increased levels of IL-17 producing cells in HCC patients was correlated with lower overall and disease-free survival[58]. Interestingly T_H17 cells have been shown to increase in certain infections as well[60].

T_{reg} are a subset of $CD4^+$ T cells that suppress T-cell immunity[61]. A major T_{reg} is $CD4^+CD25^+$ and it expresses the transcription factor FOXP3. It is activated by exposure to TGF-β in the periphery[62-64]. Another molecule commonly found on T_{reg} is cytotoxic T-lymphocyte protein 4 (CTLA-4). This molecule binds its ligands CD80 and CD86 on the antigen-presenting cell (APC) membrane and thereby blocks any stimulatory effects from the CD28 protein[65]. CTLA-4 conveys inhibitory signals on T cells and is involved in inducing T_{reg} cell activity[66]. These cells are capable of suppressing $CD8^+$ T cells similar to the T_H2 class of cells and have been found in high numbers in HCC[67]. Studies have also shown an increase proportion of T_{reg} correlated adversely with clinical outcome amongst patients with HCC[68]. A recent study investigating the roles of T_{reg} and $CD8^+$ T cells in hepatocarcinogenesis showed that T_{reg} increased in a stepwise fashion from patients with chronic viral hepatitis, pre-cirrhosis, and liver cirrhosis to precursor lesions of adenomatous hyperplasia and atypical adenomatous hyperplasia, early HCC and advanced HCC. Therefore, we can conclude that the number of T_{reg} cells increases proportionally with progressive hepatocarcinogenesis[69]. The differentiation of naïve T cells can be visualized in Figure 2.

Tumor associated macrophages

Tumor associated macrophages (TAM) are a well-known entity of the inflammatory infiltrate in tumors and are key producers of chemokines and other mediators of inflammation. These chemokines participate in triggering and maintaining the inflammatory process in tumor cells[70]. Similar to T cells, TAMs have plasticity and are

therefore able to polarize to opposite phenotypes giving rise to M1 macrophages or M2 macrophages. The M1 variety is classically involved in the Th1 response with signals from IFN-γ and produce effector molecules (reactive oxygen species) and cytokines [*e.g.*, IL-1β, IL-6 and tumor necrosis factor alpha (TNF-α)] to help provide anti-tumor immunity[71]. They also produce chemokines that attract Th1 lymphocytes in order to mount a more robust response[72]. However, once tumors are produced, the macrophages are transitioned to become protumoral and this is when the tumor microenvironment fosters change toward the opposite macrophage phenotype, M2, causing a Th2 response[73].

These M2 macrophages are activated by IL-4 and IL-13 along with growth factors such as colony stimulating factor-1 (CSF-1) which inhibit the classical activation of the M1 macrophage. M2 macrophages suppress Th1 adaptive immunity by averting anti-tumor immunity and therefore promote tumor growth and progression[74]. This tumor environment is represented by TGF-β1 and Arginase 1[75]. The current thinking is that tumors acquire mutations that subsequently lead to the production of these factors that help promote polarization of macrophages to stimulate tumor-producing cells.

HCC TAMs are identified by immunohistochemistry as human leukocyte antigen (HLA)-DR$^+$, CD163$^+$, CD206$^+$ and with the presence of elevated arginase activity. The number of TAMs found in these patients is positively correlated with poor prognosis[76].

The link between TAMs and HCC can be traced back to murine models[77]. In a Md2-knockout model, the mice automatically developed hepatitis and subsequent HCC. The TAMs produce TNF-α, activating nuclear factor kappa B (NF-κB) which has a protective role in hepatocytes by preventing apoptosis and in turn promoting tumor growth[78]. In another mouse model using the chemical carcinogen, diethylnitrosamine (DEN), hepatocyte proliferation was driven by TAM-derived TNF-α and IL-6 leading to hepatocarcinogenesis[77]. In this same model, it was found that there is a gender disparity in the formation of HCC, with DEN-induced HCC of 100% in male mice and only 10% in female mice. It is believed that the estrogen levels in females play a critical role in inhibiting IL-6 production and therefore decreasing the activation of transcription factor, NF-κB[79]. This finding supports the possible use of estrogen therapy in decreasing the risk of hepatocarcinogenesis.

TAMs have also been implicated in angiogenesis as their density in microvessels has been shown to be elevated. They secrete growth factors such as TGF-β, vascular endothelial growth factor (VEGF), fibroblast growth factor, platelet-derived growth factor (PDGF), angiogenic factor thymidine phosphorylase, angiogenesis-modulating enzyme cyclooxygenase-2 and matrix metalloproteinases (MMPs), particularly MMP-9 and 12, which all promote angiogenesis[22]. MMP-9 overexpression is associated with increased invasiveness of HCC[80].

Furthermore, TAMs move to parts of the tumor that are hypoxic[81]. The hypoxia-induced factor 1α in TAMs is necessary for its activation and migration *in vivo* studies. The hypoxia stimulates TAM chemokine production (CCL2, CCL5, IL-8, CXCL10, CXCL12 and CXCL13) which aids angiogenesis and tumor progression[82].

In addition, it has been published that TAMs produce a variety of chemokines such as CCL17, CCL18, and CCL22, which all attract T_{reg} and Th2 cells to the cancer site and therefore impede cytotoxic T cell activation (Figure 1)[83,84]. This might lead to the conclusion that a positive feedback loop exists between TAMs and T_{reg}, which provides an added layer to the immunosuppressive effects of HCC.

Finally, in cases of HBV-associated HCC, TAMs have been shown to express high levels of galectin-9 especially on Kuppfer cells (KC)[85]. The ligand for galectin-9 is T-cell immunoglobulin and mucin-domain containing-3 (TIM-3)[86]. High levels of TIM-3 have also been seen in HBV-associated HCC and are colocalized with galectin-9. When galectin-9 binds to TIM-3, it induces dormancy of these T cells and therefore effector T cell function. More importantly, blocking this pathway can recover the T cell function[85].

Similarly, TAMs decrease T cell function through the expression of the co-inhibitory molecule, PD-L1 (also known as B7-H1), which binds to PD-1 on T cells. This process reduces T cell effector function analogous to the galectin-9/Tim-3 pathway as mentioned above. Both IL-10 and TNF-α play a role in the induction of this PD-L1/PD-1 pathway in HCC (Figure 1)[76]. Likewise, blocking this pathway can help recover T cell function and its antitumor efficacy[87].

Therapy aimed at TAM reduction has been documented. In particular, zoledronic acid or clodronate-encapsulated liposomes (clodrolip), in combination with tyrosine kinase inhibitors such as sorafenib, has been shown to reduce tumor growth and angiogenesis further in mice compared to just sorafenib alone[88]. Therefore, additional therapies implementing depletion of TAMs may be a worthwhile avenue to explore.

Tumor associated neutrophils

Analogous to TAM, tumor associated neutrophils (TAN) have recently been described to contribute to carcinogenesis as well. They can produce both pro-tumoral as well as anti-tumoral effects on cancer and can be manipulated toward distinct phenotypes *via* tumor signaling[89,90].

In a similar fashion to TAMs, TANs can be divided into two main subtypes, N1 (anti-tumoral) and N2 (pro-tumoral). The plasticity of these subtypes depends on the presence of TGF-β. Neutrophils can be polarized by TGF-β to become the N2 phenotype while the inhibition of TGF-β along with increased IFN-β induces the N1 phenotype[89,91].

The pro-tumoral functions of both TAM and TAN include extracellular matrix remodeling, cancer cell invasion, angiogenesis, lymphangiogenesis, metastasis and most importantly, the inhibition of anti-tumoral immune surveillance[92]. The anti-tumoral effects of TAM and TAN include direct cytotoxic activity against the

tumor cells and the production of a variety of mediators to recruit and activate the immune system. These mediators include cytokines, chemokines and growth factors[74,93].

In HCC murine models, TANs have been shown to mediate intratumoral infiltration of macrophages and T_{reg} cells by secreting CCL2 and CCL17. This pathway stimulated neovascularization, enhanced HCC growth and metastasis and also contributed to sorafenib resistance. It has been suggested that the extent of TAN infiltration could be used as a biomarker in HCC and can predict responsiveness to sorafenib therapy and that TAN depletion can enhance sorafenib's efficacy[94].

Myeloid derived suppressor cells

In addition to the above cells, cancer growth can also be attributed to myeloid derived suppressor cells (MDSC)[95]. These cells, though not extensively studied, have shared relationships with TAM and TAN in carcinogenesis. They represent a diverse mix of immature cells of myeloid origin that are able to lessen immune responses[96].

In HCC, MDSC (specifically the $CD14^+HLA\text{-}DR^{-/low}$ phenotype) were seen in high numbers in the peripheral blood and were able to help the host evade the immune response against cancer by directly increasing arginase activity and directly suppressing the response of tumor-specific $CD4^+$ T cells. They were also seen to indirectly suppress T-cell function by inducing $CD4^+CD25^+Foxp3^+$ T_{reg} mentioned earlier[97]. Interestingly, in the presence of hypoxia or tumor-derived factors, MDSCs can differentiate toward immunosuppressive TAMs as well[98].

In a similar fashion to TAMs, MDSCs also express galectin-9 which binds to Tim-3 on T cells, thereby inducing senescence[99]. MDSCs also respond to liver macrophages and increase the levels of PD-L1, which as mentioned earlier reduces T cell effector function (Figure 1)[96]. MDSC have also been implicating in angiogenesis by producing high levels of MMP-9[100].

Natural killer cells

Natural killer (NK) cells are innate lymphoid cells that are the immune system's first line of defense against infections and tumors[101]. They are essential in the liver's immune function where they account for close to 50% of liver lymphocytes[102]. Their anti-tumor response is by direct lysis of malignant cells[101]. They express a number of immune receptors (NKRs) that are able to recognize ligands on hepatocytes, stellate cells and Kupffer cells to maintain proper immune function[103,104].

MHC class Ⅰ -related chain A (MICA) is a ligand for NK cell stimulatory receptor NKG2D and is expressed in HCC[105]. The interaction of MICA and NKG2D serves as a pathway for immune surveillance in HCC. However, a soluble form of MICA (sMICA) has been shown to isolate NKG2D and inhibit its expression. These soluble forms are increased in a variety of tumors and may serve as a tumor evasion mechanism[106].

According to the frequency of CD56 expression (dim or bright) and the presence or absence of CD16, NK cells can be divided into two subsets: $CD56^{bright}CD16^{neg}$ (produces cytokines) and $CD56^{dim}CD16^{pos}$ (cytotoxic)[107].

The density of NK cells in the peripheral blood of patients with HCC positively correlated with survival and prognosis similar to many of the other cell lines already mentioned[108]. Specifically, peripheral $CD56^{dim}CD16^{pos}$ NK subsets were dramatically reduced, which resulted in an increased ratio of $CD56^{bright}$ to $CD56^{dim}$ NK cells in HCC and therefore decreased cytotoxic capability. These cells exhibited poorer capacity to produce IFN-γ and kill target cells. This phenomenon was found to be associated with the increased $CD4^+CD25^+$ T_{reg} in the tumor environment as mentioned earlier. Decreased functionality of these cells was shown to correlate with early development and recurrence of HCC as well[109]. Murine models of hepatoma revealed that activation of NK cells can lead to clearance of the hepatoma which suggests a possible immune target in patients with liver disease[110].

Sorafenib, as mentioned earlier, is the only molecularly-targeted drug shown to have survival benefit in patients with HCC[16]. Recently, it was found to initiate liver NK cell activation and induce the anti-tumor response of these cells by triggering TAMs and increasing IFN-γ secretion[111]. The proteasome inhibitor, Bortezomib, has also been shown to stimulate cytotoxicity and IFN-γ production of NK cells by increasing the expression of MICA/B on the cell surface of hepatoma cells.

A new class of agents that have shown positive results in a variety of malignancies including HCC are the histone deacetylase inhibitors (HDACi). HDACis, such as sodium valproate, promote MICA expression on hepatoma cells and coordinate NK cell-mediated lysis. This mechanism supports this type of therapy for future treatment of HCC[112].

The mechanisms of pro-tumoral and anti-tumoral effects in hepatocarcinogenesis of the above cell types are summarized in Table 1.

ONCOGENIC PATHWAYS

Multiple growth factor signaling cascades are deregulated in hepatocarcinogenesis. Activation of oncogenes seems to be a late event in human HCC[113]. The tumor microenvironment in concert with the presence of cirrhosis induces a selection to eliminate certain checkpoint genes. This may be an early event which leads to the activation of oncogenes and tumor growth[113]. Amongst the most frequently seen pathways, the following will be discussed: Raf/mitogen-activated protein kinase (MAPK)/extracellular-signal-regulated kinase pathway (ERK), phosphatidyl-3-kinase/AKT/mammalian target or rapamycin (PI3K/AKT/MTOR), Wnt/β-catenin, NK-κB, and STAT-3.

Raf/MAPK/ERK

The Raf/MAPK/ERK plays an important role in tumor cell signaling and is involved in cell growth and differentiation. The pathway translates extracellular signals from tyrosine

Table 1 Immune cells involved in tumor response

Cell type	Mechanism	
	Pro-tumor	Anti-tumor
$CD8^+$ T cells	Exhaustion of $CD8^+$ T cells Upregulation of PD-1	Increased $CD8^+$ T cells
$CD4^+$ T cells	T_H2 response	T_H1 response
T_H17 cells	IL-17 production *via* STAT-3	
T_{reg} cells	Increased T_{reg} Impaired $CD8^+$ T cells *via* CTLA-4	
TAM	M2 (Th2 response) Increased galectin-9 *via* TIM-3 Expression of B7-H1 Induction of T_H17	M1 (Th1 response)
TAN	N2 phenotype Angiogenesis *via* T_{reg}	N1 phenotype
MDSC	Induction of T_{reg} and TAM Suppress $CD4^+$ T cells Suppress NK activity	
NK cells	Increased $CD56^{dim}CD16^{pos}$ Increased T_{reg}	Increased $CD56^{bright}CD16^{neg}$

PD-1: Programmed death 1; IL: Interleukine; STAT-3: Signal transducers and activators of transcription 3; TAM: Tumor associated macrophages; MDSC: Myeloid derived suppressor cells; TAN: Tumor associated neutrophils; NK: Natural killer; T_{reg}: Regulatory T cells.

kinase receptors, such as epidermal growth factor receptor (EGFR), vascular endothelial growth factor receptor (VEGFR), insulin-like growth factor receptor (IGFR), c-Met, and platelet-derived growth factor receptor (PDGFR), and hepatocyte growth factor receptor (MET) to the nucleus *via* a series of phosphorylation events[114]. The GTPase, RAS, and the serine/threonine kinase (Raf) are key regulators in signal transduction in this pathway[115]. Increased activation of this pathway is well known in HCC and also positively correlates with disease severity[116,117]. The increased activity of this pathway in HCC can be explained by the down-regulation of the Raf kinase inhibitor which is a suppressor of the Raf/MAPK/ERK pathway[118]. Therefore, Raf kinase targeting for treatment of HCC is a promising approach. Sorafenib is one example of a drug with inhibitory effects on Raf-1 kinase and can halt tumor progression *via* its downstream effects using this mechanism[119].

PI3K/AKT/MTOR

Similar to the Raf/MAPK/ERK pathway, the PI3K/AKT/mTOR pathway is a major intracellular signaling cascade that controls cell growth, proliferation, motility, survival, and apoptosis[120]. Activation of the AKT signaling pathway has been reported in over 40% of human HCC. This activation has prosurvival and growth-stimulatory effects by decreasing TGF-β-dependent apoptosis[121]. Evidence has demonstrated that the abnormal activation of this pathway occurs in patients with HCC. Furthermore, the expression of the mTOR downstream effector, p70s6k, is up-regulated in 45% of patients with HCC[122]. Therefore, this pathway is another important target for therapy.

Some of the inhibitors that have been studied targeting this pathway include sorafenib and PI-103 (a dual PI3K/mTOR inhibitor) alone or in combination. This was found to significantly inhibit EGF-stimulated proliferation of HCC *via* the blockage of both the PI3K/AKT/mTOR and Ras/Raf/MAPK pathways[123]. Certain mTOR inhibitors such as everolimus have been tested against placebo as second-line agents in sorafenib-refractory or intolerant HCC patients. However, there was no difference in overall survival compared to the placebo group. In subgroup analysis, patients with HBV infection derived a significant benefit [hazard ratio (HR) = 0.64; 95%CI: 0.45-0.93] as compared to those with HCV infection (HR = 0.93; 95%CI: 0.75-1.15)[124].

Wnt/β-catenin

The canonical Wnt (Wnt/β-catenin) pathway controls many processes, including cell fate determination, proliferation and stem cell maintenance. The pathway is activated by the binding of growth factors to the Frizzled (Fzd) receptors on the surface of target cells. This leads to activation of different signal transduction pathways further downstream[125]. Physiologically, β-catenin is involved in intercellular adhesion *via* interactions with E-cadherin and transmission of the proliferative signals of the Wnt pathway[126,127]. In the absence of the above, the β-catenin protein is rapidly phosphorylated by the destruction complex which consists of AXIN1, adenomatous polyposis coli (APC), glycogen synthase kinase-3β (GSK-3β), and casein kinase 1 (CK1) proteins[126,127]. This phosphorylation then leads to β-catenin becoming ready for proteolysis through the ubiquitin/proteasome system. Mechanisms that bypass this phosphorylation or mutations in the destruction complex lead to the translocation of the β-catenin to the cytoplasm and eventually the nucleus to interact with transcription factors. This translocation triggers activation of multiple genes involved in cell proliferation, survival and migration[128]. Aberrant activation of this pathway is observed in both murine and human studies of HCC[129,130].

In addition, the protein regulator of cytokinesis 1 (PRC1) is involved in cytokinesis and microtubule organization[131-133]. PRC1 has been shown to be upregulated in HCC and has an oncogenic function in promoting cancer, tumorigenesis, and metastasis. It is a direct Wnt signaling target and is necessary for Wnt signaling to mediate oncogenesis. PRC1 also potentiates Wnt signaling by promoting membrane sequestration of the destruction complex, thereby reducing APC stability and promoting stabilization of β-catenin[134].

Therefore, there is mounting evidence that β-catenin is essential for tumor cell proliferation and survival. It is obvious that targeting the Wnt/β-catenin pathway may be an attractive option for therapy and is already being investigated. Therapeutic monoclonal antibodies are useful for targeting ligands or receptors. Wnt and Fzd proteins are prime targets for these antibodies. An anti-Wnt-1 antibody has been shown to inhibit β-catenin

signaling and induce apoptosis and decrease tumor growth in HCC[135]. Furthermore, the Fzd receptor is another interesting target. A soluble Fzd peptide (sFZD-7) was shown to inhibit Wnt signaling and also sensitized the HCC to the traditional chemotherapeutic agent, doxorubicin[136].

Axin, one of the components of the destruction complex mentioned earlier is another molecular target utilizing this pathway[137]. Adenovirus-mediated gene transfer of wild-type Axin1 induced apoptosis in hepatocellular and colorectal cancer cells, once again confirming the importance of the destruction complex on the senescence of β-catenin[138]. Disruption of the actual β-catenin and transcription factor complex is a promising avenue of immunotherapeutics. However, the complexity of the transcriptional regulation of this pathway makes drug targeting a future endeavor[139].

NF-κB

Cancer, as mentioned previously, has been linked to both infection and inflammation and a variety of signaling pathways can lead to an inflammatory tumor microenvironment. Toll-like receptors (TLR) are present on the cells of the innate immune system and are activated *via* their interaction with foreign cells such as pathogens[140]. This interaction triggers the production of a variety of cytokines and chemokines that begin the inflammatory cascade[141]. The pathway controlled by the transcription factor NF-κB is essential for this to occur[142]. NF-κB is activated downstream in inflammatory cells *via* signals from TLR using the adaptor molecule myeloid differentiation primary response gene 88 (MyD88)[140] or other inflammatory cytokines such as TNF-α and IL-1β[142].

NF-κB's ties to tumorigenesis has been reported using murine models. Ablation of the gene encoding for IκB kinase β-subunit (IKK-β) which is a kinase necessary for the activation of NF-κB in myeloid cells, resulted in a reduction of tumor size in mice with colitis-associated cancer[143]. Furthermore, deletion of the gene for this kinase in Kupffer cells and hepatocytes in models of HCC resulted in reduced tumor burden and resulted in less production of inflammatory cytokines such as TNF-α and IL-6 with DEN administration. However, deletion of the kinase gene in only the hepatocytes and not in Kupffer cells resulted in a marked increase in hepatocarcinogenesis[77]. These conflicting findings underscore the complex nature of the NF-κB signaling pathway and once again the importance of immune cells in tumorigenesis.

STAT-3

Lastly, another emerging pathway in hepatocarcinogenesis is the STAT-3 signaling pathway, which has been mentioned previously and will be briefly discussed here. The STAT-3 is part of the transcription factor family and is involved in signal transduction *via* cytokines, growth factors, and oncoproteins. Once activated, STAT-3 translocates to the nucleus and binds to target genes that regulate a variety of cell activities including growth, differentiation, apoptosis, and angiogenesis[144-146]. Mostly, activated STAT-3 participates in carcinogenesis by either promoting angiogenesis or stimulating cellular proliferation[147]. Furthermore, the expression of activated STAT-3 was correlated with histological grading and intratumor microvessel density in more than 50% of patients with HCC in one study[148]. This highlights the potential importance of this pathway in tumorigenesis and progression.

It is clear that the inhibition of STAT-3 signaling could be vital in halting tumor growth[149]. This was proven in an experiment in which over-activated STAT-3 was blocked in human HCC cells. Results showed that the proliferation of HCC cells was suppressed dramatically and was associated with increased apoptosis and cell arrest[149]. Therefore, STAT-3 is an interesting target for HCC treatment and there is a current phase 1 clinical trial evaluating an inhibitor of STAT-3 in patients with HCC (NCT01839604).

IMMUNOTHERAPY

A better understanding of tumor immunobiology has led to unique avenues of targeted therapy. Below are some of the classes of therapies available in addition to ones mentioned earlier.

Vaccines

Vaccination to target TAAs such as AFP, GPC-3, NY-ESO-1, MAGEA, SSX2, and hTERT is an evolving strategy in immunotherapy. Two peptide-derived vaccines have been developed and tested. One was a nonrandomized, open-label phase 1 clinical trial against GPC3 peptides, which proved to be safe and showed a measurable immune response with higher CTL activity. However, only one of the 33 patients treated had objective tumor response[150]. The second vaccine was with an hTERT-derived peptide that revealed no signs of clinical or CTL activity[151].

Adoptive cell therapy

Adoptive cell therapy (ACT) involves the isolation and expansion of tumor-specific T cells to gain a greater number of cells than would be expected otherwise in an *ex vivo* fashion. These T cells are then infused into the patient in an attempt to boost the immune response against the tumor[152].

Autologous or allogeneic *ex vivo* expanded NK cells is an example of ACT. These NK cells can be expanded 1600-fold using new technology and can be used for their cytotoxic effects against tumor targets[153]. A Phase Ⅱ clinical trial (NCT02008929) is underway to evaluate the efficacy and safety of MG4101 (*ex vivo* expanded allogeneic NK cells) as an adjuvant treatment of advanced HCC after curative liver resection and high risk of recurrence.

Cytokine-induced killer cells (CIK) therapy using ACT has seen great results in Asia. In two randomized trials using this technique in the adjuvant setting after liver resection, showed an increase in recurrence-free survival

but no difference in overall survival[154,155].

A study that compared CIK ACT with best supportive care after combined TACE and RFA showed a dramatic reduction in the 1-year recurrence rate in the CIK-treated group (9% *vs* 30%)[156]. Furthermore, another randomized study of patients with a wide range of HCC tumor grades was treated with standard therapy or CIK in addition to standard therapy. There was an increase in overall survival and progression-free survival in the CIK-treated group but no significant improvement in patients that had undergone surgery[157]. This may indicate that CIK therapy may be more useful for those with earlier stage disease.

Immune checkpoint inhibitors

Immune checkpoints are coinhibitory molecules that block the immune response to tumor cells and decrease the overactivation of T cells. Members of this group have been mentioned earlier and include CTLA-4, PD-1, and TIM-3. Two others that will not be discussed are LAG-3 (lymphocyte activation gene-3 protein) and BTLA (B and T lymphocyte attenuator)[158,159].

Targeting these immune checkpoints has gained recent interest after three different checkpoint inhibitors were approved by the United States FDA to treat melanoma. These drugs include ipilimumab (anti-CTLA-4), pembrolizumab (anti-PD-1) and nivolumab (anti-PD-1) along with some others[160].

Anti-CTLA-4 mAbs

Two anti-CTLA-4 monoclonal antibodies (mAbs) have been developed thus far for advanced cancer such as melanoma. Ipilimumab is an IgG1 anti-CTLA-4 human mAb with a half-life of 2 wk and has shown great efficacy in metastatic melanoma[161].

Tremelimumab is an anti-CTLA-4 mAb and is from the IgG2 subclass with a longer half-life of 22 d. In a phase Ⅲ trial, the primary end-point of progression-free survival in metastatic melanoma was not met[162]. However, its use in patients with HCC and chronic HCV infection yielded partial response rate in 17.6% of patients and control of their disease in 76.4% of patients, with a median time to cancer progression of 6.48 mo. Furthermore, a significant drop in HCV viral load was seen with treatment[163].

Anti-PD-1 mAbs

PD-1 is a strong inhibitor of T cell responses as mentioned earlier. Therefore, its blockade is an important target for therapy. Another mAb that has been tested is nivolumab, which is a human IgG4 anti-PD-1 mAb that has been investigated in a variety of solid tumors with positive results[164].

A multicenter phase Ⅰ/Ⅱ trial that studied nivolumab in advanced HCC patients with intolerance to sorafenib was started in 2012. Preliminary results show that the safety profile of the treatment was acceptable and durable responses were seen and overall survival at 6 mo was 72%[165]. These results are promising and further assessment of immune checkpoint inhibitors as potential immunotherapy are being undertaken.

CONCLUSION

HCC is of increasing importance due to its rising incidence and mortality. Current therapeutic options are limited and therefore, other avenues are being pursued. The immunobiology of the tumor environment is tremendously complicated but plays a pivotal role in the development of inflammation and hepatocarcinogenesis. The multitude of ways that cancer cells can evade the immune response makes it a very difficult disease to control. The interplay amongst immune cells and tumor microenvironment determines the ultimate outcomes of patients with cancer.

Many of these cells have been well known for many years but their complex interactions are being discovered on a daily basis. Targeted therapy towards these important cells has become a fascinating topic that has fostered many studies on murine models and clinical trials on humans. The most critical aspect of immunotherapy will be to find the most appropriate patient population for treatment and testing. The advances thus far have proven to provide desirable results for hepatocarcinogenesis and will further solidify as active translational research improves.

Not all tumors are identical and individualized treatment modalities along with the current standard of care may be the future of immunotherapy in patients with HCC. These newer treatment modalities include vaccines, monoclonal antibodies against immune checkpoint inhibitors as well as ACT amplifying TIL. Enhancing the natural anti-tumor response of the body along with breaking the barriers of immune tumor evasion may be among the keys to the future of cancer treatment.

In this review, we have highlighted the role of the immune system and immunomodulatory therapy against tumors, particularly hepatocarcinomas. It is probable that we will need to develop non-immune associated therapies that together will have the most impact in treating and repressing recurrence of even the seemingly cancer-free state. This will likely be refined within the scope of precision medicine.

REFERENCES

1 **Davis GL**, Dempster J, Meler JD, Orr DW, Walberg MW, Brown B, Berger BD, O'Connor JK, Goldstein RM. Hepatocellular carcinoma: management of an increasingly common problem. *Proc* (Bayl Univ Med Cent) 2008; **21**: 266-280 [PMID: 18628926]

2 **Torre LA**, Bray F, Siegel RL, Ferlay J, Lortet-Tieulent J, Jemal A. Global cancer statistics, 2012. *CA Cancer J Clin* 2015; **65**: 87-108 [PMID: 25651787 DOI: 10.3322/caac.21262]

3 **Davis GL**, Alter MJ, El-Serag H, Poynard T, Jennings LW. Aging of hepatitis C virus (HCV)-infected persons in the United States: a multiple cohort model of HCV prevalence and disease progression. *Gastroenterology* 2010; **138**: 513-521, 521.e1-6 [PMID: 19861128 DOI: 10.1053/j.gastro.2009.09.067]

4 **Schweitzer A**, Horn J, Mikolajczyk RT, Krause G, Ott JJ. Articles

Estimations of worldwide prevalence of chronic hepatitis B virus infection: a systematic review of data published between 1965 and 2013. *Lancet* 2015; **6736**: 1-10 [DOI: 10.1016/S0140-6736(15)61412-X]
5 **El-Serag HB**. Hepatocellular carcinoma. *N Engl J Med* 2011; **365**: 1118-1127 [PMID: 21992124 DOI: 10.1056/NEJMra1001683]
6 **de Martel C**, Ferlay J, Franceschi S, Vignat J, Bray F, Forman D, Plummer M. Global burden of cancers attributable to infections in 2008: a review and synthetic analysis. *Lancet Oncol* 2012; **13**: 607-615 [PMID: 22575588 DOI: 10.1016/S1470-2045(12)70137-7]
7 **Davila JA**, Morgan RO, Shaib Y, McGlynn KA, El-Serag HB. Hepatitis C infection and the increasing incidence of hepatocellular carcinoma: a population-based study. *Gastroenterology* 2004; **127**: 1372-1380 [PMID: 15521006 DOI: 10.1053/j.gastro.2004.07.020]
8 **Fattovich G**, Stroffolini T, Zagni I, Donato F. Hepatocellular carcinoma in cirrhosis: incidence and risk factors. *Gastroenterology* 2004; **127**: S35-S50 [PMID: 15508101 DOI: 10.1038/nrgastro.2012.157]
9 European Association for Study of Liver; European Organisation for Research and Treatment of Cancer. EASL-EORTC clinical practice guidelines: management of hepatocellular carcinoma. *J Hepatol* 2012; **56**: 908-943 [PMID: 22424438 DOI: 10.1016/j.jhep.2011.12.001]
10 **Bruix J**, Sherman M. Management of hepatocellular carcinoma: an update. *Hepatology* 2011; **53**: 1020-1022 [PMID: 21374666 DOI: 10.1002/hep.24199]
11 **Omata M**, Lesmana LA, Tateishi R, Chen PJ, Lin SM, Yoshida H, Kudo M, Lee JM, Choi BI, Poon RT, Shiina S, Cheng AL, Jia JD, Obi S, Han KH, Jafri W, Chow P, Lim SG, Chawla YK, Budihusodo U, Gani RA, Lesmana CR, Putranto TA, Liaw YF, Sarin SK. Asian Pacific Association for the Study of the Liver consensus recommendations on hepatocellular carcinoma. *Hepatol Int* 2010; **4**: 439-474 [PMID: 20827404 DOI: 10.1007/s12072-010-9165-7]
12 **Llovet JM**, Brú C, Bruix J. Prognosis of hepatocellular carcinoma: the BCLC staging classification. *Semin Liver Dis* 1999; **19**: 329-338 [PMID: 10518312 DOI: 10.1055/s-2007-1007122]
13 **Dhir M**, Melin AA, Douaiher J, Lin C, Zhen WK, Hussain SM, Geschwind JF, Doyle MB, Abou-Alfa GK, Are C. A Review and Update of Treatment Options and Controversies in the Management of Hepatocellular Carcinoma. *Ann Surg* 2016; **263**: 1112-1125 [PMID: 26813914 DOI: 10.1097/SLA.0000000000001556]
14 **Tejeda-Maldonado J**, García-Juárez I, Aguirre-Valadez J, González-Aguirre A, Vilatobá-Chapa M, Armengol-Alonso A, Escobar-Penagos F, Torre A, Sánchez-Ávila JF, Carrillo-Pérez DL. Diagnosis and treatment of hepatocellular carcinoma: An update. *World J Hepatol* 2015; **7**: 362-376 [PMID: 25848464 DOI: 10.4254/wjh.v7.i3.362]
15 **Forner A**, Llovet JM, Bruix J. Hepatocellular carcinoma. *Lancet* 2012; **379**: 1245-1255 [PMID: 22353262 DOI: 10.1016/S0140-6736(11)61347-0]
16 **Llovet JM**, Ricci S, Mazzaferro V, Hilgard P, Gane E, Blanc JF, de Oliveira AC, Santoro A, Raoul JL, Forner A, Schwartz M, Porta C, Zeuzem S, Bolondi L, Greten TF, Galle PR, Seitz JF, Borbath I, Häussinger D, Giannaris T, Shan M, Moscovici M, Voliotis D, Bruix J. Sorafenib in advanced hepatocellular carcinoma. *N Engl J Med* 2008; **359**: 378-390 [PMID: 18650514 DOI: 10.1056/NEJMoa0708857]
17 **Joyce JA**, Pollard JW. Microenvironmental regulation of metastasis. *Nat Rev Cancer* 2009; **9**: 239-252 [PMID: 19279573 DOI: 10.1038/nrc2618]
18 **Grivennikov SI**, Greten FR, Karin M. Immunity, inflammation, and cancer. *Cell* 2010; **140**: 883-899 [PMID: 20303878 DOI: 10.1016/j.cell.2010.01.025]
19 **Allavena P**, Sica A, Solinas G, Porta C, Mantovani A. The inflammatory micro-environment in tumor progression: the role of tumor-associated macrophages. *Crit Rev Oncol Hematol* 2008; **66**: 1-9 [PMID: 17913510 DOI: 10.1016/j.critrevonc.2007.07.004]
20 **Coussens LM**, Werb Z. Inflammation and cancer. *Nature* 2002; **420**: 860-867 [PMID: 12490959 DOI: 10.1038/nature01322]
21 **Hanahan D**, Weinberg RA. The hallmarks of cancer. *Cell* 2000; **100**: 57-70 [PMID: 10647931 DOI: 10.1016/j.cell.2011.02.013]
22 **Balkwill FR**, Mantovani A. Cancer-related inflammation: common themes and therapeutic opportunities. *Semin Cancer Biol* 2012; **22**: 33-40 [PMID: 22210179 DOI: 10.1016/j.semcancer.2011.12.005]
23 **Balkwill F**, Charles KA, Mantovani A. Smoldering and polarized inflammation in the initiation and promotion of malignant disease. *Cancer Cell* 2005; **7**: 211-217 [PMID: 15766659 DOI: 10.1016/j.ccr.2005.02.013]
24 **Castello G**, Scala S, Palmieri G, Curley SA, Izzo F. HCV-related hepatocellular carcinoma: From chronic inflammation to cancer. *Clin Immunol* 2010; **134**: 237-250 [PMID: 19910258 DOI: 10.1016/j.clim.2009.10.007]
25 **Moradpour D**, Blum HE. Pathogenesis of hepatocellular carcinoma. *Eur J Gastroenterol Hepatol* 2005; **17**: 477-483 [PMID: 15827436]
26 **Bartosch B**, Thimme R, Blum HE, Zoulim F. Hepatitis C virus-induced hepatocarcinogenesis. *J Hepatol* 2009; **51**: 810-820 [PMID: 19545926 DOI: 10.1016/j.jhep.2009.05.008]
27 **Nakamoto Y**, Guidotti LG, Kuhlen CV, Fowler P, Chisari FV. Immune pathogenesis of hepatocellular carcinoma. *J Exp Med* 1998; **188**: 341-350 [PMID: 9670046 DOI: 10.1084/jem.188.2.341]
28 **Galon J**, Costes A, Sanchez-Cabo F, Kirilovsky A, Mlecnik B, Lagorce-Pagès C, Tosolini M, Camus M, Berger A, Wind P, Zinzindohoué F, Bruneval P, Cugnenc PH, Trajanoski Z, Fridman WH, Pagès F. Type, density, and location of immune cells within human colorectal tumors predict clinical outcome. *Science* 2006; **313**: 1960-1964 [PMID: 17008531 DOI: 10.1126/science.1129139]
29 **Zhang L**, Conejo-Garcia JR, Katsaros D, Gimotty PA, Massobrio M, Regnani G, Makrigiannakis A, Gray H, Schlienger K, Liebman MN, Rubin SC, Coukos G. Intratumoral T cells, recurrence, and survival in epithelial ovarian cancer. *N Engl J Med* 2003; **348**: 203-213 [PMID: 12529460 DOI: 10.1056/NEJMoa020177]
30 **Wilke CM**, Wu K, Zhao E, Wang G, Zou W. Prognostic significance of regulatory T cells in tumor. *Int J Cancer* 2010; **127**: 748-758 [PMID: 20473951 DOI: 10.1002/ijc.25464]
31 **Zou W**. Regulatory T cells, tumour immunity and immunotherapy. *Nat Rev Immunol* 2006; **6**: 295-307 [PMID: 16557261 DOI: 10.1038/nri1806]
32 **Curiel TJ**. Regulatory T cells and treatment of cancer. *Curr Opin Immunol* 2008; **20**: 241-246 [PMID: 18508251 DOI: 10.1016/j.coi.2008.04.008]
33 **Breous E**, Thimme R. Potential of immunotherapy for hepatocellular carcinoma. *J Hepatol* 2011; **54**: 830-834 [PMID: 21145836 DOI: 10.1016/j.jhep.2010.10.013]
34 **Abbas AK**, Lichtman AH, Pillai S. Cellular and Molecular Immunology. Häftad: Engelska, 2014
35 **Naito Y**, Saito K, Shiiba K, Ohuchi A, Saigenji K, Nagura H, Ohtani H. CD8+ T cells infiltrated within cancer cell nests as a prognostic factor in human colorectal cancer. *Cancer Res* 1998; **58**: 3491-3494 [PMID: 9721846 DOI: 10.1002/(SICI)1096-9896(199707)182: 3<318: : AID-PATH862>3.0.CO; 2-6]
36 **Gao Q**, Qiu SJ, Fan J, Zhou J, Wang XY, Xiao YS, Xu Y, Li YW, Tang ZY. Intratumoral balance of regulatory and cytotoxic T cells is associated with prognosis of hepatocellular carcinoma after resection. *J Clin Oncol* 2007; **25**: 2586-2593 [PMID: 17577038 DOI: 10.1200/JCO.2006.09.4565]
37 **Wada Y**, Nakashima O, Kutami R, Yamamoto O, Kojiro M. Clinicopathological study on hepatocellular carcinoma with lymphocytic infiltration. *Hepatology* 1998; **27**: 407-414 [PMID: 9462638 DOI: 10.1002/hep.510270214]
38 **Hiroishi K**, Eguchi J, Baba T, Shimazaki T, Ishii S, Hiraide A, Sakaki M, Doi H, Uozumi S, Omori R, Matsumura T, Yanagawa T, Ito T, Imawari M. Strong CD8(+) T-cell responses against tumor-associated antigens prolong the recurrence-free interval after tumor treatment in patients with hepatocellular carcinoma. *J Gastroenterol* 2010; **45**: 451-458 [PMID: 19936602 DOI: 10.1007/s00535-009-0155-2]
39 **Komita H**, Homma S, Saotome H, Zeniya M, Ohno T, Toda G. Interferon-gamma produced by interleukin-12-activated tumor infiltrating CD8+T cells directly induces apoptosis of mouse hepatocellular carcinoma. *J Hepatol* 2006; **45**: 662-672 [PMID: 16935390 DOI: 10.1016/j.jhep.2006.05.018]
40 **Flecken T**, Schmidt N, Hild S, Gostick E, Drognitz O, Zeiser

R, Schemmer P, Bruns H, Eiermann T, Price DA, Blum HE, Neumann-Haefelin C, Thimme R. Immunodominance and functional alterations of tumor-associated antigen-specific CD8+ T-cell responses in hepatocellular carcinoma. *Hepatology* 2014; **59**: 1415-1426 [PMID: 24002931 DOI: 10.1002/hep.26731]

41 **Gehring AJ**, Ho ZZ, Tan AT, Aung MO, Lee KH, Tan KC, Lim SG, Bertoletti A. Profile of tumor antigen-specific CD8 T cells in patients with hepatitis B virus-related hepatocellular carcinoma. *Gastroenterology* 2009; **137**: 682-690 [PMID: 19394336 DOI: 10.1053/j.gastro.2009.04.045]

42 **Agata Y**, Kawasaki A, Nishimura H, Ishida Y, Tsubata T, Yagita H, Honjo T. Expression of the PD-1 antigen on the surface of stimulated mouse T and B lymphocytes. *Int Immunol* 1996; **8**: 765-772 [PMID: 8671665 DOI: 10.1093/intimm/8.5.765]

43 **Freeman GJ**, Long AJ, Iwai Y, Bourque K, Chernova T, Nishimura H, Fitz LJ, Malenkovich N, Okazaki T, Byrne MC, Horton HF, Fouser L, Carter L, Ling V, Bowman MR, Carreno BM, Collins M, Wood CR, Honjo T. Engagement of the PD-1 immunoinhibitory receptor by a novel B7 family member leads to negative regulation of lymphocyte activation. *J Exp Med* 2000; **192**: 1027-1034 [PMID: 11015443 DOI: 10.1084/jem.192.7.1027]

44 **Dong H**, Strome SE, Salomao DR, Tamura H, Hirano F, Flies DB, Roche PC, Lu J, Zhu G, Tamada K, Lennon VA, Celis E, Chen L. Tumor-associated B7-H1 promotes T-cell apoptosis: a potential mechanism of immune evasion. *Nat Med* 2002; **8**: 793-800 [PMID: 12091876 DOI: 10.1038/nm730]

45 **Shi F**, Shi M, Zeng Z, Qi RZ, Liu ZW, Zhang JY, Yang YP, Tien P, Wang FS. PD-1 and PD-L1 upregulation promotes CD8(+) T-cell apoptosis and postoperative recurrence in hepatocellular carcinoma patients. *Int J Cancer* 2011; **128**: 887-896 [PMID: 20473887 DOI: 10.1002/ijc.25397]

46 **Zhu J**, Paul WE. Peripheral CD4+ T-cell differentiation regulated by networks of cytokines and transcription factors. *Immunol Rev* 2010; **238**: 247-262 [PMID: 20969597 DOI: 10.1111/j.1600-065X.2010.00951.x]

47 **Shuai K**. Interferon-activated signal transduction to the nucleus. *Curr Opin Cell Biol* 1994; **6**: 253-259 [PMID: 8024817]

48 **Volpes R**, van den Oord JJ, De Vos R, Depla E, De Ley M, Desmet VJ. Expression of interferon-gamma receptor in normal and pathological human liver tissue. *J Hepatol* 1991; **12**: 195-202 [PMID: 1828821]

49 **Nagao M**, Nakajima Y, Kanehiro H, Hisanaga M, Aomatsu Y, Ko S, Tatekawa Y, Ikeda N, Kanokogi H, Urizono Y, Kobayashi T, Shibaji T, Kanamura T, Ogawa S, Nakano H. The impact of interferon gamma receptor expression on the mechanism of escape from host immune surveillance in hepatocellular carcinoma. *Hepatology* 2000; **32**: 491-500 [PMID: 10960440]

50 **Johansson M**, Denardo DG, Coussens LM. Polarized immune responses differentially regulate cancer development. *Immunol Rev* 2008; **222**: 145-154 [PMID: 18363999 DOI: 10.1111/j.1600-065X.2008.00600.x]

51 **Kemp RA**, Ronchese F. Tumor-specific Tc1, but not Tc2, cells deliver protective antitumor immunity. *J Immunol* 2001; **167**: 6497-6502 [PMID: 11714817 DOI: 10.4049/jimmunol.167.11.6497]

52 **Stockinger B**, Veldhoen M, Martin B. Th17 T cells: linking innate and adaptive immunity. *Semin Immunol* 2007; **19**: 353-361 [PMID: 18023589 DOI: 10.1016/j.smim.2007.10.008]

53 **Kolls JK**, Lindén A. Interleukin-17 family members and inflammation. *Immunity* 2004; **21**: 467-476 [PMID: 15485625 DOI: 10.1016/j.immuni.2004.08.018]

54 **Park H**, Li Z, Yang XO, Chang SH, Nurieva R, Wang YH, Wang Y, Hood L, Zhu Z, Tian Q, Dong C. A distinct lineage of CD4 T cells regulates tissue inflammation by producing interleukin 17. *Nat Immunol* 2005; **6**: 1133-1141 [PMID: 16200068 DOI: 10.1038/ni1261]

55 **Numasaki M**, Fukushi J, Ono M, Narula SK, Zavodny PJ, Kudo T, Robbins PD, Tahara H, Lotze MT. Interleukin-17 promotes angiogenesis and tumor growth. *Blood* 2003; **101**: 2620-2627 [PMID: 12411307 DOI: 10.1182/blood-2002-05-1461]

56 **Tartour E**, Fossiez F, Joyeux I, Galinha A, Gey A, Claret E, Sastre-Garau X, Couturier J, Mosseri V, Vives V, Banchereau J, Fridman WH, Wijdenes J, Lebecque S, Sautès-Fridman C. Interleukin 17, a T-cell-derived cytokine, promotes tumorigenicity of human cervical tumors in nude mice. *Cancer Res* 1999; **59**: 3698-3704 [PMID: 10446984]

57 **Ma S**, Cheng Q, Cai Y, Gong H, Wu Y, Yu X, Shi L, Wu D, Dong C, Liu H. IL-17A produced by γδ T cells promotes tumor growth in hepatocellular carcinoma. *Cancer Res* 2014; **74**: 1969-1982 [PMID: 24525743 DOI: 10.1158/0008-5472.CAN-13-2534]

58 **Zhang JP**, Yan J, Xu J, Pang XH, Chen MS, Li L, Wu C, Li SP, Zheng L. Increased intratumoral IL-17-producing cells correlate with poor survival in hepatocellular carcinoma patients. *J Hepatol* 2009; **50**: 980-989 [PMID: 19329213 DOI: 10.1016/j.jhep.2008.12.033]

59 **Wang L**, Yi T, Kortylewski M, Pardoll DM, Zeng D, Yu H. IL-17 can promote tumor growth through an IL-6-Stat3 signaling pathway. *J Exp Med* 2009; **206**: 1457-1464 [PMID: 19564351 DOI: 10.1084/jem.20090207]

60 **Peck A**, Mellins ED. Precarious balance: Th17 cells in host defense. *Infect Immun* 2010; **78**: 32-38 [PMID: 19901061 DOI: 10.1128/IAI.00929-09]

61 **Sakaguchi S**, Yamaguchi T, Nomura T, Ono M. Regulatory T cells and immune tolerance. *Cell* 2008; **133**: 775-787 [PMID: 18510923 DOI: 10.1016/j.cell.2008.05.009]

62 **Rudensky AY**, Gavin M, Zheng Y. FOXP3 and NFAT: partners in tolerance. *Cell* 2006; **126**: 253-256 [PMID: 16873058 DOI: 10.1016/j.cell.2006.07.005]

63 **Sakaguchi S**. Naturally arising CD4+ regulatory t cells for immunologic self-tolerance and negative control of immune responses. *Annu Rev Immunol* 2004; **22**: 531-562 [PMID: 15032588 DOI: 10.1146/annurev.immunol.21.120601.141122]

64 **Sakaguchi S**, Miyara M, Costantino CM, Hafler DA. FOXP3+ regulatory T cells in the human immune system. *Nat Rev Immunol* 2010; **10**: 490-500 [PMID: 20559327 DOI: 10.1038/nri2785]

65 **Krummel MF**, Allison JP. CD28 and CTLA-4 have opposing effects on the response of T cells to stimulation. *J Exp Med* 1995; **182**: 459-465 [PMID: 7543139 DOI: 10.1046/j.1524-475X.1998.60506.x]

66 **Peggs KS**, Quezada SA, Chambers CA, Korman AJ, Allison JP. Blockade of CTLA-4 on both effector and regulatory T cell compartments contributes to the antitumor activity of anti-CTLA-4 antibodies. *J Exp Med* 2009; **206**: 1717-1725 [PMID: 19581407 DOI: 10.1084/jem.20082492]

67 **Fu J**, Xu D, Liu Z, Shi M, Zhao P, Fu B, Zhang Z, Yang H, Zhang H, Zhou C, Yao J, Jin L, Wang H, Yang Y, Fu YX, Wang FS. Increased regulatory T cells correlate with CD8 T-cell impairment and poor survival in hepatocellular carcinoma patients. *Gastroenterology* 2007; **132**: 2328-2339 [PMID: 17570208 DOI: 10.1053/j.gastro.2007.03.102]

68 **Hirano S**, Iwashita Y, Sasaki A, Kai S, Ohta M, Kitano S. Increased mRNA expression of chemokines in hepatocellular carcinoma with tumor-infiltrating lymphocytes. *J Gastroenterol Hepatol* 2007; **22**: 690-696 [PMID: 17444857 DOI: 10.1111/j.1440-1746.2006.04551.x]

69 **Kobayashi N**, Hiraoka N, Yamagami W, Ojima H, Kanai Y, Kosuge T, Nakajima A, Hirohashi S. FOXP3+ regulatory T cells affect the development and progression of hepatocarcinogenesis. *Clin Cancer Res* 2007; **13**: 902-911 [PMID: 17289884 DOI: 10.1158/1078-0432.CCR-06-2363]

70 **Noy R**, Pollard JW. Tumor-associated macrophages: from mechanisms to therapy. *Immunity* 2014; **41**: 49-61 [PMID: 25035953 DOI: 10.1016/j.immuni.2014.06.010]

71 **Sica A**, Mantovani A. Macrophage plasticity and polarization: in vivo veritas. *J Clin Invest* 2012; **122**: 787-795 [PMID: 22378047 DOI: 10.1172/JCI59643]

72 **Gordon S**, Taylor PR. Monocyte and macrophage heterogeneity. *Nat Rev Immunol* 2005; **5**: 953-964 [PMID: 16322748 DOI: 10.1038/nri1733]

73 **Pollard JW**. Tumour-educated macrophages promote tumour progression and metastasis. *Nat Rev Cancer* 2004; **4**: 71-78 [PMID: 14708027 DOI: 10.1038/nrc1256]

74 **Mantovani A**, Sozzani S, Locati M, Allavena P, Sica A. Macrophage polarization: tumor-associated macrophages as a paradigm for polarized M2 mononuclear phagocytes. *Trends Immunol*

2002; **23**: 549-555 [PMID: 12401408 DOI: 10.1016/S1471-4906(02)02302-5]
75 **DeNardo DG**, Barreto JB, Andreu P, Vasquez L, Tawfik D, Kolhatkar N, Coussens LM. CD4(+) T cells regulate pulmonary metastasis of mammary carcinomas by enhancing protumor properties of macrophages. *Cancer Cell* 2009; **16**: 91-102 [PMID: 19647220 DOI: 10.1016/j.ccr.2009.06.018]
76 **Wu K**, Kryczek I, Chen L, Zou W, Welling TH. Kupffer cell suppression of CD8+ T cells in human hepatocellular carcinoma is mediated by B7-H1/programmed death-1 interactions. *Cancer Res* 2009; **69**: 8067-8075 [PMID: 19826049 DOI: 10.1158/0008-5472.CAN-09-0901]
77 **Maeda S**, Kamata H, Luo JL, Leffert H, Karin M. IKKbeta couples hepatocyte death to cytokine-driven compensatory proliferation that promotes chemical hepatocarcinogenesis. *Cell* 2005; **121**: 977-990 [PMID: 15989949 DOI: 10.1016/j.cell.2005.04.014]
78 **Pikarsky E**, Porat RM, Stein I, Abramovitch R, Amit S, Kasem S, Gutkovich-Pyest E, Urieli-Shoval S, Galun E, Ben-Neriah Y. NF-kappaB functions as a tumour promoter in inflammation-associated cancer. *Nature* 2004; **431**: 461-466 [PMID: 15329734 DOI: 10.1038/nature02924]
79 **Naugler WE**, Sakurai T, Kim S, Maeda S, Kim K, Elsharkawy AM, Karin M. Gender disparity in liver cancer due to sex differences in MyD88-dependent IL-6 production. *Science* 2007; **317**: 121-124 [PMID: 17615358 DOI: 10.1126/science.1140485]
80 **Arii S**, Mise M, Harada T, Furutani M, Ishigami S, Niwano M, Mizumoto M, Fukumoto M, Imamura M. Overexpression of matrix metalloproteinase 9 gene in hepatocellular carcinoma with invasive potential. *Hepatology* 1996; **24**: 316-322 [PMID: 8690399 DOI: 10.1053/jhep.1996.v24.pm0008690399]
81 **Van Overmeire E**, Laoui D, Keirsse J, Van Ginderachter JA. Hypoxia and tumor-associated macrophages: A deadly alliance in support of tumor progression. *Oncoimmunology* 2014; **3**: e27561 [PMID: 24744977 DOI: 10.4161/onci.27561]
82 **Cramer T**, Yamanishi Y, Clausen BE, Förster I, Pawlinski R, Mackman N, Haase VH, Jaenisch R, Corr M, Nizet V, Firestein GS, Gerber HP, Ferrara N, Johnson RS. HIF-1alpha is essential for myeloid cell-mediated inflammation. *Cell* 2003; **112**: 645-657 [PMID: 12628185 DOI: 10.1016/S0092-8674(03)00154-5]
83 **Curiel TJ**, Coukos G, Zou L, Alvarez X, Cheng P, Mottram P, Evdemon-Hogan M, Conejo-Garcia JR, Zhang L, Burow M, Zhu Y, Wei S, Kryczek I, Daniel B, Gordon A, Myers L, Lackner A, Disis ML, Knutson KL, Chen L, Zou W. Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival. *Nat Med* 2004; **10**: 942-949 [PMID: 15322536 DOI: 10.1038/nm1093]
84 **Schutyser E**, Struyf S, Proost P, Opdenakker G, Laureys G, Verhasselt B, Peperstraete L, Van de Putte I, Saccani A, Allavena P, Mantovani A, Van Damme J. Identification of biologically active chemokine isoforms from ascitic fluid and elevated levels of CCL18/pulmonary and activation-regulated chemokine in ovarian carcinoma. *J Biol Chem* 2002; **277**: 24584-24593 [PMID: 11978786 DOI: 10.1074/jbc.M112275200]
85 **Li H**, Wu K, Tao K, Chen L, Zheng Q, Lu X, Liu J, Shi L, Liu C, Wang G, Zou W. Tim-3/galectin-9 signaling pathway mediates T-cell dysfunction and predicts poor prognosis in patients with hepatitis B virus-associated hepatocellular carcinoma. *Hepatology* 2012; **56**: 1342-1351 [PMID: 22505239 DOI: 10.1002/hep.25777]
86 **Zhu C**, Anderson AC, Schubart A, Xiong H, Imitola J, Khoury SJ, Zheng XX, Strom TB, Kuchroo VK. The Tim-3 ligand galectin-9 negatively regulates T helper type 1 immunity. *Nat Immunol* 2005; **6**: 1245-1252 [PMID: 16286920 DOI: 10.1038/ni1271]
87 **Kuang DM**, Zhao Q, Peng C, Xu J, Zhang JP, Wu C, Zheng L. Activated monocytes in peritumoral stroma of hepatocellular carcinoma foster immune privilege and disease progression through PD-L1. *J Exp Med* 2009; **206**: 1327-1337 [PMID: 19451266 DOI: 10.1084/jem.20082173]
88 **Zhang W**, Zhu XD, Sun HC, Xiong YQ, Zhuang PY, Xu HX, Kong LQ, Wang L, Wu WZ, Tang ZY. Depletion of tumor-associated macrophages enhances the effect of sorafenib in metastatic liver cancer models by antimetastatic and antiangiogenic effects. *Clin Cancer Res* 2010; **16**: 3420-3430 [PMID: 20570927 DOI: 10.1158/1078-0432.CCR-09-2904]
89 **Fridlender ZG**, Sun J, Kim S, Kapoor V, Cheng G, Ling L, Worthen GS, Albelda SM. Polarization of tumor-associated neutrophil phenotype by TGF-beta: "N1" versus "N2" TAN. *Cancer Cell* 2009; **16**: 183-194 [PMID: 19732719 DOI: 10.1016/j.ccr.2009.06.017]
90 **Mantovani A**, Cassatella MA, Costantini C, Jaillon S. Neutrophils in the activation and regulation of innate and adaptive immunity. *Nat Rev Immunol* 2011; **11**: 519-531 [PMID: 21785456 DOI: 10.1038/nri3024]
91 **Jablonska J**, Leschner S, Westphal K, Lienenklaus S, Weiss S. Neutrophils responsive to endogenous IFN-beta regulate tumor angiogenesis and growth in a mouse tumor model. *J Clin Invest* 2010; **120**: 1151-1164 [PMID: 20237412 DOI: 10.1172/JCI37223]
92 **Mantovani A**, Allavena P, Sica A, Balkwill F. Cancer-related inflammation. *Nature* 2008; **454**: 436-444 [PMID: 18650914 DOI: 10.1038/nature07205]
93 **Tecchio C**, Scapini P, Pizzolo G, Cassatella MA. On the cytokines produced by human neutrophils in tumors. *Semin Cancer Biol* 2013; **23**: 159-170 [PMID: 23410636 DOI: 10.1016/j.semcancer.2013.02.004]
94 **Zhou SL**, Zhou ZJ, Hu ZQ, Huang XW, Wang Z, Chen EB, Fan J, Cao Y, Dai Z, Zhou J. Tumor-Associated Neutrophils Recruit Macrophages and T-Regulatory Cells to Promote Progression of Hepatocellular Carcinoma and Resistance to Sorafenib. *Gastroenterology* 2016; **150**: 1646-1658.e17 [PMID: 26924089 DOI: 10.1053/j.gastro.2016.02.040]
95 **Gabrilovich DI**, Nagaraj S. Myeloid-derived suppressor cells as regulators of the immune system. *Nat Rev Immunol* 2009; **9**: 162-174 [PMID: 19197294 DOI: 10.1038/nri2506]
96 **Ilkovitch D**, Lopez DM. The liver is a site for tumor-induced myeloid-derived suppressor cell accumulation and immunosuppression. *Cancer Res* 2009; **69**: 5514-5521 [PMID: 19549903 DOI: 10.1158/0008-5472.CAN-08-4625]
97 **Hoechst B**, Ormandy LA, Ballmaier M, Lehner F, Krüger C, Manns MP, Greten TF, Korangy F. A new population of myeloid-derived suppressor cells in hepatocellular carcinoma patients induces CD4(+)CD25(+)Foxp3(+) T cells. *Gastroenterology* 2008; **135**: 234-243 [PMID: 18485901 DOI: 10.1053/j.gastro.2008.03.020]
98 **Corzo CA**, Condamine T, Lu L, Cotter MJ, Youn JI, Cheng P, Cho HI, Celis E, Quiceno DG, Padhya T, McCaffrey TV, McCaffrey JC, Gabrilovich DI. HIF-1α regulates function and differentiation of myeloid-derived suppressor cells in the tumor microenvironment. *J Exp Med* 2010; **207**: 2439-2453 [PMID: 20876310]
99 **Sakuishi K**, Jayaraman P, Behar SM, Anderson AC, Kuchroo VK. Emerging Tim-3 functions in antimicrobial and tumor immunity. *Trends Immunol* 2011; **32**: 345-349 [PMID: 21697013 DOI: 10.1016/j.it.2011.05.003]
100 **Yang L**, DeBusk LM, Fukuda K, Fingleton B, Green-Jarvis B, Shyr Y, Matrisian LM, Carbone DP, Lin PC. Expansion of myeloid immune suppressor Gr+CD11b+ cells in tumor-bearing host directly promotes tumor angiogenesis. *Cancer Cell* 2004; **6**: 409-421 [PMID: 15488763 DOI: 10.1016/j.ccr.2004.08.031]
101 **Caligiuri MA**. Human natural killer cells. *Blood* 2008; **112**: 461-469 [PMID: 18650461 DOI: 10.1182/blood-2007-09-077438]
102 **Gao B**, Jeong WI, Tian Z. Liver: An organ with predominant innate immunity. *Hepatology* 2008; **47**: 729-736 [PMID: 18167066 DOI: 10.1002/hep.22034]
103 **Sun H**, Sun C, Tian Z, Xiao W. NK cells in immunotolerant organs. *Cell Mol Immunol* 2013; **10**: 202-212 [PMID: 23563087 DOI: 10.1038/cmi.2013.9]
104 **Li F**, Tian Z. The liver works as a school to educate regulatory immune cells. *Cell Mol Immunol* 2013; **10**: 292-302 [PMID: 23604044 DOI: 10.1038/cmi.2013.7]
105 **Jinushi M**, Takehara T, Tatsumi T, Hiramatsu N, Sakamori R, Yamaguchi S, Hayashi N. Impairment of natural killer cell and dendritic cell functions by the soluble form of MHC class I-related chain A in advanced human hepatocellular carcinomas. *J Hepatol* 2005; **43**: 1013-1020 [PMID: 16168521 DOI: 10.1016/j.jhep.2005.05.026]

106 **Groh V**, Wu J, Yee C, Spies T. Tumour-derived soluble MIC ligands impair expression of NKG2D and T-cell activation. *Nature* 2002; **419**: 734-738 [PMID: 12384702 DOI: 10.1038/nature01112]

107 **Cooper MA**, Caligiuri MA. Isolation and characterization of human natural killer cell subsets. *Curr Protoc Immunol* 2004; **Chapter 7**: Unit 7.34 [PMID: 18432933 DOI: 10.1002/0471142735.im0734s60]

108 **Taketomi A**, Shimada M, Shirabe K, Kajiyama K, Gion T, Sugimachi K. Natural killer cell activity in patients with hepatocellular carcinoma: a new prognostic indicator after hepatectomy. *Cancer* 1998; **83**: 58-63 [PMID: 9655293]

109 **Chuang WL**, Liu HW, Chang WY. Natural killer cell activity in patients with hepatocellular carcinoma relative to early development and tumor invasion. *Cancer* 1990; **65**: 926-930 [PMID: 2153437]

110 **Miyagi T**, Takehara T, Tatsumi T, Kanto T, Suzuki T, Jinushi M, Sugimoto Y, Sasaki Y, Hori M, Hayashi N. CD1d-mediated stimulation of natural killer T cells selectively activates hepatic natural killer cells to eliminate experimentally disseminated hepatoma cells in murine liver. *Int J Cancer* 2003; **106**: 81-89 [PMID: 12794761 DOI: 10.1002/ijc.11163]

111 **Sprinzl MF**, Reisinger F, Puschnik A, Ringelhan M, Ackermann K, Hartmann D, Schiemann M, Weinmann A, Galle PR, Schuchmann M, Friess H, Otto G, Heikenwalder M, Protzer U. Sorafenib perpetuates cellular anticancer effector functions by modulating the crosstalk between macrophages and natural killer cells. *Hepatology* 2013; **57**: 2358-2368 [PMID: 23424039 DOI: 10.1002/hep.26328]

112 **Armeanu S**, Bitzer M, Lauer UM, Venturelli S, Pathil A, Krusch M, Kaiser S, Jobst J, Smirnow I, Wagner A, Steinle A, Salih HR. Natural killer cell-mediated lysis of hepatoma cells via specific induction of NKG2D ligands by the histone deacetylase inhibitor sodium valproate. *Cancer Res* 2005; **65**: 6321-6329 [PMID: 16024634 DOI: 10.1158/0008-5472.CAN-04-4252]

113 **El-Serag HB**, Lechel A, Rudolph KL. Epidemiology and Molecular Mechanisms of Hepatocarcinogenesis. 6th ed. UK: Elsevier Inc., 2012 [DOI: 10.1016/B978-1-4377-0881-3.00010-3]

114 **Avila MA**, Berasain C, Sangro B, Prieto J. New therapies for hepatocellular carcinoma. *Oncogene* 2006; **25**: 3866-3884 [PMID: 16799628 DOI: 10.1038/sj.onc.1209550]

115 **Kolch W**. Meaningful relationships: the regulation of the Ras/Raf/MEK/ERK pathway by protein interactions. *Biochem J* 2000; **351** Pt 2: 289-305 [PMID: 11023813 DOI: 10.1042/0264-6021:3510289]

116 **Ito Y**, Sasaki Y, Horimoto M, Wada S, Tanaka Y, Kasahara A, Ueki T, Hirano T, Yamamoto H, Fujimoto J, Okamoto E, Hayashi N, Hori M. Activation of mitogen-activated protein kinases/extracellular signal-regulated kinases in human hepatocellular carcinoma. *Hepatology* 1998; **27**: 951-958 [PMID: 9537433 DOI: 10.1002/hep.510270409]

117 **Huynh H**, Nguyen TT, Chow KH, Tan PH, Soo KC, Tran E. Over-expression of the mitogen-activated protein kinase (MAPK) kinase (MEK)-MAPK in hepatocellular carcinoma: its role in tumor progression and apoptosis. *BMC Gastroenterol* 2003; **3**: 19 [PMID: 12906713 DOI: 10.1186/1471-230X-3-19]

118 **Lee HC**, Tian B, Sedivy JM, Wands JR, Kim M. Loss of Raf kinase inhibitor protein promotes cell proliferation and migration of human hepatoma cells. *Gastroenterology* 2006; **131**: 1208-1217 [PMID: 17030190]

119 **Wilhelm SM**, Adnane L, Newell P, Villanueva A, Llovet JM, Lynch M. Preclinical overview of sorafenib, a multikinase inhibitor that targets both Raf and VEGF and PDGF receptor tyrosine kinase signaling. *Mol Cancer Ther* 2008; **7**: 3129-3140 [PMID: 18852116 DOI: 10.1158/1535-7163.MCT-08-0013]

120 **Roberts PJ**, Der CJ. Targeting the Raf-MEK-ERK mitogen-activated protein kinase cascade for the treatment of cancer. *Oncogene* 2007; **26**: 3291-3310 [PMID: 17496923 DOI: 10.1038/sj.onc.1210422]

121 **Chen RH**, Su YH, Chuang RL, Chang TY. Suppression of transforming growth factor-beta-induced apoptosis through a phosphatidylinositol 3-kinase/Akt-dependent pathway. *Oncogene* 1998; **17**: 1959-1968 [PMID: 9788439 DOI: 10.1038/sj.onc.1202111]

122 **Sahin F**, Kannangai R, Adegbola O, Wang J, Su G, Torbenson M. mTOR and P70 S6 kinase expression in primary liver neoplasms. *Clin Cancer Res* 2004; **10**: 8421-8425 [PMID: 15623621 DOI: 10.1158/1078-0432.CCR-04-0941]

123 **Gedaly R**, Angulo P, Hundley J, Daily MF, Chen C, Koch A, Evers BM. PI-103 and sorafenib inhibit hepatocellular carcinoma cell proliferation by blocking Ras/Raf/MAPK and PI3K/AKT/mTOR pathways. *Anticancer Res* 2010; **30**: 4951-4958 [PMID: 21187475]

124 **Zhu AX**, Kudo M, Assenat E, Cattan S, Kang YK, Lim HY, Poon RT, Blanc JF, Vogel A, Chen CL, Dorval E, Peck-Radosavljevic M, Santoro A, Daniele B, Furuse J, Jappe A, Perraud K, Anak O, Sellami DB, Chen LT. Effect of everolimus on survival in advanced hepatocellular carcinoma after failure of sorafenib: the EVOLVE-1 randomized clinical trial. *JAMA* 2014; **312**: 57-67 [PMID: 25058218 DOI: 10.1001/jama.2014.7189]

125 **Logan CY**, Nusse R. The Wnt signaling pathway in development and disease. *Annu Rev Cell Dev Biol* 2004; **20**: 781-810 [PMID: 15473860 DOI: 10.1146/annurev.cellbio.20.010403.113126]

126 **Gumbiner BM**. Signal transduction of beta-catenin. *Curr Opin Cell Biol* 1995; **7**: 634-640 [PMID: 8573337]

127 **Polakis P**. The oncogenic activation of beta-catenin. *Curr Opin Genet Dev* 1999; **9**: 15-21 [PMID: 10072352 DOI: 10.1016/S0959-437X(99)80003-3]

128 **Clevers H**, van de Wetering M. TCF/LEF factor earn their wings. *Trends Genet* 1997; **13**: 485-489 [PMID: 9433138 DOI: 10.1016/S0168-9525(97)01305-X]

129 **de La Coste A**, Romagnolo B, Billuart P, Renard CA, Buendia MA, Soubrane O, Fabre M, Chelly J, Beldjord C, Kahn A, Perret C. Somatic mutations of the beta-catenin gene are frequent in mouse and human hepatocellular carcinomas. *Proc Natl Acad Sci USA* 1998; **95**: 8847-8851 [PMID: 9671767 DOI: 10.1073/pnas.95.15.8847]

130 **Harada N**, Oshima H, Katoh M, Tamai Y, Oshima M, Taketo MM. Hepatocarcinogenesis in mice with beta-catenin and Ha-ras gene mutations. *Cancer Res* 2004; **64**: 48-54 [PMID: 14729607 DOI: 10.1158/0008-5472.CAN-03-2123]

131 **Salinas PC**. Modulation of the microtubule cytoskeleton: a role for a divergent canonical Wnt pathway. *Trends Cell Biol* 2007; **17**: 333-342 [PMID: 17643305 DOI: 10.1016/j.tcb.2007.07.003]

132 **Jiang W**, Jimenez G, Wells NJ, Hope TJ, Wahl GM, Hunter T, Fukunaga R. PRC1: a human mitotic spindle-associated CDK substrate protein required for cytokinesis. *Mol Cell* 1998; **2**: 877-885 [PMID: 9885575]

133 **Subramanian R**, Wilson-Kubalek EM, Arthur CP, Bick MJ, Campbell EA, Darst SA, Milligan RA, Kapoor TM. Insights into antiparallel microtubule crosslinking by PRC1, a conserved nonmotor microtubule binding protein. *Cell* 2010; **142**: 433-443 [PMID: 20691902 DOI: 10.1016/j.cell.2010.07.012]

134 **Chen J**, Rajasekaran M, Xia H, Zhang X, Kong SN, Sekar K, Seshachalam VP, Deivasigamani A, Goh BK, Ooi LL, Hong W, Hui KM. The microtubule-associated protein PRC1 promotes early recurrence of hepatocellular carcinoma in association with the Wnt/β-catenin signalling pathway. *Gut* 2016; **pii**: gutjnl-2015-310625 [PMID: 26941395 DOI: 10.1136/gutjnl-2015-310625]

135 **Wei W**, Chua MS, Grepper S, So SK. Blockade of Wnt-1 signaling leads to anti-tumor effects in hepatocellular carcinoma cells. *Mol Cancer* 2009; **8**: 76 [PMID: 19778454 DOI: 10.1186/1476-4598-8-76]

136 **Wei W**, Chua MS, Grepper S, So SK. Soluble Frizzled-7 receptor inhibits Wnt signaling and sensitizes hepatocellular carcinoma cells towards doxorubicin. *Mol Cancer* 2011; **10**: 16 [PMID: 21314951 DOI: 10.1186/1476-4598-10-16]

137 **Stamos JL**, Weis WI. The β-catenin destruction complex. *Cold Spring Harb Perspect Biol* 2013; **5**: a007898 [PMID: 23169527 DOI: 10.1101/cshperspect.a007898]

138 **Satoh S**, Daigo Y, Furukawa Y, Kato T, Miwa N, Nishiwaki T, Kawasoe T, Ishiguro H, Fujita M, Tokino T, Sasaki Y, Imaoka S, Murata M, Shimano T, Yamaoka Y, Nakamura Y. AXIN1 mutations in hepatocellular carcinomas, and growth suppression in cancer cells by virus-mediated transfer of AXIN1. *Nat Genet* 2000; **24**: 245-250 [PMID: 10700176 DOI: 10.1038/73448]

139 **Dahmani R**, Just PA, Perret C. The Wnt/β-catenin pathway as a therapeutic target in human hepatocellular carcinoma. *Clin Res Hepatol Gastroenterol* 2011; **35**: 709-713 [PMID: 21778132 DOI: 10.1016/j.clinre.2011.05.010]

140 **Akira S**, Uematsu S, Takeuchi O. Pathogen recognition and innate immunity. *Cell* 2006; **124**: 783-801 [PMID: 16497588 DOI: 10.1016/j.cell.2006.02.015]

141 **Karin M**, Lawrence T, Nizet V. Innate immunity gone awry: linking microbial infections to chronic inflammation and cancer. *Cell* 2006; **124**: 823-835 [PMID: 16497591 DOI: 10.1016/j.cell.2006.02.016]

142 **Karin M**. Nuclear factor-kappaB in cancer development and progression. *Nature* 2006; **441**: 431-436 [PMID: 16724054 DOI: 10.1038/nature04870]

143 **Greten FR**, Eckmann L, Greten TF, Park JM, Li ZW, Egan LJ, Kagnoff MF, Karin M. IKKbeta links inflammation and tumorigenesis in a mouse model of colitis-associated cancer. *Cell* 2004; **118**: 285-296 [PMID: 15294155 DOI: 10.1016/j.cell.2004.07.013]

144 **Bromberg J**, Darnell JE. The role of STATs in transcriptional control and their impact on cellular function. *Oncogene* 2000; **19**: 2468-2473 [PMID: 10851045 DOI: 10.1038/sj.onc.1203476]

145 **Yu H**, Jove R. The STATs of cancer--new molecular targets come of age. *Nat Rev Cancer* 2004; **4**: 97-105 [PMID: 14964307 DOI: 10.1038/nrc1275]

146 **Bowman T**, Garcia R, Turkson J, Jove R. STATs in oncogenesis. *Oncogene* 2000; **19**: 2474-2488 [PMID: 10851046 DOI: 10.1038/sj.onc.1203527]

147 **Calò V**, Migliavacca M, Bazan V, Macaluso M, Buscemi M, Gebbia N, Russo A. STAT proteins: from normal control of cellular events to tumorigenesis. *J Cell Physiol* 2003; **197**: 157-168 [PMID: 14502555 DOI: 10.1002/jcp.10364]

148 **Yang SF**, Wang SN, Wu CF, Yeh YT, Chai CY, Chunag SC, Sheen MC, Lee KT. Altered p-STAT3 (tyr705) expression is associated with histological grading and intratumour microvessel density in hepatocellular carcinoma. *J Clin Pathol* 2007; **60**: 642-648 [PMID: 16901975 DOI: 10.1136/jcp.2006.036970]

149 **Sun X**, Zhang J, Wang L, Tian Z. Growth inhibition of human hepatocellular carcinoma cells by blocking STAT3 activation with decoy-ODN. *Cancer Lett* 2008; **262**: 201-213 [PMID: 18248786 DOI: 10.1016/j.canlet.2007.12.009]

150 **Sawada Y**, Yoshikawa T, Nobuoka D, Shirakawa H, Kuronuma T, Motomura Y, Mizuno S, Ishii H, Nakachi K, Konishi M, Nakagohri T, Takahashi S, Gotohda N, Takayama T, Yamao K, Uesaka K, Furuse J, Kinoshita T, Nakatsura T. Phase I trial of a glypican-3-derived peptide vaccine for advanced hepatocellular carcinoma: immunologic evidence and potential for improving overall survival. *Clin Cancer Res* 2012; **18**: 3686-3696 [PMID: 22577059 DOI: 10.1158/1078-0432.CCR-11-3044]

151 **Greten TF**, Forner A, Korangy F, N'Kontchou G, Barget N, Ayuso C, Ormandy LA, Manns MP, Beaugrand M, Bruix J. A phase II open label trial evaluating safety and efficacy of a telomerase peptide vaccination in patients with advanced hepatocellular carcinoma. *BMC Cancer* 2010; **10**: 209 [PMID: 20478057]

152 **Humphries C**. Adoptive cell therapy: Honing that killer instinct. *Nature* 2013; **504**: S13-S15 [PMID: 24352359 DOI: 10.1038/504S13a]

153 **Alici E**, Sutlu T, Björkstrand B, Gilljam M, Stellan B, Nahi H, Quezada HC, Gahrton G, Ljunggren HG, Dilber MS. Autologous antitumor activity by NK cells expanded from myeloma patients using GMP-compliant components. *Blood* 2008; **111**: 3155-3162 [PMID: 18192509 DOI: 10.1182/blood-2007-09-110312]

154 **Hui D**, Qiang L, Jian W, Ti Z, Da-Lu K. A randomized, controlled trial of postoperative adjuvant cytokine-induced killer cells immunotherapy after radical resection of hepatocellular carcinoma. *Dig Liver Dis* 2009; **41**: 36-41 [PMID: 18818130 DOI: 10.1016/j.dld.2008.04.007]

155 **Takayama T**, Sekine T, Makuuchi M, Yamasaki S, Kosuge T, Yamamoto J, Shimada K, Sakamoto M, Hirohashi S, Ohashi Y, Kakizoe T. Adoptive immunotherapy to lower postsurgical recurrence rates of hepatocellular carcinoma: a randomised trial. *Lancet* 2000; **356**: 802-807 [PMID: 11022927 DOI: 10.1016/S0140-6736(00)02654-4]

156 **Weng DS**, Zhou J, Zhou QM, Zhao M, Wang QJ, Huang LX, Li YQ, Chen SP, Wu PH, Xia JC. Minimally invasive treatment combined with cytokine-induced killer cells therapy lower the short-term recurrence rates of hepatocellular carcinomas. *J Immunother* 2008; **31**: 63-71 [PMID: 18157013 DOI: 10.1097/CJI.0b013e31815a121b]

157 **Yu X**, Zhao H, Liu L, Cao S, Ren B, Zhang N, An X, Yu J, Li H, Ren X. A randomized phase II study of autologous cytokine-induced killer cells in treatment of hepatocellular carcinoma. *J Clin Immunol* 2014; **34**: 194-203 [PMID: 24337625 DOI: 10.1007/s10875-013-9976-0]

158 **Fourcade J**, Sun Z, Pagliano O, Guillaume P, Luescher IF, Sander C, Kirkwood JM, Olive D, Kuchroo V, Zarour HM. CD8(+) T cells specific for tumor antigens can be rendered dysfunctional by the tumor microenvironment through upregulation of the inhibitory receptors BTLA and PD-1. *Cancer Res* 2012; **72**: 887-896 [PMID: 22205715 DOI: 10.1158/0008-5472.CAN-11-2637]

159 **Nguyen LT**, Ohashi PS. Clinical blockade of PD1 and LAG3--potential mechanisms of action. *Nat Rev Immunol* 2015; **15**: 45-56 [PMID: 25534622 DOI: 10.1038/nri3790]

160 **Pardoll DM**. The blockade of immune checkpoints in cancer immunotherapy. *Nat Rev Cancer* 2012; **12**: 252-264 [PMID: 22437870 DOI: 10.1038/nrc3239]

161 **Hodi FS**, O'Day SJ, McDermott DF, Weber RW, Sosman JA, Haanen JB, Gonzalez R, Robert C, Schadendorf D, Hassel JC, Akerley W, van den Eertwegh AJ, Lutzky J, Lorigan P, Vaubel JM, Linette GP, Hogg D, Ottensmeier CH, Lebbé C, Peschel C, Quirt I, Clark JI, Wolchok JD, Weber JS, Tian J, Yellin MJ, Nichol GM, Hoos A, Urba WJ. Improved survival with ipilimumab in patients with metastatic melanoma. *N Engl J Med* 2010; **363**: 711-723 [PMID: 20525992 DOI: 10.1056/NEJMoa1003466]

162 **Ribas A**, Kefford R, Marshall MA, Punt CJ, Haanen JB, Marmol M, Garbe C, Gogas H, Schachter J, Linette G, Lorigan P, Kendra KL, Maio M, Trefzer U, Smylie M, McArthur GA, Dreno B, Nathan PD, Mackiewicz J, Kirkwood JM, Gomez-Navarro J, Huang B, Pavlov D, Hauschild A. Phase III randomized clinical trial comparing tremelimumab with standard-of-care chemotherapy in patients with advanced melanoma. *J Clin Oncol* 2013; **31**: 616-622 [PMID: 23295794 DOI: 10.1200/JCO.2012.44.6112]

163 **Sangro B**, Gomez-Martin C, de la Mata M, Iñarrairaegui M, Garralda E, Barrera P, Riezu-Boj JI, Larrea E, Alfaro C, Sarobe P, Lasarte JJ, Pérez-Gracia JL, Melero I, Prieto J. A clinical trial of CTLA-4 blockade with tremelimumab in patients with hepatocellular carcinoma and chronic hepatitis C. *J Hepatol* 2013; **59**: 81-88 [PMID: 23466307 DOI: 10.1016/j.jhep.2013.02.022]

164 **Topalian SL**, Hodi FS, Brahmer JR, Gettinger SN, Smith DC, McDermott DF, Powderly JD, Carvajal RD, Sosman JA, Atkins MB, Leming PD, Spigel DR, Antonia SJ, Horn L, Drake CG, Pardoll DM, Chen L, Sharfman WH, Anders RA, Taube JM, McMiller TL, Xu H, Korman AJ, Jure-Kunkel M, Agrawal S, McDonald D, Kollia GD, Gupta A, Wigginton JM, Sznol M. Safety, activity, and immune correlates of anti-PD-1 antibody in cancer. *N Engl J Med* 2012; **366**: 2443-2454 [PMID: 22658127 DOI: 10.1056/NEJMoa1200690]

165 **Elkhoueiry AB**, Melero I, Crocenzi TS, Welling TH, Yau TC, Yeo W, Chopra A, Grosso J, Lang L, Anderson J, Cruz CMD, Sangro B. Phase I/II safety and antitumor activity of nivolumab in patients with advanced hepatocellular carcinoma (HCC): CA209-040. *J Clin Oncol* (Meeting Abstracts) May 2015; **33**: no. 15_supplLBA101. Available from: URL: http://meetinglibrary.asco.org/content/146146-156

P- Reviewer: Fava G, Hung LY, Ooi LLPJ **S- Editor**: Ji FF
L- Editor: A **E- Editor**: Lu YJ

World Journal of Gastrointestinal Pathophysiology

Submit a Manuscript: http://www.wjgnet.com/esps/
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.4291/wjgp.v7.i3.256

World J Gastrointest Pathophysiol 2016 August 15; 7(3): 256-265
ISSN 2150-5330 (online)

REVIEW

Update on diagnostic value of breath test in gastrointestinal and liver diseases

Imran Siddiqui, Sibtain Ahmed, Shahab Abid

Imran Siddiqui, Sibtain Ahmed, Department of Pathology and Laboratory Medicine, Aga Khan University, Karachi 74800, Pakistan

Shahab Abid, Section of Gastroenterology, Department of Medicine, Aga Khan University, Karachi 74800, Pakistan

Author contributions: Siddiqui I performed the literature search, data accusation and majority of the writing work in first draft, prepared the figures and tables; Ahmed S performed the literature search, data accusation and contributed in writing first draft, help with figures and tables; Abid S conceived the idea, designed the outline and coordinated the writing of the paper; all authors have reviewed the final draft and agreed upon.

Conflict-of-interest statement: There is no conflict of interest associated with any of the senior author or other coauthors who contributed their efforts in this manuscript.

Manuscript source: Invited manuscript

Correspondence to: Dr. Shahab Abid, Professor and Head, Section of Gastroenterology, Department of Medicine, Aga Khan University, Stadium Road, PO Box 3500, Karachi 74800, Pakistan. shahab.abid@aku.edu
Telephone: +92-21-34864656
Fax: +92-21-34934294

Received: March 4, 2016
Peer-review started: March 7, 2016
First decision: April 15, 2016
Revised: April 22, 2016
Accepted: May 10, 2016
Article in press: May 11, 2016
Published online: August 15, 2016

Abstract

In the field of gastroenterology, breath tests (BTs) are used intermittently as diagnostic tools that allow indirect, non-invasive and relatively less cumbersome evaluation of several disorders by simply quantifying the appearance in exhaled breath of a metabolite of a specific substrate administered. The aim of this review is to have an insight into the principles, methods of analysis and performance parameters of various hydrogen, methane and carbon BTs which are available for diagnosing gastrointestinal disorders such as Helicobacter pylori infection, small intestinal bacterial overgrowth, and carbohydrate malabsorption. Evaluation of gastric emptying is routinely performed by scintigraphy which is however, difficult to perform and not suitable for children and pregnant women, this review has abridged the 13C-octanoic acid test in comparison to scintigraphy and has emphasized on its working protocol and challenges. A new development such as electronic nose test is also highlighted. Moreover we have also explored the limitations and constraints restraining the wide use of these BT. We conclude that breath testing has an enormous potential to be used as a diagnostic modality. In addition it offers distinct advantages over the traditional invasive methods commonly employed.

Key words: Breath tests; Diagnostic techniques; Lactase deficiency; Gastrointestinal tract; *Helicobacter pylori*

Core tip: The aim of this review is to have an insight into the principles, methods of analysis and performance parameters of various breath tests available for diagnosing gastrointestinal disorders. Furthermore we have also explored the limitations and constraints restricting the wide use of these tests.

Siddiqui I, Ahmed S, Abid S. Update on diagnostic value

of breath test in gastrointestinal and liver diseases. *World J Gastrointest Pathophysiol* 2016; 7(3): 256-265 Available from: URL: http://www.wjgnet.com/2150-5330/full/v7/i3/256.htm DOI: http://dx.doi.org/10.4291/wjgp.v7.i3.256

INTRODUCTION

Composition of human breath is a blend of various inert gases as well as nitrogen, oxygen and carbon dioxide (CO_2). In addition, researchers have also revealed several other trace volatile organic compounds (VOCs) in breath with concentrations varying from parts per million (ppm) to trillion (ppt)[1,2]. Commonly present VOCs in breath include, ethane, hydrogen, and methanol which are harvests of primary metabolic processes in the body and can play a pivotal role for various medical diagnostics[3].

In the current era of advanced human diagnostics, breath analysis is widely gaining attentiveness of clinicians and laboratories as a noninvasive diagnostic option. Gas analysis sensors and sensor systems are now available, as a product of rapid development in micro and nanotechnology. These tools are being progressively amended for laboratory testing and the more recent discovery of new gas volatile compound biomarkers have opened new horizons for researchers[4].

Speaking from an analytical point of view composition of breath is less complex than serum and urine thus making it a preferable matrix for a comprehensive analysis. Furthermore, these procedures can be easily repeated if the need arises for a recheck.

To identify the disease processes occurring in the gastrointestinal (GI) tract the use of endoscopy and colonoscopy are commonly on the rise, however these modalities are not only invasive and costly but the patients are also more at risk of suffering from complications with significant morbidities. Breath testing provides a solution to some of the practical issues faced in GI testing, although suffers from its own limitations.

METHODOLOGY

We selected articles from the PubMed database and Google scholar by using the search terms "breath test" (BT), "*Helicobacter pylori*" (*H. pylori*), "carbon breath test" and "urea breath test" (UBT). Inclusion criteria were articles published in English, in peer-reviewed journals, between 1966 and 2011. The articles were further filtered in a team meeting, keeping in view the ideology behind this mini review, *i.e.*, the current practices, the new advancements and factors limiting the wide use of BTs.

BASIC MECHANISM OF BT

BTs are based on the consumption of numerous substrates that undergo processing at different points in the GI tract. The concept revolves around the fact that the metabolized substrate leads to the production of gases (*e.g.*, CO_2, H_2) that become part of the blood stream, are expelled and measured in exhaled breath *via* the different analyzers available.

Moreover hydrogen and carbon BTs are the most widely known and practice, methane BT are also gaining popularity based on the fact that its production is prevalent in 36%-50% of healthy subjects in comparison with hydrogen which is more pervasive. Literature review has shown that a noticeable amount of subjects do not produce hydrogen in spite of having small intestinal bacterial overgrowth (SIBO) because of the presence of the bacterium *Methanobrevibacter smithii* (*M. smithii*) which converts hydrogen into methane.

There is a significant rise in the utility of breath testing since their development considering the fact that they are non-invasive and relatively simpler and safer tools for the diagnosis of various disorders of GI tract such as *H. pylori* infection, gastric motility, SIBO, and sugar malabsorption. Different available BT are summarized in (Table 1).

Table 1 List of breath tests available for clinical utilization

Indications
Tests for small intestinal bacterial overgrowth
Glucose hydrogen breath test
Lactulose hydrogen breath test
13C-glycocholate breath test
13C-xylose breath test
Tests for carbohydrate malabsorption
Fructose hydrogen breath test
Lactose hydrogen breath test
Saccharose hydrogen breath test
13C-lactose breath test
13C-fructose breath test
13C-saccharose breath test
Methane breath test
Tests for Helicobacter pylori infection
13C-urea breath test
14C-urea breath test
Tests for the evaluation of gastric emptying
13C-octanoic acid breath test
Tests for the evaluation of exocrine pancreatic insufficiency
13C-mixed triglycerides breath test
13C-starch breath test
13C-egg protein breath test
Tests for the evaluation of hepatocellular function
13C-aminopyrine breath test
13C-ethacetin breath test

UBT: Urea breath test; 14C: 14 carbon; 13C: 13 carbon.

HYDROGEN BT

Principle

Hydrogen is a product of the intestinal bacterial overgrowth when dietary carbohydrates encounter malabsorption in the small intestine. Hydrogen producing bacteria chiefly reside in the colon. A quantifiable amount of this colonic hydrogen is absorbed into the bloodstream and is exhaled and eventually detected by breath

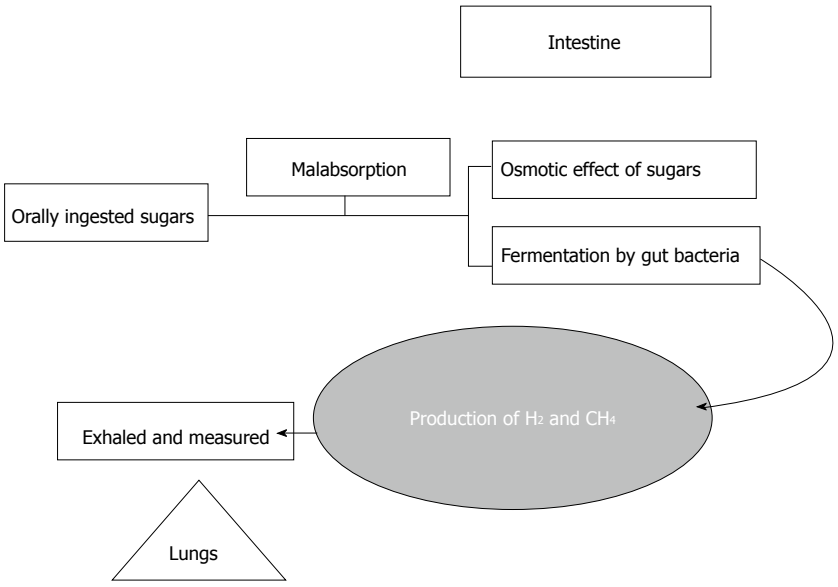


Figure 1 Principle of hydrogen breath test.

testing[5] (Figure 1).

Analysis

Hydrogen concentrations are commonly measured using gas chromatography or electrochemical cells. With the rising entity of point of care testing (POCT), portable even pocket sized breath analyzers are now being developed which enable a reliable direct measurement in practice or at bedside[6].

Points to consider

Hydrogen BTs lack standardization in laboratories worldwide which renders the comparison of test results difficult. The dosage of the carbohydrate, the volume of the dissolving fluid, the duration of the test period, the interval of breath samples collection as well as the optimal cut-offs used for reporting differs among test providers.

Practical application of hydrogen breath testing

Hydrogen breath testing for SIBO: Glucose is a preferred substrate to detect SIBO as it follows a prompt reabsorption in the proximal small bowel. The recommended cut-off point diverges between 10 and 20 ppm. In the presence of bacteria in the small intestine, glucose get fermented and liberated in the high quantity and can be detected easily in breath.

Protocol: Subjects are made to undergo an overnight fast. Prerequisites of the test include teeth brushing and use of disinfecting mouth wash and gargles, keeping in mind the fact that oral bacteria can lead to false increment on hydrogen peaks. With the commencement of breath hydrogen sampling basal breath hydrogen is recorded. In circumstance when basal values of breath hydrogen are recorded in excess of 16 ppm, substrates are not given and test is abandoned as according to few researchers high basal hydrogen values are diagnostic of SIBO but this finding remains contentious. A diagnosis of SIBO is made on glucose hydrogen BT if there is an upsurge in breath hydrogen by 12 ppm above the base line levels. Reportedly sensitivity and specificity of this test are 62% and 83% respectively, when compared with culture from jejunal aspirate[7].

Some studies have also suggested lactulose BT for making a diagnosis of SIBO but it was found to be less specific compared to the glucose BT[8].

Hydrogen breath testing for carbohydrate malabsorption

Lactose hydrogen BT: Four variants of lactase deficiency have been identified, *i.e.*, primary, secondary, developmental and congenital lactase deficiency. Statistics suggest that primary lactase deficiency predominates affecting more than 50% of the world's population[9,10]. Ethnicity and amount of dairy consumption are the contributing factors, whereas risk is reportedly higher in Asian and American Indian people compared to Europeans[11,12].

Protocol: Baseline hydrogen measurements are taken in expired breath. Fasting subjects are given 50 g lactose orally mixed with water. Further samples to detect the hydrogen quantity are taken at 15-30 min time intervals continued over a period of 4 h. Detection of more than 10-20 ppm over the baseline hydrogen value (detected in at least 2 breath samples) indicates lactose malabsorption.

Improvement is sensitivity have been reported by studies if the test is extended for a period of 6 h with hourly sample collection from 3 to 6 h. However, this is not yet extensively applied as standard clinical practice protocol[13].

False-positive results are seen with recent smoking or inadequate pre-test fasting (high carbohydrate load). False-negative results may arise following recent use of antibiotics, in patients with lung disorders, or in approximately 10% to 20% of patients who are hydrogen non-producers.

Other tests for carbohydrate metabolism use fructose or saccharose as substrate but are not popular for clinical use[14-16].

METHANE (CH_4) BT

The addition of methane to hydrogen measurement has improved the diagnostic accuracy of these BTs by capturing the 20% to 30% of the general population which produces methane as a main byproduct of carbohydrate fermentation[17]. Furthermore, Methane testing has also potentially contributed towards an increment in sensitivity of lactose BT[18].

Methane production is prevalent in 36%-50% of healthy subjects in comparison with hydrogen which is more pervasive[19-21]. *M. smithii* are the chief producers of methane in humans. This process takes place chiefly in the left colon.

Methane production is more disease specific as suggested by different studies, for example: Methane excretion is not found in diarrheal states such as ulcerative colitis or Crohn's disease and on the other hand it is more frequently observed in diverticulosis[22] and encoparesis[23] related with constipation.

Furthermore literature review revealed significant association between delayed gut motility and CH_4. Reportedly mean of transit time in CH_4 producers was 84.6 h and in non-producers was 48.6 h[24].

Analysis

More or less follows the same protocol as hydrogen breath testing. The only difference is established while sample analysis is done for methane. Gas chromatography equipped with range of detectors based on flame ionization[25-28], thermal conductivity[29], pulsed helium discharge ionization[30] and mass spectrometry[31] are available for methane analysis. Furthermore, selective ion flow transfer mass spectrometry (SIFT-MS) methane analysis is also practiced which is relatively a more convenient technique[32].

CARBON BTS

Carbon exists in various isotopic forms; the most well-known forms being the 12C, 13C and 14C isotopes. 14C is a radioactive isotope and is instable. It has a half time decay of 5730 years, whereas only 12C and 13C are stable forms.

Principle

This technique is based on the use of either the radioactive isotope of carbon, 14C or the safer and preferable nonradioactive 13C isotope[33,34]. 13C differs by only one neutron from the naturally more common 12C-atom. The detection of 13C-carbondioxide ($13CO_2$) in breath is the time limiting step from ingestion of the substrate to its complete metabolism, till the final outbreath of the end product $13CO_2$.

Analysis

Breath samples are collected at intervals ranging from 4 to 24 h after ingestion of the substrate[31,35]. Most centers utilize the high resolution isotope ratio mass spectrometers (IRMS) for the differentiation of $13CO_2$ and $12CO_2$. The introduction of non-dispersive isotope selective infrared spectrometers (NDIRS) has simplified the use of 13C-BTs and have paved the way for analysis in small centers as well[36-38].

Points to consider

This technique has got an edge in favor of non-hydrogen-producers. Furthermore lesser quantity of substrate is required compared with other tests. However, the costs of some substrates still limits the wide spread use. Endogenous CO_2 production, which fluctuated extensively in the numerous diseases, has resulted in declining diagnostic accuracy.

Practical application of carbon breath testing

UBT for *H. pylori* infection: A meta-analysis by Ferwana *et al*[39] has reported pooled sensitivity and specificity of UBT to be 96% and 93% respectively. Similar results were also the outcome of a multicenter Japanese study conducted in 2002, making UBT a reliable test for *H. pylori* infections[40]. Study from developing world also suggest that UBT is a highly accurate and reliable diagnostic modality as reflected by another study form Egypt that revealed a sensitivity and specificity of UBT to be 98% and 89% respectively[41].

Principle: Begins with the oral administration of 13C or 14C labeled urea. *H. pylori* produce the urea splitting enzyme Urease, which ultimately cleaves the labeled urea to ammonia and bicarbonate. Bicarbonate is the precursor of CO_2 that is incorporated into breath (Figure 2).

Owing to the radioactive hazard of 14C, here also 13C UBT is the preferred method of detection. A large multicenter study evaluated the accuracy of 13C-UBT in children taking biopsy as gold standard and stated a sensitivity ranging from 96%-98% and specificity 96%-99%[42].

Analysis: The test underwent various reforms regarding substrate dose, fasting state, test meal and breath sample intervals[43]. Commonly used protocol uses 75 mg 13C-urea administered to fasting subjects mixed with 200 mL citric acid solution. Breath samples are taken at baseline, followed by re-sampling at 20 or 30 min after ingestion of the substrate. A delta over baseline in breath 13C-enrichment above 3.5%-5% is considered positive.

Beginning of the 21th century has marked the advancement of UBT with the introduction of bench top analyzers based on the principle of molecular correlation spectrometry pooled with infrared spectrometer[44,45]. Campuzano-Maya *et al*[46] developed a simplified 13C-UBT protocol which when evaluated yielded an accuracy of

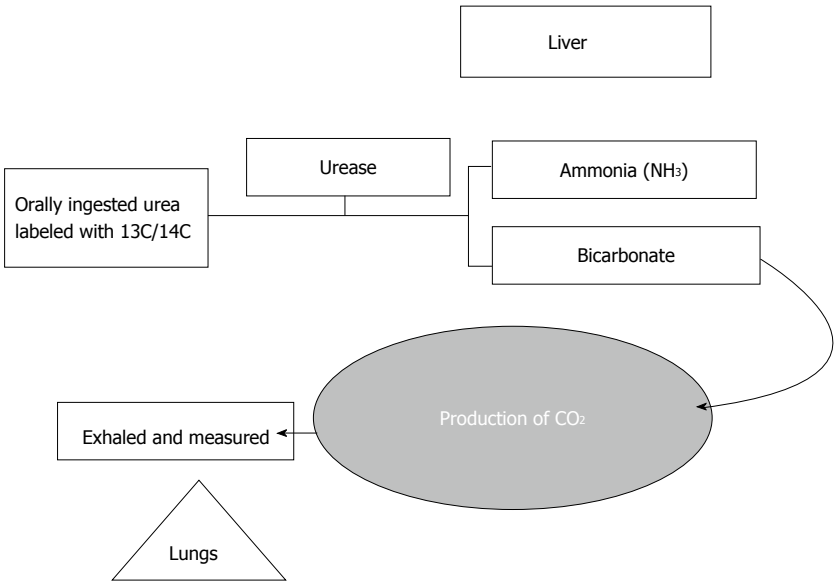


Figure 2 Principle of urea breath test.

100% for the diagnosis of *H. pylori*. This version required only 50 mg 13C-urea, no prior test meal, and more importantly a single breath sample collected at 10 min[46].

Points to consider: High cost of substrate is a drawback of this test. The use of bismuth-based preparations, drugs including proton pump inhibitors several antibiotics can effects the results of this test[47]. Gargles or mouth wash are routinely advised before the commencement of the test as oral contamination could lead to false positive results.

The 14C-UBT owing to its radioactivity potential has not been promoted for use in children and women of reproductive age group. However, the amount of radioactivity delivered to the patient is low, arising the question of its prescription to the pediatric and pregnant population by some researchers[48]. On the brighter side of the picture, the 13C-UBT can be safely used in these patient groups[49]. A comparison of 13C and 14C UBT is summarized in (Table 2).

13C-Octanoic acid BT for the evaluation of gastric emptying

The gold standard test to assess gastric emptying is Scintigraphy using radioactive tracers. The other alternative available is 13C-BT that uses the 13C-octanoate to label the solid components of test meals and the 13C-acetate which is utilized to label fluids.

Principle: This test is established on the fact that time taken up by the transport of the tracer substance is considered the rate limiting state together with the ingested food from the stomach into the duodenum, while the remaining processes till the elimination of CO_2 follows at a constant rate[50].

Protocol: Egg yolk is used as a test meal labeled with 13C-octanoic acid. 13C-octanoic acid is absorbed upon its passage through the duodenum, and eventually oxidized by the liver to $13CO_2$. Gastric emptying of the egg yolk into the duodenum serves as a rate limiting step which in turn influences the detection of 13C in exhaled breath samples.

Most studies have validated 13C-octanoic acid test against scintigraphy and have found acceptable correlation. However scintigraphy itself suffers from lack of standardization. Differences in test meals, position of the patient, rate, and extent of imaging are the factors that affect test results.

Choi *et al*[51] in 1997 evaluated the performance of simultaneous OBT and scintigraphy in 15 healthy participants and revealed that these tests do not significantly correlate with each other. However acceptable reproducibility was obtained with a mean coefficient of variation of $t_{1/2}$ of 20% between individuals and 12% within the same individual[51]. They put forward that OBT is only a reliable tool for intra-individual comparisons. However in the coming years this study faced immense criticism and findings were not adopted[52].

There is abundance of data that has compared breath testing with scintigraphy, however as magnetic resonance imaging (MRI) is potentially the most valid method; stable isotope BT should preferably be compared with MRI. In respect to this a study by Haans *et al*[53] evaluated MRI against breath testing for gastric emptying and revealed a strong correlation between the two techniques for liquid emptying compared for solid gastric emptying.

Advantages: Provides a better alternative test to scintigraphy as it is free from radioactive hazards, particularly in children, women in reproductive age group and subjects requiring repetitive testing. It overcomes certain limitations such as operator dependence and time constraint of ultrasonography. Furthermore it's less costly compared to magnetic resonance imaging.

Limitations of 13C-octanoic acid BT: There are certain limitations of 13C-octanoic acid breath test (OBT). Firstly it suffers from lack of standardization. Furthermore

Table 2 Comparison of the 14C and 13C urea breath test

	14C-UBT	13C-UBT
Test performed at	Nuclear medicine department	No specific location required
Analysis	Specialized nuclear medicine department and β-scintillation counters	Mass spectrometry analysis (in a hospital or mailed to the manufacturer)
Radioactive hazard	Yes	No
Patient selection	Not suitable for children or pregnant women	Safer for children or pregnant women

UBT: Urea breath test; 14C: 14 carbon; 13C: 13 carbon.

protocol followed at different centers has certain variations. Literature review further highlighted that there also exists a difference in gastric emptying time taken as part of the methodology. Moreover collections of breath samples every fifteen minutes for four hours while the patients with minimal physical activities is difficult.

Methods of analysis: In most instances mass spectrophotometry (MS) is the technique of choice for measuring sampled $13CO_2$. Potential hindrances associated with MS are high cost and large operating units. Based on the convenience of its application Non-dispersive infrared isotope spectrometry (NDIRS) is potential substitute of MS[54].

13C-*spirulina platensis* (*S. platensis*) is also reported to be used as a marker of gastric emptying by some researchers[54,55]. *S. platensis* belongs to the family of algae used as a food product in distinct locations worldwide including the United States. It's a blend of protein, starch and lipids. Egg yolk is used as a test meal mixed with 13C-labeled *S. platensis*. After undergoing the process of emptying in the stomach and finally absorption, 13C enriched CO_2 is exhaled from the 13C-labeled *S. platensis*. This phenomenon is aimed at assessing the solid phase of gastric emptying.

Carbon BT to evaluate exocrine pancreatic insufficiency

Amylase, Lipase and secretin pancreozymin tests are the more reliable used in evaluation of patients with pancreatic disorders. The other testing modalities that can be included in practice are.

13C-triglyceride BT: This test utilizes the substrate using Lipase activity. The thoroughly investigated triglyceride BT is the "mixed" triglyceride test using 1,3-distearyl, 2-[13C-carboxyl] octanoyl glycerol; demonstrating a sensitivity and specificity of 89% and 81% respectively[56].

Literature review recommends 200-300 mg of the mixed triglycerides is the predominantly used doses for adults (15 mg/kg body weight for children). Before administration of test meal breath samples are collected, followed by successive sampling at 30 min intervals for 5 h[57].

Other carbon BTs for exocrine pancreatic insufficiency are

13C-starch BT and 13C egg protein BT which are based on Amylase and proteases activity respectively.

13C-BTs to evaluate hepatocellular function: 13C Aminopyrine BT for hepatocellular microsomal enzyme function: This test explores hepatic microsomal enzyme function. Literature review suggests a protocol that is based on the ingestion of 2 mg/kg aminopyrine with water. The recovery of the tracer after 60 or 120 min can be used as a diagnostic marker[58].

13C-methacetin BT for assessment of microsomal liver function: 13C-methacetin is a combination of a methyl group labeled with the non-radioactive isotope 13CN-demethylase, which is cytochrome P450 dependent enzyme responsible for de-methylation of 13C-Methacetin after oral absorption. The magnitude of appearance of 13C in breath analysis is correlated with the process of de-methylation. This test has been reported to be a reliable marker for the differentiation between early cirrhotic (Child A) and noncirrhotic patients but its performance for the detection of liver fibrosis remains questionable[59].

Carbon-breath tests to determine small intestine bacterial overgrowth

13C D-xylose was suggested as a marker of SIBO in the 1980s. Orally ingested D-xylose labeled with 13C, after modification by gut flora yields labeled CO_2 measured in the breath[35]. Reportedly D-xylose is a poor metabolic substrate for common coliform bacteria including *Escherichia coli, enterococci,* and *clostridia*, increasing the risk of false-negative results. Due to variation found in literature in sensitivities and specificities in comparison to hydrogen BT its use still remains controversial[34].

Glycocholate BT which is considered the forerunner of BTs for the evaluation of suspected SIBO used glycocholic acid labeled with 14C[60]. Owing to its inability to distinguish small bowel from colonic bacterial deconjugation and high risk of radiation hazard this test has been mostly out of practice[61].

13C-BT assessing carbohydrate malabsorption

Though the substrates required for 13C-BTs are costly compared to the hydrogen BT, but doses required are much less and the problem of non-hydrogen producers does not exist.

Specific intestinal enzyme activities can be tested using the appropriate 13C-substrate.

Lactase: 13C-lactose[62], saccharase: 13C-saccharose[62],

Table 3 Representative diagnostic accuracy in terms of sensitivity and specificity of various breath tests available for clinical use

Test	Indication	Sensitivity and specificity
Glucose hydrogen breath test	SIBO	62% and 83%[7]
Lactulose hydrogen breath test	SIBO	31% and 86%[5]
13C-glycocholate breath test	SIBO	76% and 33%[69]
13C-xylose breath test	SIBO	89% and 30%[69]
Fructose hydrogen breath test	Carbohydrate malabsorption	98% and 86%[65]
Lactose hydrogen breath test	Carbohydrate malabsorption	80% and 100%[66]
13C-lactose breath test	Carbohydrate malabsorption	84% and 96%[70]
13C-urea breath test	*H. pylori* infection	96% and 93%[39]
13C-aminopyrine breath test	Evaluation of liver function	85.7% and 67.5%[58]
13C-methacetin breath test	Evaluation of liver function	92.6% and 94.1%[67]
13C-phenacetin breath test	Evaluation of liver function	98% and 60%[68]
13C-mixed triglycerides breath test	Evaluation of exocrine pancreatic insufficiency	89% and 81%[56]
13C-octanoic acid breath test	Assessment of gastric emptying	67% and 80%[71]

SIBO: Small intestinal bacterial overgrowth; 13C: 13 carbon.

carbohydrate absorption: 13C-fructose[63].

LIMITATIONS OF BREATH TESTING

Though breath testing provides a near perfect alternative, it writhes from its own limitations. The lack of standardization of various analytical methodologies adopted by laboratories worldwide is a major issue reflected in literature search when it comes to breath testing. Similarly there is extensive variation in results and cut offs reported.

When it comes to the question of availability of resources, instruments for breath analysis are expensive and comprehensive operator training is an important requirement.

Even the most thoroughly proven 13C UBT has certain limitations. Especially in a resource limited areas, the availability of mass spectrometry based analyzers to evaluate the breath samples and computing a ratio of 12C to 13C are major hindrances as these instruments are quite expensive[64]. In most cases when 13C UBT is undertaken, samples are sent to a central processing laboratory for analysis which further adds to the test cost and increases the timeline for rapid delivery of results.

Mukhopadhyay[3] reported that lack of proven associations between the various elements detected in breath and disease the testing is aimed at. As in certain instances the analyte detected in breath is exhaled in response to various other metabolic processes rather than the disease for which the test is being conducted[3].

Various available BTs which are in use in clinical practice with their reported sensitivities and specificities are listed in (Table 3).

FUTURE DEVELOPMENTS

Contemporary bench top analyzers are being quickly replaced by POCT systems. One such development in the breath testing technology is the introduction of electronic nose (E-nose) test[4]. These instruments are designed on a highly specific sensor technology based on diverse micro sensor arrays. Each sensor is aimed at detecting a specific chemical exhaled in breath.

Principle

A specific pattern of response in recorded on to the sensor array when odor molecules from a breath sample are passed through. This signal yield is then sorted out utilizing the power of artificial neural networks to generate a specific output pattern, aimed at a particular diagnosis.

Though various researchers have studies the use of E-nose test and have suggested its role as a highly efficient diagnostic technique, further studies and validation studies concerning this entity are still required.

CONCLUSION

We conclude that BTs are useful modalities which are currently underutilized. With the advancement of new diagnostic tools especially desktop equipment for gas analysis, use of BTs is going to rise in near future.

What is the current knowledge?

The most frequently utilized BTs in GI disorders worldwide are UBT and Hydrogen BT. Breath testing remains underutilized due to the widespread belief that these test requires expensive instrumentation involving a complex analytic process and highly skilled operators. There is a perception that though these tests are less invasive, they possess radioactive hazards. In cases when samples are sent to reference labs for analysis it leads to elongation of the turnaround time of results.

What is new in this review?

13C carbon test are highlighted in this review, mostly based on mass spectrometry. Such compounds are safe because they are non-radioactive. Advent of Bench top analyzers and point of care testing will further pave the way for utilization of breath testing, leading to rapid production of results. Future development is aimed at development of specific sensor based hand

held analyzers like the E-nose tests. Thus to conclude these diagnostic modality can be effectively used as it is relatively safer and noninvasive compared to the contemporary tests in use and tests can be easily repeated if need arises.

REFERENCES

1 **Phillips M**, Herrera J, Krishnan S, Zain M, Greenberg J, Cataneo RN. Variation in volatile organic compounds in the breath of normal humans. *J Chromatogr B Biomed Sci Appl* 1999; **729**: 75-88 [PMID: 10410929 DOI: 10.1016/S0378-4347(99)00127-9]

2 **Schubert JK**, Miekisch W, Geiger K, Nöldge-Schomburg GF. Breath analysis in critically ill patients: potential and limitations. *Expert Rev Mol Diagn* 2004; **4**: 619-629 [PMID: 15347256]

3 **Mukhopadhyay R**. Don't waste your breath. Researchers are developing breath tests for diagnosing diseases, but how well do they work? *Anal Chem* 2004; **76**: 273A-276A [PMID: 15326722]

4 **Arasaradnam RP**, Covington JA, Harmston C, Nwokolo CU. Review article: next generation diagnostic modalities in gastroenterology--gas phase volatile compound biomarker detection. *Aliment Pharmacol Ther* 2014; **39**: 780-789 [PMID: 24612215 DOI: 10.1111/apt.12657]

5 **Ghoshal UC**. How to interpret hydrogen breath tests. *J Neurogastroenterol Motil* 2011; **17**: 312-317 [PMID: 21860825 DOI: 10.5056/jnm.2011.17.3.312]

6 **Braden B**. Methods and functions: Breath tests. *Best Pract Res Clin Gastroenterol* 2009; **23**: 337-352 [PMID: 19505663 DOI: 10.1016/j.bpg.2009.02.014]

7 **Corazza GR**, Menozzi MG, Strocchi A, Rasciti L, Vaira D, Lecchini R, Avanzini P, Chezzi C, Gasbarrini G. The diagnosis of small bowel bacterial overgrowth. Reliability of jejunal culture and inadequacy of breath hydrogen testing. *Gastroenterology* 1990; **98**: 302-309 [PMID: 2295385]

8 **Rhodes JM**, Middleton P, Jewell DP. The lactulose hydrogen breath test as a diagnostic test for small-bowel bacterial overgrowth. *Scand J Gastroenterol* 1979; **14**: 333-336 [PMID: 441681 DOI: 10.3109/00365527909179892]

9 **Kretchmer N**. Lactose and lactase--a historical perspective. *Gastroenterology* 1971; **61**: 805-813 [PMID: 4947662]

10 **Kretchmer N**. On the homology between human development and pediatrics. *Pediatr Res* 1968; **2**: 283-286 [PMID: 5669665 DOI: 10.1203/00006450-196807000-00007]

11 **Sahi T**. Genetics and epidemiology of adult-type hypolactasia. *Scand J Gastroenterol Suppl* 1994; **202**: 7-20 [PMID: 8042019 DOI: 10.3109/00365529409091740]

12 **Heyman MB**. Lactose intolerance in infants, children, and adolescents. *Pediatrics* 2006; **118**: 1279-1286 [PMID: 16951027 DOI: 10.1542/peds.2006-1721]

13 **Matthews SB**, Waud JP, Roberts AG, Campbell AK. Systemic lactose intolerance: a new perspective on an old problem. *Postgrad Med J* 2005; **81**: 167-173 [PMID: 15749792 DOI: 10.1136/pgmj.2004.025551]

14 **Beaugerie L**, Flourié B, Lémann M, Achour L, Franchisseur C, Rambaud JC. Sorbitol absorption in the healthy human small intestine is increased by the concomitant ingestion of glucose or lipids. *Eur J Gastroenterol Hepatol* 1995; **7**: 125-128 [PMID: 7712303]

15 **Jain NK**, Rosenberg DB, Ulahannan MJ, Glasser MJ, Pitchumoni CS. Sorbitol intolerance in adults. *Am J Gastroenterol* 1985; **80**: 678-681 [PMID: 4036946]

16 **Perman JA**, Barr RG, Watkins JB. Sucrose malabsorption in children: noninvasive diagnosis by interval breath hydrogen determination. *J Pediatr* 1978; **93**: 17-22 [PMID: 650340 DOI: 10.1016/S0022-3476(78)80592-7]

17 **Levitt MD**, Furne JK, Kuskowski M, Ruddy J. Stability of human methanogenic flora over 35 years and a review of insights obtained from breath methane measurements. *Clin Gastroenterol Hepatol* 2006; **4**: 123-129 [PMID: 16469670 DOI: 10.1016/j.cgh.2005.11.006]

18 **Waud JP**, Matthews SB, Campbell AK. Measurement of breath hydrogen and methane, together with lactase genotype, defines the current best practice for investigation of lactose sensitivity. *Ann Clin Biochem* 2008; **45**: 50-58 [PMID: 18275674 DOI: 10.1258/acb.2007.007147]

19 **McKay LF**, Eastwood MA, Brydon WG. Methane excretion in man--a study of breath, flatus, and faeces. *Gut* 1985; **26**: 69-74 [PMID: 3965369 DOI: 10.1136/gut.26.1.69]

20 **Peled Y**, Weinberg D, Hallak A, Gilat T. Factors affecting methane production in humans. Gastrointestinal diseases and alterations of colonic flora. *Dig Dis Sci* 1987; **32**: 267-271 [PMID: 3816480 DOI: 10.1007/BF01297052]

21 **Melcher EA**, Levitt MD, Slavin JL. Methane production and bowel function parameters in healthy subjects on low- and high-fiber diets. *Nutr Cancer* 1991; **16**: 85-92 [PMID: 1665560 DOI: 10.1080/01635589109514147]

22 **Weaver GA**, Krause JA, Miller TL, Wolin MJ. Incidence of methanogenic bacteria in a sigmoidoscopy population: an association of methanogenic bacteria and diverticulosis. *Gut* 1986; **27**: 698-704 [PMID: 3721294 DOI: 10.1136/gut.27.6.698]

23 **Fiedorek SC**, Pumphrey CL, Casteel HB. Breath methane production in children with constipation and encopresis. *J Pediatr Gastroenterol Nutr* 1990; **10**: 473-477 [PMID: 2162940 DOI: 10.1097/00005176-199005000-00010]

24 **Lin HC**, Pimentel M, Chen JH. Intestinal transit is slowed by luminal methane. *Neurogastroenterol Motil* 2002; **14**: 437

25 **Weiss RF**. Determinations of carbon dioxide and methane by dual catalyst flame ionization chromatography and nitrous oxide by electron capture chromatography. *J Chromatogr Sci* 1981; **19**: 611-616 [DOI: 10.1093/chromsci/19.12.611]

26 **Loftfield N**, Flessa H, Augustin J, Beese F. Automated gas chromatographic system for rapid analysis of the atmospheric trace gases methane, carbon dioxide, and nitrous oxide. *J Environ Qual* 1997; **26**: 560-564 [DOI: 10.2134/jeq1997.00472425002600020030x]

27 **Zimmermann S**, Krippner P, Vogel A, Muller Jr. Miniaturized flame ionization detector for gas chromatography. *Sensors and Actuators B: Chemical* 2002; **83**: 285-289 [DOI: 10.1016/S0925-4005(01)01060-7]

28 **Thammakhet C**, Thavarungkul P, Brukh R, Mitra S, Kanatharana P. Microtrap modulated flame ionization detector for on-line monitoring of methane. *J Chromatogr A* 2005; **1072**: 243-248 [PMID: 15887494 DOI: 10.1016/j.chroma.2005.03.041]

29 **Guilbot P**, Valtz A, Legendre H, Richon D. Rapid on-line sampler-injector: a reliable tool for HT-HP sampling and on-line GC analysis. *Analusis* 2000; **28**: 426-431 [DOI: 10.1051/analusis:2000128]

30 **Eijkel JCT**, Stoeri H, Manz A. A molecular emission detector on a chip employing a direct current microplasma. *Analytical Chemistry* 1999; **71**: 2600-2606 [DOI: 10.1021/ac990257j]

31 **Niemann HB**, Atreya SK, Bauer SJ, Carignan GR, Demick JE, Frost RL, Gautier D, Haberman JA, Harpold DN, Hunten DM, Israel G, Lunine JI, Kasprzak WT, Owen TC, Paulkovich M, Raulin F, Raaen E, Way SH. The abundances of constituents of Titan's atmosphere from the GCMS instrument on the Huygens probe. *Nature* 2005; **438**: 779-784 [PMID: 16319830 DOI: 10.1038/nature04122]

32 **Dryahina K**, Smith D, Spanel P. Quantification of methane in humid air and exhaled breath using selected ion flow tube mass spectrometry. *Rapid Commun Mass Spectrom* 2010; **24**: 1296-1304 [PMID: 20391601 DOI: 10.1002/rcm.4513]

33 **Stotzer PO**, Kilander AF. Comparison of the 1-gram (14)C-D-xylose breath test and the 50-gram hydrogen glucose breath test for diagnosis of small intestinal bacterial overgrowth. *Digestion* 2000; **61**: 165-171 [PMID: 10773721 DOI: 10.1159/000007753]

34 **Dellert SF**, Nowicki MJ, Farrell MK, Delente J, Heubi JE. The 13C-xylose breath test for the diagnosis of small bowel bacterial overgrowth in children. *J Pediatr Gastroenterol Nutr* 1997; **25**: 153-158 [PMID: 9252901 DOI: 10.1097/00005176-199708000-00005]

35 **King CE**, Toskes PP. Breath tests in the diagnosis of small intestine

bacterial overgrowth. *Crit Rev Clin Lab Sci* 1984; **21**: 269-281 [PMID: 6439469 DOI: 10.3109/10408368409165785]
36 **Braden B**, Haisch M, Duan LP, Lembcke B, Caspary WF, Hering P. Clinically feasible stable isotope technique at a reasonable price: analysis of 13CO2/12CO2-abundance in breath samples with a new isotope selective-nondispersive infrared spectrometer. *Z Gastroenterol* 1994; **32**: 675-678 [PMID: 7871857]
37 **Braden B**, Caspary WF, Lembcke B. Nondispersive infrared spectrometry for 13CO2/12CO2-measurements: a clinically feasible analyzer for stable isotope breath tests in gastroenterology. *Z Gastroenterol* 1999; **37**: 477-481 [PMID: 10427653]
38 **Koletzko S**, Haisch M, Seeboth I, Braden B, Hengels K, Koletzko B, Hering P. Isotope-selective non-dispersive infrared spectrometry for detection of Helicobacter pylori infection with 13C-urea breath test. *Lancet* 1995; **345**: 961-962 [PMID: 7715299 DOI: 10.1016/S0140-6736(95)90704-1]
39 **Ferwana M**, Abdulmajeed I, Alhajiahmed A, Madani W, Firwana B, Hasan R, Altayar O, Limburg PJ, Murad MH, Knawy B. Accuracy of urea breath test in Helicobacter pylori infection: meta-analysis. *World J Gastroenterol* 2015; **21**: 1305-1314 [PMID: 25632206 DOI: 10.3748/wjg.v21.i4.1305]
40 **Kato S**, Ozawa K, Konno M, Tajiri H, Yoshimura N, Shimizu T, Fujisawa T, Abukawa D, Minoura T, Iinuma K. Diagnostic accuracy of the 13C-urea breath test for childhood Helicobacter pylori infection: a multicenter Japanese study. *Am J Gastroenterol* 2002; **97**: 1668-1673 [PMID: 12135016 DOI: 10.1111/j.1572-0241.2002.05825.x]
41 **Frenck RW**, Fathy HM, Sherif M, Mohran Z, El Mohammedy H, Francis W, Rockabrand D, Mounir BI, Rozmajzl P, Frierson HF. Sensitivity and specificity of various tests for the diagnosis of Helicobacter pylori in Egyptian children. *Pediatrics* 2006; **118**: e1195-e1202 [PMID: 16982805 DOI: 10.1542/peds.2005-2925]
42 **Elitsur Y**, Tolia V, Gilger MA, Reeves-Garcia J, Schmidt-Sommerfeld E, Opekun AR, El-Zimaity H, Graham DY, Enmei K. Urea breath test in children: the United States prospective, multicenter study. *Helicobacter* 2009; **14**: 134-140 [PMID: 19298341 DOI: 10.1111/j.1523-5378.2009.00670.x]
43 **Gatta L**, Ricci C, Tampieri A, Osborn J, Perna F, Bernabucci V, Vaira D. Accuracy of breath tests using low doses of 13C-urea to diagnose Helicobacter pylori infection: a randomised controlled trial. *Gut* 2006; **55**: 457-462 [PMID: 16162678 DOI: 10.1136/gut.2005.078626]
44 **Israeli E**, Ilan Y, Meir SB, Buenavida C, Goldin E. A novel 13C-urea breath test device for the diagnosis of Helicobacter pylori infection: continuous online measurements allow for faster test results with high accuracy. *J Clin Gastroenterol* 2003; **37**: 139-141 [PMID: 12869884 DOI: 10.1097/00004836-200308000-00009]
45 **Chen TS**, Chang FY, Chen PC, Huang TW, Ou JT, Tsai MH, Wu MS, Lin JT. Simplified 13C-urea breath test with a new infrared spectrometer for diagnosis of Helicobacter pylori infection. *J Gastroenterol Hepatol* 2003; **18**: 1237-1243 [PMID: 14535979 DOI: 10.1046/j.1440-1746.2003.03139.x]
46 **Campuzano-Maya G**. An optimized 13C-urea breath test for the diagnosis of H pylori infection. *World J Gastroenterol* 2007; **13**: 5454-5464 [PMID: 17907288 DOI: 10.3748/wjg.v13.i41.5454]
47 **Chey WD**, Wong BC. American College of Gastroenterology guideline on the management of Helicobacter pylori infection. *Am J Gastroenterol* 2007; **102**: 1808-1825 [PMID: 17608775 DOI: 10.1111/j.1572-0241.2007.01393.x]
48 **Gunnarsson M**, Leide-Svegborn S, Stenström K, Skog G, Nilsson LE, Hellborg R, Mattsson S. No radiation protection reasons for restrictions on 14C urea breath tests in children. *Br J Radiol* 2002; **75**: 982-986 [PMID: 12515707 DOI: 10.1259/bjr.75.900.750982]
49 **Hunt R**, Fallone C, Veldhuyzan van Zanten S, Sherman P, Smaill F, Flook N, Thomson A. Canadian Helicobacter Study Group Consensus Conference: Update on the management of Helicobacter pylori--an evidence-based evaluation of six topics relevant to clinical outcomes in patients evaluated for H pylori infection. *Can J Gastroenterol* 2004; **18**: 547-554 [PMID: 15457293 DOI: 10.1155/2004/326767]
50 **Uchida M**, Shimizu K. 13C-acetic acid is more sensitive than 13C-octanoic acid for evaluating gastric emptying of liquid enteral nutrient formula by breath test in conscious rats. *Biol Pharm Bull* 2007; **30**: 487-489 [PMID: 17329843 DOI: 10.1248/bpb.30.487]
51 **Choi MG**, Camilleri M, Burton DD, Zinsmeister AR, Forstrom LA, Nair KS. [13C]octanoic acid breath test for gastric emptying of solids: accuracy, reproducibility, and comparison with scintigraphy. *Gastroenterology* 1997; **112**: 1155-1162 [PMID: 9097998 DOI: 10.1016/S0016-5085(97)70126-4]
52 **Perri F**, Pastore MR, Annese V. 13C-octanoic acid breath test for measuring gastric emptying of solids. *Eur Rev Med Pharmacol Sci* 2005; **9**: 3-8 [PMID: 16457123]
53 **Haans JJL**, Paridaans NPM, Wong C, Eilers PHC, Doornbos J, de Roos A, Masclee AAM. Magnetic Resonance Imaging for Evaluation of Gastric Motor Function. Chapter 7: Comparison of gastric emptying determined by stable isotope breath test and Magnetic Resonance Imaging during simultaneous recording. Evaluation of Gastric Motor Function 8. Available from: URL: http://digitalarchive.maastrichtuniversity.nl/fedora/get/guid: a32b0e97-332d-41f3-8695-f3a6912ef077/ASSET1
54 **Szarka LA**, Camilleri M, Vella A, Burton D, Baxter K, Simonson J, Zinsmeister AR. A stable isotope breath test with a standard meal for abnormal gastric emptying of solids in the clinic and in research. *Clin Gastroenterol Hepatol* 2008; **6**: 635-643.e1 [PMID: 18406670 DOI: 10.1016/j.cgh.2008.01.009]
55 **Lee JS**, Camilleri M, Zinsmeister AR, Burton DD, Kost LJ, Klein PD. A valid, accurate, office based non-radioactive test for gastric emptying of solids. *Gut* 2000; **46**: 768-773 [PMID: 10807886 DOI: 10.1136/gut.46.6.768]
56 **Kalivianakis M**, Verkade HJ, Stellaard F, van der Were M, Elzinga H, Vonk RJ. The 13C-mixed triglyceride breath test in healthy adults: determinants of the 13CO2 response. *Eur J Clin Invest* 1997; **27**: 434-442 [PMID: 9179552 DOI: 10.1046/j.1365-2362.1997.1310678.x]
57 **Löser C**, Brauer C, Aygen S, Hennemann O, Fölsch UR. Comparative clinical evaluation of the 13C-mixed triglyceride breath test as an indirect pancreatic function test. *Scand J Gastroenterol* 1998; **33**: 327-334 [PMID: 9548629 DOI: 10.1080/00365529850170946]
58 **Giannini EG**, Fasoli A, Borro P, Botta F, Malfatti F, Fumagalli A, Testa E, Polegato S, Cotellessa T, Milazzo S, Risso D, Testa R. 13C-galactose breath test and 13C-aminopyrine breath test for the study of liver function in chronic liver disease. *Clin Gastroenterol Hepatol* 2005; **3**: 279-285 [PMID: 15765448 DOI: 10.1016/S1542-3565(04)00720-7]
59 **Braden B**, Faust D, Sarrazin U, Zeuzem S, Dietrich CF, Caspary WF, Sarrazin C. 13C-methacetin breath test as liver function test in patients with chronic hepatitis C virus infection. *Aliment Pharmacol Ther* 2005; **21**: 179-185 [PMID: 15679768 DOI: 10.1111/j.1365-2036.2005.02317.x]
60 **Hofmann AF**, Fromm H. New breath test for bile acid deconjugation. *N Engl J Med* 1971; **285**: 686-687 [PMID: 5563482 DOI: 10.1056/NEJM197109162851211]
61 **Gunnarsson M**, Leide-Svegborn S, Stenström K, Skog G, Nilsson LE, Thorsson O, Hellborg R, Mattsson S. Long-term biokinetics and radiation exposure of patients undergoing 14C-glycocholic acid and 14C-xylose breath tests. *Cancer Biother Radiopharm* 2007; **22**: 762-771 [PMID: 18158767 DOI: 10.1089/cbr.2007.0350]
62 **Hiele M**, Ghoos Y, Rutgeerts P, Vantrappen G. Measurement of the rate of assimilation of oligo- and polysaccharides by 13CO2 breath tests and isotope ratio mass spectrometry. *Biomed Environ Mass Spectrom* 1988; **16**: 133-135 [PMID: 3149533]
63 **Hoekstra JH**, van den Aker JH, Kneepkens CM, Stellaard F, Geypens B, Ghoos YF. Evaluation of 13CO2 breath tests for the detection of fructose malabsorption. *J Lab Clin Med* 1996; **127**: 303-309 [PMID: 9273364 DOI: 10.1016/S0022-2143(96)90099-2]
64 **Romagnuolo J**, Schiller D, Bailey RJ. Using breath tests wisely in a gastroenterology practice: an evidence-based review of indications and pitfalls in interpretation. *Am J Gastroenterol* 2002; **97**: 1113-1126 [PMID: 12014715 DOI: 10.1111/j.1572-0241.2002.05664.x]
65 **Götze H**, Mahdi A. [Fructose malabsorption and dysfunctional

gastrointestinal manifestations]. *Monatsschr Kinderheilkd* 1992; **140**: 814-817 [PMID: 1470188]

66 **Rosado JL**, Solomons NW. Sensitivity and specificity of the hydrogen breath-analysis test for detecting malabsorption of physiological doses of lactose. *Clin Chem* 1983; **29**: 545-548 [PMID: 6687450]

67 **Schneider A**, Caspary WF, Saich R, Dietrich CF, Sarrazin C, Kuker W, Braden B. 13C-methacetin breath test shortened: 2-point-measurements after 15 minutes reliably indicate the presence of liver cirrhosis. *J Clin Gastroenterol* 2007; **41**: 33-37 [PMID: 17198062 DOI: 10.1097/MCG.0b013e31802dd4b9]

68 **Lara Baruque S**, Razquin M, Jimenez I, Vazquez A, Gisbert JP, Pajares JM. 13C-phenylalanine and 13C-methacetin breath test to evaluate functional capacity of hepatocyte in chronic liver disease. *Dig Liver Dis* 2000; **32**: 226-232 [PMID: 10975773 DOI: 10.1016/S1590-8658(00)80825-7]

69 **Donald IP**, Kitchingmam G, Donald F, Kupfer RM. The diagnosis of small bowel bacterial overgrowth in elderly patients. *J Am Geriatr Soc* 1992; **40**: 692-696 [PMID: 1607585 DOI: 10.1111/j.1532-5415.1992.tb01961.x]

70 **Wetzel K**, Fischer H. 13C-breath tests in medical research and clinical diagnosis. Leipzig: Fischer Analysen Instrumente GmbH, 2005

71 **Delbende B**, Perri F, Couturier O, Leodolter A, Mauger P, Bridgi B, Bizais Y, des Varannes SB, Andriulli A, Galmiche JP. 13C-octanoic acid breath test for gastric emptying measurement. *Eur J Gastroenterol Hepatol* 2000; **12**: 85-91 [PMID: 10656216 DOI: 10.1097/00042737-200012010-00016]

P- Reviewer: Bernhardt GA, Koch TR, Quigley EMM, Rosenthal P
S- Editor: Ji FF **L- Editor**: A **E- Editor**: Lu YJ

World Journal of Gastrointestinal Pathophysiology

Submit a Manuscript: http://www.wjgnet.com/esps/
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.4291/wjgp.v7.i3.266

World J Gastrointest Pathophysiol 2016 August 15; 7(3): 266-275
ISSN 2150-5330 (online)

Novel biomarkers of fibrosis in Crohn's disease

Gianluca Pellino, Pierlorenzo Pallante, Francesco Selvaggi

Gianluca Pellino, Francesco Selvaggi, Unit of Colorectal Surgery, Department of Medical, Surgical, Neurological, Metabolic and Ageing Sciences, Second University of Naples, 80138 Naples, Italy

Pierlorenzo Pallante, Institute of Experimental Endocrinology and Oncology, National Research Council, c/o Department of Molecular Medicine and Medical Biotechnology, University of Naples "Federico II", 80131 Naples, Italy

Author contributions: Pellino G and Pallante P performed research and wrote the paper; Selvaggi F contributed critical revision of the manuscript for important intellectual content.

Conflict-of-interest statement: The authors have no conflict of interest to disclose for this manuscript.

Manuscript source: Invited manuscript

Correspondence to: Francesco Selvaggi, MD, EBSQ (Colo.), Associate Professor of General Surgery, Unit of Colorectal Surgery, Department of Medical, Surgical, Neurological, Metabolic and Ageing Sciences, Second University of Naples, Piazza Miraglia 2, 80138 Naples, Italy. fselvaggi@hotmail.com
Telephone: +39-81-5667919
Fax: +39-81-5667919

Received: April 21, 2016
Peer-review started: April 22, 2016
First decision: June 6, 2016
Revised: June 27, 2016
Accepted: July 20, 2016
Article in press: July 22, 2016
Published online: August 15, 2016

Abstract

Fibrosis represents a major challenge in Crohn's disease (CD), and many CD patients will develop fibrotic strictures requiring treatment throughout their lifetime. There is no drug that can reverse intestinal fibrosis, and so endoscopic balloon dilatation and surgery are the only effective treatments. Since patients may need repeated treatments, it is important to obtain the diagnosis at an early stage before strictures become symptomatic with extensive fibrosis. Several markers of fibrosis have been proposed, but most need further validation. Biomarkers can be measured either in biological samples obtained from the serum or bowel of CD patients, or using imaging tools and tests. The ideal tool should be easily obtained, cost-effective, and reliable. Even more challenging is fibrosis occurring in ulcerative colitis. Despite the important burden of intestinal fibrosis, including its detrimental effect on outcomes and quality of life in CD patients, it has received less attention than fibrosis occurring in other organs. A common mechanism that acts *via* a specific signaling pathway could underlie both intestinal fibrosis and cancer. A comprehensive overview of recently introduced biomarkers of fibrosis in CD is presented, along with a discussion of the controversial areas remaining in this field.

Key words: Crohn's disease; Fibrosis; Inflammatory bowel diseases; Diagnosis; Biomarkers

Core tip: Fibrosis occurs in a disturbingly large proportion of patients suffering from Crohn's disease (CD), and invasive procedures may be required for both its diagnosis and treatment. Several biomarkers of intestinal fibrosis have recently been proposed. Most of them still need to be validated, but they could be useful for obtaining an early diagnosis of fibrosis, thereby allowing timely treatment and delaying or even avoiding surgery. A comprehensive overview of recently introduced bio-

markers of fibrosis in CD is presented, along with a discussion of the controversial areas remaining in this field.

Pellino G, Pallante P, Selvaggi F. Novel biomarkers of fibrosis in Crohn's disease. *World J Gastrointest Pathophysiol* 2016; 7(3): 266-275 Available from: URL: http://www.wjgnet.com/2150-5330/full/v7/i3/266.htm DOI: http://dx.doi.org/10.4291/wjgp.v7.i3.266

INTRODUCTION

Fibrosis represents a major challenge in Crohn's disease (CD). Approximately 50% of patients suffering from CD will develop penetrating or fibrotic strictures, and up to 75% of them will eventually need surgery[1-4]. However, fibrosis occurring in the bowel in inflammatory bowel diseases (IBD) is a problem that has been largely neglected by the scientific community, particularly compared with fibrosis occurring in other organs, such as the liver, lung, kidney, and heart[5]. Insufficient resources are allocated for research in intestinal fibrosis, and there is currently no available medical treatment for preventing or reversing fibrosis. All current efforts are focused on improving the ability to obtain an early diagnosis and apply timely treatment, ideally with the aid of noninvasive biomarkers of fibrosis (Table 1, Figure 1).

CHALLENGES IN INTESTINAL FIBROSIS

Several problems should be considered when investigating biomarkers of intestinal fibrosis in CD[1]. No validated quantitative or qualitative scores are currently available for diagnosing the presence of fibrosis and its extent. There is also no agreement on how to perform biopsies in strictured bowel segments, and the number and depth of samples have varied among the published studies. Furthermore, no standard anatomopathological scoring system has been developed, which increases the difficulties of data interpretation. Lastly, no medical treatment is currently able to reverse intestinal fibrosis once it has occurred.

NONINVASIVE BIOMARKERS OF FIBROSIS

Genetic markers of fibrosis

The pathogenesis of CD is complex, involving interactions between host-predisposing factors and environmental agents. Genetic factors controlling the immune system and the intestinal microbiome are likely to be involved, given that several genetic polymorphisms and variations have been associated with an increased susceptibility to IBD. However, genetic variations are present in fewer than one-quarter of CD patients[6]. Rather than considering chromosomes and genes themselves, it might be better to investigate the mechanisms that control their expression in order to understand and potentially modulate the pathways leading to fibrosis in CD.

There is accumulating evidence that circulating single-stranded, noncoding RNA molecules (microRNA) modulate adaptive immune responses[7]. This is potentially highly significant since it may make it possible to diagnose those patients who are more likely to develop fibrotic strictures at an earlier stage, or to monitor the response to treatment.

NOD2/CARD15 gene polymorphisms are the most widely investigated in intestinal fibrogenesis. They have been associated with a higher risk of developing stricturing CD[8,9], and their expression could be influenced by race[10]. The underlying mechanism could be impairment of barrier function by such genetic mutations[11]. It has been suggested that more than half of the patients carrying an *NOD2/CARD15* mutation will develop stricturing CD, with the findings being similar for patients from Europe[9] and North America[12]. One large study investigated the presence of the SNP13 polymorphism of *NOD2/CARD15* in patients with ulcerative colitis (UC) and CD, and found that homozygosis was only observed in the latter[12]. Most studies have suggested that the risk of developing strictures increases with the number of mutations[13]. Although clinical decision-making in this field is almost completely unexplored, *NOD2* mutations have been associated with a greater need for the resection of strictures and with surgical recurrence[14]. The detection of genetic biomarkers in asymptomatic patients may therefore lead to changes in the management of such patients.

Other genetic and epigenetic factors may also play a role in intestinal fibrogenesis, and they have recently been investigated thoroughly. Genes controlling the expression of several cytokines - particularly interleukin (IL)-10[15] and IL-23[8] - have been associated with an increased risk of bowel stricture, but the evidence is conflicting, and so relying on these genes cannot be recommended for routine clinical practice[13]. Other molecules that are involved in maintaining the homeostasis between profibrotic and antifibrotic mechanisms have been proposed as candidates for diagnosing fibrotic CD at an early stage [*e.g.*, transforming growth factor (TGF) and metalloproteinase][1,5,16], but their possible role as biomarkers needs to be further elucidated.

Obtaining more reliable findings requires prospective, collaborative studies involving several centers across multiple countries aimed at identifying genetic factors underlying fibrosis. Such an approach would lower the costs for each participating unit, make the results more consistent, facilitate the inclusion of a large patient sample, and allow the application of genome-wide analysis to a wide spectrum of genes simultaneously. Moreover, population-based cohorts would be easier to establish and would allow comparison with non-CD individuals.

Table 1 Overview of fibrosis biomarkers and relative references

Biomarkers	Alteration/finding	Ref.
Genetic markers		
NOD2/CARD15	Polymorphisms	Barrett *et al*[8], 2008, Lesage *et al*[9] 2002, Yamazaki *et al*[10] 2002, Buhner *et al*[11] 2006, Abreu *et al*[12] 2002, Jürgens *et al*[13] 2010, Alvarez-Lobos *et al*[14] 2005
Epigenetic markers		
MD-2	Demethylation	Vamadevan *et al*[39] 2010
IFN-γ	Methylation	Gonsky *et al*[40] 2011
TH1	????	Gonsky *et al*[40] 2011
miR-200b	Increase	Chen *et al*[41] 2012
miR-29a	Decrease	Nijhuis *et al*[42] 2014
Serological markers		
ASCA	High concentration	Vasiliauskas *et al*[17] 2000, Forcione *et al*[18] 2004, Mow *et al*[20] 2004, Ferrante *et al*[21] 2007, Rieder *et al*[22] 2010, Seow *et al*[23] 2009, Arnott *et al*[25] 2004, Papp *et al*[26] 2008, Simondi *et al*[24] 2008
Anti-OmpC	High concentration	Mow *et al*[20] 2004, Arnott *et al*[25] 2004, Ferrante *et al*[21] 2007, Papp *et al*[26] 2008
Anti-I2	High concentration	Mow *et al*[20] 2004, Arnott *et al*[25] 2004
Anti-CBir1	High concentration	Targan *et al*[19] 2005
ALCA	High concentration	Ferrante *et al*[21] 2007, Rieder *et al*[22] 2010, Seow *et al*[23] 2009, Papp *et al*[26] 2008, Simondi *et al*[24] 2008
AMCA	High concentration	Ferrante *et al*[21] 2007, Rieder *et al*[22] 2010, Seow *et al*[23] 2009, Papp *et al*[26] 2008
ACCA	High concentration	Ferrante *et al*[21] 2007, Seow *et al*[23] 2009, Papp *et al*[26] 2008
Anti-C	High concentration	Rieder *et al*[22] 2010, Seow *et al*[23] 2009
Anti-L	High concentration	Rieder *et al*[22] 2010, Seow *et al*[23] 2009
CRP	Increase	Henriksen *et al*[29] 2008
Laminin	Increase	Koutroubakis *et al*[30] 2003a
Collagen Ⅳ	Decrease	Koutroubakis *et al*[30] 2003a
Fibronectin	Decrease	Allan *et al*[28] 1989
YKL-40	Increase	Koutroubakis *et al*[33] 2003b
bFGF	Increase	Di Sabatino *et al*[34] 2004
Radiological markers		
MRI	Enhancement patterns	Rimola *et al*[46] 2015
PET-MRI	Quantitative-qualitative analysis	Catalano *et al*[53] 2016, Pellino *et al*[49] 2016

ASCA: Anti-*Saccaromyces cerevisiae* antibody; anti-OmpC: Anti-*Escherichia coli* outer membrane porine C antibody; Anti-I2: Anti-*Pseudomonas fluorescens* associated sequence I2 antibody; Anti-CBir1: Anti-bacterial flagellin antibody; ALCA: Anti-laminaribioside carbohydrate antibody; AMCA: Anti-mannobioside carbohydrate antibody; ACCA: Anti-chitobioside carbohydrate antibody; Anti-C: Anti-chitin antibody; Anti-L: Anti-laminarin antibody; CRP: C-reactive protein; YKL-40: Chitinase-like glycoprotein; bFGF: Basic fibroblast growth factor; MRI: Magnetic resonance imaging; PET: Positron emission tomography.

Serological markers of fibrosis

Probably one of the first and best-characterized class of serological biomarkers of fibrosis in CD pathology is antibody molecules directed against microbial proteic products of the intestinal tract. These mainly comprise the following antibodies: Anti-*Saccaromyces-cerevisiae* (ASCA), anti-*Escherichia-coli* outer membrane porine C (anti-OmpC), anti-*Pseudomonas*-associated sequence-I2 antibodies (anti-I2), antibacterial flagellin antibodies, antilaminaribioside carbohydrate antibodies (ALCA), antimannobioside carbohydrate antibodies, antichitobioside carbohydrate antibodies, antichitin antibody, and antilaminarin antibody. In detail, high levels of ASCA have been found to be associated with fibrostenosis and penetrating disease and, more generally, with a greater need for surgery within 3 years from diagnosis compared with ASCA-negative patients[17-20].

On the other hand, with the exception of ALCA, glycan markers have been associated with complicated CD manifestations (fistulae and strictures) and surgery[21-24]. However, none of these markers has been specifically associated with fibrostenosis or penetrating disease[21-24]. In addition, it has been inferred that the intensity of immune responses influences the CD manifestations and need for surgery[19-22]. However, despite high levels of these markers being found in serum, none of them is currently recommended for use in following the course of the disease[19,20,22].

The antibodies directed against products of *Escherichia coli* (anti-OmpC and anti-flagellin) and *Pseudomonas fluorescens* (anti-I2) have been also successfully associated with the presence of fibrosis in CD[19-21,25,26]. It has been also reported that positivity for these three markers is associated with a higher frequency of fibrostenosis or penetrating disease[27].

Other markers have been also described, but diagnostically they are less useful than gut microbial markers. C-reactive protein (CRP) and proteins of the extracellular matrix (ECM; fibronectin) have been associated with fibrostenosis[28-30]. In particular, a prospective study found that the CRP level at diagnosis is closely associated with the subsequent risk of surgery in patients with CD[29].

The ECM plays a key role in disease processes, which has led to fibrinogenesis products related to its accumulation being considered as possible markers.

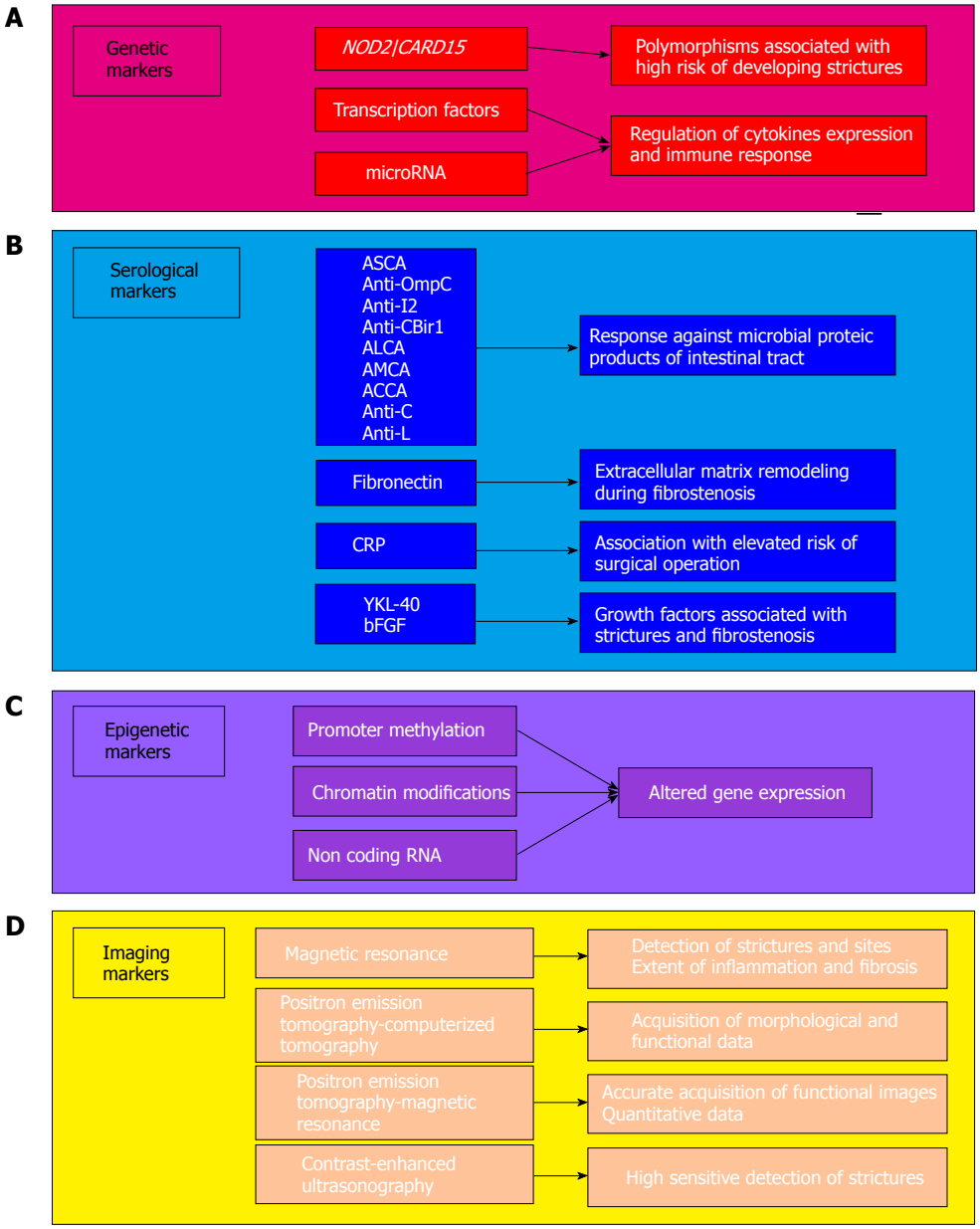


Figure 1 Biomarkers for assessment of fibrosis in Crohn's disease. The assessment of fibrosis currently relies on several biomarkers. Genetic markers (A) such as polymorphisms of the *NOD2*/*CARD15* gene and transcription factors/microRNAs are currently associated with the presence of strictures and altered immune response. Serological markers (B) are evaluated to assess the presence of fibrosis in a minimally invasive fashion. Epigenetic markers (C) are useful to understand the mechanism underlying development of fibrosis. Markers evaluated through sophisticated imaging technologies (D) allow to acquire and elaborate precise course of the disease.

While collagen types Ⅰ and Ⅲ are important in the intestinal fibrinogenesis process, the serum levels of direct procollagen precursors were not elevated in CD patients[1,5,16,31,32]. One study of the basement membrane found that while levels of laminin were increased, levels of collagen Ⅳ were decreased in CD patients relative to controls, and there was no association with fibrostenosis[30]. Lastly, like collagen Ⅳ, the levels of fibronectin were decreased in CD patients relative to controls, but higher levels of fibronectin were associated with the presence of stricture formations in the intestine[28].

Another class of markers is growth factor molecules, the best representatives of which are chitinase-like glycoprotein (YKL-40) and basic fibroblast growth factor (bFGF). YKL-40 has been associated with CD patients affected by strictures[33], and bFGF serum levels are strictly associated with a fibrostenosis phenotype[34].

Epigenetic markers of fibrosis

Biological information is encoded in the genetic pool of each individual, but gene expression can be altered by the environment, making variations inheritable *via* a mechanism called epigenetics[35]. These epigenetic variations that culminate in the modulation of gene expression are mainly due to a tightly regulated and complex mechanism based on the methylation of DNA,

modification of chromatin, and regulation on noncoding RNA, with the last mechanism mainly involving microRNAs. This complex mechanism is also observed in IBD, but its functioning relative to inflammation is not well understood. Nevertheless, it is known that it generally relies on the suppression of gene transcription through methylation of the promoter region. Epigenetic alteration during inflammation could be directly reflected in the activation, tolerance regulation, and regulation of T-cells[36].

Methylation of the promoter DNA and modification of histone proteins represent the main alterations observed in IBD. Several types of DNA methylation typical of intestinal disease that are observed in IBD are likely to be responsible for the altered expression of crucial genes that, in turn, induce the onset and progression of IBD. In this view, an altered methylation profile could be associated with CD. It has also been reported that at least seven CpG islands are differentially methylated in IBD patients[37] and that the pattern of DNA methylation affecting the IL-12 and IL-23 pathways in IBD is subtype-specific[38].

However, little is known about the mechanism through which methylation alters the gene expression and progression of IBD. It has been reported that demethylation of the MD-2 promoter is sufficient to induce the expression of this gene, which is crucial for the Toll-like receptor 4/MD-2 complex[39]. Additionally, it has been observed that methylation alters the levels of interferon-γ in IBD patients, resulting in them being correlated with immune responses[40]. Moreover, the expression profiles of cytokine TH1 have been associated with the epigenome setup of IBD patients[40]. It is therefore clear that genetic factors are involved in the etiology of IBD.

There is compelling evidence for the involvement of microRNAs in the regulation of immune responses in autoimmune pathologies, including CD[7]. This single-stranded noncoding class of RNA is able - *via* base complementarity - to regulate mRNA translation and stability. In particular, microRNAs belonging to the miR-200 family have been associated with different pathologies *via* their ability to modulate the key genes involved in the epithelial-to-mesenchymal transition (EMT).

More-promising candidate biomarkers for the pathology of fibrosis are currently represented by the class of noncoding molecules of circulating microRNAs. However, few studies have exploited microRNAs as fibrosis biomarkers. One study found that miR-200b levels were higher in CD patients with fibrosis than in their counterparts without fibrosis, and that TGF-β1 was able to induce the expression of miR-200b[41]. On the other hand, another study found that miR-29a was down-regulated in the serum of CD patients with stricture formations[42]. These two studies are valuable since they demonstrate that some microRNAs can be used to precisely discriminate between inflammatory and fibrotic disease, and thereby aid decisions about the use of therapy or surgery. It would therefore be very useful to find an accurate microRNA biomarker for monitoring the outcome during the course of therapy. Five microRNAs were found to be up-regulated in CD patients after 6 wk of treatment with infliximab, and two of them (let-7d and let-7e) were particularly elevated in patients exhibiting complete clinical remission[43]. These findings clearly suggest the usefulness of microRNA monitoring as a biomarker of the response to therapy in CD patients with intestinal fibrosis.

Because of the complexity of the disease and the continuous characterization of novel biomarkers, it is necessary to create structured collaboration networks for collecting and cataloging the findings of biopsies (including liquid biopsies), and to allocate appropriate resources for translational research. The results obtained on the laboratory bench could rapidly be applied to patients in hospital beds. Networks of this type have already been realized, and new ones focusing on colorectal cancer are being implemented.

Imaging markers of fibrosis

Almost all IBD patients need cross-sectional imaging for guiding their clinical management. Magnetic resonance imaging (MRI) is currently considered the ideal tool for identifying fibrosis in CD[44-47]. Studies have found significant variations in the sensitivity and specificity of MRI in detecting strictures. The clinical relevance of detecting stricture is intuitive, but it might be even more important for (1) detecting pathological sites where the stricture of the lumen is too minor to be detected using current MRI technologies; and (2) assessing the extent of inflammation and fibrosis at each detected stricture.

Since there is currently no treatment method for reversing fibrosis once it has settled in the bowel wall in humans, it would be useful to be able to detect asymptomatic sites. This would allow the physician to start medical treatment or modulate an existing treatment based on other indexes of CD activity and clinical parameters.

Inflammation and fibrosis represent two sides of the same coin in CD, and most strictures show both features but to different extents[1,47]. A certain degree of inflammation can be observed even in strictures with an extensive fibrotic component, and *vice versa*[1]. It is consequently more likely that strictures are predominantly fibrotic or inflammatory, rather than showing features of only inflammation or fibrosis. The latter can occur in CD, but this is extremely rare in practice. This observation has significant clinical implications, because it is now well known that strictures with active inflammation - irrespective of a fibrotic component - can be effectively managed with anti-inflammatory medications[1,48,49]. Knowing the exact proportions of these two components could delay or even avoid surgery in selected patients. At the same time this could identify those patients who are very likely to not respond to medical treatment because they have no active inflammation, and should instead be treated immediately with surgery to avoid unnecessary exposure to drugs.

The focus of recent studies has moved toward quantifying fibrosis in CD using imaging, with MRI

showing promise[46]. Such quantification requires both morphological and functional data to be obtained during cross-sectional imaging, which means combining conventional cross-sectional imaging with examinations that are able to detect sites of active inflammation. The most-used cross-sectional hybrid test is the ^{18}fluoro-deoxiglucose positron-emission tomography (PET) combined with computed tomography (CT). The method can provide both functional images, provided by PET, and anatomic images, provided by CT. Studies have shown that PET-CT offers advantages over PET alone in CD[50,51]. However, CT involves exposure to ionizing radiation, and most of the information provided by PET-CT can be obtained with current MRI scanning technologies in experienced hands.

PET-MRI is a new hybrid tool that was recently tested in patients with cancers[52,53]. It was shown that a machine that can perform PET and MRI scans simultaneously changed the management of cancer patients[52]. This has led to PET-MRI being used to assess patients with CD. PET-MRI is superior to both MRI alone and PET-CT because it provides functional images that are not available with conventional MRI, and the quality of images is significantly higher for PET-MRI than for PET-CT[49]. PET-MRI is more accurate than PET-CT at detecting extraluminal disease, and may be used to identify patients who are more likely to need fecal diversion during surgery. Moreover, PET-MRI can more reliably identify distant CD sites, hence aiding the selection of patients in whom surgery should either start open or with hand-assisted laparoscopy, reducing the intraoperative time that would be associated with starting with minimally invasive surgery that would eventually need to be converted to open surgery[49]. PET-MRI has been reported to detect fibrosis more accurately than both PET-CT and MRI alone. The use of PET-MRI to select patients suitable for a trial with rescue medical treatment before surgery found that over 70% of them did not require surgery[49]. Even more importantly, PET-MRI can produce quantitative data[53]. A direct correlation has been observed between PET standard uptake values and the degree of inflammation, by testing simultaneously each stricture detected with PET-MRI. Furthermore, some variables can be used to grade the extent of fibrosis quantitatively, ultimately resulting in a more reliable and reproducible way to diagnose CD patients[53]. This results in better patient management, based on agreed criteria, thereby also reducing interobserver and intraobserver variability.

Questions could be raised about the safety of PET-MRI, due to it involving exposure to radioactive nuclides, especially in young patients. However, a PET-MRI examination can be effective even at low radiation doses, and MRI alone can involve exposing patients to higher radiation levels during the reconstruction phase[54].

Shortcomings of PET-MRI are the high costs of individual tests, the requirement for a hybrid machine, and the long acquisition times, which make this technique unsuitable for patients needing immediate treatment and those who cannot tolerate long examinations[49,51,52].

A novel tool that is now being used frequently is contrast-enhanced ultrasonography (US), which has been associated with good sensitivity in detecting strictures[55,56]. However, concerns exist about the ability of US to discriminate between inflammation and fibrosis, and the implementation of a US scan with elastography has not yet been validated. Moreover, the physical shape of a patient can significantly influence the efficacy of US, which may be relevant given that many CD patients show mesenteric obesity (often drug-related), and the capabilities of US are affected by the operator's ability.

Fibrosis in UC

While fibrotic strictures are key features of CD, recent studies have suggested that UC patients can also develop fibrosis. This occurs in the large bowel and raises the concern of malignancy, justifying a surgical approach. In addition to concerns about cancer, thickening and increased stiffness of the large bowel cause several types of dysfunction since they affect intestinal motility[57,58].

Fibrosis in UC was neglected until very recently[57,58], and investigating it further would improve our understanding of the mechanisms underlying fibrosis development. The fibrosis that occurs in UC is particularly intriguing since it might occur without mucosal inflammation[58]. It is known that fibrosis results from chronic inflammation, but cases with a fibrostenosis pattern of CD are often observed. This could reflect a link between CD and UC fibrogenesis. Serological markers of fibrosis in the diagnosis of these patients could also be applied to medical treatments, assessing responses, and the following up of these patients. As an example, mucosal healing is currently used to assess the effectiveness of treatment in IBD, but this pathway might not be involved in patients who progress to fibrosis without significant mucosal involvement.

In addition, investigating UC-related fibrosis could highlight possible differences among CD and UC patients who develop fibrosis and facilitate the identification of biomarkers of fibrosis specific to each of these two entities. In UC patients it could be important to look beyond the mucosal surface[58], and this represents a further challenge in IBD patients that researchers involved in intestinal fibrosis development should take into account.

Wnt signaling and fibrosis: Any link with bowel cancer in CD?

The aberrant activation of the Wnt pathway has recently been associated with the pathogenesis of IBD[59]. It has also been reported that several molecules involved in Wnt signaling are down-regulated in CD tissues: Wnt2, Wnt5a, Wnt5b, Fzd2, Fzd4, Fzd6, LRP6, Dvl, and SFRP1[60,61]. In addition, it has been reported that attenuation of the Wnt pathway by alteration of TCF4 and LRP6 is directly responsible for the diminished production of alpha-defensins by Paneth cells[62] and the dysfunction of the barrier[63]. This represents a hallmark of CD, and hence

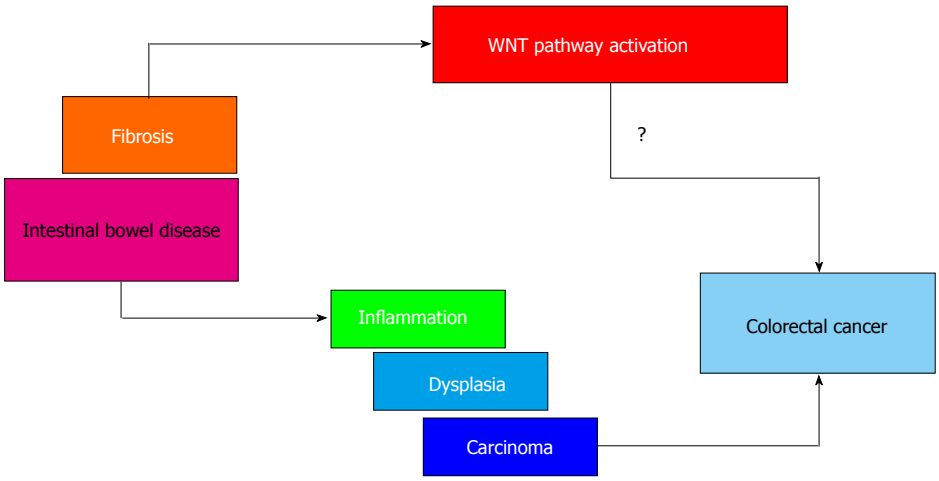


Figure 2 The involvement of Wnt pathway in the progression from fibrosis to cancer. Patients affected by intestinal bowel disease are highly susceptible to a specific type of colorectal carcinogenesis characterized by the inflammation-dysplasia-carcinoma progression, without the appearance of adenoma. The activation of Wnt pathway in colitis could represent a possible link between fibrosis and the development of colorectal cancer.

could potentially represent a therapeutic target[64]. Furthermore, activation of the Wnt/β-catenin pathway is able to induce the EMT and is important for fibrogenesis mediated by TGF-β[65]. In particular, TGF-β is able to stimulate Wnt signaling by suppressing the expression of DKK-1, a Wnt inhibitor. In addition, activation of the Wnt/β-catenin pathway is able to increase ECM synthesis and regulate several MMP genes[66]. It is also reported that blocking the Wnt pathway can reverse the fibrosis, thus representing a useful therapeutic target[67].

The Wnt pathway represents an important signaling pathway during development[68]. Wnt ligands are able to activate either canonical or noncanonical pathways, with the former based on the crucial role of the β-catenin protein[69]. The Wnt pathway is of course also important in adult tissue homeostasis, and it is down-regulated in cancer. In particular, about 80% of colorectal cancer patients carry mutations in key components of the pathway, and in particular activating mutations in β-catenin and inactivating mutations in the adenomatous polyposis coli gene[70]. The Wnt pathway could be activated either directly through mutations of its components, or indirectly through the secretion of triggering ligands or the depletion of inhibitors.

It is well known that the lifetime susceptibility to colorectal cancer is higher among IBD patients than the rest of the population[71,72]. This specific type of carcinogenesis is characterized by the well-defined progression from inflammation to dysplasia to carcinoma, without the appearance of adenoma, as observed in sporadic cancer[72-74], and with several differences in the pathogenesis with respect to the sporadic counterpart. p53 mutations are frequently found in colitis-associated carcinogenesis, whereas in sporadic cases they are only found in advanced disease[75,76]. Moreover, these kinds of mutations are also found in the colonic mucosa adjacent to the area with dysplastic colitis[77,78]. It has also been found that the Wnt pathway is frequently activated very early in colitis-associated carcinogenesis[59], as well in the adjacent nondysplastic mucosa. This clearly suggests that early activation of the Wnt pathway in the area surrounding a dysplastic or malignant lesion in colitis could represent a link between fibrosis and the future development of colorectal cancer (Figure 2).

CONCLUSION

In recent years several biomarkers of fibrosis have been proposed, tested, and verified in patients with CD. However, validating studies are still needed to confirm the reliability of these markers. This would eventually allow for the development of noninvasive tools to detect them and perform early diagnoses of incipient fibrosis, and hence implement prompt treatment. Moreover, such researches could lead to a better understanding of the mechanisms underlying intestinal fibrogenesis and clarify the potential links between CD fibrosis and cancer.

REFERENCES

1 **Rieder F**, Latella G, Magro F, Yuksel ES, Higgins PD, Di Sabatino A, de Bruyn JR, Rimola J, Brito J, Bettenworth D, Van Assche G, Bemelman W, D'Hoore A, Pellino G, Dignass AU. European Crohn's and Colitis Organisation Topical Review on Prediction, Diagnosis and Management of Fibrostenosing Crohn's Disease. *J Crohns Colitis* 2016 Feb 29; Epub ahead of print [PMID: 26928961 DOI: 10.1093/ecco-jcc/jjw055]

2 **Latella G**, Papi C. Crucial steps in the natural history of inflammatory bowel disease. *World J Gastroenterol* 2012; **18**: 3790-3799 [PMID: 22876029 DOI: 10.3748/wjg.v18.i29.3790]

3 **Bernell O**, Lapidus A, Hellers G. Risk factors for surgery and postoperative recurrence in Crohn's disease. *Ann Surg* 2000; **231**: 38-45 [PMID: 10636100]

4 **Siassi M**, Weiger A, Hohenberger W, Kessler H. Changes in surgical therapy for Crohn's disease over 33 years: a prospective longitudinal study. *Int J Colorectal Dis* 2007; **22**: 319-324 [PMID: 16733647 DOI: 10.1007/s00384-006-0150-5]

5 **Latella G**, Rogler G, Bamias G, Breynaert C, Florholmen J, Pellino G, Reif S, Speca S, Lawrance IC. Results of the 4th scientific

workshop of the ECCO (I): pathophysiology of intestinal fibrosis in IBD. *J Crohns Colitis* 2014; **8**: 1147-1165 [PMID: 24731838 DOI: 10.1016/j.crohns.2014.03.008]

6 **Franke A**, McGovern DP, Barrett JC, Wang K, Radford-Smith GL, Ahmad T, Lees CW, Balschun T, Lee J, Roberts R, Anderson CA, Bis JC, Bumpstead S, Ellinghaus D, Festen EM, Georges M, Green T, Haritunians T, Jostins L, Latiano A, Mathew CG, Montgomery GW, Prescott NJ, Raychaudhuri S, Rotter JI, Schumm P, Sharma Y, Simms LA, Taylor KD, Whiteman D, Wijmenga C, Baldassano RN, Barclay M, Bayless TM, Brand S, Büning C, Cohen A, Colombel JF, Cottone M, Stronati L, Denson T, De Vos M, D'Inca R, Dubinsky M, Edwards C, Florin T, Franchimont D, Gearry R, Glas J, Van Gossum A, Guthery SL, Halfvarson J, Verspaget HW, Hugot JP, Karban A, Laukens D, Lawrance I, Lemann M, Levine A, Libioulle C, Louis E, Mowat C, Newman W, Panés J, Phillips A, Proctor DD, Regueiro M, Russell R, Rutgeerts P, Sanderson J, Sans M, Seibold F, Steinhart AH, Stokkers PC, Torkvist L, Kullak-Ublick G, Wilson D, Walters T, Targan SR, Brant SR, Rioux JD, D'Amato M, Weersma RK, Kugathasan S, Griffiths AM, Mansfield JC, Vermeire S, Duerr RH, Silverberg MS, Satsangi J, Schreiber S, Cho JH, Annese V, Hakonarson H, Daly MJ, Parkes M. Genome-wide meta-analysis increases to 71 the number of confirmed Crohn's disease susceptibility loci. *Nat Genet* 2010; **42**: 1118-1125 [PMID: 21102463 DOI: 10.1038/ng.717]

7 **Dalal SR**, Kwon JH. The Role of MicroRNA in Inflammatory Bowel Disease. *Gastroenterol Hepatol* (NY) 2010; **6**: 714-722 [PMID: 21437020]

8 **Barrett JC**, Hansoul S, Nicolae DL, Cho JH, Duerr RH, Rioux JD, Brant SR, Silverberg MS, Taylor KD, Barmada MM, Bitton A, Dassopoulos T, Datta LW, Green T, Griffiths AM, Kistner EO, Murtha MT, Regueiro MD, Rotter JI, Schumm LP, Steinhart AH, Targan SR, Xavier RJ, Libioulle C, Sandor C, Lathrop M, Belaiche J, Dewit O, Gut I, Heath S, Laukens D, Mni M, Rutgeerts P, Van Gossum A, Zelenika D, Franchimont D, Hugot JP, de Vos M, Vermeire S, Louis E, Cardon LR, Anderson CA, Drummond H, Nimmo E, Ahmad T, Prescott NJ, Onnie CM, Fisher SA, Marchini J, Ghori J, Bumpstead S, Gwilliam R, Tremelling M, Deloukas P, Mansfield J, Jewell D, Satsangi J, Mathew CG, Parkes M, Georges M, Daly MJ. Genome-wide association defines more than 30 distinct susceptibility loci for Crohn's disease. *Nat Genet* 2008; **40**: 955-962 [PMID: 18587394 DOI: 10.1038/ng.175]

9 **Lesage S**, Zouali H, Cézard JP, Colombel JF, Belaiche J, Almer S, Tysk C, O'Morain C, Gassull M, Binder V, Finkel Y, Modigliani R, Gower-Rousseau C, Macry J, Merlin F, Chamaillard M, Jannot AS, Thomas G, Hugot JP. CARD15/NOD2 mutational analysis and genotype-phenotype correlation in 612 patients with inflammatory bowel disease. *Am J Hum Genet* 2002; **70**: 845-857 [PMID: 11875755 DOI: 10.1086/339432]

10 **Yamazaki K**, Takazoe M, Tanaka T, Kazumori T, Nakamura Y. Absence of mutation in the NOD2/CARD15 gene among 483 Japanese patients with Crohn's disease. *J Hum Genet* 2002; **47**: 469-472 [PMID: 12202985 DOI: 10.1007/s100380200067]

11 **Buhner S**, Buning C, Genschel J, Kling K, Herrmann D, Dignass A, Kuechler I, Krueger S, Schmidt HH, Lochs H. Genetic basis for increased intestinal permeability in families with Crohn's disease: role of CARD15 3020insC mutation? *Gut* 2006; **55**: 342-347 [PMID: 16000642 DOI: 10.1136/gut.2005.065557]

12 **Abreu MT**, Taylor KD, Lin YC, Hang T, Gaiennie J, Landers CJ, Vasiliauskas EA, Kam LY, Rojany M, Papadakis KA, Rotter JI, Targan SR, Yang H. Mutations in NOD2 are associated with fibrostenosing disease in patients with Crohn's disease. *Gastroenterology* 2002; **123**: 679-688 [PMID: 12198692 DOI: 10.1053/gast.2002.35393]

13 **Jürgens M**, Brand S, Laubender RP, Seiderer J, Glas J, Wetzke M, Wagner J, Pfennig S, Tillack C, Beigel F, Weidinger M, Schnitzler F, Kreis ME, Göke B, Lohse P, Herrmann K, Ochsenkühn T. The presence of fistulas and NOD2 homozygosity strongly predict intestinal stenosis in Crohn's disease independent of the IL23R genotype. *J Gastroenterol* 2010; **45**: 721-731 [PMID: 20428899 DOI: 10.1007/s00535-010-0231-7]

14 **Alvarez-Lobos M**, Arostegui JI, Sans M, Tassies D, Plaza S, Delgado S, Lacy AM, Pique JM, Yagüe J, Panés J. Crohn's disease patients carrying Nod2/CARD15 gene variants have an increased and early need for first surgery due to stricturing disease and higher rate of surgical recurrence. *Ann Surg* 2005; **242**: 693-700 [PMID: 16244543]

15 **Wynn TA**. Cellular and molecular mechanisms of fibrosis. *J Pathol* 2008; **214**: 199-210 [PMID: 18161745 DOI: 10.1002/path.2277]

16 **Lawrance IC**, Rogler G, Bamias G, Breynaert C, Florholmen J, Pellino G, Reif S, Speca S, Latella G. Cellular and Molecular Mediators of Intestinal Fibrosis. *J Crohns Colitis* 2015 Nov 2; Epub ahead of print [PMID: 25306501 DOI: 10.1016/j.crohns.2014.09.00]

17 **Vasiliauskas EA**, Kam LY, Karp LC, Gaiennie J, Yang H, Targan SR. Marker antibody expression stratifies Crohn's disease into immunologically homogeneous subgroups with distinct clinical characteristics. *Gut* 2000; **47**: 487-496 [PMID: 10986208 DOI: 10.1136/gut.47.4.487]

18 **Forcione DG**, Rosen MJ, Kisiel JB, Sands BE. Anti-Saccharomyces cerevisiae antibody (ASCA) positivity is associated with increased risk for early surgery in Crohn's disease. *Gut* 2004; **53**: 1117-1122 [PMID: 15247177 DOI: 10.1136/gut.2003.030734]

19 **Targan SR**, Landers CJ, Yang H, Lodes MJ, Cong Y, Papadakis KA, Vasiliauskas E, Elson CO, Hershberg RM. Antibodies to CBir1 flagellin define a unique response that is associated independently with complicated Crohn's disease. *Gastroenterology* 2005; **128**: 2020-2028 [PMID: 15940634 DOI: 10.1053/j.gastro.2005.03.046]

20 **Mow WS**, Vasiliauskas EA, Lin YC, Fleshner PR, Papadakis KA, Taylor KD, Landers CJ, Abreu-Martin MT, Rotter JI, Yang H, Targan SR. Association of antibody responses to microbial antigens and complications of small bowel Crohn's disease. *Gastroenterology* 2004; **126**: 414-424 [PMID: 14762777 DOI: 10.1053/j.gastro.2003.11.015]

21 **Ferrante M**, Henckaerts L, Joossens M, Pierik M, Joossens S, Dotan N, Norman GL, Altstock RT, Van Steen K, Rutgeerts P, Van Assche G, Vermeire S. New serological markers in inflammatory bowel disease are associated with complicated disease behaviour. *Gut* 2007; **56**: 1394-1403 [PMID: 17456509 DOI: 10.1136/gut.2006.108043]

22 **Rieder F**, Schleder S, Wolf A, Dirmeier A, Strauch U, Obermeier F, Lopez R, Spector L, Fire E, Yarden J, Rogler G, Dotan N, Klebl F. Association of the novel serologic anti-glycan antibodies anti-laminarin and anti-chitin with complicated Crohn's disease behavior. *Inflamm Bowel Dis* 2010; **16**: 263-274 [PMID: 19653286 DOI: 10.1002/ibd.21046]

23 **Seow CH**, Stempak JM, Xu W, Lan H, Griffiths AM, Greenberg GR, Steinhart AH, Dotan N, Silverberg MS. Novel anti-glycan antibodies related to inflammatory bowel disease diagnosis and phenotype. *Am J Gastroenterol* 2009; **104**: 1426-1434 [PMID: 19491856 DOI: 10.1038/ajg.2009.79]

24 **Simondi D**, Mengozzi G, Betteto S, Bonardi R, Ghignone RP, Fagoonee S, Pellicano R, Sguazzini C, Pagni R, Rizzetto M, Astegiano M. Antiglycan antibodies as serological markers in the differential diagnosis of inflammatory bowel disease. *Inflamm Bowel Dis* 2008; **14**: 645-651 [PMID: 18240283 DOI: 10.1002/ibd.20368]

25 **Arnott ID**, Landers CJ, Nimmo EJ, Drummond HE, Smith BK, Targan SR, Satsangi J. Sero-reactivity to microbial components in Crohn's disease is associated with disease severity and progression, but not NOD2/CARD15 genotype. *Am J Gastroenterol* 2004; **99**: 2376-2384 [PMID: 15571586 DOI: 10.1111/j.1572-0241.2004.40417.x]

26 **Papp M**, Altorjay I, Dotan N, Palatka K, Foldi I, Tumpek J, Sipka S, Udvardy M, Dinya T, Lakatos L, Kovacs A, Molnar T, Tulassay Z, Miheller P, Norman GL, Szamosi T, Papp J; Hungarian IBD Study Group, Lakatos PL. New serological markers for inflammatory bowel disease are associated with earlier age at onset, complicated disease behavior, risk for surgery, and NOD2/CARD15 genotype in a Hungarian IBD cohort. *Am J Gastroenterol* 2008; **103**: 665-681 [PMID: 18047543 DOI: 10.1111/j.1572-0241.2007.01652.x]

27 **Dubinsky MC**, Kugathasan S, Mei L, Picornell Y, Nebel J, Wrobel I, Quiros A, Silber G, Wahbeh G, Katzir L, Vasiliauskas E, Bahar R, Otley A, Mack D, Evans J, Rosh J, Hemker MO, Leleiko N, Crandall W, Langton C, Landers C, Taylor KD, Targan SR,

Rotter JI, Markowitz J, Hyams J. Increased immune reactivity predicts aggressive complicating Crohn's disease in children. *Clin Gastroenterol Hepatol* 2008; **6**: 1105-1111 [PMID: 18619921 DOI: 10.1016/j.cgh.2008.04.032]

28 **Allan A**, Wyke J, Allan RN, Morel P, Robinson M, Scott DL, Alexander-Williams J. Plasma fibronectin in Crohn's disease. *Gut* 1989; **30**: 627-633 [PMID: 2731755 DOI: 10.1136/gut.30.5.627]

29 **Henriksen M**, Jahnsen J, Lygren I, Stray N, Sauar J, Vatn MH, Moum B; IBSEN Study Group. C-reactive protein: a predictive factor and marker of inflammation in inflammatory bowel disease. Results from a prospective population-based study. *Gut* 2008; **57**: 1518-1523 [PMID: 18566104 DOI: 10.1136/gut.2007.146357]

30 **Koutroubakis IE**, Petinaki E, Dimoulios P, Vardas E, Rousso-moustakaki M, Maniatis AN, Kouroumalis EA. Serum laminin and collagen IV in inflammatory bowel disease. *J Clin Pathol* 2003; **56**: 817-820 [PMID: 14600124 DOI: 10.1136/jcp.56.11.817]

31 **Rieder F**, Fiocchi C. Intestinal fibrosis in IBD--a dynamic, multifactorial process. *Nat Rev Gastroenterol Hepatol* 2009; **6**: 228-235 [PMID: 19347014 DOI: 10.1038/nrgastro.2009.31]

32 **Loeschke K**, Kaltenthaler P. [Procollagen-III-peptide in the serum of patients with Crohn disease]. *Z Gastroenterol* 1989; **27**: 137-139 [PMID: 2718533]

33 **Koutroubakis IE**, Petinaki E, Dimoulios P, Vardas E, Rousso-moustakaki M, Maniatis AN, Kouroumalis EA. Increased serum levels of YKL-40 in patients with inflammatory bowel disease. *Int J Colorectal Dis* 2003; **18**: 254-259 [PMID: 12673492 DOI: 10.1007/s00384-002-0446-z]

34 **Di Sabatino A**, Ciccocioppo R, Armellini E, Morera R, Ricevuti L, Cazzola P, Fulle I, Corazza GR. Serum bFGF and VEGF correlate respectively with bowel wall thickness and intramural blood flow in Crohn's disease. *Inflamm Bowel Dis* 2004; **10**: 573-577 [PMID: 15472517]

35 **Bonasio R**, Tu S, Reinberg D. Molecular signals of epigenetic states. *Science* 2010; **330**: 612-616 [PMID: 21030644 DOI: 10.1126/science.1191079]

36 **Scarpa M**, Stylianou E. Epigenetics: Concepts and relevance to IBD pathogenesis. *Inflamm Bowel Dis* 2012; **18**: 1982-1996 [PMID: 22407855 DOI: 10.1002/ibd.22934]

37 **Lin Z**, Hegarty JP, Cappel JA, Yu W, Chen X, Faber P, Wang Y, Kelly AA, Poritz LS, Peterson BZ, Schreiber S, Fan JB, Koltun WA. Identification of disease-associated DNA methylation in intestinal tissues from patients with inflammatory bowel disease. *Clin Genet* 2011; **80**: 59-67 [PMID: 20950376 DOI: 10.1111/j.1399-0004.2010.01546.x]

38 **Lin Z**, Hegarty JP, Yu W, Cappel JA, Chen X, Faber PW, Wang Y, Poritz LS, Fan JB, Koltun WA. Identification of disease-associated DNA methylation in B cells from Crohn's disease and ulcerative colitis patients. *Dig Dis Sci* 2012; **57**: 3145-3153 [PMID: 22821069 DOI: 10.1007/s10620-012-2288-z]

39 **Vamadevan AS**, Fukata M, Arnold ET, Thomas LS, Hsu D, Abreu MT. Regulation of Toll-like receptor 4-associated MD-2 in intestinal epithelial cells: a comprehensive analysis. *Innate Immun* 2010; **16**: 93-103 [PMID: 19710105 DOI: 10.1177/1753425909339231]

40 **Gonsky R**, Deem RL, Landers CJ, Derkowski CA, Berel D, McGovern DP, Targan SR. Distinct IFNG methylation in a subset of ulcerative colitis patients based on reactivity to microbial antigens. *Inflamm Bowel Dis* 2011; **17**: 171-178 [PMID: 20848535 DOI: 10.1002/ibd.21352]

41 **Chen Y**, Ge W, Xu L, Qu C, Zhu M, Zhang W, Xiao Y. miR-200b is involved in intestinal fibrosis of Crohn's disease. *Int J Mol Med* 2012; **29**: 601-606 [PMID: 22294131 DOI: 10.3892/ijmm.2012.894]

42 **Nijhuis A**, Biancheri P, Lewis A, Bishop CL, Giuffrida P, Chan C, Feakins R, Poulsom R, Di Sabatino A, Corazza GR, MacDonald TT, Lindsay JO, Silver AR. In Crohn's disease fibrosis-reduced expression of the miR-29 family enhances collagen expression in intestinal fibroblasts. *Clin Sci* (Lond) 2014; **127**: 341-350 [PMID: 24641356 DOI: 10.1042/CS20140048]

43 **Fujioka S**, Nakamichi I, Esaki M, Asano K, Matsumoto T, Kitazono T. Serum microRNA levels in patients with Crohn's disease during induction therapy by infliximab. *J Gastroenterol Hepatol* 2014; **29**: 1207-1214 [PMID: 24447044 DOI: 10.1111/jgh.12523]

44 **Panes J**, Bouhnik Y, Reinisch W, Stoker J, Taylor SA, Baumgart DC, Danese S, Halligan S, Marincek B, Matos C, Peyrin-Biroulet L, Rimola J, Rogler G, van Assche G, Ardizzone S, Ba-Ssalamah A, Bali MA, Bellini D, Biancone L, Castiglione F, Ehehalt R, Grassi R, Kucharzik T, Maccioni F, Maconi G, Magro F, Martín-Comín J, Morana G, Pendsé D, Sebastian S, Signore A, Tolan D, Tielbeek JA, Weishaupt D, Wiarda B, Laghi A. Imaging techniques for assessment of inflammatory bowel disease: joint ECCO and ESGAR evidence-based consensus guidelines. *J Crohns Colitis* 2013; **7**: 556-585 [PMID: 23583097 DOI: 10.1016/j.crohns.2013.02.020]

45 **Ha CY**, Kumar N, Raptis CA, Narra VR, Ciorba MA. Magnetic resonance enterography: safe and effective imaging for stricturing Crohn's disease. *Dig Dis Sci* 2011; **56**: 2906-2913 [PMID: 21688128 DOI: 10.1007/s10620-011-1781-0]

46 **Rimola J**, Planell N, Rodríguez S, Delgado S, Ordás I, Ramírez-Morros A, Ayuso C, Aceituno M, Ricart E, Jauregui-Amezaga A, Panés J, Cuatrecasas M. Characterization of inflammation and fibrosis in Crohn's disease lesions by magnetic resonance imaging. *Am J Gastroenterol* 2015; **110**: 432-440 [PMID: 25623654 DOI: 10.1038/ajg.2014.424]

47 **Rieder F**, de Bruyn JR, Pham BT, Katsanos K, Annese V, Higgins PD, Magro F, Dotan I. Results of the 4th scientific workshop of the ECCO (Group II): markers of intestinal fibrosis in inflammatory bowel disease. *J Crohns Colitis* 2014; **8**: 1166-1178 [PMID: 24726695 DOI: 10.1016/j.crohns.2014.03.009]

48 **Yaffe BH**, Korelitz BI. Prognosis for nonoperative management of small-bowel obstruction in Crohn's disease. *J Clin Gastroenterol* 1983; **5**: 211-215 [PMID: 6306093]

49 **Pellino G**, Nicolai E, Catalano OA, Campione S, D'Armiento FP, Salvatore M, Cuocolo A, Selvaggi F. PET/MR Versus PET/CT Imaging: Impact on the Clinical Management of Small-Bowel Crohn's Disease. *J Crohns Colitis* 2016; **10**: 277-285 [PMID: 26574490 DOI: 10.1093/ecco-jcc/jjv207]

50 **Lenze F**, Wessling J, Bremer J, Ullerich H, Spieker T, Weckesser M, Gonschorrek S, Kannengiesser K, Rijcken E, Heidemann J, Luegering A, Schober O, Domschke W, Kucharzik T, Maaser C. Detection and differentiation of inflammatory versus fibromatous Crohn's disease strictures: prospective comparison of 18F-FDG-PET/CT, MR-enteroclysis, and transabdominal ultrasound versus endoscopic/histologic evaluation. *Inflamm Bowel Dis* 2012; **18**: 2252-2260 [PMID: 22359277 DOI: 10.1002/ibd.22930]

51 **Maccioni F**, Patak MA, Signore A, Laghi A. New frontiers of MRI in Crohn's disease: motility imaging, diffusion-weighted imaging, perfusion MRI, MR spectroscopy, molecular imaging, and hybrid imaging (PET/MRI). *Abdom Imaging* 2012; **37**: 974-982 [PMID: 22743838 DOI: 10.1007/s00261-012-9890-6]

52 **Catalano OA**, Rosen BR, Sahani DV, Hahn PF, Guimaraes AR, Vangel MG, Nicolai E, Soricelli A, Salvatore M. Clinical impact of PET/MR imaging in patients with cancer undergoing same-day PET/CT: initial experience in 134 patients--a hypothesis-generating exploratory study. *Radiology* 2013; **269**: 857-869 [PMID: 24009348 DOI: 10.1148/radiol.13131306]

53 **Catalano OA**, Gee MS, Nicolai E, Selvaggi F, Pellino G, Cuocolo A, Luongo A, Catalano M, Rosen BR, Gervais D, Vangel MG, Soricelli A, Salvatore M. Evaluation of Quantitative PET/MR Enterography Biomarkers for Discrimination of Inflammatory Strictures from Fibrotic Strictures in Crohn Disease. *Radiology* 2016; **278**: 792-800 [PMID: 26436860 DOI: 10.1148/radiol.2015150566]

54 **Jones T**, Budinger TF. The potential for low-dose functional studies in maternal-fetal medicine using PET/MR imaging. *J Nucl Med* 2013; **54**: 2016-2017 [PMID: 24029653 DOI: 10.2967/jnumed.113.123919]

55 **Castiglione F**, de Sio I, Cozzolino A, Rispo A, Manguso F, Del Vecchio Blanco G, Di Girolamo E, Castellano L, Ciacci C, Mazzacca G. Bowel wall thickness at abdominal ultrasound and the one-year-risk of surgery in patients with Crohn's disease. *Am J Gastroenterol* 2004; **99**: 1977-1983 [PMID: 15447760 DOI: 10.1111/j.1572-0241.2004.40267.x]

56 **Castiglione F**, Testa A, Rea M, De Palma GD, Diaferia M, Musto D, Sasso F, Caporaso N, Rispo A. Transmural healing evaluated by

bowel sonography in patients with Crohn's disease on maintenance treatment with biologics. *Inflamm Bowel Dis* 2013; **19**: 1928-1934 [PMID: 23835441 DOI: 10.1097/MIB.0b013e31829053ce]
57 **Gordon IO**, Agrawal N, Goldblum JR, Fiocchi C, Rieder F. Fibrosis in ulcerative colitis: mechanisms, features, and consequences of a neglected problem. *Inflamm Bowel Dis* 2014; **20**: 2198-2206 [PMID: 24892966 DOI: 10.1097/MIB.0000000000000080]
58 **Latella G**, Rieder F. Time to Look Underneath the Surface: Ulcerative Colitis-Associated Fibrosis. *J Crohns Colitis* 2015; **9**: 941-942 [PMID: 26303632 DOI: 10.1093/ecco-jcc/jjv142]
59 **Claessen MM**, Schipper ME, Oldenburg B, Siersema PD, Offerhaus GJ, Vleggaar FP. WNT-pathway activation in IBD-associated colorectal carcinogenesis: potential biomarkers for colonic surveillance. *Cell Oncol* 2010; **32**: 303-310 [PMID: 20208143 DOI: 10.3233/CLO-2009-0503]
60 **Tuller T**, Atar S, Ruppin E, Gurevich M, Achiron A. Common and specific signatures of gene expression and protein-protein interactions in autoimmune diseases. *Genes Immun* 2013; **14**: 67-82 [PMID: 23190644 DOI: 10.1038/gene.2012.55]
61 **Hughes KR**, Sablitzky F, Mahida YR. Expression profiling of Wnt family of genes in normal and inflammatory bowel disease primary human intestinal myofibroblasts and normal human colonic crypt epithelial cells. *Inflamm Bowel Dis* 2011; **17**: 213-220 [PMID: 20848536 DOI: 10.1002/ibd.21353]
62 **Koslowski MJ**, Kübler I, Chamaillard M, Schaeffeler E, Reinisch W, Wang G, Beisner J, Teml A, Peyrin-Biroulet L, Winter S, Herrlinger KR, Rutgeerts P, Vermeire S, Cooney R, Fellermann K, Jewell D, Bevins CL, Schwab M, Stange EF, Wehkamp J. Genetic variants of Wnt transcription factor TCF-4 (TCF7L2) putative promoter region are associated with small intestinal Crohn's disease. *PLoS One* 2009; **4**: e4496 [PMID: 19221600 DOI: 10.1371/journal.pone.0004496]
63 **Beisner J**, Teltschik Z, Ostaff MJ, Tiemessen MM, Staal FJ, Wang G, Gersemann M, Perminow G, Vatn MH, Schwab M, Stange EF, Wehkamp J. TCF-1-mediated Wnt signaling regulates Paneth cell innate immune defense effectors HD-5 and -6: implications for Crohn's disease. *Am J Physiol Gastrointest Liver Physiol* 2014; **307**: G487-G498 [PMID: 24994854 DOI: 10.1152/ajpgi.00347.2013]
64 **Koslowski MJ**, Teltschik Z, Beisner J, Schaeffeler E, Wang G, Kübler I, Gersemann M, Cooney R, Jewell D, Reinisch W, Vermeire S, Rutgeerts P, Schwab M, Stange EF, Wehkamp J. Association of a functional variant in the Wnt co-receptor LRP6 with early onset ileal Crohn's disease. *PLoS Genet* 2012; **8**: e1002523 [PMID: 22393312 DOI: 10.1371/journal.pgen.1002523]
65 **Akhmetshina A**, Palumbo K, Dees C, Bergmann C, Venalis P, Zerr P, Horn A, Kireva T, Beyer C, Zwerina J, Schneider H, Sadowski A, Riener MO, MacDougald OA, Distler O, Schett G, Distler JH. Activation of canonical Wnt signalling is required for TGF-β-mediated fibrosis. *Nat Commun* 2012; **3**: 735 [PMID: 22415826 DOI: 10.1038/ncomms1734]
66 **Wu B**, Crampton SP, Hughes CC. Wnt signaling induces matrix metalloproteinase expression and regulates T cell transmigration. *Immunity* 2007; **26**: 227-239 [PMID: 17306568]
67 **Henderson WR**, Chi EY, Ye X, Nguyen C, Tien YT, Zhou B, Borok Z, Knight DA, Kahn M. Inhibition of Wnt/beta-catenin/CREB binding protein (CBP) signaling reverses pulmonary fibrosis. *Proc Natl Acad Sci USA* 2010; **107**: 14309-14314 [PMID: 20660310 DOI: 10.1073/pnas.1001520107]
68 **Fodde R**, Smits R, Clevers H. APC, signal transduction and genetic instability in colorectal cancer. *Nat Rev Cancer* 2001; **1**: 55-67 [PMID: 11900252 DOI: 10.1038/35094067]
69 **Valenta T**, Hausmann G, Basler K. The many faces and functions of β-catenin. *EMBO J* 2012; **31**: 2714-2736 [PMID: 22617422 DOI: 10.1038/emboj.2012.150]
70 **White BD**, Chien AJ, Dawson DW. Dysregulation of Wnt/β-catenin signaling in gastrointestinal cancers. *Gastroenterology* 2012; **142**: 219-232 [PMID: 22155636 DOI: 10.1053/j.gastro.2011.12.001]
71 **Annese V**, Beaugerie L, Egan L, Biancone L, Bolling C, Brandts C, Dierickx D, Dummer R, Fiorino G, Gornet JM, Higgins P, Katsanos KH, Nissen L, Pellino G, Rogler G, Scaldaferri F, Szymanska E, Eliakim R. European Evidence-based Consensus: Inflammatory Bowel Disease and Malignancies. *J Crohns Colitis* 2015; **9**: 945-965 [PMID: 26294789 DOI: 10.1093/ecco-jcc/jjv141]
72 **Jess T**, Rungoe C, Peyrin-Biroulet L. Risk of colorectal cancer in patients with ulcerative colitis: a meta-analysis of population-based cohort studies. *Clin Gastroenterol Hepatol* 2012; **10**: 639-645 [PMID: 22289873 DOI: 10.1016/j.cgh.2012.01.010]
73 **Itzkowitz SH**, Yio X. Inflammation and cancer IV. Colorectal cancer in inflammatory bowel disease: the role of inflammation. *Am J Physiol Gastrointest Liver Physiol* 2004; **287**: G7-17 [PMID: 15194558 DOI: 10.1152/ajpgi.00079.2004]
74 **Egan L**, D'Inca R, Jess T, Pellino G, Carbonnel F, Bokemeyer B, Harbord M, Nunes P, Van der Woude J, Selvaggi F, Triantafillidis J. Non-colorectal intestinal tract carcinomas in inflammatory bowel disease: results of the 3rd ECCO Pathogenesis Scientific Workshop (II). *J Crohns Colitis* 2014; **8**: 19-30 [PMID: 23664498 DOI: 10.1016/j.crohns.2013.04.009]
75 **Risques RA**, Rabinovitch PS, Brentnall TA. Cancer surveillance in inflammatory bowel disease: new molecular approaches. *Curr Opin Gastroenterol* 2006; **22**: 382-390 [PMID: 16760754 DOI: 10.1097/01.mog.0000231812.95525.a7]
76 **Vogelstein B**, Fearon ER, Hamilton SR, Kern SE, Preisinger AC, Leppert M, Nakamura Y, White R, Smits AM, Bos JL. Genetic alterations during colorectal-tumor development. *N Engl J Med* 1988; **319**: 525-532 [PMID: 2841597 DOI: 10.1056/NEJM198809013190901]
77 **Braakhuis BJ**, Tabor MP, Kummer JA, Leemans CR, Brakenhoff RH. A genetic explanation of Slaughter's concept of field cancerization: evidence and clinical implications. *Cancer Res* 2003; **63**: 1727-1730 [PMID: 12702551]
78 **Brentnall TA**, Crispin DA, Rabinovitch PS, Haggitt RC, Rubin CE, Stevens AC, Burmer GC. Mutations in the p53 gene: an early marker of neoplastic progression in ulcerative colitis. *Gastroenterology* 1994; **107**: 369-378 [PMID: 8039614]

P- Reviewer: Cario E, Cheifetz AS, Seicean A **S- Editor**: Gong ZM
L- Editor: A **E- Editor**: Lu YJ

World Journal of Gastrointestinal Pathophysiology

Submit a Manuscript: http://www.wjgnet.com/esps/
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.4291/wjgp.v7.i3.276

World J Gastrointest Pathophysiol 2016 August 15; 7(3): 276-282
ISSN 2150-5330 (online)

Pancreatic disorders in inflammatory bowel disease

Filippo Antonini, Raffaele Pezzilli, Lucia Angelelli, Giampiero Macarri

Filippo Antonini, Giampiero Macarri, Department of Gastroenterology, A.Murri Hospital, Polytechnic University of Marche, 63900 Fermo, Italy

Raffaele Pezzilli, Department of Digestive Diseases and Internal Medicine, Sant'Orsola-Malpighi Hospital, 40138 Bologna, Italy

Lucia Angelelli, Medical Oncology, Mazzoni Hospital, 63100 Ascoli Piceno, Italy

Author contributions: Antonini F designed the research; Antonini F and Pezzilli R did the data collection and analyzed the data; Antonini F, Pezzilli R and Angelelli L wrote the paper; Macarri G revised the paper and granted the final approval.

Conflict-of-interest statement: The authors declare no conflict of interest.

Manuscript source: Invited manuscript

Correspondence to: Filippo Antonini, MD, Department of Gastroenterology, A.Murri Hospital, Polytechnic University of Marche, Ospedale "A.Murri", 63900 Fermo, Italy. filippore@yahoo.it
Telephone: +39-0734-6252249
Fax: +39-0734-6252252

Received: May 24, 2016
Peer-review started: May 25, 2016
First decision: June 17, 2016
Revised: July 8, 2016
Accepted: July 20, 2016
Article in press: July 22, 2016
Published online: August 15, 2016

Abstract

An increased incidence of pancreatic disorders either acute pancreatitis or chronic pancreatitis has been recorded in patients with inflammatory bowel disease (IBD) compared to the general population. Although most of the pancreatitis in patients with IBD seem to be related to biliary lithiasis or drug induced, in some cases pancreatitis were defined as idiopathic, suggesting a direct pancreatic damage in IBD. Pancreatitis and IBD may have similar presentation therefore a pancreatic disease could not be recognized in patients with Crohn's disease and ulcerative colitis. This review will discuss the most common pancreatic diseases seen in patients with IBD.

Key words: Pancreas; Pancreatitis; Extraintestinal manifestations; Exocrine pancreatic insufficiency; Ulcerative colitis; Crohn's disease; Inflammatory bowel disease

Core tip: Pancreatic disorders are not uncommon in patients with inflammatory bowel disease (IBD). The most frequent manifestation is acute pancreatitis (AP). Causes of AP are mainly a concomitant biliary lithiasis or drugs used in the treatment of IBD. However for some IBD-related pancreatitis, idiopathic by definition, where no relationship with lithiasis or drugs can be recognized, a direct pancreatic damage would be hypothesized.

Antonini F, Pezzilli R, Angelelli L, Macarri G. Pancreatic disorders in inflammatory bowel disease. *World J Gastrointest Pathophysiol* 2016; 7(3): 276-282 Available from: URL: http://www.wjgnet.com/2150-5330/full/v7/i3/276.htm DOI: http://dx.doi.org/10.4291/wjgp.v7.i3.276

INTRODUCTION

Crohn's disease and ulcerative colitis are the two major clinically defined forms of inflammatory bowel disease (IBD). Although they affect mainly the bowel, often present with systemic manifestations and involvement of organs other than the gastrointestinal tract. However distinguishing between proper extra-intestinal manifestations (EIMs), *i.e.*, systemic alterations directly

related to the disease, and extra-intestinal complications, *i.e.*, conditions secondary to metabolic disturbance, anatomic alterations or side effects of treatment, is not always straightforward. Although the most common EIMs are mucocutaneous, ophtalmologic, arthritic, hepato-biliary, pulmonary, an increased incidence of pancreatic disorders either acute pancreatitis (AP) or chronic pancreatitis (CP) has been recorded in patients with IBD compared to the general population[1]. The first report of association between IBD and pancreatitis dates back to 1950, when Ball *et al*[2] published a post-mortem study of 86 patients with ulcerative colitis where pancreatic lesions either macroscopic or microscopic were detected in 14% and 53% respectively. A similar autopsy study of 39 patients with Crohn's disease revealed pancreatic fibrosis in 38% of them[3]. No one of the cases in both studies had presented with symptoms or signs of pancreatitis, what suggests pancreatic disease had been subclinical or silent. Increasing number of cases of either acute or CP have been reported since[4-7].

In the pediatric population pancreatic disease is rare (0.7%-1.6%) but higher than in the general population. Incidence of AP is higher in females than in males with a greater occurrence in those with active and severe IBD. Children with IBD also have an increased risk of developing CP and asymptomatic hyperamylasemia[8].

However most of the pancreatitis in patients with IBD, both adults and children, seem to be related to biliary lithiasis or drug induced[9].

AP

Epidemiology

AP is the most common pancreatic disease associated with IBD; it is also the one associated with the highest morbidity. No definitive data are available regarding incidence of idiopathic AP in IBD. Figures obtained from small series estimate an incidence between 1.2% and 3.1%, higher in Crohn's disease than in ulcerative colitis[10,11]. The odd for AP seems to be as high as 4.3 and 2.1 times in Crohn's disease and in ulcerative respectively compared to the general population[12]. One study including patients with Crohn's disease after a 10 years follow up showed a significantly higher incidence of AP than in the general population (1.4% *vs* 0.007%)[13]. A large series of patients with Crohn's disease looked at etiology of AP: 21% and 15% of cases were due to biliary lithiasis and alcohol respectively; 12% and 13% of cases were associated with drugs or Crohn's disease to the duodenum; 8% of AP were defined as "idiopathic"[7]. In Seyrig's study idiopathic AP was only 1.5% (5 in 331 patients with IBD), but not all patients were investigated with ERCP[10]. In Heikius's study incidence of idiopathic AP was 3% in patients with IBD but 4% in the subgroup with Crohn's disease[13].

Diagnosis

The main issue in analyzing association between AP and IBD is the number of communal either clinical features either laboratory abnormalities. Two of following three criteria are required to make a diagnosis of AP: (1) typical abdominal pain; (2) threefold or more elevation of serum pancreatic enzymes; and (3) imaging confirming inflammation of the pancreatic gland[14]. However abdominal pain is also one of the cornerstone in the diagnosis of IBD and also typical symptoms of pancreatitis, like nausea, vomiting and diarrhea, may be present in Crohn's disease and ulcerative colitis. Moreover elevation of pancreatic enzymes may be found in patients with IBD with no clinical evidence of pancreatic disease[15]. Therefore an exacerbation of IBD might be mistaken for AP and *vice versa*.

Etiology

Biliary lithiasis: Incidence of biliary lithiasis increases in IBD compared to the general population, in particular in Crohn's disease with figures between 13% and 34%, where this variability depends either on study design either on selection criteria[16-19]. Nonetheless it seems also related to site and extension of intestinal disease (distal *vs* proximal ileum). Risk of biliary lithiasis is as high as three times in patients with extensive ilieal involvement, and this may be explained by a reduced enterohepatic circulation as a result of inflammation[18]. In fact, after surgery involving small bowel where the absorbing surface for biliary acids results reduced, incidence of biliary lithiasis is about 24%[20].

Drugs: Most of the drugs used to treat IBD may be associated with an increased risk of pancreatitis[21-23]. Azathioprine (AZA) and its active metabolite 6-mercaptopurine have AP as well-known side effect[24-27]. However other drugs as mesalamine, salazopyrin, metronidazole and steroids are reported to induce pancreatitis[28-35]. Toxic pancreatitis typically arises within the first weeks of treatment, presents with a mild clinical course and resolves quickly as soon as the drug is discontinued[21-23]. A prospective multicentric registration study has been recently carried out in 510 patients with IBD of which 338 with Crohn's disease, 117 with ulcerative colitis and 15 with unspecified colitis[36]. All patients were enrolled as soon as they started AZA; AP were diagnosed in accordance with international guidelines. AZA was stopped by 186 patients (36.5%). The most common cause of discontinuation was nausea (12.2%). Pancreatitis occurred in 37 patients (7.3%): 43% were admitted with a median inpatient length of stay of 5 d (10% had peripancreatic fluid collection, 24% had vomiting and 14% had fever). No patient underwent non-surgical or surgical interventions. At univariate and multivariate analysis smoking was found to be the strongest risk factor for AZA-induced AP[36]. However a meta-analysis showed that the risk of AZA-induced AP is very low[37].

However drug-induced pancreatitis could develop any time during the course of the treatment and it is not

always easy to establish a direct correlation between resolution of symptoms and drug withdrawal. Rechallenge test may be attempted in some cases of mild pancreatitis, as defined according to the CT severity index[38].

Duodenal obstruction: Finally, a cause of AP may be recognized in the duodenal involvement, found in 0.5%-4% of patients with Crohn's disease, often presenting with duodenal stenosis[39,40]. How a pancreatic obstruction can cause pancreatitis, it is not clear, but other conditions associated with similar anatomic alteration like stenosing cancer or annular pancreas can cause pancreatitis[41]. Overall, cases of pancreatitis associated with duodenal Crohn's disease are a small number in literature.

Natural history and management

No data are available regarding natural history of idiopathic AP in IBD but it has generally a benign course[9]. The youngest patient recorded is a 6 years old girl with underlying ulcerative colitis[42]. Whereas AP occurs in patients with Crohn's disease when they have an established diagnosis, in patients with ulcerative colitis it may either anticipate the diagnosis of colitis or arise later during the course of disease, or appear at the onset of the intestinal disease itself[43,44]. The record of a number of cases where an effective treatment of the underlying IBD produced improvement in the concomitant AP would support the theory of a direct pancreatic involvement in IBD, although it needs to be confirmed by longitudinal studies[45].

In most cases, AP in IBD patients are mild. The management should be the same of that in the general population, and involves supportive care with fluid therapy, electrolyte replacement, pain control, and nutritional support[46]. Treatment of active IBD in a patient with AP could be challenging because most of the drugs used for IBD (including total parenteral nutrition) can result in exacerbation of pancreatitis. A case of successful use of Infliximab for the treatment of idiopathic AP in a young male patient with a severe active Crohn's disease has been reported[47].

CP

IBD-related CP seems to be a different disease from calcific chronic pancreatitis (CCP) associated with alcohol abuse. First of all clinical presentation is different. In fact abdominal pain, the earliest and most common symptom in CCP (> 80%), is rarely present in IBD-related CP (16% in ulcerative colitis and 48% in Crohn's disease)[48]. Moreover CCP is more frequent in males whereas in IBD-CP ratio male/female is 3/10 in ulcerative colitis and 6/10 in Crohn's disease[48]. In addition, pseudocysts and pancreatic calcifications are typical in CCP but are almost absent in IBD-related CP[43]. Idiopathic CP in IBD is reported in 1.2%-1.5% of the cases, varying according with the diagnostic technique[43].

Exocrine pancreatic insufficiency

Pancreatic insufficiency is reported in patients with IBD between 18 and 80% of the cases[15,43,44]. Maconi *et al*[49] showed reduced fecal elastase level in 18% of patients with IBD. Heikius *et al*[50] found that 19% of not selected patients with IBD had signs of pancreatic insufficiency either by paraminobenzoic acid (PABA) test either by secretin-cerulein test. During a secretin-cerulein test Angelini *et al*[51] observed a decrease in plasma bicarbonate and serum enzymes in 35% of patients with Crohn's disease and 50% of patients with ulcerative colitis whereas isolated decrease in lipase was noted in 58% of patients with Crohn's disease and in 80% with ulcerative colitis. In a larger series Hegnohj *et al*[52] confirmed that output of amylase and lipase can be proved significantly reduced by Lundh meal test. Pancreatic insufficiency seemed to be related to extension of Crohn's disease, mainly if with ileal location and in active phase.

Asymptomatic pancreatic hyperenzymemia

Serum pancreatic enzymes may be elevated, normal or reduced in CP. In patients with IBD, elevation in amylase and lipase has been noticed along with alterations in the pancreatic duct system, mainly in patients with concomitant primary sclerosing cholangitis (PSC)[50-52]. Katz *et al*[53] described hyperamylasemia with no pancreatitis in 8% of patients affected by Crohn's disease. Tromm *et al*[54] found asymptomatic hyperamylasemia and hyperlipasemia in 16% of patients with Crohn's disease and 21% of patients with ulcerative colitis, in absence of pancreatic morphological abnormalities on ultrasound and with no association with disease activity, duration or medication. On the other hand, in Heikius's study elevation in serum amylase and lipase (in 11% and 7% for Crohn's disease and ulcerative colitis respectively) linked to a more extensive and active disease as well as a concomitant PSC[13]. Whether slightly elevated serum pancreatic enzymes may be an early indicator of significant pancreatic damage, it should be evaluated by longitudinal studies and extended follow.

Moreover, it must be considered that in case of absent urinary amylase the source of elevated amylase could be the salivary glands. Even lipase, that is known to be pancreatitis-specific, sometimes can be found elevated without any symptoms.

Duct system abnormality in IBD

What the prevalence is of alterations within the duct system in IBD, it is controversial, however there does not always seem to be correlation with pancreatic insufficiency. Abnormalities may be like wall irregularity, short stenosis or dilation of the main pancreatic duct. Heikius *et al*[50] found duct abnormalities in 8.4% (20 in 237) of patients with IBD, investigated with ERCP (gold standard imaging). However ERCP has limitations, being an invasive study and causing pancreatitis itself: As a

Table 1 Cardinal features for differential diagnosis

Characteristics	Drug-induced pancreatitis	Idiopatic IBD-associated pancreatitis	Autoimmune pancreatitis
Epidemiology	Pediatric patients Elderly patients Females > males	Males >> females	Male-female 2:1 (type 1) Male-female: 1:1 (type 2)
Age at presentation of pancreatitis	Any ages	20-40 yr	60-65 yr (type 1) 45-50 yr (type 2)
Clinical presentation	Abdominal pain	Abdominal pain Exocrine pancreatic insufficiency	Jaundice Mild abdominal pain Diabetes
Sierology	Elevated pancreatic enzymes Normal IgG4	Elevated pancreatic enzymes Normal IgG4	Normal or slightly elevated pancreatic enzymes Elevated IgG4 (in type 1)
Imaging	Normal pancreas or oedematous pancreatitis	Normal pancreas or oedematous pancreatitis Diffuse pancreatic enlargement or long/multiple MPD narrowing No calcifications or pseudocysts	Diffuse pancreatic enlargement or long/multiple MPD narrowing No calcifications or pseudocysts
Key point	Direct correlation between resolution of symptoms and drug withdrawal Symptoms recurrence with re-challenge test	Exclusion of other causes of pancreatitis (drug, lithiasis, alcohol...)	Rapid response to steroid with radiologically demonstrable resolution or marked clinical improvement

MPD: Main pancreatic duct; IBD: Inflammatory bowel disease.

result it would be offered to a selected population, what may underestimate results. In two studies secretine-enhanced MR Cholangiopancreatography has been used: Toda *et al*[55] assessed 79 patients with ulcerative colitis, with no pancreatic symptoms and with or without alteration in pancreatic function tests: 16.4% had lesions suggestive of CP, half of them with normal amylase. On the other hand Barthet *et al*[44] found duct abnormality in 11% of 79 patients with IBD, but did recognize neither a link with history of pancreatitis nor pancreatic insufficiency.

PATHOPHYSIOLOGY

Pathogenesis of IBD-related pancreatitis is unknown. An immune pathogenesis has been proposed, but whether idiopathic pancreatitis in IBD is a type of autoimmune pancreatitis (AIP), this is still debatable[56] (Table 1).

AIP is a rare, distinct and increasingly recognized disorder of presumed autoimmune etiology and is classified into two types, which are mainly differentiated by clinical and histological features[57]. Type 1 AIP is the most common type worldwide, affects predominantly males in the sixth decade and pancreas is involved as part of a systemic IgG4-positive disease, often associated with involvement of other organs, accompanying conditions such as sclerosing cholangitis, sclerosing sialadenitis and retroperitoneal fibrosis. Treatment with steroids is usually effective and when a rapid clinico-radiological response occurs, this could be considered as a diagnostic criterion[58]. Type 2 AIP, on the contrary, is not characterized by elevated IgG4 levels, affects younger patients equally distributed between genders and is frequently associated with IBD. Both types of AIP may present with painless jaundice, weight loss, diabetes and mild abdominal pain[57]. Clinical presentation of AP with high serum amylase is rare. Prevalence of IBD in patients with AIP seems to be higher than in the general population[59]. The relationship between IBD and AIP mainly involves ulcerative colitis and type 2 AIP. Specifically, the rate of ulcerative colitis in patients with AIP is up to 35%[60,61]. On the other hand, incidence of AIP in patients with IBD is low. A Japanese study conducted on 1751 patients with IBD found a 0.4% prevalence of AIP type 2 (n = 7)[62].

There is similarity between idiopathic pancreatitis in IBD (IBD-related pancreatitis) and AIP, either in imaging (alteration of duct system) either in pathology (when available). Duct system alteration with narrowing (segmental or diffuse) of the main pancreatic duct is a distinctive feature of AIP, nevertheless this is also a frequent finding in IBD-related pancreatitis[63,64]. Moreover in AIP as in IBD-related pancreatitis, calcifications and pseudocysts are absent. In a recent retrospective study of 71 patients with AIP, 4 (5.6%) had IBD (3 ulcerative colitis and 1 Crohn's disease) and IBD was diagnosed before or concomitantly to AIP[59]. In the intestinal specimen of one patient with ulcerative colitis IgG4 tissue infiltration was found, but was absent in the specimen of the only patient with Crohn's disease. In a French multicentric study as many as 38% of patients with AIP had a diagnosis of IBD[65]. These data suggest that AIP may be considered an EIM when found along with IBD. IgG4 elevation is considered typical of AIP: In 71%-92% of patients this tend to drop on steroids[63,64]. On the contrary in IBD-related pancreatitis IgG4 are almost always into the normal range. In the previously mentioned study of Barthet *et al*[44], 5 patients presented with diffuse narrowing of the duct system and 1 patient had pathology suggestive of AIP. Nevertheless all these patients had low level of IgG4, what may suggest that idiopathic pancreatitis found alongside IBD could be

considered a distinct but possibly closely related type of AIP. Moreover, in a recent retrospective study of 104 patients with AIP, 6 were founds to have ulcerative colitis, of which 2 showed colonic tissue with increased infiltration of IgG4-positive plasma cells whereas no infiltration was found in 24 patients with ulcerative colitis without AIP (P = 0.006): This suggests that ulcerative colitis may be an extra-pancreatic manifestation of AIP[66].

What also supports immune pathogenesis is the finding of antibodies anti-pancreas (PABs), mainly in Crohn's disease, where they are present in as many as 27%-39% of cases, whereas in ulcerative colitis they are rarely found (0%-5%)[9]. In IBD, PABS are usually found in about 20% of cases, but without association with pancreatic insufficiency or alteration of duct system[43,44]. Stöcker *et al*[67] described presence of PABs in 39% of patients with Crohn's disease and 4% with ulcerative colitis, whereas Seibold *et al*[68] found PABs in 27% of 212 patients with Crohn's disease. These antibodies are directed against exocrine pancreas and are located in the acinar lumen or in the acinar cells. However a clear pathogenetic role for PAB in IBD-related pancreatitis has not been proved yet and they don't seem to be connected with activity of disease neither with the onset of pancreatitis. Their presence may reflect immune deregulation caused by intestinal inflammation or cross reactivity, as well as for other auto-antibodies[69]. Another evidence supporting a direct association between IBD and pancreatic inflammation comes from a study conducted on animals, looking at MUC1, a transmembrane glycoprotein expressed on the apical surface of ductal epithelial cells in many organs and also present on colonic epithelium of humans with IBD[70]. MUC1 was abnormally expressed and hypoglycosylated and showed chemotactic properties for cells of the innate immune system thus promoting acute and chronic inflammation. In this paper, MUC1-specific T cells migrated not only to the colon, but also to the pancreas of mice with IBD, suggesting that pancreatic inflammation could be an EIM of IBD, characterized by pro-inflammatory, abnormal MUC1 expression[70].

Why pancreatic secretion is reduced in IBD, it is not clear. In Crohn's disease, one reason may be malnutrition, common in many patients, or a reduced hormone secretion by the intestinal wall, because of inflammation or consequences of scarring[13]. An important issue is whether increase of serum pancreatic enzymes is due to direct pancreatic damage or other causes as, for example, increased intestinal passage of intraluminal enzymes. Hyperenzymemia during more extensive and more severe intestinal disease supports the latter, as for analogy with hyperamylasemia found during other intestinal inflammatory conditions like ischemic colitis[13].

Other mechanisms have been speculated to explain a direct pancreatic damage in IBD. A single case was reported of a patient with Crohn's disease, found with a pancreatic granuloma on pathology: This may be the first case of histologically proved extra-intestinal localization of disease[71].

Finally, a possible coexistence of IBD with other autoimmune condition, like lupus mesenteric vasculitis, should be considered in case of pancreatitis, since IBD predominantly affects gastrointestinal tract, while lupus mesenteric vasculitis may also present extraintestinal involvement such as pancreatitis[72].

CONCLUSION

Patients with diagnosis of IBD have increased risk of either acute or CP. Causes are mainly a concomitant biliary lithiasis or drugs used in the treatment of IBD. However a number of pancreatitis, "idiopathic" by definition, should be considered EIM. Pancreatitis and IBD may have similar presentation therefore a pancreatic disease could not be recognized in patients with Crohn's disease and ulcerative colitis. However elevation of pancreatic enzymes only, without symptoms nor imaging suggestive of pancreatitis, is not an indication to start a treatment but should recommend follow up in first instance. On the other hand patients with IBD presenting with pancreatitis-like abdominal pain should be investigated to rule out a concomitant pancreatic disease.

REFERENCES

1 **Rothfuss KS**, Stange EF, Herrlinger KR. Extraintestinal manifestations and complications in inflammatory bowel diseases. *World J Gastroenterol* 2006; **12**: 4819-4831 [PMID: 16937463 DOI: 10.3748/wjg.v12.i30.4819]

2 **Ball WP**, Baggenstoss AH, Bargen JA. Pancreatic lesions associated with chronic ulcerative colitis. *Arch Pathol* (Chic) 1950; **50**: 347-358 [PMID: 15433704]

3 **Chapin LE**, Scudamore HH, Baggenstoss AH, Bargen JA. Regional enteritis: associated visceral changes. *Gastroenterology* 1956; **30**: 404-415 [PMID: 13305757]

4 **Legge DA**, Hoffman HN, Carlson HC. Pancreatitis as a complication of regional enteritis of the duodenum. *Gastroenterology* 1971; **61**: 834-837 [PMID: 5125685]

5 **Altman HS**, Phillips G, Bank S, Klotz H. Pancreatitis associated with duodenal Crohn's disease. *Am J Gastroenterol* 1983; **78**: 174-177 [PMID: 6829539]

6 **Matsumoto T**, Matsui T, Iida M, Nunoi K, Fujishima M. Acute pancreatitis as a complication of Crohn's disease. *Am J Gastroenterol* 1989; **84**: 804-807 [PMID: 2741892]

7 **Moolsintong P**, Loftus EV, Chari ST, Egan LJ, Tremaine WJ, Sandborn WJ. Acute pancreatitis in patients with Crohn's disease: clinical features and outcomes. *Inflamm Bowel Dis* 2005; **11**: 1080-1084 [PMID: 16306770 DOI: 10.1097/01.MIB.0000186485.30623.ad]

8 **Cardile S**, Randazzo A, Valenti S, Romano C. Pancreatic involvement in pediatric inflammatory bowel diseases. *World J Pediatr* 2015; **11**: 207-211 [PMID: 26253411 DOI: 10.1007/s12519-015-0029-z]

9 **Pitchumoni CS**, Rubin A, Das K. Pancreatitis in inflammatory bowel diseases. *J Clin Gastroenterol* 2010; **44**: 246-253 [PMID: 20087199 DOI: 10.1097/MCG.0b013e3181cadbe1]

10 **Seyrig JA**, Jian R, Modigliani R, Golfain D, Florent C, Messing B, Bitoun A. Idiopathic pancreatitis associated with inflammatory bowel disease. *Dig Dis Sci* 1985; **30**: 1121-1126 [PMID: 2866072 DOI: 10.1007/BF01314044]

11 **Niemelä S**, Lehtola J, Karttunen T, Lähde S. Pancreatitis in patients with chronic inflammatory bowel disease. *Hepatogastroenterology* 1989; **36**: 175-177 [PMID: 2753464]

12 **Rasmussen HH**, Fonager K, Sørensen HT, Pedersen L, Dahlerup JF, Steffensen FH. Risk of acute pancreatitis in patients with

chronic inflammatory bowel disease. A Danish 16-year nationwide follow-up study. *Scand J Gastroenterol* 1999; **34**: 199-201 [PMID: 10192201 DOI: 10.1080/00365529950173096]
13 **Heikius B**, Niemelä S, Lehtola J, Karttunen TJ. Elevated pancreatic enzymes in inflammatory bowel disease are associated with extensive disease. *Am J Gastroenterol* 1999; **94**: 1062-1069 [PMID: 10201484 DOI: 10.1111/j.1572-0241.1999.01015.x]
14 **Tenner S**. Initial management of acute pancreatitis: critical issues during the first 72 hours. *Am J Gastroenterol* 2004; **99**: 2489-2494 [PMID: 15571599 DOI: 10.1111/j.1572-0241.2004.40329.x]
15 **Bokemeyer B**. Asymptomatic elevation of serum lipase and amylase in conjunction with Crohn's disease and ulcerative colitis. *Z Gastroenterol* 2002; **40**: 5-10 [PMID: 11803494 DOI: 10.1055/s-2002-19636]
16 **Lorusso D**, Leo S, Mossa A, Misciagna G, Guerra V. Cholelithiasis in inflammatory bowel disease. A case-control study. *Dis Colon Rectum* 1990; **33**: 791-794 [PMID: 2202567 DOI: 10.1007/BF02052328]
17 **Hutchinson R**, Tyrrell PN, Kumar D, Dunn JA, Li JK, Allan RN. Pathogenesis of gall stones in Crohn's disease: an alternative explanation. *Gut* 1994; **35**: 94-97 [PMID: 8307459 DOI: 10.1136/gut.35.1.94]
18 **Lapidus A**, Bångstad M, Aström M, Muhrbeck O. The prevalence of gallstone disease in a defined cohort of patients with Crohn's disease. *Am J Gastroenterol* 1999; **94**: 1261-1266 [PMID: 10235204 DOI: 10.1111/j.1572-0241.1999.01076.x]
19 **Parente F**, Pastore L, Bargiggia S, Cucino C, Greco S, Molteni M, Ardizzone S, Porro GB, Sampietro GM, Giorgi R, Moretti R, Gallus S. Incidence and risk factors for gallstones in patients with inflammatory bowel disease: a large case-control study. *Hepatology* 2007; **45**: 1267-1274 [PMID: 17464998 DOI: 10.1002/hep.21537]
20 **Kangas E**, Lehmusto P, Matikainen M. Gallstones in Crohn's disease. *Hepatogastroenterology* 1990; **37**: 83-84 [PMID: 2312044]
21 **Lankisch PG**, Dröge M, Gottesleben F. Drug induced acute pancreatitis: incidence and severity. *Gut* 1995; **37**: 565-567 [PMID: 7489946 DOI: 10.1136/gut.37.4.565]
22 **Trivedi CD**, Pitchumoni CS. Drug-induced pancreatitis: an update. *J Clin Gastroenterol* 2005; **39**: 709-716 [PMID: 16082282 DOI: 10.1097/01.mcg.0000173929.60115.b4]
23 **Eland IA**, van Puijenbroek EP, Sturkenboom MJ, Wilson JH, Stricker BH. Drug-associated acute pancreatitis: twenty-one years of spontaneous reporting in The Netherlands. *Am J Gastroenterol* 1999; **94**: 2417-2422 [PMID: 10484002 DOI: 10.1111/j.1572-0241.1999.01367.x]
24 **Bermejo F**, Lopez-Sanroman A, Taxonera C, Gisbert JP, Pérez-Calle JL, Vera I, Menchén L, Martín-Arranz MD, Opio V, Carneros JA, Van-Domselaar M, Mendoza JL, Luna M, López P, Calvo M, Algaba A. Acute pancreatitis in inflammatory bowel disease, with special reference to azathioprine-induced pancreatitis. *Aliment Pharmacol Ther* 2008; **28**: 623-628 [PMID: 18513380 DOI: 10.1111/j.1365-2036.2008.03746.x]
25 **Weersma RK**, Peters FT, Oostenbrug LE, van den Berg AP, van Haastert M, Ploeg RJ, Posthumus MD, Homan van der Heide JJ, Jansen PL, van Dullemen HM. Increased incidence of azathioprine-induced pancreatitis in Crohn's disease compared with other diseases. *Aliment Pharmacol Ther* 2004; **20**: 843-850 [PMID: 15479355 DOI: 10.1111/j.1365-2036.2004.02197.x]
26 **Floyd A**, Pedersen L, Nielsen GL, Thorlacius-Ussing O, Sorensen HT. Risk of acute pancreatitis in users of azathioprine: a population-based case-control study. *Am J Gastroenterol* 2003; **98**: 1305-1308 [PMID: 12818274 DOI: 10.1111/j.1572-0241.2003.07459.x]
27 **Cappell MS**, Das KM. Rapid development of pancreatitis following reuse of 6-mercaptopurine. *J Clin Gastroenterol* 1989; **11**: 679-681 [PMID: 2584670 DOI: 10.1097/00004836-198912000-00017]
28 **Block MB**, Genant HK, Kirsner JB. Pancreatitis as an adverse reaction to salicylazosulfapyridine. *N Engl J Med* 1970; **282**: 380-382 [PMID: 4391490 DOI: 10.1056/NEJM197002122820710]
29 **Rubin R**. Sulfasalazine-induced fulminant hepatic failure and necrotizing pancreatitis. *Am J Gastroenterol* 1994; **89**: 789-791 [PMID: 7909645]
30 **Garau P**, Orenstein SR, Neigut DA, Kocoshis SA. Pancreatitis associated with olsalazine and sulfasalazine in children with ulcerative colitis. *J Pediatr Gastroenterol Nutr* 1994; **18**: 481-485 [PMID: 7520936 DOI: 10.1097/00005176-199405000-00015]
31 **Plotnick BH**, Cohen I, Tsang T, Cullinane T. Metronidazole-induced pancreatitis. *Ann Intern Med* 1985; **103**: 891-892 [PMID: 2415031 DOI: 10.7326/0003-4819-103-6-891]
32 **Nigwekar SU**, Casey KJ. Metronidazole-induced pancreatitis. A case report and review of literature. *JOP* 2004; **5**: 516-519 [PMID: 15536294]
33 **Tsesmeli NE**, Giannoulis KE, Savopoulos CG, Vretou EE, Ekonomou IA, Giannoulis EK. Acute pancreatitis as a possible consequence of metronidazole during a relapse of ulcerative colitis. *Eur J Gastroenterol Hepatol* 2007; **19**: 805-806 [PMID: 17700268 DOI: 10.1097/MEG.0b013e328133f2fb]
34 **Kaplan MH**, Dreiling DA. Steroids revisited. II. Was cortisone responsible for the pancreatitis? *Am J Gastroenterol* 1977; **67**: 141-147 [PMID: 324269]
35 **Caldarola V**, Hassett JM, Hall AH, Bronstein AB, Kulig KW, Rumack BH. Hemorrhagic pancreatitis associated with acetaminophen overdose. *Am J Gastroenterol* 1986; **81**: 579-582 [PMID: 3717123]
36 **Teich N**, Mohl W, Bokemeyer B, Bündgens B, Büning J, Miehlke S, Hüppe D, Maaser C, Klugmann T, Kruis W, Siegmund B, Helwig U, Weismüller J, Drabik A, Stallmach A. Azathioprine-induced Acute Pancreatitis in Patients with Inflammatory Bowel Diseases--A Prospective Study on Incidence and Severity. *J Crohns Colitis* 2016; **10**: 61-68 [PMID: 26468141 DOI: 10.1093/ecco-jcc/jjv188]
37 **Timmer A**, Patton PH, Chande N, McDonald JW, MacDonald JK. Azathioprine and 6-mercaptopurine for maintenance of remission in ulcerative colitis. *Cochrane Database Syst Rev* 2016; **5**: CD000478 [PMID: 27192092 DOI: 10.1002/14651858.CD000478.pub4]
38 **Nitsche CJ**, Jamieson N, Lerch MM, Mayerle JV. Drug induced pancreatitis. *Best Pract Res Clin Gastroenterol* 2010; **24**: 143-155 [PMID: 20227028 DOI: 10.1016/j.bpg.2010.02.002]
39 **Spiess SE**, Braun M, Vogelzang RL, Craig RM. Crohn's disease of the duodenum complicated by pancreatitis and common bile duct obstruction. *Am J Gastroenterol* 1992; **87**: 1033-1036 [PMID: 1642205]
40 **Eisner TD**, Goldman IS, McKinley MJ. Crohn's disease and pancreatitis. *Am J Gastroenterol* 1993; **88**: 583-586 [PMID: 8470642]
41 **Creutzfeldt W**, Lankisch PG. Acute pancreatitis: etiology and pathogenesis. In: Berk J, Haubrich W, Kalser M, editors. Bockus Gastroenterology. 4th ed. Philadelphia: WB Saunders Co, 1985: 3971-3992
42 **Halabi IM**. Ulcerative colitis presenting as acute pancreatitis in a 6-year-old patient. *Gastroenterol Hepatol* (NY) 2009; **5**: 429-430 [PMID: 20574502]
43 **Barthet M**, Hastier P, Bernard JP, Bordes G, Frederick J, Allio S, Mambrini P, Saint-Paul MC, Delmont JP, Salducci J, Grimaud JC, Sahel J. Chronic pancreatitis and inflammatory bowel disease: true or coincidental association? *Am J Gastroenterol* 1999; **94**: 2141-2148 [PMID: 10445541 DOI: 10.1111/j.1572-0241.1999.01287.x]
44 **Barthet M**, Lesavre N, Desplats S, Panuel M, Gasmi M, Bernard JP, Dagorn JC, Grimaud JC. Frequency and characteristics of pancreatitis in patients with inflammatory bowel disease. *Pancreatology* 2006; **6**: 464-471 [PMID: 16847384 DOI: 10.1159/000094564]
45 **Papanikolaou IS**, Liatsos C, Dourakis SS, Mavrogiannis C. A case of acute pancreatitis associated with Crohn's disease. *Ann Saudi Med* 2002; **22**: 70-72 [PMID: 17259771]
46 **Ramos LR**, Sachar DB, DiMaio CJ, Colombel JF, Torres J. Inflammatory Bowel Disease and Pancreatitis: A Review. *J Crohns Colitis* 2016; **10**: 95-104 [PMID: 26351384 DOI: 10.1093/ecco-jcc/jjv153]
47 **Triantafillidis JK**, Cheracakis P, Hereti IA, Argyros N, Karra E. Acute idiopathic pancreatitis complicating active Crohn's disease: favorable response to infliximab treatment. *Am J Gastroenterol* 2000; **95**: 3334-3336 [PMID: 11095387 DOI: 10.1111/j.1572-0241.2000.03332.x]
48 **Steer ML**, Waxman I, Freedman S. Chronic pancreatitis. *N Engl J Med* 1995; **332**: 1482-1490 [PMID: 7739686 DOI: 10.1056/NEJM199506013322206]

49 **Maconi G**, Dominici R, Molteni M, Ardizzone S, Bosani M, Ferrara E, Gallus S, Panteghini M, Bianchi Porro G. Prevalence of pancreatic insufficiency in inflammatory bowel diseases. Assessment by fecal elastase-1. *Dig Dis Sci* 2008; **53**: 262-270 [PMID: 17530399 DOI: 10.1007/s10620-007-9852-y]

50 **Heikius B**, Niemelä S, Lehtola J, Karttunen T, Lähde S. Pancreatic duct abnormalities and pancreatic function in patients with chronic inflammatory bowel disease. *Scand J Gastroenterol* 1996; **31**: 517-523 [PMID: 8734352 DOI: 10.3109/00365529609006775]

51 **Angelini G**, Cavallini G, Bovo P, Brocco G, Castagnini A, Lavarini E, Merigo F, Tallon N, Scuro LA. Pancreatic function in chronic inflammatory bowel disease. *Int J Pancreatol* 1988; **3**: 185-193 [PMID: 3361159]

52 **Hegnhøj J**, Hansen CP, Rannem T, Søbirk H, Andersen LB, Andersen JR. Pancreatic function in Crohn's disease. *Gut* 1990; **31**: 1076-1079 [PMID: 1698692 DOI: 10.1136/gut.31.9.1076]

53 **Katz S**, Bank S, Greenberg RE, Lendvai S, Lesser M, Napolitano B. Hyperamylasemia in inflammatory bowel disease. *J Clin Gastroenterol* 1988; **10**: 627-630 [PMID: 2466072 DOI: 10.1097/00004836-198812000-00010]

54 **Tromm A**, Höltmann B, Hüppe D, Kuntz HD, Schwegler U, May B. [Hyperamylasemia, hyperlipasemia and acute pancreatitis in chronic inflammatory bowel diseases]. *Leber Magen Darm* 1991; **21**: 15-16, 19-22 [PMID: 1709251]

55 **Toda N**, Akahane M, Kiryu S, Matsubara Y, Yamaji Y, Okamoto M, Minagawa N, Ohgi K, Komatsu Y, Yahagi N, Yoshida H, Kawabe T, Ohtomo K, Omata M. Pancreas duct abnormalities in patients with ulcerative colitis: a magnetic resonance pancreatography study. *Inflamm Bowel Dis* 2005; **11**: 903-908 [PMID: 16189420 DOI: 10.1097/01.MIB.0000183419.17563.17]

56 **Barthet M**. Acute pancreatitis: an emerging presentation for autoimmune pancreatitis in patients with inflammatory bowel disease. *Gastroenterol Hepatol* (NY) 2009; **5**: 431-433 [PMID: 20574503]

57 **Madhani K**, Farrell JJ. Autoimmune Pancreatitis: An Update on Diagnosis and Management. *Gastroenterol Clin North Am* 2016; **45**: 29-43 [PMID: 26895679 DOI: 10.1016/j.gtc.2015.10.005]

58 **Pezzilli R**, Pagano N. Pathophysiology of autoimmune pancreatitis. *World J Gastrointest Pathophysiol* 2014; **5**: 11-17 [PMID: 24891971 DOI: 10.4291/wjgp.v5.i1.11]

59 **Ravi K**, Chari ST, Vege SS, Sandborn WJ, Smyrk TC, Loftus EV. Inflammatory bowel disease in the setting of autoimmune pancreatitis. *Inflamm Bowel Dis* 2009; **15**: 1326-1330 [PMID: 19235915 DOI: 10.1002/ibd.20898]

60 **Srinath AI**, Gupta N, Husain SZ. Probing the Association of Pancreatitis in Inflammatory Bowel Disease. *Inflamm Bowel Dis* 2016; **22**: 465-475 [PMID: 26535870 DOI: 10.1097/MIB.0000000000000611]

61 **Roque Ramos L**, DiMaio CJ, Sachar DB, Atreja A, Colombel JF, Torres J. Autoimmune pancreatitis and inflammatory bowel disease: Case series and review of literature. *Dig Liver Dis* 2016; **48**: 893-898 [PMID: 27260331 DOI: 10.1016/j.dld.2016.05.008]

62 **Ueki T**, Kawamoto K, Otsuka Y, Minoda R, Maruo T, Matsumura K, Noma E, Mitsuyasu T, Otani K, Aomi Y, Yano Y, Hisabe T, Matsui T, Ota A, Iwashita A. Prevalence and clinicopathological features of autoimmune pancreatitis in Japanese patients with inflammatory bowel disease. *Pancreas* 2015; **44**: 434-440 [PMID: 25469544 DOI: 10.1097/MPA.0000000000000261]

63 **Yoshida K**, Toki F, Takeuchi T, Watanabe S, Shiratori K, Hayashi N. Chronic pancreatitis caused by an autoimmune abnormality. Proposal of the concept of autoimmune pancreatitis. *Dig Dis Sci* 1995; **40**: 1561-1568 [PMID: 7628283 DOI: 10.1007/BF02285209]

64 **Ito T**, Nakano I, Koyanagi S, Miyahara T, Migita Y, Ogoshi K, Sakai H, Matsunaga S, Yasuda O, Sumii T, Nawata H. Autoimmune pancreatitis as a new clinical entity. Three cases of autoimmune pancreatitis with effective steroid therapy. *Dig Dis Sci* 1997; **42**: 1458-1468 [PMID: 9246047 DOI: 10.1023/A: 1018862626221]

65 **Barthet M**, Rebours V, Buscail L, Palazzo L, Alric L. Autoimmune pancreatitis: results of a multicenter study from the French pancreatic club. *Gut* 2007; **39** (S1): A177

66 **Park SH**, Kim D, Ye BD, Yang SK, Kim JH, Yang DH, Jung KW, Kim KJ, Byeon JS, Myung SJ, Kim MH, Kim JH. The characteristics of ulcerative colitis associated with autoimmune pancreatitis. *J Clin Gastroenterol* 2013; **47**: 520-525 [PMID: 23426453 DOI: 10.1097/MCG.0b013e31827fd4a2]

67 **Stöcker W**, Otte M, Ulrich S, Normann D, Finkbeiner H, Stöcker K, Jantschek G, Scriba PC. Autoimmunity to pancreatic juice in Crohn's disease. Results of an autoantibody screening in patients with chronic inflammatory bowel disease. *Scand J Gastroenterol Suppl* 1987; **139**: 41-52 [PMID: 3324299 DOI: 10.3109/00365528709089774]

68 **Seibold F**, Mörk H, Tanza S, Müller A, Holzhüter C, Weber P, Scheurlen M. Pancreatic autoantibodies in Crohn's disease: a family study. *Gut* 1997; **40**: 481-484 [PMID: 9176075 DOI: 10.1136/gut.40.4.481]

69 **Targan SR**, Landers CJ, Cobb L, MacDermott RP, Vidrich A. Perinuclear anti-neutrophil cytoplasmic antibodies are spontaneously produced by mucosal B cells of ulcerative colitis patients. *J Immunol* 1995; **155**: 3262-3267 [PMID: 7673739]

70 **Kadayakkara DK**, Beatty PL, Turner MS, Janjic JM, Ahrens ET, Finn OJ. Inflammation driven by overexpression of the hypoglycosylated abnormal mucin 1 (MUC1) links inflammatory bowel disease and pancreatitis. *Pancreas* 2010; **39**: 510-515 [PMID: 20084048 DOI: 10.1097/MPA.0b013e3181bd6501]

71 **Gschwantler M**, Kogelbauer G, Klose W, Bibus B, Tscholakoff D, Weiss W. The pancreas as a site of granulomatous inflammation in Crohn's disease. *Gastroenterology* 1995; **108**: 1246-1249 [PMID: 7698591 DOI: 10.1016/0016-5085(95)90226-0]

72 **Katsanos KH**, Voulgari PV, Tsianos EV. Inflammatory bowel disease and lupus: a systematic review of the literature. *J Crohns Colitis* 2012; **6**: 735-742 [PMID: 22504032 DOI: 10.1016/j.crohns.2012.03.005]

P- Reviewer: Akyuz F, Hokama A, Sipos F **S- Editor**: Ji FF
L- Editor: A **E- Editor**: Lu YJ

World Journal of Gastrointestinal Pathophysiology

Submit a Manuscript: http://www.wjgnet.com/esps/
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.4291/wjgp.v7.i3.283

World J Gastrointest Pathophysiol 2016 August 15; 7(3): 283-287
ISSN 2150-5330 (online)

ORIGINAL ARTICLE

Retrospective Cohort Study

"Magic" of our gastric cancer results on perioperative chemotherapy

Carlos León-Espinoza, Fernando López-Mozos, Roberto Marti-Obiol, Marina Garces-Albir, Joaquin Ortega-Serrano

Carlos León-Espinoza, Fernando López-Mozos, Roberto Marti-Obiol, Marina Garces-Albir, Joaquin Ortega-Serrano, Department of Digestive and General Surgery, Hospital Clínico Universitario de Valencia, 46010 Valencia, Spain

Author contributions: All authors contributed equally to this work.

Institutional review board statement: Institutional Review Board's approval is unnecessary for this manuscript, because of its retrospective character. Individual subjects cannot be identified in any way by the data collected.

Informed consent statement: Informed consent is unnecessary for this manuscript, because of its retrospective character. Individual subjects cannot be identified in any way by the data collected.

Conflict-of-interest statement: All authors declare having no conflicts of interest of any kind.

Data sharing statement: The original anonymous dataset is available on request from the corresponding author at garalma@hotmail.com.

Manuscript source: Invited manuscript

Correspondence to: Marina Garces-Albir, MD, PhD, Department of Digestive and General Surgery, Hospital Clínico Universitario de Valencia, Avd. Blasco Ibañez, 17, 46010 Valencia, Spain. garalma@hotmail.com
Telephone: +34-96-2051717
Fax: +34-96-3868864

Received: March 22, 2016
Peer-review started: March 23, 2016
First decision: April 15, 2016
Revised: April 28, 2016
Accepted: May 17, 2016
Article in press: May 27, 2016
Published online: August 15, 2016

Abstract

AIM: To determine reproducibility of perioperative chemotherapy for gastric cancer (GC) on our settings by identifying patient's overall survival and comparing them to larger studies.

METHODS: Retrospective analysis of our series, where we present our eleven-year's experience on GC managed according to perioperative approach of three preoperative chemotherapy cycles followed by surgery and finally three postoperative chemotherapy cycles. Chemotherapic scheme used was Xelox (Oxaliplatin and Capecitabine). Epidemiologic parameters as well as surgical variables were analysed, presented, and compared to other series with similar approaches. Survival was estimated by Kaplan Meier/log rank method and also compared to these studies.

RESULTS: Mean age was 65 years old. Overall survival in our series was 37.7%, similar to other groups using perioperative schemes. Mortality was 4% and morbidity 30%, which are also similar to those groups. Survival curves were compared to larger studies, finding similarities on them. Subgroup survival analysis between chemotherapy responders and non-responders didn't reach statically significant differences.

CONCLUSION: Perioperative chemotherapic scheme can be reproduced on our setting with good results and

without increasing morbidity or mortality.

Key words: Stomach neoplasms; Perioperative period; Chemotherapy

Core tip: This is a retrospective study that evaluates and compares survival after perioperative chemotherapy on gastric cancer patients managed similarly in other settings. We confirmed reproducibility of this scheme on smaller settings than classic studies.

León-Espinoza C, López-Mozos F, Marti-Obiol R, Garces-Albir M, Ortega-Serrano J. "Magic" of our gastric cancer results on perioperative chemotherapy. *World J Gastrointest Pathophysiol* 2016; 7(3): 283-287 Available from: URL: http://www.wjgnet.com/2150-5330/full/v7/i3/283.htm DOI: http://dx.doi.org/10.4291/wjgp.v7.i3.283

INTRODUCTION

Global gastric cancer's (GC's) incidence has notably change in western countries during the last decades. Nowadays, GC stands for about 6.8% of all cancers, and represents about 8.8% of all deaths for cancer. Among all cancers, it is the fourth on incidence in men and the fifth in women, and is the third and fifth cause of cancer deaths in men and women respectively[1].

GC's survival mostly depends on stage at time of diagnosis. The 5-year survival after radical surgery on a localized GC is about 70%-95%, while metastatic disease's survival reaches about 3-4 mo.

Surgery is the only curative treatment for GC. Either total or partial gastrectomies associated to lymphadenectomy are the gold standard. Local, regional and distant recurrence may occur despite a correct oncologic resection. Therefore, medical therapy against micrometastasis is crucial. Several treatment schemes have developed during the pre-, peri- and post-operatory phases in order to improve long term survival. Perioperative treatment is a common scheme with proofed efficacy, but concern about disease progression by delaying surgery and increased surgical morbidity has also developed.

In our unit we use a pre- and post-operatory chemotherapy (QT) scheme following recommendations published by Cunningham *et al*[2], assuming it has long-term benefits, with an acceptable morbidity and mortality rates.

This retrospective review aimed to describe the overall survival as our primary endpoint and compare it with previous publications. Secondary endpoints were: Analysis of clinical response after perioperative chemotherapy, postoperative morbidity/mortality and re-intervention rates, comparing them to papers published previously by other groups using a similar approach.

Table 1 Patient's age, gender and clinical stage

		n	%
Gender	Male	74	74.7
	Female	25	25.3
Clinical stage	Ⅱ	24	24.2
	Ⅲa	57	57.6
	Ⅲb	18	18.2
Age	65 ± 9 yr		

MATERIALS AND METHODS

Patients included in our database were introduced prospectively since 2004, after multidisciplinary esophagogastric unit was created. From a total of 211 patients diagnosed of GC, ninety-nine received perioperative QT and were analyzed. The excluded 112 patients were either T1-2 N0 M0 or had metastatic disease. Epidemiologic variables are described on Table 1, including Age, gender, and clinical stage.

GC's diagnosis was based on endoscopic-biopsy results. We assessed the extension by performing a CT scan and endoscopic ultrasonography (EUS). Some particular cases needed further explorations as MRI, PET-CT or diagnostic laparoscopy. TNM sixth edition classification was used to determine clinical stage[3].

Once identified, we included patients catalogued as T3-T4 and/or N (+), without distant disease and started perioperative Chemotherapy based on Xelox (Oxaliplatin and Capecitabine). They received 3 cycles preoperatively and 3 cycles at least 6 wk after surgery, depending on performance status.

Variables as surgical technique, surgical intention, post-operatory morbidity, re-intervention rate and post-operatory mortality (starting on surgery until 30 d after hospital dismiss) were analyzed. Reassessment of stage was performed 2 wk after last pre-operatory chemotherapy cycle, and classified as follows: (1) complete regression; (2) partial regression; (3) stable disease/no response; and (4) progressive disease, following recommendations published on RECIST guideline (version 1.1)[4].

Survival estimation was analyzed by Kaplan-Meier/log rank method comparing subgroups of patients with response (Complete and Partial regression) and without response (Stable or progressive disease) to pre-operative QT, and comparing results to similar previous trials.

RESULTS

Patient's variables

Mean age on our group was 65 ± 9 years, divided on 74 (74.7%) male and 25 female (25.3%). Distribution of preoperative clinical stage is specified on Table 1.

Surgery

Open surgery was the rule for every patient who received

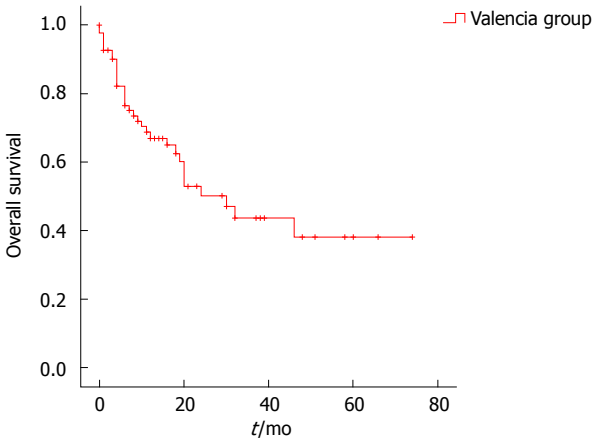


Figure 1 Overall survival estimated by Kaplan-Meier in our group.

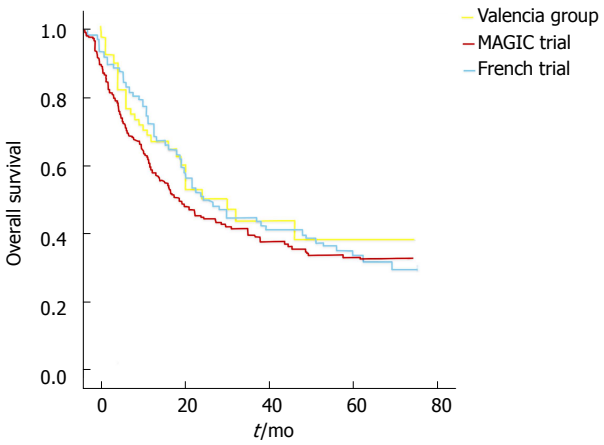


Figure 2 Comparison of survival curves of Valencia group with MAGIC and French Group trials.

preoperative QT. Surgery with curative intent was practiced on 78 patients, while palliative procedures were practiced on 21 patients.

Among all surgeries, we identified 34 distal gastrectomies, 53 total gastrectomies, 3 proximal gastrectomies, 3 gastro-jejunostomies and 6 exploratory laparotomies. D2 Lymphadenectomy was the rule for curative intent surgery. Extended resections for T4 tumors were performed on 20 patients.

Preoperative response

Eighty percent of patients who received preoperative QT completed the programmed three cycles. Uncompleted cycles were due to several reasons (toxicity, patient decision, *etc.*). After reassessment we found that 4 patients had a complete response, 55 had partial response, 35 had a stable disease, and 5 presented progressive disease, following the criteria of RECIST Guidelines[4].

Post-operatory morbidity

After surgery, 30 patients had complications, which represented 30% of study group. Most of them were either superficial surgical site infections (9 patients) or cardio-pulmonary disorders (10 patients).

Nineteen patients suffered from major surgical morbidities, finding six anastomotic leaks, two duodenal stump leaks, two acute intestinal obstructions, two iatrogenic small bowel perforations, four left sub-phrenic abscesses and three hemoperitoneums.

Due to mayor post-operatory complications, 6 patients underwent reoperation (Reoperation rate: 6%): One anastomotic leak, two iatrogenic perforations, one obstruction, one hemoperitoneum and one sub-phrenic abscess (managed initially percutaneous without improvement). The other patients having major surgical complications underwent medical, percutaneous or endoscopic management.

Post-operatory mortality

Mortality on post-operatory period was 4% (4 patients), secondary to peritonitis and multisystem failure after mayor surgical complications.

Follow up, survival and recurrence

Mean follow up in patients who underwent surgery was 16.1 mo. Overall survival by Kaplan-Meier method was analyzed and represented on Figure 1. Mean survival was 37.7 ± 4.3 mo.

We also compared our survival curves with the ones published on MAGIC trial and French Group trial, finding similar results (Figure 2).

We compared subgroup survival, based on preoperative chemotherapy response. Results are compared on Figure 3. This analysis failed to reach statistical differences ($P = 0.1$). During this time, fifteen recurrences were occurred. Between responders, 8 recurrences we detected, while on non-responders there were 7 recurrences.

DISCUSSION

Many strategies for improving survival on GC have been proposed during last years. Initially, surgery was considered the only beneficial treatment on GC management. The development of newer chemotherapic agents and their combination have opened some perspectives on survival improvement. Moreover, radiotherapy has also evolved, limiting side effects and minimizing radiation fields, becoming also an interesting alternative. When deciding on which therapy to choose for improving survival and the proper time to apply it, we must balance their benefits and disadvantages.

Neoadjuvant therapy has the theoretical advantage of allowing *in vivo* chemo-sensitivity tests, thus facilitating the choice of the most appropriate postoperative regimens. It can also increase compliance rate and maintain better nutritional status, while eliminating hidden micrometastases and reducing tumor size[5].

On the other hand, preoperative therapy could delay surgical treatment increasing the risk of disease progression and increasing post operatory morbidity and mortality rates.

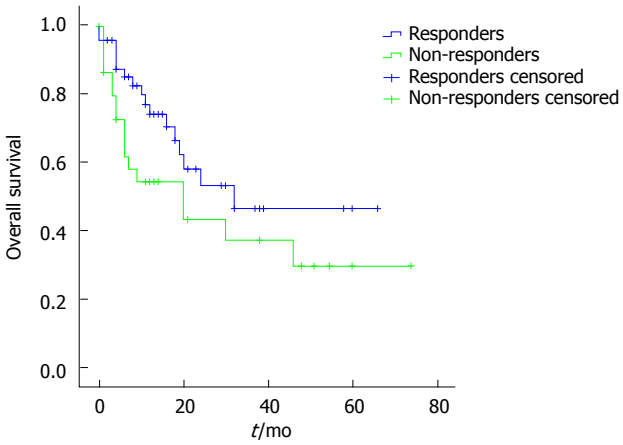


Figure 3 Overall survival in responder and non-responder subgroups in our study.

Another approach to chemotherapy is the post-operatory treatment. Adjuvant therapy has the advantage of immediate surgical excision with no chance of local progression. Nonetheless, surgery as first step of treatment has it's own disadvantages. It can stimulate neoplastic proliferation, shorten tumor's doubling time, decrease circulation/chemotherapy penetration on surgical bed and increase probability of chemo-resistant cellular clone development[6]. Adjuvant therapy also depends on performance status, which can be importantly altered after surgery.

Several meta-analyses have been conducted for identifying which therapy contributes better to both overall and disease-free survival. Initial studies on adjuvant therapy in western countries showed no benefits on overall or disease-free survival[7,8]. Nevertheless, on Asia, studies have concluded some benefits on patients receiving chemotherapy based on mitomycin (MMC), showing prolonged 5-year survival rate, becoming later MMC ± fluorouracile (FU) the standard regimen[9]. Further RCT have shown some advantages on adjuvant therapy based on capecitabine plus oxaliplatin in patients with stage Ⅱ-ⅢB GC[10].

Radiotherapy in addition to adjuvant chemotherapy is an approach supported by McDonald *et al*[11] on the Intergroup 0116 trial. It demonstrated overall and disease-free survival benefits and has been ratified by many smaller studies. Nevertheless, surgical quality on this trial has been criticized, based on low D2 or D1 rate. It has been also reported that adjuvant radio-chemotherapy has benefits over chemotherapy alone[12]. American NCCN guidelines recommend this regimen on patient's tumors recognized as T2 or higher tumors, which didn't receive preoperative treatment.

Perioperative chemotherapy is another regimen studied and supported. It was tested on Cunningham's MAGIC trial, and details are described on his paper[2]. It has two phases; the first is a 3-cycle preoperative therapy, followed by surgery and finally another 3-cycle postoperative therapy.

Many benefits were recognized on this trial, starting from increased R0 surgeries without increasing complication rate, and increased overall and disease free survival.

A French trial[13] comparing perioperative chemotherapy with surgery alone, showed results similar to the ones obtained on MAGIC trial, confirming reproducibility of the first trial. As our team have surgical similarities with French and English groups and believe on potential benefits of preoperative chemotherapy, we included patients on this regimen since 2006.

On MAGIC trial primary endpoint was overall survival, reaching a significant improvement with a hazard ratio of 0.75 (95%CI: 0.6-0.93; P = 0.009) on favor of perioperative chemotherapy group. Five-year survival also showed improvement, prolonging survival from 23% on surgery alone group, to 36% on perioperative group.

In the last years some perspectives have arisen on treatment of advanced GC. Results on locally advanced GC and peritoneal carcinomatosis (PC) secondary to GC have still disappointing results, but researches on selected patients with advanced GC have been conducted during last years.

Due to advances on PC surgery, patients with CG have been included on protocols for complete cytoreductive surgery (CRS) and hyperthermic intraperitoneal chemotherapy, with encouraging results. Glehen *et al*[14] conducted a multicenter retrospective study on France, finding benefits on selected cases of PC from gastric origin, reaching a 13% on 5 year overall survival. Nevertheless, they recommend strict inclusion criteria such as limited seeding extension (PCI < 12), following response to neoadjuvant chemotherapy and with no diffuse small bowel involvement[14]. More recently an American group conducted a randomized controlled trial[15], comparing overall survival between patients following CRS and HIPEC and patients treated only with systemic chemotherapy. They also found overall survival benefits on CRS + HIPEC group, but emphasized on careful patient selection.

On our revision, overall survival was the primary endpoint and we found a 37.7%, which is similar to survival found by Cunningham on MAGIC trial, confirming reproducibility of MAGIC trial, and reassuring our confidence on the perioperative approach. We think that our follow up should be longer to let us conclude survival benefits.

Our complication rate (30%) reached percentages similar than other series, as Dutch Gastric Cancer trial (43%), German Gastric Cancer trial (22%) or MRC Gastric Cancer Surgical trial (46%).

Post-operatory mortality rate of 4% on our series is slightly inferior to previous published trials, which stay between 5%-13%. We performed subgroup analysis in order to establish if pre-operative tumor response to chemotherapy has implications on long-term survival rates. We couldn't reach significant differences between both groups but detected a slight benefit in favor of responders. If this difference was real, patient might benefit of a different post-operative QT scheme. Further studies should be design to investigate on this issue.

In conclusion, perioperative therapy on GC in our institution, has similar survival rates as previous published

papers, without increasing complication or mortality rates, supporting safe reproducibility of this approach. Survival benefits among patients categorized by preoperative response have not reached statically significant differences. Prolonged follow-up and increasing number of patients would be appropriate to met stronger conclusions.

COMMENTS

Background

Gastric cancer (GC) is one of the most prevalent and lethal neoplasia. Different approaches are currently followed around the world, with different results. Since the authors' group started applying the MAGIC trial with the intent to reproduce their results in different settings and compare the final results with theirs.

Research frontiers

The authors' group supports perioperative approach as the best one available nowadays. Since started applying this scheme, the authors believed in its benefits and started on data collect for posterior analysis and local validation of this approach.

Innovations and breakthroughs

The theoretical risks of preoperative chemotherapy affecting surgical results, doesn't reflect neither in morbidity nor in mortality rates. Survival rates obtained are similar to the ones published before, reassuring reproducibility of this scheme.

Applications

Application of this scheme in smaller settings as the authors, show similar results as emblematic studies of perioperative approach.

Terminology

Perioperative chemotherapy is an oncologic approach consistent with preoperative chemotherapy, followed by surgery and finally post-operative chemotherapy. On GC, this approach was described by Cunningham *et al* on his MAGIC trial, and supported by several posterior studies.

Peer-review

The author of this retrospective analysis aimed to evaluate effect of perioperative chemotherapy for GC. And authors found the conclusion that "perioperative chemotherapic scheme can be reproduced on the setting with good results and without increasing morbidity or mortality". The authors raised up a valuable issue in this study. Also there were enough results to support opinion of authors.

REFERENCES

1 **Torre LA**, Bray F, Siegel RL, Ferlay J, Lortet-Tieulent J, Jemal A. Global cancer statistics, 2012. *CA Cancer J Clin* 2015; **65**: 87-108 [PMID: 25651787 DOI: 10.3322/caac.21262]

2 **Cunningham D**, Allum WH, Stenning SP, Thompson JN, Van de Velde CJ, Nicolson M, Scarffe JH, Lofts FJ, Falk SJ, Iveson TJ, Smith DB, Langley RE, Verma M, Weeden S, Chua YJ. Perioperative chemotherapy versus surgery alone for resectable gastroesophageal cancer. *N Engl J Med* 2006; **355**: 11-20 [PMID: 16822992 DOI: 10.1056/NEJMoa055531]

3 **Green FL**, Page DL, Fleming ID, Fritz AG, Balch CM, Haller DG, Morrow M. AJCC Cancer Staging Manual. 6th ed. New York: Springer, 2002: 99-106 [DOI: 10.1007/978-1-4757-3656-4]

4 **Eisenhauer EA**, Therasse P, Bogaerts J, Schwartz LH, Sargent D, Ford R, Dancey J, Arbuck S, Gwyther S, Mooney M, Rubinstein L, Shankar L, Dodd L, Kaplan R, Lacombe D, Verweij J. New response evaluation criteria in solid tumours: revised RECIST guideline (version 1.1). *Eur J Cancer* 2009; **45**: 228-247 [PMID: 19097774 DOI: 10.1016/j.ejca.2008.10.026]

5 **D'Ugo D**, Rausei S, Biondi A, Persiani R. Preoperative treatment and surgery in gastric cancer: friends or foes? *Lancet Oncol* 2009; **10**: 191-195 [PMID: 19185837 DOI: 10.1016/S1470-2045(09)70021-X]

6 **Fisher B**, Gunduz N, Coyle J, Rudock C, Saffer E. Presence of a growth-stimulating factor in serum following primary tumor removal in mice. *Cancer Res* 1989; **49**: 1996-2001 [PMID: 2702641]

7 **Coombes RC**, Schein PS, Chilvers CE, Wils J, Beretta G, Bliss JM, Rutten A, Amadori D, Cortes-Funes H, Villar-Grimalt A. A randomized trial comparing adjuvant fluorouracil, doxorubicin, and mitomycin with no treatment in operable gastric cancer. International Collaborative Cancer Group. *J Clin Oncol* 1990; **8**: 1362-1369 [PMID: 2199622]

8 **Macdonald JS**, Fleming TR, Peterson RF, Berenberg JL, McClure S, Chapman RA, Eyre HJ, Solanki D, Cruz AB, Gagliano R. Adjuvant chemotherapy with 5-FU, adriamycin, and mitomycin-C (FAM) versus surgery alone for patients with locally advanced gastric adenocarcinoma: A Southwest Oncology Group study. *Ann Surg Oncol* 1995; **2**: 488-494 [PMID: 8591078 DOI: 10.1007/BF02307081]

9 **Lim L**, Michael M, Mann GB, Leong T. Adjuvant therapy in gastric cancer. *J Clin Oncol* 2005; **23**: 6220-6232 [PMID: 16135489 DOI: 10.1200/JCO.2005.11.593]

10 **Bang YJ**, Kim YW, Yang HK, Chung HC, Park YK, Lee KH, Lee KW, Kim YH, Noh SI, Cho JY, Mok YJ, Kim YH, Ji J, Yeh TS, Button P, Sirzén F, Noh SH. Adjuvant capecitabine and oxaliplatin for gastric cancer after D2 gastrectomy (CLASSIC): a phase 3 open-label, randomised controlled trial. *Lancet* 2012; **379**: 315-321 [PMID: 22226517 DOI: 10.1016/S0140-6736(11)61873-4]

11 **Macdonald JS**, Smalley SR, Benedetti J, Hundahl SA, Estes NC, Stemmermann GN, Haller DG, Ajani JA, Gunderson LL, Jessup JM, Martenson JA. Chemoradiotherapy after surgery compared with surgery alone for adenocarcinoma of the stomach or gastroesophageal junction. *N Engl J Med* 2001; **345**: 725-730 [PMID: 11547741 DOI: 10.1056/NEJMoa010187]

12 **Lee J**, Lim do H, Kim S, Park SH, Park JO, Park YS, Lim HY, Choi MG, Sohn TS, Noh JH, Bae JM, Ahn YC, Sohn I, Jung SH, Park CK, Kim KM, Kang WK. Phase III trial comparing capecitabine plus cisplatin versus capecitabine plus cisplatin with concurrent capecitabine radiotherapy in completely resected gastric cancer with D2 lymph node dissection: the ARTIST trial. *J Clin Oncol* 2012; **30**: 268-273 [PMID: 22184384 DOI: 10.1200/JCO.2011.39.1953]

13 **Ychou M**, Boige V, Pignon JP, Conroy T, Bouché O, Lebreton G, Ducourtieux M, Bedenne L, Fabre JM, Saint-Aubert B, Genève J, Lasser P, Rougier P. Perioperative chemotherapy compared with surgery alone for resectable gastroesophageal adenocarcinoma: an FNCLCC and FFCD multicenter phase III trial. *J Clin Oncol* 2011; **29**: 1715-1721 [PMID: 21444866 DOI: 10.1200/JCO.2010.33.0597]

14 **Glehen O**, Gilly FN, Arvieux C, Cotte E, Boutitie F, Mansvelt B, Bereder JM, Lorimier G, Quenet F, Elias D. Peritoneal carcinomatosis from gastric cancer: a multi-institutional study of 159 patients treated by cytoreductive surgery combined with perioperative intraperitoneal chemotherapy. *Ann Surg Oncol* 2010; **17**: 2370-2377 [PMID: 20336386 DOI: 10.1245/s10434-010-1039-7]

15 **Rudloff U**, Langan RC, Mullinax JE, Beane JD, Steinberg SM, Beresnev T, Webb CC, Walker M, Toomey MA, Schrump D, Pandalai P, Stojadinovic A, Avital I. Impact of maximal cytoreductive surgery plus regional heated intraperitoneal chemotherapy (HIPEC) on outcome of patients with peritoneal carcinomatosis of gastric origin: results of the GYMSSA trial. *J Surg Oncol* 2014; **110**: 275-284 [PMID: 25042700 DOI: 10.1002/jso.23633]

P- Reviewer: Li Y, Ulasoglu C **S- Editor**: Ji FF **L- Editor**: A **E- Editor**: Lu YJ

World Journal of Gastrointestinal Pathophysiology

Submit a Manuscript: http://www.wjgnet.com/esps/
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.4291/wjgp.v7.i3.288

World J Gastrointest Pathophysiol 2016 August 15; 7(3): 288-295
ISSN 2150-5330 (online)

ORIGINAL ARTICLE

Retrospective Cohort Study

Does the antibody production ability affect the serum anti-*Helicobacter pylori* IgG titer?

Hyun Ah Chung, Sun-Young Lee, Hee Won Moon, Jeong Hwan Kim, In-Kyung Sung, Hyung Seok Park, Chan Sup Shim, Hye Seung Han

Hyun Ah Chung, Sun-Young Lee, Jeong Hwan Kim, In-Kyung Sung, Hyung Seok Park, Chan Sup Shim, Department of Internal Medicine, Konkuk University School of Medicine, Seoul 05030, South Korea

Hee Won Moon, Department of Laboratory Medicine, Konkuk University School of Medicine, Seoul 05030, South Korea

Hye Seung Han, Department of Pathology, Konkuk University School of Medicine, Seoul 05030, South Korea

Author contributions: Chung HA and Lee SY wrote the manuscript; Lee SY designed research; Chung HA, Moon HW and Han HS analyzed data; Kim JH, Sung IK, Park HS and Shim CS supervised the study.

Supported by Konkuk University in 2015, No. KU2015-A019-0270.

Institutional review board statement: The study was reviewed and approved for publication by the institutional review board of the Konkuk University School of Medicine (KUH1010625).

Clinical trial registration statement: This study was registered at ClinicalTrials.gov ID: KCT0001302 (https://cris.nih.go.kr) after the approval by the institutional review board of the Konkuk University School of Medicine (KUH1010625).

Informed consent statement: Written informed consent was obtained from all the participants before the procedure as described in the MATERIALS AND METHODS section.

Conflict-of-interest statement: No authors have any conflict of interest.

Data sharing statement: The original anonymized database is available for collaborative studies *via* the corresponding author, sunyoung@kuh.ac.kr.

Manuscript source: Invited manuscript

Correspondence to: Sun-Young Lee, MD, PhD, Department of Internal Medicine, Konkuk University School of Medicine, 120-1 Neungdong-ro, Gwangjin-gu, Seoul 05030, South Korea. sunyoung@kuh.ac.kr
Telephone: +82-2-20307747
Fax: +82-2-20307748

Received: December 27, 2015
Peer-review started: December 28, 2015
First decision: January 30, 2016
Revised: June 5, 2016
Accepted: July 11, 2016
Article in press: July 13, 2016
Published online: August 15, 2016

Abstract

AIM: To investigate the relationship between serum titers of anti-*Helicobacter pylori* (*H. pylori*) immunoglobulin G (IgG) and hepatitis B virus surface antibody (HBsAb).

METHODS: Korean adults were included whose samples had positive Giemsa staining on endoscopic biopsy and were studied in the hepatitis B virus surface antigen (HBsAg)/HBsAb serologic assay, pepsinogen (PG) assay, and *H. pylori* serologic test on the same day. Subjects were excluded if they were positive for HBsAg, had a recent history of medication, or had other medical condition(s). We analyzed the effects of the following factors on serum titers of HBsAb and the anti-*H. pylori* IgG: Age, density of *H. pylori* infiltration in biopsy samples,

serum concentrations of PG Ⅰ and PG Ⅱ, PG Ⅰ/Ⅱ ratio, and white blood cell count.

RESULTS: Of 111 included subjects, 74 (66.7%) exhibited a positive HBsAb finding. The serum anti-*H. pylori* IgG titer did not correlate with the serum HBsAb titer (P = 0.185); however, it correlated with the degree of *H. pylori* infiltration on gastric biopsy (P < 0.001) and serum PG Ⅱ concentration (P = 0.042). According to the density of *H. pylori* infiltration on gastric biopsy, subjects could be subdivided into those with a marked (median: 3.95, range 0.82-4.00) (P = 0.458), moderate (median: 3.37, range 1.86-4.00), and mild *H. pylori* infiltrations (median: 2.39, range 0.36-4.00) (P < 0.001). Subjects with a marked *H. pylori* infiltration on gastric biopsy had the highest serological titer, whereas in subjects with moderate and mild *H. pylori* infiltrations titers were correspondingly lower (P < 0.001). After the successful eradication, significant decreases of the degree of *H. pylori* infiltration (P < 0.001), serum anti-*H. pylori* IgG titer (P < 0.001), and serum concentrations of PG I (P = 0.028) and PG Ⅱ (P = 0.028) were observed.

CONCLUSION: The anti-*H. pylori* IgG assay can be used to estimate the burden of bacteria in immunocompetent hosts with *H. pylori* infection, regardless of the HBsAb titer after HBV vaccination.

Key words: Antibody; *Helicobacter pylori*; Hepatitis B; Immunoglobulin G; Pepsinogen

Core tip: Koreans receive a routine childhood immunization program, including hepatitis B vaccinations, but serum hepatitis B virus (HBV) surface antibody responses are variable. It is unclear whether the beneficial functional immune aspects inherent in vaccine responders can be translated into a robust immune response after *Helicobacter pylori* (*H. pylori*) infection. In this study, the serum anti-*H. pylori* immunoglobulin G (IgG) titer appears to be significantly linked to the bacterial load of the stomach, regardless of the ability of antibody production after HBV vaccination. The serum anti-*H. pylori* IgG assay can be used to estimate the burden of bacteria in immunocompetent hosts with *H. pylori* infection.

Chung HA, Lee SY, Moon HW, Kim JH, Sung IK, Park HS, Shim CS, Han HS. Does the antibody production ability affect the serum anti-*Helicobacter pylori* IgG titer? *World J Gastrointest Pathophysiol* 2016; 7(3): 288-295 Available from: URL: http://www.wjgnet.com/2150-5330/full/v7/i3/288.htm DOI: http://dx.doi.org/10.4291/wjgp.v7.i3.288

INTRODUCTION

Helicobacter pylori (*H. pylori*) infection triggers inflammatory and immune responses[1,2]. The serum anti-*H. pylori* immunoglobulin G (IgG) titer is affected by various factors, including bacterial colonization, persistence, virulence, and host immune responses[3,4]. However, the persistence of *H. pylori* over decades in infected individuals suggests that the anti-*H. pylori* IgG does not play a role in the host immune response.

Serum antibody titers depend on the ability of individuals to produce antibodies. It is known that in Koreans, serum titers of the surface antibody against the hepatitis B virus (HBsAb) vary after hepatitis B virus (HBV) vaccinations[5]. Approximately 10% of Koreans do not develop an adequate immune response after they have received a vaccination series, and the rate of non-responsiveness correlates with older age, smoking, male gender, and the presence of chronic diseases[6,7]. Similarly, variable anti-*H. pylori* IgG titers may reflect different immune statuses in individuals with a similar *H. pylori* burden. Taken together with an established link between the HBV vaccine response and immune constitution[8,9], these findings suggest that the evaluation of the HBsAb response in HBV-vaccinated individuals could provide useful information regarding their immune states.

The immune response *via* the activation of helper T cells may stimulate production of both the *H. pylori* IgG and HBsAb[2,8], although the theoretical background underlying this mechanism remains uncertain. Little is known about the serum anti-*H. pylori* IgG titer as a parameter of the immune response to *H. pylori* infection because the knowledge of the *H. pylori* immunopathogenesis is limited. In addition, it is unclear whether the beneficial functional immune aspects inherent in vaccine responders can be translated into a robust immune response after *H. pylori* infection.

In the present study, gastric biopsy samples were analyzed to determine whether there is a correlation between the serum titers of the anti-*H. pylori* IgG and HBsAb in conditions with a similar *H. pylori* burden. In addition, variables that significantly correlated with the serum titers of the anti-*H. pylori* IgG and HBsAb were analyzed.

MATERIALS AND METHODS

Study population

In this cross-sectional study, Korean adults who underwent upper esophagogastroduodenoscopy (EGD) with gastric biopsies for pathology and Giemsa staining, serum pepsinogen (PG) assay, serum anti-*H. pylori* IgG assay and serum HBV surface antigen (HBsAg)/HBsAb assay on the same day at our center were included (Figure 1). The subjects were excluded in following conditions: (1) negative Giemsa staining; (2) positive HBsAg finding; (3) recent medication; (4) history of *H. pylori* eradication; (5) serum anti-*H. pylori* IgG testing other than the Vidas assay; or (6) the presence of disease(s) including any condition related to immunosuppressed state. This study was registered at ClinicalTrials.gov ID: KCT0001302 (https://cris.nih.go.kr) after the approval

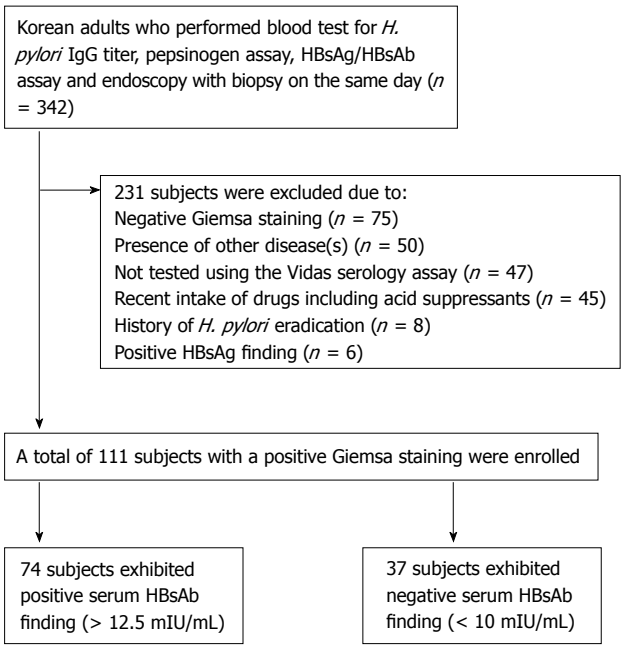


Figure 1 Flow of this study. Of the 342 Korean adults, only the subjects with a positive Giemsa staining were included in the study. *H. pylori*: *Helicobacter pylori*; HBsAg: Hepatitis B surface antigen; HBsAb: Hepatitis B surface antibody.

by the institutional review board of the Konkuk University School of Medicine (KUH1010625).

Serum anti-H. pylori IgG assay

Venous blood was sampled after 12 h of fasting for serum anti-*H. pylori* IgG assay, serum PG assay and serum HBsAg/HBsAb assay. The *H. pylori* serology titer was measured using the Vidas *H. pylori* IgG assay (BioMérieux, Marcy-l' Etoile, France) according to the manufacturer's instruction. Based on the Vidas *H. pylori* IgG assay package insert, positive finding was defined as a serum IgG titer equal or over 1.00 with sensitivity of 98.1% and specificity of 90.8%.

Serum PG assay

For serum PG Ⅰ and PG Ⅱ concentrations, the fasting blood samples were centrifuged and measured using the latex-enhanced turbidimetic immunoassay (HBi Co., Anyang, South Korea)[10]. Gastric corpus atrophy was diagnosed if the serum PG Ⅰ/Ⅱ ratio was less than 3.0 and the serum PG Ⅰ concentration was less than 70 ng/mL.

Serum HBsAg and HBsAb assay

Fasting blood sample was analyzed for the serum HBsAg and HBsAb levels using the ADVIA Centaur system (Siemens Healthcare Diagnostics Inc., Deerfield, IL, United States) as described in the previous study[11]. According to the manufacturer's instructions, negative findings were provided if the index value of HBsAg was of < 1.0 and if HBsAb was of < 7.5 mIU/mL on this chemiluminescent immunoassay. For HBsAg, equivocal findings were provided if the index value was equal to 1.0, while positive findings were provided if it was of > 1.0. For HBsAb, equivocal findings were provided if the index value was between 7.5 and 12.5 mIU/mL, while positive findings were provided if it was of > 12.5 mIU/mL.

Upper gastrointestinal endoscopy and gastric biopsy

Each participant underwent EGD on the same day of blood sampling at our center using GIF-H260 (Olympus, Tokyo, Japan) endoscope. During EGD, gastric biopsy was performed for pathology, histologic assay of *H. pylori* density and Giemsa staining. The biopsied specimens were fixed in 95% ethanol and embedded in paraffin blocks. Thereafter, the samples were sectioned and stained with hematoxylin and eosin (HE) and Giemsa. Histologic assay of *H. pylori* density were graded as mild, moderate and marked infiltration. If the density differed according to the biopsied site, the highest density and location were collected for the statistical analysis. Based on the Updated Sydney System, the grades were scored as either none (0), mild (1), moderate (2), or marked (3) for activity (the intensity of acute polymorphonuclear cell infiltrates), inflammation (the intensity of chronic mononuclear cell infiltrates), atrophy, and intestinal metaplasia.

H. pylori eradication and follow-up tests

A first-line therapy was performed with amoxicillin 1 g, clarithromycin 500 mg, and a proton pump inhibitor 20 mg twice daily to the subjects who agreed on *H. pylori* eradication. Four weeks after the eradication, a urease breath test was carried out. If it was positive, a second-line therapy was performed with tetracycline 500 mg, bismuth 300 mg four times a day, metronidazole 500 mg and a proton pump inhibitor twice a day. Follow-up tests for EGD and serum assays were performed as the initial tests described above.

Statistical analysis

For the statistical analysis, SPSS version 19.0 (SPSS Inc., Chicago, IL, United States) were used. A *P*-value less than 0.05 was considered statistically significant. Continuous variables were summarized as mean ± SD using the Student's *t*-test, while categorical variables were summarized as frequency (%) using the χ^2 test. The differences between the groups were compared using the ANOVA test for continuous variables.

The strength of correlation between the serum anti-*H. pylori* IgG titer and variables were estimated by correlation analysis. For continuous variables that were found to be related to severe *H. pylori* infiltration on gastric biopsy, a receiver operating characteristic (ROC) curve was constructed by plotting sensitivity (true-positive rate) against 1-specificity (false-positive rate). Accuracies of the significant variables were measured based on the area under the ROC curve (AUC) analysis with a 95%CI and standard error (SE) values.

Follow-up data were analyzed to compare the changes between the subjects with successful eradication

Table 1 Baseline characteristics of the included subjects *n* (%)

Variables	Subjects (*n* = 111)
Age (years old, mean ± SD)	55.3 ± 9.7
Gender (male:female)	66:45
Serum anti-*H. pylori* IgG titer (AU/mL, mean ± SD)	3.26 ± 0.97
Serum PG Ⅰ level (ng/mL, mean ± SD)	72.0 ± 28.8
Serum PG Ⅱ level (ng/mL, mean ± SD)	22.0 ± 9.2
Serum PG ratio (mean ± SD)	3.5 ± 1.2
Presence of corpus gastric atrophy as reflected by serum PG assay	23 (20.7)
Degree of *H. pylori* infiltration on biopsy	
Mild	14 (12.6)
Moderate	23 (20.7)
Marked	74 (66.7)
Scores based on Updated Sydney system	
Activity (mean ± SD)	1.92 ± 0.69
Chronic inflammation (mean ± SD)	2.04 ± 0.38
Atrophy (median with ranges)	0.97 (0-3)
Intestinal metaplasia (median with ranges)	0.64 (0-3)
Biopsied site	
Antrum	69 (62.2)
Body or angle	36 (32.4)
Fundus or cardia	6 (5.4)
Serum HBsAb titer (mIU/mL, median with ranges)	102.19 (1-1000)
Positive HBsAb assay	78 (70.3)
Platelet (× 10^3/μL, mean ± SD)	235.3 ± 48.2
White blood cell count (× 10^3/μL, mean ± SD)	5853.8 ± 1595.9
Neutrophil (%)	56.0 ± 9.3
Lymphocyte (%)	36.4 ± 8.7
Monocyte (%)	4.7 ± 1.6
Eosinophil (%)	1.84 (0-13)
Basophil (%)	0.41 (0-5)

HBsAb: Hepatitis B surface antibody; PG: Pepsinogen; *H. pylori*: *Helicobacter pylori*.

and those with persistent *H. pylori* infection. For the eradicated subjects, differences between pre- and post-eradication were analyzed using the Wilcoxon signed rank test. In similar, differences between initial and follow-up data were analyzed using the Wilcoxon signed-rank test in the subjects with persistent *H. pylori* infection.

RESULTS

Characteristics of the subjects

A total of 111 Korean adults were tested with the Vidas assay, and 74 (66.7%) subjects exhibited a positive HBsAb finding. The degrees of *H. pylori* infiltration on gastric biopsy were mild in 14 subjects, moderate in 23 subjects, and marked in 74 subjects (Table 1). The serum HBsAb findings did not differ between the groups (Table 2). Of all variables, marked degree of *H. pylori* infiltration showed the highest serum anti-*H. pylori* IgG titer ($P < 0.001$) and serum PG Ⅱ concentration ($P = 0.021$).

Variables correlated with serum HBsAb titer

There was no significant correlation between serum anti-*H. pylori* IgG titer and serum HBsAb titer ($P = 0.557$). The serum HBsAb titer was not related to any of the tested variables including the counts of platelet and white blood cell (Table 3).

Variables correlated with serum anti-H. pylori IgG titer

The serum anti-*H. pylori* IgG titer was positively correlated with the density of *H. pylori* infiltration on gastric biopsy ($P < 0.001$) and the serum PG Ⅱ concentrations ($P = 0.042$) using the correlation analysis. However, it was neither related to the positive HBsAb finding ($P = 0.905$) nor the serum HBsAb titer ($P = 0.557$). Distribution of serum anti-*H. pylori* IgG titers according to the *H. pylori* infiltration are shown in Figure 2.

Significant variables for *H. pylori* infiltration were analyzed using the ROC curve analysis (Figure 3). The cut-off value of serum anti-*H. pylori* IgG titer for correlating with severe density of *H. pylori* infiltration was 2.9 AU/mL with sensitivity and specificity values 81.1% and 51.4% (AUC = 0.659, 95%CI: 0.548-0.770, SE = 0.057, $P = 0.007$). However, serum PG Ⅱ concentration showed no statistical significance (AUC = 0.593, 95%CI: 0.481-0.705, SE = 0.057, $P = 0.111$) on the ROC analysis.

Subgroup analysis of the followed-up subjects

Of 111 included subjects, 41 were followed up for EGD and serum assays. Of these 41 followed-up subjects, 29 underwent *H. pylori* eradication therapy, and 4 failed on eradication. Therefore, a comparison was made between 25 subjects with successful eradication and 16 subjects with persistent infection (including 4 who failed on eradication). There was no difference on the initial test findings between the eradicated and persistent groups (Table 4).

After *H. pylori* eradication, significant decreases were noticed on the degree of *H. pylori* infiltration ($P < 0.001$), serum PG Ⅰ concentration ($P = 0.028$) and serum PG Ⅱ concentration ($P = 0.028$). As a consequence, the serum PG Ⅰ/Ⅱ ratio was significantly increased after eradication ($P = 0.028$). On the contrary, there was no significant differences between the initial and follow-up data on *H. pylori* infiltration ($P = 0.335$) and serum PG Ⅰ/Ⅱ ratio ($P = 0.395$) in the subjects with persistent *H. pylori* infection.

DISCUSSION

A significant link has been found between the serum anti-*H. pylori* IgG titer and the bacterial load of the stomach, regardless of the antibody producing capability of the host. Furthermore, significant decreases of the degree of *H. pylori* infiltration, serum anti-*H. pylori* IgG titer, and serum concentrations of PG Ⅰ and PG Ⅱ in the subjects with successfully eradicated *H. pylori* infection were observed. At the same time, such changes were not observed in the subjects with persistent *H. pylori* infection. Based on these results, the serum anti-*H. pylori* IgG titer could be considered an indicator of the

Table 2 Characteristics of the subjects according to the degree of *H. pylori* infiltration on gastric biopsy *n* (%)

Variables	Mild *H. pylori* infiltration (*n* = 14)	Moderate *H. pylori* infiltration (*n* = 23)	Marked *H. pylori* infiltration (*n* = 74)	*P* value
Age (yr, mean ± SD)	54.7 ± 8.4	57.7 ± 12.0	54.7 ± 9.0	0.428
Gender (male)	6 (42.9)	16 (69.6)	44 (59.5)	0.276
Serum anti-*H. pylori* IgG titer (AU/mL)[1]	2.39 (0.36-4.00)	3.37 (1.86-4.00)	3.95 (0.82-4.00)	< 0.001
Serum PG Ⅰ level (ng/mL, mean ± SD)	58.0 ± 19.3	75.5 ± 32.6	73.6 ± 28.7	0.146
Serum PG Ⅱ level (ng/mL, mean ± SD)	15.6 ± 6.3	22.9 ± 9.5	22.9 ± 9.2	0.021
Serum PG Ⅰ / Ⅱ ratio (mean ± SD)	4.1 ± 1.7	3.4 ± 1.0	3.4 ± 1.1	0.118
Presence of corpus gastric atrophy as reflected by PG assay	3 (21.4)	5 (21.7)	15 (20.3)	0.986
Biopsied site (antrum:body or angle:fundus or cardia)	1:3:0	15:7:1	43:26:5	0.625
Scores based on Updated Sydney system				
Activity (mean ± SD)	1.5 ± 0.7	1.9 ± 0.5	2.0 ± 0.7	0.034
Inflammation (mean ± SD)	1.9 ± 0.5	2.0 ± 0.2	2.1 ± 0.4	0.052
Atrophy (median with ranges)	0.8 (0-3)	1.3 (0-3)	0.9 (0-3)	0.589
Intestinal metaplasia (median with ranges)	0.6 (0-3)	0.9 (0-3)	0.6 (0-3)	0.771
Positive HBsAb finding	9 (64.3)	17 (73.9)	52 (70.3)	0.824
HBsAb titer (mIU/mL)[1]	174.9 (1-1000)	120.7 (1-1000)	86.8 (1-1000)	0.601
Platelet (× 10^3/μL, mean ± SD)	252.2 ± 41.9	231.9 ± 51.9	233.2 ± 48.1	0.375
White blood cell count (× 10^3/μL, mean ± SD)	5688.6 ± 1552.3	5780.0 ± 1390.9	5908.0 ± 1678.0	0.869
Neutrophil (%)	55.1 ± 9.3	54.9 ± 10.3	45.5 ± 9.1	0.702
Lymphocyte (%)	36.8 ± 8.7	37.0 ± 9.8	36.2 ± 8.4	0.919
Monocyte (%)	4.9 ± 1.6	4.9 ± 2.1	4.6 ± 1.5	0.732
Eosinophil (%)	2.4 (0-6)	2.0 (0-1)	1.8 (0-1)	0.819
Basophil (%)	0.4 (0-1)	0.5 (0-1)	0.4 (0-5)	0.771

[1]Values are shown as median with ranges due to asymmetrical distribution. HBsAb: Hepatitis B surface antibody; PG: Pepsinogen; *H. pylori*: *Helicobacter pylori*.

Table 3 Correlation analysis for the serum anti-*H. pylori* IgG titer and serum HBsAb titer

Variables	Correlation coefficient	*P* value
Serum anti-*H. pylori* IgG titer		
Old age	-0.009	0.924
Increased density of *H. pylori* infiltration	0.389	< 0.001
Increased serum PG Ⅰ level	0.116	0.224
Increased serum PG Ⅱ level	0.194	0.042
Increased serum PG Ⅰ / Ⅱ ratio	-0.18	0.059
Higher degree of activity	0.272	0.004
Higher degree of inflammation	0.125	0.192
Higher degree of atrophy	0.021	0.826
Higher degree of intestinal metaplasia	-0.047	0.624
Presence of gastric corpus atrophy as reflected by PG assay	-0.015	0.876
Increased HBsAb titer	-0.056	0.557
Increased platelet count	-0.061	0.522
Increased white blood cell count	-0.078	0.417
Serum HBsAb titer		
Old age	-0.088	0.358
Increased density of *H. pylori* infiltration	-0.07	0.466
Increased serum PG Ⅰ level	0.046	0.634
Increased serum PG Ⅱ level	-0.054	0.572
Increased serum PG Ⅰ / Ⅱ ratio	0.136	0.154
Higher degree of activity	0.077	0.42
Higher degree of inflammation	-0.112	0.24
Higher degree of atrophy	0.036	0.706
Higher degree of intestinal metaplasia	0.054	0.573
Presence of gastric corpus atrophy as reflected by PG assay	-0.164	0.086
Increased platelet count	0.008	0.935
Increased white blood cell count	-0.069	0.473

HBsAb: Hepatitis B surface antibody; PG: Pepsinogen; *H. pylori*: *Helicobacter pylori*.

bacterial burden in infected subjects. This finding may lead to novel opportunities toward enhancing *H. pylori* eradication.

H. pylori has the ability to persist despite a vast array of host immune responses, which appear to differ between infected subjects[12]. The present findings

Table 4 Findings of the followed-up subjects *n* (%)

	Successful *H. pylori* eradication (*n* = 25)	Persistent *H. pylori* infection (*n* = 16)	*P* value	Before eradication	After eradication	*P* value (Z[1])	Initial	Follow-up	*P* value
Initial test findings									
Age (yr, mean ± SD)	52.3 ± 7.9	55.3 ± 12.7	0.351						
Gender (male:female)	15:10	9:07	1						
Degree of *H. pylori* infiltration on biopsy (mild:moderate: marked)	4:05:16	2:04:10	0.907						
Anti-*H. pylori* IgG titer (AU/mL, mean ± SD)	3.00 ± 1.17	3.17 ± 0.92	0.632						
PG Ⅰ level (ng/mL, mean ± SD)	79.3 ± 31.7	64.9 ± 23.4	0.126						
PG Ⅱ level (ng/mL, mean ± SD)	23.4 ± 8.5	18.9 ± 9.1	0.117						
PG ratio (mean ± SD)	3.5 ± 1.1	3.9 ± 1.5	0.419						
Presence of corpus gastric atrophy as reflected by serum PG assay	4 (16.0)	2 (12.5)	0.566						
HBsAb titer (mIU/mL, median with ranges)	72.2 (3.1-1000)	170.3 (3.1-1000)	0.632						
Positive HBsAb assay	16 (64)	12 (75)	0.513						
Duration of the follow-up period (months, median with ranges)	18.1 (2-61)	20.2 (6-41)	0.887						
Subjects with successful *H. pylori* eradication (*n* = 25)									
Follow-up test findings									
Degree of *H. pylori* infiltration on biopsy (none:mild:moderate: marked)				0:4:5:16	25:0:0:0	< 0.001 (-4.520)			
Anti-*H. pylori* IgG assay (negative:lowest:middle:highest quartiles)[2]				3:2:14:6	20:4:1:0	< 0.001 (-4.171)			
PG Ⅰ level (ng/mL, mean ± SD)				79.3 ± 31.7	54.2 ± 14.5	0.028 (-2.201)			
PG Ⅱ level (ng/mL, mean ± SD)				23.4 ± 8.5	7.0 ± 1.7	0.028 (-2.201)			
PG ratio (mean ± SD)				3.5 ± 1.1	7.8 ± 1.6	0.028 (-2.200)			
HBsAb titer (mIU/mL, median with ranges)				72.5 (3.1-1000)	18.4 (3.1-1000)	0.308			
Subjects with persistent *H. pylori* infection (*n* = 16)									
Follow-up test findings									
Degree of *H. pylori* infiltration on biopsy (none:mild:moderate: marked)							0:2:4:10	1:3:2:10	0.335
Anti-*H. pylori* IgG assay (negative:lowest:middle:highest quartiles)[2]							1:0:8:7	0:2:7:7	1.18
PG Ⅰ level (ng/mL, mean ± SD)							64.9 ± 23.4	71.8 ± 35.2	1
PG Ⅱ level (ng/mL, mean ± SD)							18.9 ± 9.1	21.4 ± 10.3	0.779
PG ratio (mean ± SD)							3.9 ± 1.5	3.6 ± 0.8	0.395
HBsAb titer (mIU/mL, median with ranges)							170.3 (3.1-1000)	202.1 (3.1-1000)	0.314

[1]Z values are shown for the significant variables using Wilcoxon signed rank test; [2]The serum anti-*H. pylori* IgG titer was compared using the quartiles because it was measured using the Vidas *H. pylori* IgG assay until 2012, and using the Chorus *H. pylori* IgG assay thereafter. SD: Standard deviation; HBsAb: Hepatitis B surface antibody; PG: Pepsinogen; *H. pylori*: *Helicobacter pylori*.

suggest that the serum anti-*H. pylori* IgG titer is related to the burden of *H. pylori* antigens, because lymphocytes are sensitized to the *H. pylori* antigens and IgG is produced by B cells against a variety of *H. pylori* surface (flagellar) proteins and bacterial toxins. Furthermore, the development of the positive HBV vaccine antibody response involves not only the T cell functions, but also other functional pathways, including B cell activity and antigen presentation of the peptide-based vaccine[6,7,13]. These findings suggest that the amount of IgG production *via* the host immune response upon *H. pylori* infection is more closely related to the burden of *H. pylori* antigens than to the ability of the host to produce antibodies, which is gauged by the serum HBsAb titer.

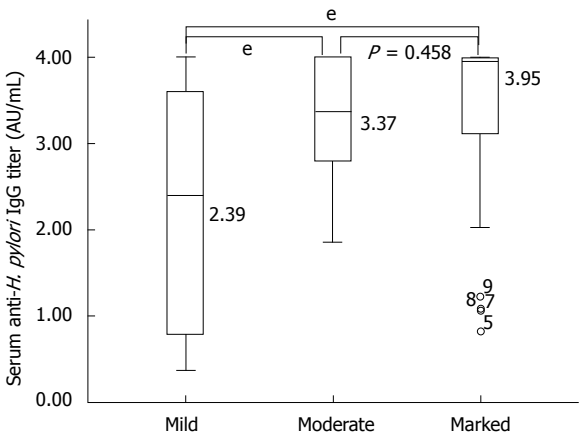


Figure 2 The serum anti-*H. pylori* IgG titer according to the degree of *H. pylori* infiltration on gastric biopsy. Subjects with marked *H. pylori* infiltration showed the highest serology titer followed by those with moderate and mild infiltrations.

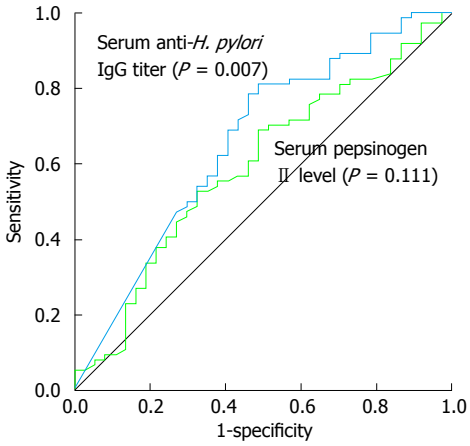


Figure 3 Receiver operating characteristic curves for correlating with the density of *H. pylori* infiltration. The cut-off value of the serum anti-*H. pylori* IgG titer for correlating with severe density of *H. pylori* infiltration was 2.9 AU/ml (AUC = 0.659, 95%CI: 0.548-0.770, SE = 0.057, *P* = 0.007). There was no significant finding with regard to the serum PG Ⅱ concentration (AUC = 0.593, 95%CI: 0.481-0.705, SE = 0.057, *P* = 0.111).

In the present study, it was found that the serum anti-*H. pylori* IgG titer positively correlated with the degree of *H. pylori* infiltration on the biopsied specimen, regardless of the biopsied site of the stomach. This finding is consistent with the results of previous studies, in which the significance of the serum anti-*H. pylori* IgG titer was demonstrated, and indirectly indicates the relationship between the severity of histological changes and mucosal bacterial density[14-16]. Evaluation of the serum anti-*H. pylori* IgG titer can detect *H. pylori* infection in patients with marked atrophic gastritis and metaplastic gastritis, even in the event of negative biopsy specimens, and provide an indicator of the efficacy of *H. pylori* eradication[17-20].

Serum PG assays are widely used for the measurements of gastric inflammation[21,22] and in combination with the serum anti-*H. pylori* IgG assay during gastric cancer screening[10,23]. The link between the immune response and *H. pylori* infection-induced gastric inflammation, as measured by the serum PG assay, has been established[24]. In that study, the *Salmonella typhi* (*S. typhi*) IgG seroconversion was more common in the subjects with the *H. pylori* infection than in those without it after anti-*S. typhi* vaccination. In the present study, the serum anti-*H. pylori* IgG titer positively correlated with the serum PG levels and *H. pylori* infiltration in biopsy samples, regardless of the HBsAb titer. This suggests that the bacterial burden directly correlates with the degree of gastric inflammation, despite the differential development and recruitment of specifically committed cells that occurred after the *H. pylori* infection in the subjects.

The limitation of this study is that only 41 subjects underwent the follow-up tests. Furthermore, the serum anti-*H. pylori* IgG titer was followed up using the Chorus *H. pylori* IgG assay (DIESSE Diagnostica Senese, Siena, Italy) because the initially used Vidas *H. pylori* IgG assay was not available after 2012. Despite these limitations, significant differences in the follow-up findings of serum assays and *H. pylori* infiltration were found only in the subjects in whom *H. pylori* eradication was successfully achieved. In support of these observations, a recent study described a high rate of concurrence and similar diagnostic accuracy between the Vidas *H. pylori* IgG assay and the Chorus *H. pylori* IgG assay[25].

In conclusion, the findings of this study show that the serum anti-*H. pylori* IgG titer is significantly associated with the bacterial load of the stomach, regardless of the antibody producing capability of the host. Although the anti-*H. pylori* IgG response requires preserved function of several immune pathways, it appears that the serum anti-*H. pylori* IgG titer correlates with the intragastric bacterial load rather than with the HBsAb titer. The serum anti-*H. pylori* IgG titer is therefore useful for estimating the bacterial burden of *H. pylori* infection.

COMMENTS

Background

Serum antibody titers depend on the ability of individuals to produce antibodies. It is unclear whether the beneficial functional immune aspects inherent in hepatitis B virus vaccine responders can be translated into a robust immune response after *Helicobacter pylori* (*H. pylori*) infection.

Research frontiers

In this cross-sectional study, consecutive Korean adults were included whose samples had positive Giemsa staining on endoscopic biopsy and were studied in the hepatitis B virus surface antigen (HBsAg)/HBsAb serologic assay, pepsinogen (PG) assay, and anti-*H. pylori* immunoglobulin G (IgG) assay on the same day. This approach allows the authors to demonstrate correlation between serum HBsAb titer and anti-*H. pylori* IgG titer.

Innovations and breakthrough

In this study the authors demonstrated that the serum anti-*H. pylori* IgG titer correlates with the intragastric bacterial load rather than with the HBsAb titer.

Applications

The serum anti-*H. pylori* IgG titer is therefore useful for estimating the bacterial

burden of *H. pylori* infection.

Terminology

Serologic testing for IgG antibodies to *H. pylori* is commonly used noninvasive method to diagnose *H. pylori* infection. The IgG antibody titer is indicative of the severity of gastritis and the presence of *H. pylori*.

Peer-review

This is a novel look at a very interesting topic. In the clinical finding presented in this manuscript, the authors showed that the serum anti-*H. pylori* IgG assay can be used to estimate the burden of bacteria in immunocompetent hosts with *H. pylori* infection.

REFERENCES

1 **McNamara D**, El-Omar E. Helicobacter pylori infection and the pathogenesis of gastric cancer: a paradigm for host-bacterial interactions. *Dig Liver Dis* 2008; **40**: 504-509 [PMID: 18486572 DOI: 10.1016/j.dld.2008.02.031]

2 **Wilson KT**, Crabtree JE. Immunology of Helicobacter pylori: insights into the failure of the immune response and perspectives on vaccine studies. *Gastroenterology* 2007; **133**: 288-308 [PMID: 17631150 DOI: 10.1053/j.gastro.2007.05.008]

3 **Portal-Celhay C**, Perez-Perez GI. Immune responses to Helicobacter pylori colonization: mechanisms and clinical outcomes. *Clin Sci* (Lond) 2006; **110**: 305-314 [PMID: 16464172 DOI: 10.1042/CS20050232]

4 **Robinson K**, Kenefeck R, Pidgeon EL, Shakib S, Patel S, Polson RJ, Zaitoun AM, Atherton JC. Helicobacter pylori-induced peptic ulcer disease is associated with inadequate regulatory T cell responses. *Gut* 2008; **57**: 1375-1385 [PMID: 18467372 DOI: 10.1136/gut.2007.137539]

5 **Yeo Y**, Gwack J, Kang S, Koo B, Jung SJ, Dhamala P, Ko KP, Lim YK, Yoo KY. Viral hepatitis and liver cancer in Korea: an epidemiological perspective. *Asian Pac J Cancer Prev* 2013; **14**: 6227-6231 [PMID: 24377509 DOI: 10.7314/APJCP.2013.14.11.6227]

6 **Wiedmann M**, Liebert UG, Oesen U, Porst H, Wiese M, Schroeder S, Halm U, Mössner J, Berr F. Decreased immunogenicity of recombinant hepatitis B vaccine in chronic hepatitis C. *Hepatology* 2000; **31**: 230-234 [PMID: 10613751]

7 **Altunöz ME**, Senateş E, Yeşil A, Calhan T, Ovünç AO. Patients with inflammatory bowel disease have a lower response rate to HBV vaccination compared to controls. *Dig Dis Sci* 2012; **57**: 1039-1044 [PMID: 22147248 DOI: 10.1007/s10620-011-1980-8]

8 **Yoon JH**, Shin S, In Jw, Chang JY, Song EY, Roh EY. Association of HLA alleles with the responsiveness to hepatitis B virus vaccination in Korean infants. *Vaccine* 2014; **32**: 5638-5644 [PMID: 25148772 DOI: 10.1016/j.vaccine.2014.08.007]

9 **Martinetti M**, De Silvestri A, Belloni C, Pasi A, Tinelli C, Pistorio A, Salvaneschi L, Rondini G, Avanzini MA, Cuccia M. Humoral response to recombinant hepatitis B virus vaccine at birth: role of HLA and beyond. *Clin Immunol* 2000; **97**: 234-240 [PMID: 11112362 DOI: 10.1006/clim.2000.4933]

10 **Choi HS**, Lee SY, Kim JH, Sung IK, Park HS, Shim CS, Jin CJ. Combining the serum pepsinogen level and Helicobacter pylori antibody test for predicting the histology of gastric neoplasm. *J Dig Dis* 2014; **15**: 293-298 [PMID: 24602176 DOI: 10.1111/1751-2980.12144]

11 **Kim H**, Hur M, Moon HW, Park CM, Cho JH, Park KS, Lee K, Chang S. Pre- and post-transfusion testing for hepatitis B virus surface antigen and antibody in blood recipients: a single-institution experience in an area of high endemicity. *Ann Lab Med* 2012; **32**: 73-78 [PMID: 22259782 DOI: 10.3343/alm.2012.32.1.73]

12 **Genta RM**. The immunobiology of Helicobacter pylori gastritis. *Semin Gastrointest Dis* 1997; **8**: 2-11 [PMID: 9000497]

13 **Goncalves L**, Albarran B, Salmen S, Borges L, Fields H, Montes H, Soyano A, Diaz Y, Berrueta L. The nonresponse to hepatitis B vaccination is associated with impaired lymphocyte activation. *Virology* 2004; **326**: 20-28 [PMID: 15262491 DOI: 10.1016/j.virol.2004.04.042]

14 **Tu H**, Sun L, Dong X, Gong Y, Xu Q, Jing J, Yuan Y. Serum anti-Helicobacter pylori immunoglobulin G titer correlates with grade of histological gastritis, mucosal bacterial density, and levels of serum biomarkers. *Scand J Gastroenterol* 2014; **49**: 259-266 [PMID: 24329006 DOI: 10.3109/00365521.2013.869352]

15 **Sheu BS**, Shiesh SC, Yang HB, Su IJ, Chen CY, Lin XZ. Implications of Helicobacter pylori serological titer for the histological severity of antral gastritis. *Endoscopy* 1997; **29**: 27-30 [PMID: 9083733 DOI: 10.1055/s-2007-1004057]

16 **Gong YH**, Sun LP, Jin SG, Yuan Y. Comparative study of serology and histology based detection of Helicobacter pylori infections: a large population-based study of 7,241 subjects from China. *Eur J Clin Microbiol Infect Dis* 2010; **29**: 907-911 [PMID: 20440530 DOI: 10.1007/s10096-010-0944-9]

17 **Koizumi W**, Tanabe S, Imaizumi H, Hibi K, Kida M, Ohida M, Okayasu I, Saigenji K. Effect of anti-Helicobacter pylori IgG antibody titer following eradication of Helicobacter pylori infection. *Hepatogastroenterology* 2003; **50**: 293-296 [PMID: 12630044]

18 **Fanti L**, Ieri R, Mezzi G, Testoni PA, Passaretti S, Guslandi M. Long-term follow-up and serologic assessment after triple therapy with omeprazole or lansoprazole of Helicobacter-associated duodenal ulcer. *J Clin Gastroenterol* 2001; **32**: 45-48 [PMID: 11154169 DOI: 10.1097/00004836-200101000-00011]

19 **Marchildon P**, Balaban DH, Sue M, Charles C, Doobay R, Passaretti N, Peacock J, Marshall BJ, Peura DA. Usefulness of serological IgG antibody determinations for confirming eradication of Helicobacter pylori infection. *Am J Gastroenterol* 1999; **94**: 2105-2108 [PMID: 10445535 DOI: 10.1111/j.1572-0241.1999.01285.x]

20 **Hirschl AM**, Brandstätter G, Dragosics B, Hentschel E, Kundi M, Rotter ML, Schütze K, Taufer M. Kinetics of specific IgG antibodies for monitoring the effect of anti-Helicobacter pylori chemotherapy. *J Infect Dis* 1993; **168**: 763-766 [PMID: 8354918 DOI: 10.1093/infdis/168.3.763]

21 **Sun LP**, Gong YH, Wang L, Yuan Y. Serum pepsinogen levels and their influencing factors: a population-based study in 6990 Chinese from North China. *World J Gastroenterol* 2007; **13**: 6562-6567 [PMID: 18161928 DOI: 10.3748/wjg.13.6562]

22 **Shiota S**, Murakami K, Okimoto T, Kodama M, Yamaoka Y. Serum Helicobacter pylori CagA antibody titer as a useful marker for advanced inflammation in the stomach in Japan. *J Gastroenterol Hepatol* 2014; **29**: 67-73 [PMID: 24033876 DOI: 10.1111/jgh.12359]

23 **Kishikawa H**, Nishida J, Ichikawa H, Kaida S, Takarabe S, Matsukubo T, Miura S, Morishita T, Hibi T. Fasting gastric pH of Japanese subjects stratified by IgG concentration against Helicobacter pylori and pepsinogen status. *Helicobacter* 2011; **16**: 427-433 [PMID: 22059393 DOI: 10.1111/j.1523-5378.2011.00868.x]

24 **Muhsen K**, Pasetti MF, Reymann MK, Graham DY, Levine MM. Helicobacter pylori infection affects immune responses following vaccination of typhoid-naive U.S. adults with attenuated Salmonella typhi oral vaccine CVD 908-htrA. *J Infect Dis* 2014; **209**: 1452-1458 [PMID: 24273182 DOI: 10.1093/infdis/jit625]

25 **Lee SY**, Moon HW, Hur M, Yun YM. Validation of western Helicobacter pylori IgG antibody assays in Korean adults. *J Med Microbiol* 2015; **64**: 513-518 [PMID: 25752852 DOI: 10.1099/jmm.0.000050]

P- Reviewer: Slomiany BL **S- Editor**: Gong ZM **L- Editor**: A **E- Editor**: Lu YJ

World Journal of Gastrointestinal Pathophysiology

Submit a Manuscript: http://www.wjgnet.com/esps/
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.4291/wjgp.v7.i3.296

World J Gastrointest Pathophysiol 2016 August 15; 7(3): 296-299
ISSN 2150-5330 (online)

CASE REPORT

Culprit for recurrent acute gastrointestinal massive bleeding: "Small bowel Dieulafoy's lesions" - a case report and literature review

Anjana Sathyamurthy, Jessica N Winn, Jamal A Ibdah, Veysel Tahan

Anjana Sathyamurthy, Jessica N Winn, Jamal A Ibdah, Veysel Tahan, Division of Gastroenterology and Hepatology, University of Missouri, Columbia, MO 65201, United States

Author contributions: All authors contributed to the acquisition of data, writing, and revision of this manuscript.

Institutional review board statement: This case report was approved by the Institutional Review Board of University of Missouri-Columbia. IRB #2004644.

Informed consent statement: The patient was consented and we got our institutional Review Board approval at University of Missouri-Columbia.

Conflict-of-interest statement: All the authors have no conflicts of interests to declare.

Manuscript source: Invited manuscript

Correspondence to: Veysel Tahan, MD, Assistant Professor, Division of Gastroenterology and Hepatology, University of Missouri, 1 Hospital Dr, Columbia, MO 65201, United States. tahanv@health.missouri.edu
Telephone: +1-573-8846044
Fax: +1-573-8844595

Received: March 24, 2016
Peer-review started: March 24, 2016
First decision: May 16, 2016
Revised: June 2, 2016
Accepted: July 14, 2016
Article in press: July 18, 2016
Published online: August 15, 2016

Abstract

A Dieulafoy's lesion is a dilated, aberrant, submucosal vessel that erodes the overlying epithelium without evidence of a primary ulcer or erosion. It can be located anywhere in the gastrointestinal tract. We describe a case of massive gastrointestinal bleeding from Dieulafoy's lesions in the duodenum. Etiology and precipitating events of a Dieulafoy's lesion are not well known. Bleeding can range from being self-limited to massive life- threatening. Endoscopic hemostasis can be achieved with a combination of therapeutic modalities. The endoscopic management includes sclerosant injection, heater probe, laser therapy, electrocautery, cyanoacrylate glue, banding, and clipping. Endoscopic tattooing can be helpful to locate the lesion for further endoscopic re-treatment or intraoperative wedge resection. Therapeutic options for re-bleeding lesions comprise of repeated endoscopic hemostasis, angiographic embolization or surgical wedge resection of the lesions. We present a 63-year-old Caucasian male with active bleeding from the two small bowel Dieulafoy's lesions, which was successfully controlled with epinephrine injection and clip applications.

Key words: Dieulafoy's lesion; Small intestine; Massive bleeding; Endoscopic treatment; Endoscopy; Surgery

Core tip: Small bowel Dieulafoy's lesion is a rare entity that can cause severe life threatening gastrointestinal hemorrhage. It is difficult to diagnose and treat a small bowel Dieulafoy's lesion by initial endoscopy unlike most gastric Dieulafoy's lesions. We report a rare presentation of small bowel Dieulafoy's lesions in a 63-year-old male. The hemorrhage was successfully controlled by epinephrine injection and clip applications. We also reviewed small bowel Dieulafoy's lesion studies and reports in the literature.

Sathyamurthy A, Winn JN, Ibdah JA, Tahan V. Culprit for recurrent acute gastrointestinal massive bleeding: "Small bowel Dieulafoy's lesions" - a case report and literature review. *World J Gastrointest Pathophysiol* 2016; 7(3): 296-299 Available from: URL: http://www.wjgnet.com/2150-5330/full/v7/i3/296.htm DOI: http://dx.doi.org/10.4291/wjgp.v7.i3.296

INTRODUCTION

Dieulafoy's lesion (DL) is a dilated, aberrant, submucosal vessel that erodes the overlying epithelium without evidence of a primary ulcer or erosion[1]. Dieulafoy's lesions account for 0.5% to 14% of all acute upper gastrointestinal bleeding events[2,3]. They develop throughout the gastrointestinal tract. However, approximately 98% of the lesions occur in the stomach and 80% of the gastric lesions are located in the proximal lesser curvature[4]. Although bleeding from small bowel DLs is rare[3], it may cause recurrent life-threatening, massive acute gastrointestinal bleeding[5]. Endoscopic tattooing can be helpful in locating the lesions for further re-treatment such as bowel resection when endoscopic treatments fail[6]. There are limited data regarding endoscopic treatment of small bowel DLs. We report an unusual case of small bowel DLs that we encountered in our clinical practice.

CASE REPORT

A 63-year-old Caucasian male who had: (1) an end-stage renal disease on hemodialysis; (2) radiation for squamous cell carcinoma of the throat; (3) gastrostomy tube insertion; (4) an episode of deep vein thrombosis (DVT) diagnosed a month prior to clinical presentation and on warfarin anticoagulation; (5) a recent diagnosis of invasive adenocarcinoma of the ascending colon; and (6) a history of intermittent melena/hematochezia with known duodenal bulb ulcers for which *Helicobacter pylori* eradication therapy was carried out one year prior to presentation. The patient presented with acute blood loss anemia and melena. Upon initial admission, he underwent two upper endoscopies with injection of epinephrine, deployment of hemostatic clips, and application of thermal cautery for duodenal bulb ulcers, then coil embolization of the gastroduodenal artery by interventional radiology. Despite these therapeutic interventions, he continued bleeding. Subsequently, he underwent tagged red blood cell scan to locate the source of bleeding, which was non-informative. He then had a colonoscopy, which revealed a previously noted an oozing friable and ulcerated colonic mass with a diameter of 2.5 cm that was treated with epinephrine. Further management of the colonic mass was deferred to the surgical oncology team due to the patient's on-going bleeding and co-morbidities, the patient experienced no further bleeding during the hospitalization and were subsequently discharged without anticoagulation. However, 2 d following discharge, the patient presented again with anemia and melena. He was re-admitted for recurrent upper gastrointestinal bleeding. We performed an urgent esophagogastroduodenoscopy (EGD) on admission which revealed two DLs 5 cm distal to the previous clips on the posterior wall of the second part of the duodenum. One of these DLs was a spurting artery while the other was oozing. They were treated with 5 mL epinephrine injection followed by placement of four clips, which successfully controlled the massive bleeding (Figure 1). Subsequent superior mesenteric angiograms demonstrated no active gastrointestinal hemorrhage. No further episodes of bleeding reported after the patient's discharge. The patient did not re-bleed during the same hospitalization for 5 d. He was discharged on hospice in view of his recurrent bleeding episodes and overall poor prognosis, then to home 2 d later.

DISCUSSION

The diameters of a DL vessels vary from 1 mm to 5 mm, which are about 10 times those of mucosal capillaries. They are usually located on the lesser curvature side of the gastroesophageal junction, although other areas of the gastrointestinal tract can be involved, including small intestines as in our case[3,7]. Etiology and precipitating events are not well known. Non-steroidal anti-inflammatory drugs may cause mucosal atrophy and ischemia[6]. Usually, the patients are males with co-morbidities such as coronary artery disease, diabetes, hypertension, chronic kidney disease, and alcohol abuse. Intensity of bleeding can vary from self-limited to life-threatening. The DL can be difficult to identify and it should be considered in the differential diagnosis of gastrointestinal bleeding without an obvious source.

Bleeding from small bowel DLs may be life-threatening and was treated only surgically before 1990[8]. The first published report of bleeding from small bowel DLs in 1978 involved 2 cases that were surgically treated[9]. Goldenberg *et al*[10] were the first to report a case of successful treatment of bleeding from duodenal DLs with a dual therapy with epinephrine injection and electrocoagulation. Recently, Lipka *et al*[3] recommended mechanical clipping as the therapy of choice since their three cases were successfully treated with clips after thermal therapy and/or radiological embolization failed. Furthermore, endoscopic hemostasis can be successfully achieved with a combination of epinephrine injection, hemostatic clip placement, bipolar coagulation, thermal coagulation, band ligation, argon plasma coagulation, and cyanoacrylate[10-13]. Endoscopic tattooing can be helpful in locating the lesions for endoscopic retreatment or intraoperative wedge resection. Therapeutic options for re-bleeding include repeat endoscopic hemostasis, angiographic embolization, or surgical wedge resection of the lesions. In general, endoscopic and angiographic interventions are initial attempted in controlling bleeding from DLs while a surgical approach is carried out if the others fail[12-16].

Table 1 summarizes the five case studies/reports about the management of the small bowel Dieulafoy's

Table 1 Small bowel Dieulafoy's lesion case studies and reports

Ref.	Dieulafoy's Lesion (*n*)	Incidence (%)	Duodenum DL (*n*)	Jejunum DL (*n*)	Ileum DL (*n*)	Endoscopic treatment	Repeated endoscopic treatment	Surgery needed (*n*)
Lipka *et al*[3]	8	2.6	1	7	0	8/8; Epi, bipolar, clips, APC	1	0
Prachayakul *et al*[17]	5	4.31	0	5	0	5/5; Epi, clips, APC	0	0
Dulic-Lakovic *et al*[12]	10	3.5	0	9	1	10/10; Epi, APC, clips	3	2
Chen *et al*[18]	4	2.6	0	4	0	4/4; Epi and clips	1	1
Kozan *et al*[19]	1	N/A	0	1	0	0	0	1
Han *et al*[20]	1	N/A	0	1	0	0	0	1
Present report	2 DLs in 1 case	N/A	2	0	0	2/2; Epi and clips	0	0

DL: Dieulafoy's lesion; N/A: Not applicable; Epi: Epinephrine injection; APC: Argon plasma coagulation.

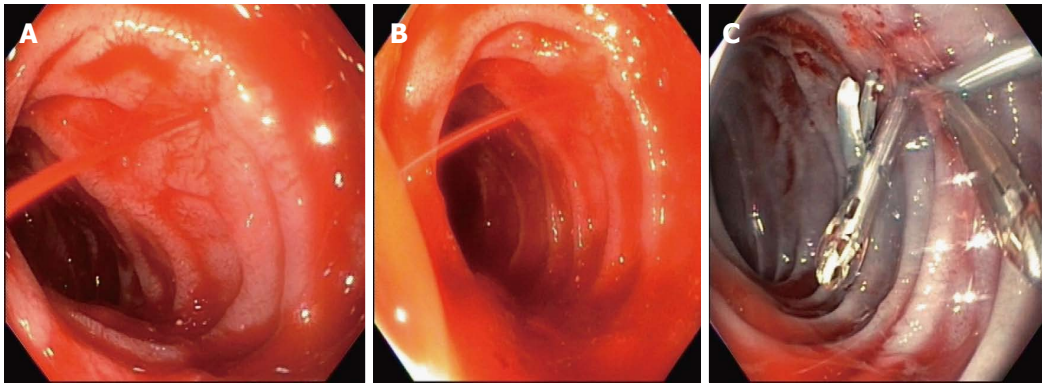


Figure 1 Small bowel Dieulafoy's lesions (A, B) and the active spurting hemorrhage was successful controlled (C).

lesions that were predominantly located in the jejunum. The bleeding was controlled with endoscopic interventions which reduced the need for surgery[3,12,18-20].

In conclusion, our case report and the current limited literature demonstrate that early aggressive endoscopic hemostasis of the small bowel DLs can be a successful alternative to surgery, with a low rate of re-bleeding.

COMMENTS

Case characteristics

A 63-year-old male who was on hemodialysis for end stage renal disease and warfarin for recent deep vein thrombosis, received radiation for squamous cell carcinoma of the throat, had gastrostomy tube insertion, had invasive adenocarcinoma of ascending colon, and had *Helicobacter pylori* eradication therapy for duodenal bulb ulcers. He presented with massive gastrointestinal hemorrhage.

Clinical diagnosis

Massive hemorrhage from the Dieulafoy's lesions at the distal duodenum.

Differential diagnosis

Hemorrhage from peptic ulcers or erosions, colon cancer, diverticuli, arterio-venous malformation, and aorto-enteric fistula.

Laboratory diagnosis

The patient was admitted to the intensive care unit with an acute hemoglobin drop of 5 g/dL.

Imaging diagnosis

Upper endoscopy revealed active hemorrhage from the small bowel Dieulafoy's lesions.

Treatment

Submucosal epinephrine injection and placement of four clips' application successfully controlled the hemorrhage.

Related reports

Previous small bowel Dieulafoy's lesion reports and studies were summarized in Table 1. The present report underlined the importance of the dual treatment for a successful outcome.

Term explanation

Dieulafoy's lesion is a dilated aberrant submucosal vessel that erodes the overlying epithelium without evidence of a primary ulcer or erosion. Small bowel Dieulafoy's lesion is rare but can cause life-threatening hemorrhage.

Experiences and lessons

Small bowel Dieulafoy's lesion hemorrhage is rare. When it is diagnosed, early aggressive endoscopic approach can be a successful alternative to extensive surgical resection.

Peer-review

The strongest point of this manuscript is reporting on a very rare diagnosis, successfully solved. The manuscript is concise and clear. The pictures are illustrative. The English language is fairly good. The experience presented in the manuscript could improve the readers' practice.

REFERENCES

1 **Lee YT**, Walmsley RS, Leong RW, Sung JJ. Dieulafoy's lesion. *Gastrointest Endosc* 2003; **58**: 236-243 [PMID: 12872092 DOI: 10.1067/mge.2003.328]

2 **Stark ME**, Gostout CJ, Balm RK. Clinical features and endoscopic management of Dieulafoy's disease. *Gastrointest Endosc* 1992; **38**: 545-550 [PMID: 1397908 DOI: 10.1016/S0016-5107(92)70513-6]

3 **Lipka S**, Rabbanifard R, Kumar A, Brady P. A single-center United States experience with bleeding Dieulafoy lesions of the small bowel: diagnosis and treatment with single-balloon enteroscopy. *Endosc Int Open* 2015; **3**: E339-E345 [PMID: 26356602 DOI: 10.1055/s-0034-1391901]

4 **Baettig B**, Haecki W, Lammer F, Jost R. Dieulafoy's disease: endoscopic treatment and follow up. *Gut* 1993; **34**: 1418-1421 [PMID: 8244112 DOI: 10.1136/gut.34.10.1418]

5 **Parra-Blanco A**, Takahashi H, Méndez Jerez PV, Kojima T, Aksoz K, Kirihara K, Palmerín J, Takekuma Y, Fuijta R. Endoscopic management of Dieulafoy lesions of the stomach: a case study of 26 patients. *Endoscopy* 1997; **29**: 834-839 [PMID: 9476766 DOI: 10.1055/s-2007-1004317]

6 **Jeon HK**, Kim GH. Endoscopic Management of Dieulafoy's Lesion. *Clin Endosc* 2015; **48**: 112-120 [PMID: 25844338 DOI: 10.5946/ce.2015.48.2.112]

7 **Pollack R**, Lipsky H, Goldberg RI. Duodenal Dieulafoy's lesion. *Gastrointest Endosc* 1993; **39**: 820-822 [PMID: 8293911 DOI: 10.1016/S0016-5107(93)70276-X]

8 **Choudari CP**, Palmer KR. Dieulafoy's lesion of the duodenum; successful endoscopic therapy. *Endoscopy* 1993; **25**: 371 [PMID: 8348889 DOI: 10.1055/s-2007-1010334]

9 **Matuchansky C**, Babin P, Abadie JC, Payen J, Gasquet C, Barbier J. Jejunal bleeding from a solitary large submucosal artery. Report of two cases. *Gastroenterology* 1978; **75**: 110-113 [PMID: 401085]

10 **Goldenberg SP**, DeLuca VA, Marignani P. Endoscopic treatment of Dieulafoy's lesion of the duodenum. *Am J Gastroenterol* 1990; **85**: 452-454 [PMID: 2248637]

11 **Lara LF**, Sreenarasimhaiah J, Tang SJ, Afonso BB, Rockey DC. Dieulafoy lesions of the GI tract: localization and therapeutic outcomes. *Dig Dis Sci* 2010; **55**: 3436-3441 [PMID: 20848205 DOI: 10.1007/s10620-010-1385-0]

12 **Dulic-Lakovic E**, Dulic M, Hubner D, Fuchssteiner H, Pachofszky T, Stadler B, Maieron A, Schwaighofer H, Püspök A, Haas T, Gahbauer G, Datz C, Ordubadi P, Holzäpfel A, Gschwantler M. Bleeding Dieulafoy lesions of the small bowel: a systematic study on the epidemiology and efficacy of enteroscopic treatment. *Gastrointest Endosc* 2011; **74**: 573-580 [PMID: 21802676 DOI: 10.1016/j.gie.2011.05.027]

13 **Iacopini F**, Petruzziello L, Marchese M, Larghi A, Spada C, Familiari P, Tringali A, Riccioni ME, Gabbrielli A, Costamagna G. Hemostasis of Dieulafoy's lesions by argon plasma coagulation (with video). *Gastrointest Endosc* 2007; **66**: 20-26 [PMID: 17591469 DOI: 10.1016/j.gie.2006.11.022]

14 **Mumtaz R**, Shaukat M, Ramirez FC. Outcomes of endoscopic treatment of gastroduodenal Dieulafoy's lesion with rubber band ligation and thermal/injection therapy. *J Clin Gastroenterol* 2003; **36**: 310-314 [PMID: 12642736 DOI: 10.1097/00004836-200304000-00006]

15 **Nadal E**, Burra P, Senzolo M. Cyanoacrylate injection to treat recurrent bleeding from Dieulafoy's lesion. *Gastrointest Endosc* 2013; **78**: 964-965.e2 [PMID: 24237954 DOI: 10.1016/j.gie.2013.08.015]

16 **D'Imperio N**, Papadia C, Baroncini D, Piemontese A, Billi P, Dal Monte PP. N-butyl-2-cyanoacrylate in the endoscopic treatment of Dieulafoy ulcer. *Endoscopy* 1995; **27**: 216 [PMID: 7601062 DOI: 10.1055/s-2007-1005671]

17 **Prachayakul V**, Deesomsak M, Aswakul P, Leelakusolvong S. The utility of single-balloon enteroscopy for the diagnosis and management of small bowel disorders according to their clinical manifestations: a retrospective review. *BMC Gastroenterol* 2013; **13**: 103 [PMID: 23800178 DOI: 10.1186/1471-230X-13-103]

18 **Chen TH**, Chiu CT, Lin WP, Su MY, Hsu CM, Chen PC. Application of double-balloon enteroscopy in jejunal diverticular bleeding. *World J Gastroenterol* 2010; **16**: 5616-5620 [PMID: 21105196 DOI: 10.3748/wjg.v16.i44.5616]

19 **Kozan R**, Gülen M, Yılmaz TU, Leventoğlu S, Yılmaz E. Massive lower gastrointestinal bleeding from a jejunal Dieulafoy lesion. *Ulus Cerrahi Derg* 2014; **30**: 225-227 [PMID: 25931935 DOI: 10.5152/UCD.2014.2271]

20 **Han MS**, Park BK, Lee SH, Yang HC, Hong YK, Choi YJ. [A case of Dieulafoy lesion of the jejunum presented with massive hemorrhage]. *Korean J Gastroenterol* 2013; **61**: 279-281 [PMID: 23756670 DOI: 10.4166/kjg.2013.61.5.279]

P- Reviewer: Serban DE, Vradelis S, Wang BM **S- Editor**: Qiu S
L- Editor: A **E- Editor**: Lu YJ

Published by **Baishideng Publishing Group Inc**
8226 Regency Drive, Pleasanton, CA 94588, USA
Telephone: +1-925-223-8242
Fax: +1-925-223-8243
E-mail: bpgoffice@wjgnet.com
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
http://www.wjgnet.com

ISSN 2150-5330 (online)

World Journal of *Gastrointestinal Pathophysiology*

World J Gastrointest Pathophysiol 2016 November 15; 7(4): 300-319

Published by Baishideng Publishing Group Inc

WJGP World Journal of Gastrointestinal Pathophysiology

A peer-reviewed, online, open-access journal of gastrointestinal pathophysiology

Editorial Board

2016-2019

The *World Journal of Gastrointestinal Pathophysiology* Editorial Board consists of 503 members, representing a team of worldwide experts in gastrointestinal pathophysiology. They are from 45 countries, including Argentina (2), Australia (13), Austria (2), Belgium (7), Brazil (9), Brunei Darussalam (1), Canada (21), China (30), Croatia (1), Czech Repoublic (2), Denmark (3), Egypt (1), Estonia (1), Finland (1), France (8), Germany (24), Greece (7), Hungary (5), India (12), Indonesia (1), Iran (2), Ireland (2), Israel (8), Italy (38), Japan (45), Lebanon (3), Malaysia (1), Mexico (2), Netherlands (7), Norway (1), Poland (4), Portugal (1), Romania (1), Russia (1), Singapore (4), South Korea (12), Spain (24), Sweden (11), Switzerland (3), Thailand (2), Turkey (7), Ukraine (1), United Kingdom (10), United States (161) and Venezuela (1).

Zhan-Ju Liu, *Shanghai*
Shi Liu, *Wuhan*
Zhen-Ning Wang, *Shenyang*
Xiao-Hong Wang, *Beijing*
Wei Wei, *Hefei*
Dong-Ping Xie, *Shanghai*
Wen-Xie Xu, *Shanghai*
Xiao Yang, *Beijing*
Hua Yang, *Chongqing*
Wei-Zhen Zhang, *Beijing*
Hua-Chuan Zheng, *Shenyang*
Min-Sheng Zhu, *Nanjing*
Jinxia Zhu, *Beijing*
Da-Ling Zhu, *Harbin*
Yong-Liang Zhu, *Hangzhou*

Croatia

Alen Protic, *Rijeka*

Czech Republic

Pavel Hladik, *Semily*
Martin Vokurka, *Prague*

Denmark

Lars Arendt-Nielsen, *Aalborg*
Frank V Schiodt, *Copenhagen*
Jonas Worsoe, *Aarhus*

Egypt

Mahmoud Aboelneen Khattab, *Minia*

Estonia

Enn Seppet, *Tartu*

Finland

Pauli Antero Puolakkainen, *Turku*

France

Bruno Bonaz, *Grenoble*
Pierre M Dechelotte, *Rouen*
Jean-Paul Lallès, *Saint-Gilles*
Charles-Henri Malbert, *Saint-Gilles*
Thierry Piche, *Nice*
Pascale Plaisancié, *Lyon*
Michelina Plateroti, *Lyon*
Véronique Vitton, *Marseille*

Germany

Hans G Beger, *Ulm*
Carsten Bergmann, *Ingelheim*
Elke Cario, *Essen*
Arno J Dormann, *Koln*
Nikolaus Gassler, *Aachen*
Andreas Geier, *Wuerzburg*
Werner Hartwig, *Heidelberg*
Marion Hewicker-Trautwein, *Hannover*
Jens Hoeppner, *Freiburg*
Tobias Keck, *Freiburg*
Jorg Kleeff, *Munich*
Peter Malfertheiner, *Magdeburg*
Oliver Mann, *Hamburg*
Christoph W Michalski, *Munich*
Andreas K Nussler, *Munich*
Christian Pehl, *Vilsbiburg*
Peter Schemmer, *Heidelberg*
Manuel A Silva, *Penzberg*
Marc Stemmler, *Freiburg*
Frank Tacke, *Aachen*
Sya N Ukena, *Braunschweig*
Brigitte Vollmar, *Rostock*
Thomas M Wex, *Magdeburg*
Margot Zoller, *Heidelberg*

Greece

Stelios F Assimakopoulos, *Patras*
George N Dalekos, *Larissa*
Alkiviadis Efthymiou, *thessaloniki*
Maria Gazouli, *Athens*
Ioannis E Koutroubakis, *Heraklion*
Gerassimos J Mantzaris, *Athens*
George V Papatheodoridis, *Athens*

Hungary

Mária Bagyánszki, *Szeged*
Mihály Boros, *Szeged*
Laszlo Czako, *Szeged*
Pal Miheller, *Budapest*
Zoltan Rakonczay, *Szeged*

India

Anil K Agarwal, *Delhi*
Uday Bandyopadhyay, *Kolkata*
Sriparna Basu, *Varanasi*
Chandra K Chakraborti, *Rourkela*
Nilesh M Dagia, *Mumbai*
Rajeev Garg, *Punjab*
Akhtar Mahmood, *Chandigarh*
Veerareddy P Reddy, *Andhra Pradesh*
Chandra P Sharma, *Thiruvananthapuram*
Shailesh V Shrikhande, *Mumbai*
Virendra Singh, *Chandigarh*
Nicholas J Skill, *Indianapolis*

Indonesia

Laurentius A Lesmana, *Jakarta*

Iran

Gholamreza Roshandel, *Gorgan*
Shahram Shahabi, *Urmia*

Ireland

Billy Bourke, *Dublin*
Stephen J Keely, *Dublin*

Israel

Yosefa Avraham, *Jerusalem*
Yaron Bar-Dayan, *Holon*
Shomron Ben-Horin, *Tel-Hashomer*
Boris Kirshtein, *Beer Sheva*
Stephen Malnick, *Rehovot*
Yaakov Maor, *Tel-Hashomer*
Rifaat Safadi, *Jerusalem*
Nachum Vaisman, *Tel Aviv*

Italy

Rosaria Acquaviva, *Catania*
Alessandro Antonelli, *Pisa*
Giacosa Attilio, *Genova*
Salavtore Auricchio, *Naples*
Guido Basilisco, *Milan*
Antonio Basoli, *Rome*
Massimo Bellini, *Pisa*
Luigi Bonavina, *Milano*
Alfio Brogna, *Catania*
Giuseppe Calamita, *Bari*
Raffaele Capasso, *Naples*
Ignazio Castagliuolo, *Padova*
Francesco Cresi, *Torino*
Rosario Cuomo, *Napoli*
Salvatore Cuzzocrea, *Gazzi*
Mario M D'Elios, *Florence*
Cinzia Domeneghini, *Milan*
Luca Elli, *Milano*
Walter Fries, *Messina*
Eugenio Gaudio, *Rome*
Marco Gobbetti, *Bari*
Fabio Grizzi, *Rozzano*
Enzo Grossi, *Milanese*
Enzo Ierardi, *Foggia*
Pietro Invernizzi, *Monza*
Angelo A Izzo, *Naples*
Anna Kohn, *Rome*
Giovanni Latella, *L'Aquila*
Massimo Marignani, *Rome*
Sergio Morini, *Rome*
Raffaele Pezzilli, *Bologna*
Cristiano Rumio, *Milan*
Giovanni Sarnelli, *Naples*
Edoardo V Savarino, *Padua*
Pierpaolo Sileri, *Roma*
Annamaria Staiano, *Naples*
Giacomo C Sturniolo, *Padua*
Claudio Tiribelli, *Trieste*

Japan

Hirotada Akiho, *Fukuoka*
Akihiro Asakawa, *Kagoshima*
Hisashi Aso, *Sendai*
Yasu-Taka Azuma, *Osaka*
Shotaro Enomoto, *Wakayama*
Mikihiro Fujiya, *Hokkaido*
Takahisa Furuta, *Hamamatsu*
Akira Hokama, *Nishihara*
Ryota Hokari, *Saitama*
Yuichi Hori, *Hyogo*
Masahiro Iizuka, *Akita*
Shoji Ikuo, *Kobe*
Motohiro Imano, *Osaka*

Hajime Isomoto, *Nagasaki*
Tatehiro Kagawa, *Isehara*
Haruki Kitazawa, *Sendai*
Shigeru BH Ko, *Tokyo*
Xiao-Kang Li, *Tokyo*
Noriaki Manabe, *Okayama*
Atsushi Masamune, *Sendai*
Hiroyuki Matsubayashi, *Suntogun*
Kazuyuki Matsushita, *Inohana Chuo-ku*
Reiko Miyazawa, *Gunma*
Kazunari Murakami, *Oita*
Hikaru Nagahara, *Tokyo*
Yuji Naito, *Kyoto*
Atsushi Nakajima, *Yokohama*
Shoji Natsugoe, *Kagoshima City*
Tsutomu Nishida, *Suita*
Koji Nomoto, *Tokyo*
Shouji Shimoyama, *Tokyo*
Goshi Shiota, *Yonago*
Hidekazu Suzuki, *Tokyo*
Hitoshi Takagi, *Gunma*
Toru Takahashi, *Okayama*
Yoshihisa Takahashi, *Tokyo*
Kan Uchiyama, *Chiba*
Yoshiyuki Ueno, *Sendai*
Takato Ueno, *Fukuoka*
Hisayuki Uneyama, *Kwasaki*
Mitsunori Yamakawa, *Yamagata*
Takayuki Yamamoto, *Mie*
Yutaka Yata, *Maebashi-city*
Naohisa Yoshida, *Kyoto*
Hitoshi Yoshiji, *Kashihara*

Lebanon

Costantine F Daher, *Byblos*
Assaad M Soweid, *Beirut*
Julnar Usta, *Beirut*

Malaysia

Andrew CS Boon, *Ipoh*

Mexico

José M de la Roca-Chiapas, *Leon*
Maria-Raquel Huerta-Franco, *Leon*

Netherland

Wouter J de Jonge, *Amsterdam*
Aldo Grefhorst, *Groningen*
Daniel Keszthelyi, *Maastricht*
Cornelis FM Sier, *ZA Leiden*
Pieter J Tanis, *AZ Amsterdam*
Luc JW van der Laan, *Rotterdam*
Sander van der Marel, *Leiden*

Norway

Anne M Bakke, *Oslo*

Poland

Stanistaw A Hac, *Gdansk*
Stanislaw J Kowalski, *Krakow*
Agata Mulak, *Wroclaw*
Napoleon Waszkiewicz, *Choroszcz*

Portugal

Ricardo Marcos, *Porto*

Romania

Mihai Ciocirlan, *Bucharest*

Russia

Ludmila Filaretova, *Petersburg*

Singapore

Madhav Bhatia, *Singapore*
Brian K Goh, *Singapore*
Khek-Yu Ho, *Singapore*
Cliff KS Ong, *Singapore*

South Korea

Jae Hee Cheon, *Seoul*
Myung-Haing Cho, *Seoul*
Ki-Baik Hahm, *Seongnam*
HO Jae Han, *Gwangju*
Chang-Duk Jun, *Gwangju*
Sang Geon Kim, *Seoul*
Hong Joo Kim, *Seoul*
Jin-Kyung Kim, *Gyeongsan-Si*
Won-Jae Lee, *Seoul*
Sung-Joo Park, *Iksan*
Seung Ha Park, *Gangwon Do*
Kwan-Kyu Park, *Daegu*

Spain

Raquel Abalo, *Madrid*
Juan G Abraldes, *Barcelona*
Dario Acuna-Castroviejo, *Armilla*
Agustin Albillos, *Madrid*
Maria-Angeles Aller, *Madrid*
Fernando Azpiroz, *Barcelona*
Ramón Bataller, *Barcelona*
Marco Bustamante-Balén, *Valencia*
Andres Cardenas, *Barcelona*
Joan Claria, *Barcelona*
Pere Clavé, *Barcelona*
Manuel B de Acosta, *Santiago*
Manuel Giner, *Madrid*
Borja Hernandez-Breijo, *Madrid*
Angel I Lanas, *Zaragoza*
Maite T Martin, *Barcelona*
Vicente Martinez, *Bellaterra*
Jose M Mates, *Malaga*
Julio Mayol, *Madrid*
Marcal Pastor-Anglada, *Barcelona*
María E Sáez, *Seville*
Yolanda Sanz, *Burjassot*
Carlos Taxonera, *Madrid*
Maria D Yago, *Granada*

Sweden

Marco Del Chiaro, *Stockholm*
Frida Fak, *Gothenburg*
Gunnar FA Flemstrom, *Uppsala*
Evangelos Kalaitzakis, *Gothenburg*
Kristina Lamas, *Umea*
Bob R Olsson, *Goteborg*
Sara M Regnér, *Malmo*
Peter T Schmidt, *Stockholm*
Xiao-Feng Sun, *Linkoping*
Henrik Thorlacius, *Malmo*
Curt Tysk, *Orebro*

Switzerland

Jyrki J Eloranta, *Zurich*
Remy Meier, *Liestal*
Catherine M Pastor, *Geneva*

Thailand

Thawatchai Akaraviputh, *Bangkok*
Weekitt Kittisupamongkol, *Bangkok*

Turkey

Mehmet Bektas, *Ankara*
Ugur Duman, *Bursa*
Mukaddes Esrefoglu, *Istanbul*
Ahmet Guven, *Ankara*
Muammer Karadeniz, *Bornova-Izmir*
Elvan Ozbek, *Sakarya*
Ilhami Yuksel, *Ankara*

Ukraine

Oksana S Zayavhkivska, *Lviv*

United Kingdom

Geoffrey Burnstock, *London*
Janice E Drew, *Aberdeen*
Girish L Gupte, *Birmingham*
David C Hay, *Edinburgh*
Nusrat Husain, *Manchester*
Michael L Lucas, *Glasgow*
Jamie Murphy, *London*
Vadim Sumbayev, *Chatham Maritime*
Wing-Kin Syn, *Birmingham*
Andrea Varro, *Liverpool*

United States

Sami R Achem, *Florida*
Tauseef Ali, *Oklahoma City*
Shrikant Anant, *Oklahoma*
Mohammed S Anwer, *North Grafton*
Andrew Aronsohn, *Chicago*
Toms Augustin, *Sayre*

WJGP

World Journal of Gastrointestinal Pathophysiology

Contents

Quarterly Volume 7 Number 4 November 15, 2016

ORIGINAL ARTICLE

Basic Study

Retrospective Study

META-ANALYSIS

World Journal of Gastrointestinal Pathophysiology
Volume 7 Number 4 November 15, 2016

Contents

NAME OF JOURNAL
World Journal of Gastrointestinal Pathophysiology

ISSN
ISSN 2150-5330 (online)

LAUNCH DATE
April 15, 2010

Frequency
Quarterly

EDITORIAL OFFICE
Xiu-Xia Song, Director
Fang-Fang Ji, Vice Director
World Journal of Gastrointestinal Pathophysiology
Baishideng Publishing Group Inc
8226 Regency Drive, Pleasanton, CA 94588, USA
Telephone: +1-925-2238242
Fax: +1-925-2238243
E-mail: editorialoffice@wjgnet.com
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
http://www.wjgnet.com

PUBLISHER
Baishideng Publishing Group Inc
8226 Regency Drive,
Pleasanton, CA 94588, USA
Telephone: +1-925-223-8242
Fax: +1-925-223-8243
E-mail: bpgoffice@wjgnet.com
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
http://www.wjgnet.com

PUBLICATION DATE
November 15, 2016

INSTRUCTIONS TO AUTHORS
http://www.wjgnet.com/bpg/gerinfo/204

ONLINE SUBMISSION
http://www.wjgnet.com/esps/

World Journal of Gastrointestinal Pathophysiology

Submit a Manuscript: http://www.wjgnet.com/esps/
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.4291/wjgp.v7.i4.300

World J Gastrointest Pathophysiol 2016 November 15; 7(4): 300-306
ISSN 2150-5330 (online)

ORIGINAL ARTICLE

Basic Study

Anti-*Helicobacter pylori* effect of CaG-NANA, a new sialic acid derivative

Yun-Hee Rhee, Hyun-Jeong Ku, Hye-Ji Noh, Hyang-Hyun Cho, Hee-Kyong Kim, Jin-Chul Ahn

Yun-Hee Rhee, Hyun-Jeong Ku, Jin-Chul Ahn, Beckman Laser Institute Korea, Dankook University, Chungnam 31116, South Korea

Hye-Ji Noh, Hyang-Hyun Cho, Hee-Kyong Kim, MEDINUTROL Co., Jeonnam 57024, South Korea

Jin-Chul Ahn, Department of Biomedical Science, College of Medicine, Dankook University, Chungnam 31116, South Korea

Jin-Chul Ahn, Medical Laser Research Center, Dankook University, Chungnam 31116, South Korea

Author contributions: Rhee YH performed the majority of experiments, analyzed the data, and wrote the manuscript; Ku HJ and Noh HJ participated in animal experiments; Cho HH, Kim HK and Ahn JC designed and coordinated the research.

Institutional animal care and use committee statement: All procedures involving animals were reviewed and approved by the Institutional Animal Care and Use Committee of the Dankook University (IACUC protocol number: DKU-14-036).

Animal care and use statement: The ICR mice (20-22 g) were housed in pathogen free environment and had free access to sterile neutral water and standard mouse feed. Oral administration was performed with conscious animals using sonde appropriate for the animal size.

Conflict-of-interest statement: The authors have no conflict of interest to declare.

Data sharing statement: Statistics and histology analysis of data are available from the corresponding author at jcahn@dankook.ac.kr.

Manuscript source: Invited manuscript

Correspondence to: Jin-Chul Ahn, MD, Department of Biomedical Science, College of Medicine, Dankook University, 119 Dandae-ro, Cheonan, Chungnam 31116, South Korea. jcahn@dankook.ac.kr
Telephone: +82-1899-3700

Received: June 28, 2016
Peer-review started: June 29, 2016
First decision: August 10, 2016
Revised: August 12, 2016
Accepted: September 13, 2016
Article in press: September 18, 2016
Published online: November 15, 2016

Abstract

AIM

To investigate the bactericidal effects of calcium chelated N-acetylneuraminic acid-glycomacropeptide (CaG-NANA) against *Helicobacter pylori* (*H. pylori*).

METHODS

For manufacture of CaG-NANA, calcium (Ca) was combined with glycomacropeptide (GMP) by chelating, and N-acetylneuraminic acid (NANA) was produced with Ca-GMP substrate by an enzymatic method. The final concentration of each component was 5% Ca, 7% NANA, 85% GMP, and 3% water. For *in vitro* study, various concentrations of CaG-NANA were investigated under the minimal inhibitory concentration (MIC). For *in vivo* study, CaG-NANA was administered orally for 3 wk after *H. pylori* infection. The levels of inflammatory cytokines in blood were analyzed by enzyme-linked immunosorbent assay and eradication of *H. pylori* was assessed by histological observation.

RESULTS

The time-kill curves showed a persistent decrease in cell

numbers, which depended on the dose of CaG-NANA, and MIC of CaG-NANA against *H. pylori* was 0.5% *in vitro*. Histopathologic observation revealed no obvious inflammation or pathologic changes in the gastric mucosa in the CaG-NANA treatment group *in vivo*. The colonization of *H. pylori* was reduced after CaG-NANA treatment. The levels of interleukin (IL)-6, IL-1β, tumor necrosis factor-α, and IL-10 were also decreased by CaG-NANA.

CONCLUSION

CaG-NANA demonstrates effective anti-bactericidal activity against *H. pylori* both *in vitro* and *in vivo*.

Key words: *Helicobacter pylori*; Calcium chealated N-acetylneuraminic acid-glycomacropeptide

Core tip: Calcium chelated N-acetylneuraminic acid-glycomacropeptide demonstrates effective anti-bactericidal activity against *Helicobacter pylori* both *in vitro* and *in vivo*.

Rhee YH, Ku HJ, Noh HJ, Cho HH, Kim HK, Ahn JC. Anti-*Helicobacter pylori* effect of CaG-NANA, a new sialic acid derivative. *World J Gastrointest Pathophysiol* 2016; 7(4): 300-306 Available from: URL: http://www.wjgnet.com/2150-5330/full/v7/i4/300.htm DOI: http://dx.doi.org/10.4291/wjgp.v7.i4.300

INTRODUCTION

Helicobacter pylori (*H. pylori*) has been reported to be associated with many gastrointestinal diseases such as chronic gastritis, peptic ulcers, gastric carcinoma, and mucosa-associated lymphoid tissue lymphoma[1-3]. Until now, standard triple therapy consisting of a proton pump inhibitor and two broad-spectrum antibiotics, usually amoxicillin, clarithromycin or metronidazole, has been able to achieve eradication[3-5]. However, the Maastricht IV Consensus report recommended the careful choice of the antibiotic combination for treatment according to local *H. pylori* antibiotic resistance patterns due to multidrug resistance of *H. pylori*. For example, the concentrations of antibiotics for treatment of *H. pylori* infection were required at four times when *H. pylori* had local clarithromycin resistance[6]. Since antibiotic abuse had side effects and allowed development of resistance to antibiotics, alternative strategies have been proposed to counteract *H. pylori* infection. As an alternative approach, preservation of mucus from *H. pylori* attachment is emphasized because *H. pylori* infection disrupted the epithelial barrier which resulted in inflammation or cancer[7]. For example, dietary inhibitors such as lacto-oligosaccharide has been suggested as a solution for *H. pylori* infections[8]. Although oligosaccharides specific for the *H. pylori* lectins may potentially act as inhibitors of adhesion to mucus, their production in commercial amounts as anti-adhesion therapeutic agents is still a problem[9,10]. Here, we introduce a new N-acetylneuraminic acid (NANA) combined glycomacropeptide (GMP) which was made from milk serum hydrolysis protein powder as an anti-*H. pylori* agent. Wadström *et al*[11] reported that NANA derivative had an adhesion potential to *H. pylori* lectins and Hirmo *et al*[12] reported that milk glycoprotein had an anti-*H. pylori* effect. We designed a new material with NANA, GMP, and calcium (CaG-NANA), and anti-*H. pylori* activities of CaG-NANA were investigated both *in vitro* and *in vivo*.

MATERIALS AND METHODS

H. pylori strain and experimental animals

H. pylori strain (SS1-passed-5) was kindly provided by Helicobacter pylori Korean Type Culture Collection (HpKTCC, Jinju, South Korea) and grown in 1.5% agar added Brucella broth with 10% horse serum (No. CM0169; OXOID, Waltham, MA, United States). The pathogen-free (SPF) male ICR mice (20-22 g) were purchased from Orient Bio (Daejeon, South Korea). All the animals were housed in an SPF environment and had free access to sterile neutral water and standard mouse feed. The experimental procedures in this study were approved by the Experimental Animal Ethics Committee of Dankook University, South Korea (No. DKU14-036).

Supply of CaG-NANA

CaG-NANA and each component of CaG-NANA were provided by MediNutrol (Kwangju, South Korea). As shown in Figure 1A, CaG-NANA was manufactured from calcium (Ca), GMP, and NANA by chelating and enzyme methods where the component concentrations were 5%, 85%, and 7%, respectively. For comparing each produced compound, we wrote abbreviated form as standard NANA (S-NANA), GMP linked NANA (G-NANA), and calcium chelated G-NANA (CaG-NANA).

High performance liquid chromatography for confirmation of NANA

For confirmation of NANA component, we analyzed CaG-NANA using high performance liquid chromatography (HPLC) method. HPLC analysis was performed using Agilent 1260 model equipped with a pump (G1311C), an auto sampler (G1329B), a column (G1316A), and a ultraviolet detector (G1314F), which was purchased from Agilent (Santa Clara, CA, United States). The condition of analysis was described in Table 1.

Culture and collection of *H. pylori*

H. pylori was incubated in Brucella medium contained the selective supplements (No. SR0083; OXOID, Waltham, MA, United States) under microaerobic environment (15% CO_2, 5% O_2, 80% N_2) at 37 ℃ for 72 h. The bacterial colonies were collected and identified

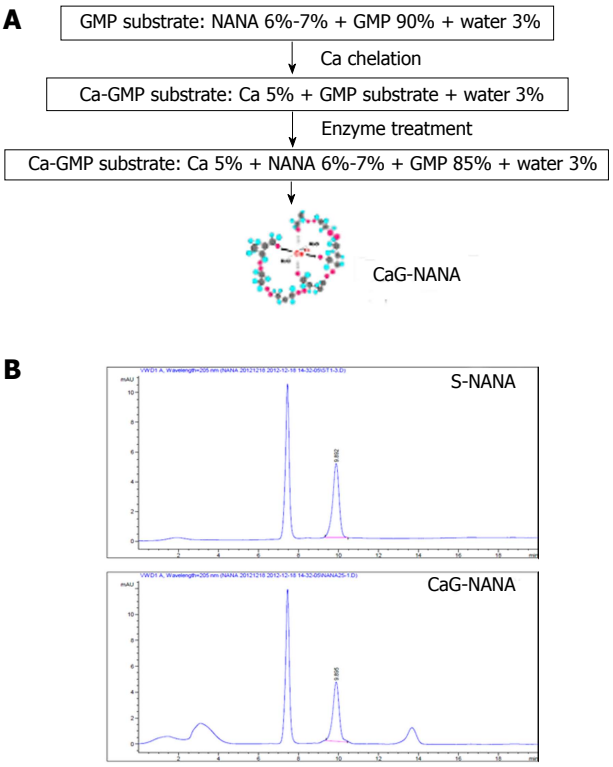


Figure 1 Confirmation of N-acetylneuraminic acid. A: The scheme of CaG-NANA manufacture; B: HPLC analysis of NANA content in CaG-NANA. The condition of HPLC analysis is described in Table 1. GMP: Glycomacropeptide; HPLC: High performance liquid chromatography; CaG-NANA: Calcium-glycomacropeptide-N-acetylneuraminic acid; S-NANA: Standard N-acetylneuraminic acid.

with a Pronto-Dry infection kit (Kokab Enterprise, Karachi, Pakistan) for bactericidal tests *in vitro*.

Inhibitory effect of CaG-NANA on H. pylori in vitro

The inhibitory activity of CaG-NANA against the growth of *H. pylori* was assessed using an agar dilution method. Briefly, 0.1%, 0.25%, or 0.5% of CaG-NANA and its components were added to the Brucella agar. The non-drug agar served as a negative control. Agar plates were inoculated with *H. pylori* at serial concentrations of 1×10^8, 1×10^7, and 1×10^6 colony forming units (CFU)/mL and cultured for 72 h. The minimal inhibitory concentration (MIC) was defined as the minimal concentration of CaG-NANA required for complete inhibition of *H. pylori* growth. The colonies were counted using image J (https://imagej.nih.gov/ij/) after 72 h incubation and the average number was calculated. Bactericidal activity was evaluated using time-kill curves with 0.5, 1.0 and 2.0 × MIC of CaG-NANA compared with the blank controls.

Experimental design in vivo

Mice (n = 40) were randomly divided into four groups: Blank control, *H. pylori* infected control, antibiotic treatment, and CaG-NANA treatment. *H. pylori* (1×10^9 /mL) was administered by gastric intubation to mice three times in a week except the blank control, and *H. pylori* infection was detected with gastric irrigation from randomly selected mice using an *H. pylori* detection test kit which was purchased from Pronto Dry (Brignais, France; Supple 1A). The antibiotic treatment was performed with a suspension of amoxicillin (12.33 mg/kg), metronidazole (164.40 mg/kg), and clarithromycin (205.54 mg/kg) which were equivalent to clinical administration (Dankook University hospital, Korea). Treatments with CaG-NANA and each component including NANA, S-NANA, and G-NANA were performed by oral administration every day for 3 wk, and the experimental design is shown in Figure 2A.

Table 1 High performance liquid chromatography analysis condition

Detector	UV detector
Wavelength	205 nm
Column	Aminex HPX-87H Ion Exclusion column (300 mm × 7.8 mm, 9 μm)
Mobile phase	10 mmol/L H_2SO_4 in water (isocratic)
Running time	20 min
Flow rate	0.5 mL/min
Injection volume	10 mL
Temperature	40 ℃

Histological observation

The animals were deprived of feed but allowed free access to water for 24 h before sacrificed. At the end of sacrifice, blood was collected from mouse and gastric tissues were removed and fixed in 10% formalin. After fixation, tissues were processed and embedded in paraffin. The paraffin blocks were cut into 4 μm sections and stained with Harris' hematoxylin and eosin for histological observation under a microscope (BX51, Olympus, Miami, FL, United States).

Enzyme-linked immunosorbent assay

The expression levels of interleukin (IL)-1β, IL-6, tumor necrosis factor (TNF)-α, and IL-10 in blood serum were determined using enzyme-linked immunosorbent assay (ELISA). Mouse ELISA kits were purchased from R&D Systems (Minneapolis, MN, United States). The assays were performed according to the manufacturer's instructions and repeated in triplicate.

Statistical analysis

Eradication rates were compared among groups by one-way analysis of variance (Kruskal-Wallis) and Dunn's multiple comparison test (GraphPad Software Inc., La Jolla, CA, United States). $P < 0.05$ was considered statistically significant.

RESULTS

Confirmation of NANA

As shown Figure 1B, the NANA component of CaG-NANA showed the same purity as standard NANA.

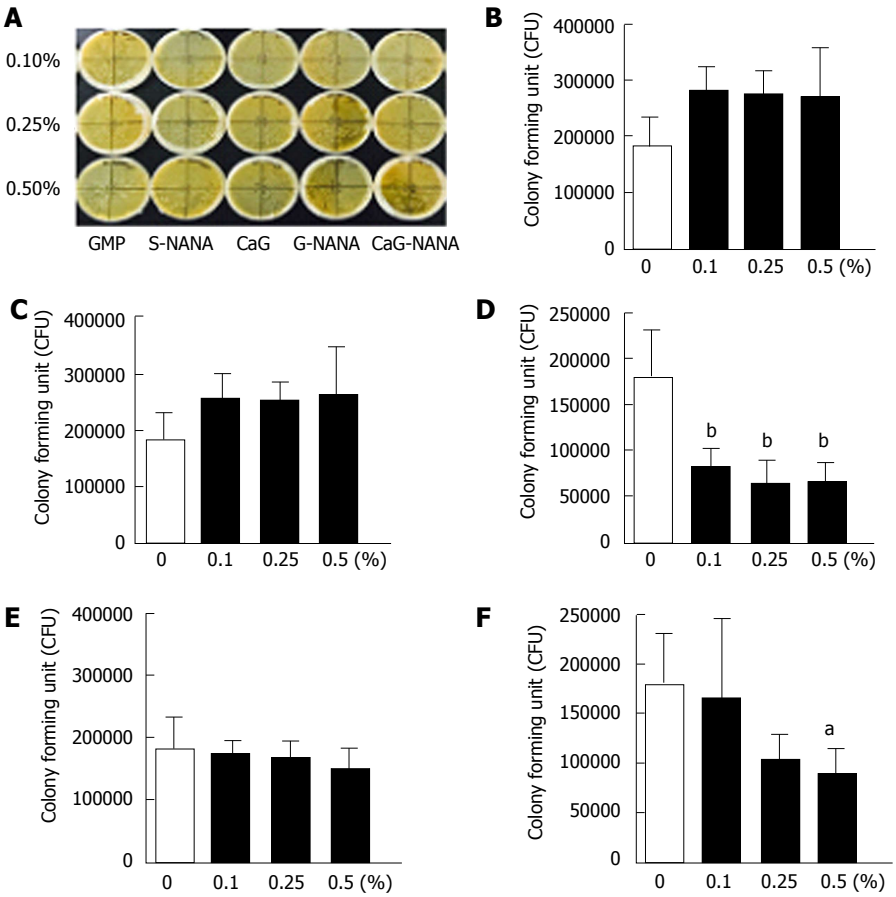


Figure 2 Inhibitory effect of calcium-glycomacropeptide-N-acetylneuraminic acid on *Helicobacter pylori in vitro*. Agar plates were inoculated with *Helicobacter pylori* (*H. pylori*) at serial concentrations of 1 × 10^8, 1 × 10^7, and 1 × 10^6 CFU/mL and cultured for 72 h. The minimal inhibitory concentration (MIC) was defined as the minimal concentration of materials required for complete inhibition of *H. pylori* growth. Bactericidal activity was evaluated using time-kill curves with 0.5, 1.0 and 2.0 × MIC of CaG-NANA compared with blank controls. All experiments were performed three times and significance was set at $^aP < 0.1$ and $^bP < 0.05$. A: Picture of colony forming unit assay; B: GMP; C: CaG; D: S-NANA; E: G-NANA; F: CaG-NANA. GMP: Glycomacropeptide; CaG: Calcium-glycomacropeptide; S-NANA: Ststandard N-acetylneuraminic acid; CaG-NANA: Calcium-glycomacropeptide-N-acetylneuraminic acid.

Inhibitory effect of CaG-NANA on H. pylori in vitro

The bactericidal effects of CaG-NANA and each component against *H. pylori* were assessed *in vitro*. We described previously that CaG-NANA is composed of NANA, GMP and calcium, thus we tested the anti-bacterial effects of every component used in CaG-NANA synthesis. As shown in Figure 1, S-NANA, G-NANA and CaG-NANA had an anti-bacterial effect (Figure 1D, E and F) whereas GMP and GMP with calcium (CaG) had no activity in the colony forming assay (Figure 1B and C). This result indicated that only NANA included compound had anti-bacterial activity.

Inhibitory effect of CaG-NANA on H. pylori in vivo

For the *in vivo* study, we divided mice into four groups and 1 × 10^9/mL CFU of *H. pylori* was administered into mice three times for one week except the negative group. *H. pylori* infection was confirmed by gastric irrigation using an *H. pylori* detection test kit (Pronto Dry). After confirmation, treatments with antibiotics, CaG-NANA and its components were orally administered every day for 3 wk. The doses of antibiotics were described in the "Material and Methods" section (Figure 3A). The doses of CaG-NANA and its components were fixed at 0.5% (v/w). We followed the mouse weight after treatment. As shown in Figure 3B, mouse weight was decreased in the S-NANA and *H. pylori* positive control groups. From this result, S-NANA was considered to have toxicity at 0.5% of concentration.

Next, we observed the gastric mucus layer of mice. The majority of the *H. pylori* population reside in the mucus which binds the organisms *via* specific interactions such as pathogen adherence. Normal gastric mucosa has a uniform surface epithelium (Figure 4A), whereas *H. pylori* infected gastric mucosa has an irregular outline of the epithelium layer along with neutrophil and macrophage infiltrations (Figure 4B). In addition, bacterial attachment is mediated by outer membrane adhesions that bind to glycoconjugates present in the gastric mucus layer, lining the surface epithelium of the gastric mucosa in the *H. pylori* infected group. As shown in Figure 4C, the antibiotic treatment group had decreased infiltration of macrophages and adhesion of *H. pylori* colonization, however, the destruction of surface epithelium layers was also observed. Meanwhile, every NANA including group showed uniformed surface epithelium layers. Interestingly, many macrophages and neutrophils were observed in the S-NANA treatment

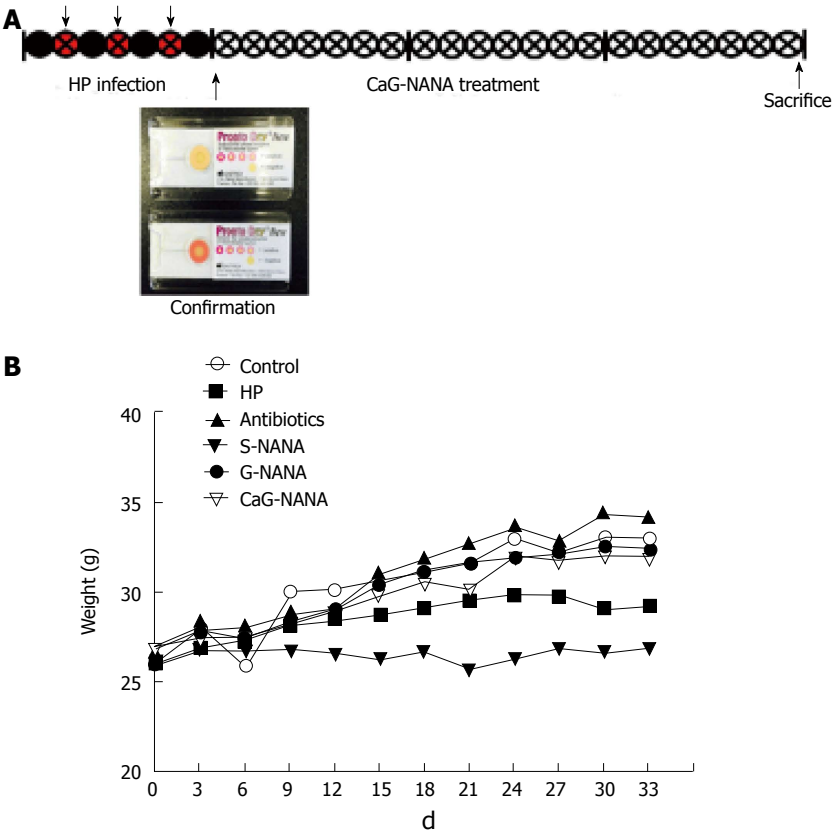


Figure 3 Inhibitory effect of calcium-glycomacropeptide-N-acetylneuraminic acid on *Helicobacter pylori in vivo*. Antibiotic doses are described in the "materials and methods" section. A: Schematic design of *in vivo* study; B: Weight changes of mice. HP: Helicobacter pyroli; S-NANA: Standard N-acetyln-euraminic acid; G-NANA: Glycomacropeptide-N-acetylneuraminic acid; CaG-NANA: Calcium-glycomacropeptide-N-acetylneuraminic acid.

group (Figure 4D). The damage to surface epithelium in the antibiotics treatment group and inflammation observed in the S-NANA treatment group might be related to mouse weight loses. Both the G-NANA and CaG-NANA treatment groups showed the detachment of *H. pylori* colonization without any damage to surface epithelium or inflammation (Figure 4E and F).

We also assessed the levels of IL-1β, IL-6, TNF-α, and IL-10 in blood by ELISA. PBS was used as a control because every compound was dissolved in PBS. Both of S-NANA and CaG-NANA showed a potent ability to decrease inflammatory cytokines. The level of IL-1β was reduced from 7.6 ng/mL to 0.8 ng/mL after S-NANA treatment and 2.36 ng/mL after CaG-NANA treatment (Figure 5A). The level of IL-6 was remarkedly decreased from 113.4 ng/mL to 33.5 ng/mL after S-NANA treatment and 1.26 ng/mL after CaG-NANA treatment (Figure 5B). The level of TNF-α was also reduced to 8.21 ng/mL after S-NANA treatment and 1.44 ng/mL after CaG-NANA treatment (Figure 5C). Meanwhile, the level of IL-10 was reduced only in the CaG-NANA treatment group from 7.62 ng/mL to 6.3 ng/mL (Figure 5D).

DISCUSSION

The anti-*H. pylori* effects of antibiotic formulas have been investigated, but the findings are limited by varying drug quality and sources, as well as the numerous and complicated components of formulas. Thus, it has been suggested that dietary inhibitors may be a solution for certain infections as an alternative approach[8]. Eradication of *H. pylori* has remained difficult for reasons that lie in the biology and environment of the organism. *H. pylori* populations colonize epithelial cells that line the antrum of the stomach and survive in the acidic environment[13]. Most anti-microbial agents are poorly secreted in the gastric mucosa because of the stomach environment[14], and the residues expressed on *H. pylori* enable specific binding to the mucus layers. Thus, CaG-NANA was excogitated to overcome the acidic environment of the stomach and have bactericidal activity by interrupting glycol-conjugation of *H. pylori* on the mucosa.

CaG-NANA, a compound of N-acetylneuraminic acid with calcium combined glycomacropeptide, demonstrated an anti-bacterial effect against *H. pylori*. We manufactured CaG-NANA as a dietary inhibitor against *H. pylori*; NANA was demonstrated as an *H. pylori* adhesion blocker[9,15,16], glycomacropeptide was used for modulating the acidic phase of the stomach, and calcium was inserted for improvement as functional food. NANA content in CaG-NANA was evaluated compared to S-NANA by HPLC, which confirmed its content and purity (Figure 1B). CaG-NANA at concentrations > 0.25% regulated the population of *H. pylori in vitro*. S-NANA also decreased the population of *H. pylori in vitro* (Figure 2D); however, S-NANA resulted in a severe weight loss *in vivo* (Figure 3B), which demonstrated that S-NANA treatment only had negative utility. Meanwhile, G-NANA and CaG-NANA had bactericidal and anti-adhesion effects on glyco-conjugation of the mucosa without toxicity. Bacterial attachment is mediated by outer membrane adhesions that bind to glycoconjugates present in the gastric mucus layer and lining the surface epithelium of

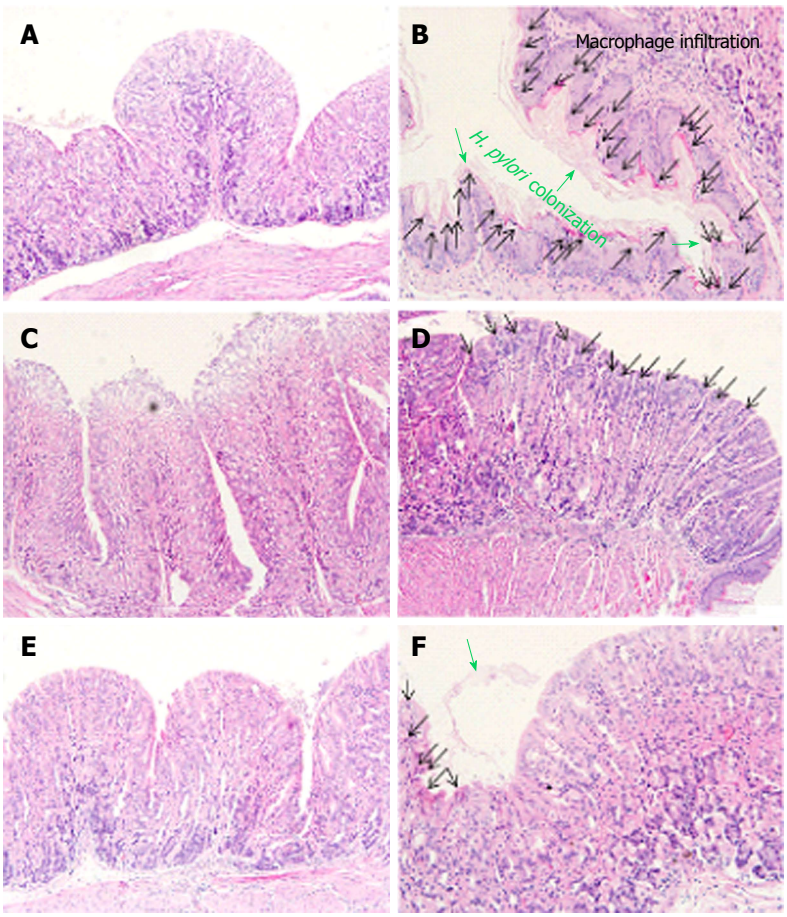


Figure 4 Histology of the gastric mucosa. Mouse stomachs were removed and underwent hematoxylin and eosin staining. *H. pylori* colonization and macrophage infiltration were observed under a light microscope. A: Normal group; B: *H. pylori* infected group; C: Antibiotics treated group; D: S-NANA treated group; E: G-NANA treated group; F: CaG-NANA treated group. S-NANA: Standard N-acetylneuraminic acid; G-NANA: Glycomacropeptide-N-acetylneuraminic acid; CaG-NANA: Calcium-glycomacropeptide-N-acetylneuraminic acid.

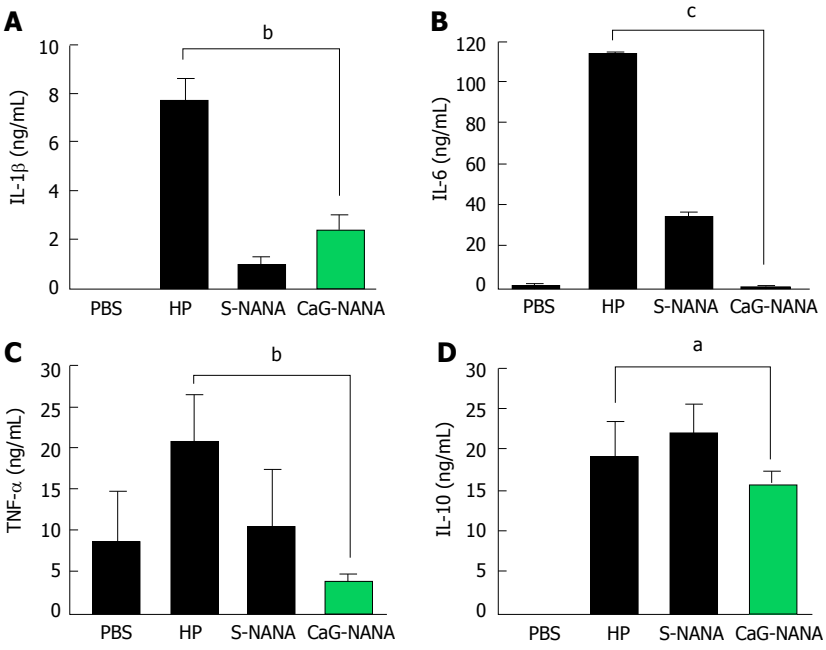


Figure 5 Expression levels of interleukine-1β, interleukine-6, tumor necrosis factor-α, and interleukine-10 in blood serum determined by enzyme-linked immunosorbent assay. The assays were performed according to the manufacturer's instructions and all experiments were repeated in triplicate. Statistical analysis was performed using Dunn's multiple comparison test with significance set at $^{a}P < 0.1$ and $^{b}P < 0.05$, $^{c}P < 0.01$. A: IL-1β; B: IL-6; C: TNF-α; D: IL-10. IL: Interleukine; TNF: Tumor necrosis factor; S-NANA: Standard N-acetylneuraminic acid; G-NANA: Glycomacropeptide-N-acetylneuraminic acid; CaG-NANA: Calcium-glycomacropeptide-N-acetylneuraminic acid.

the gastric mucosa[17].

H. pylori is also known to induce inflammatory response that includes the up-regulation of pro-inflammatory cytokines. CaG-NANA exerted a down-regulatory effect on pro-inflammatory cytokines such as IL-1β, IL-6, TNF-α and IL-10. Reduction of pro-inflammatory cytokines induced by *H. pylori* is responsible for the recruitment of macrophages and neutrophils in the lamina propria. CaG-NANA exerted an antagonistic effect against *H. pylori*, which is an anti-microbial effect *via* different mechanisms from those of antibiotics.

This study suggests that it is possible to decrease the number of *H. pylori* or its activation in the stomach through a regular ingestion of N-acetylneuraminic acid containing glycomacropeptide as dietary products. Moreover, CaG-NANA is a complex compound which can be manufactured in large scale at a low cost under good manufacturing practice (GMP). In conclusion, the anti-*H. pylori* effects of CaG-NANA were confirmed both *in vitro* and *in vivo*, which provided experimental support for future human trials.

COMMENTS

Background

Helicobacter pylori (*H. pylori*) has been reported to be associated with many gastrointestinal diseases such as chronic gastritis, peptic ulcers, gastric carcinoma, and mucosa-associated lymphoid tissue lymphoma. Until now, standard triple therapy consisting of a proton pump inhibitor and two broad-spectrum antibiotics, usually amoxicillin, clarithromycin or metronidazole, has been able to achieve eradication.

Research frontiers

The authors introduce a new N-acetylneuraminic acid (NANA) combined glycomacropeptide (GMP) which was made from milk serum hydrolysis protein powder as an anti-*H. pylori* agent. Wadstorm *et al* reported that NANA derivative had an adhesion potential to *H. pylori* lectins and Hirmo *et al* reported that milk glycoprotein had an anti-*H. pylori* effect. The authors designed a new material with NANA, GMP, and calcium (CaG-NANA), and anti-*H. pylori* activities of CaG-NANA were investigated both *in vitro* and *in vivo*.

Innovations and breakthroughs

The anti-*H. pylori* effects of CaG-NANA were confirmed both *in vitro* and *in vivo*, which provided experimental support for future human trials.

Applications

CaG-NANA is a complex compound which can be manufactured in a large scale at a low cost under GMP.

Peer-review

In the present paper, entitled "Anti-*Helicobacter pylori* effect of CaG-NANA, a new sialic acid derivative", Rhee *H et al* have investigated the effect of a compound named Cag-NANA in an animal model of *H. pylori*-associated gastritis and, *in vitro*, in bacterial cultures.

REFERENCES

1 **Simon PM**, Goode PL, Mobasseri A, Zopf D. Inhibition of Helicobacter pylori binding to gastrointestinal epithelial cells by sialic acid-containing oligosaccharides. *Infect Immun* 1997; **65**: 750-757 [PMID: 9009338]

2 **Logan RP**. Adherence of Helicobacter pylori. *Aliment Pharmacol Ther* 1996; **10** Suppl 1: 3-15 [PMID: 8730255 DOI: 10.1046/j.1365-2036.1996.22164001.x]

3 **Opekun AR**, El-Zaimaity HM, Osato MS, Gilger MA, Malaty HM, Terry M, Headon DR, Graham DY. Novel therapies for Helicobacter pylori infection. *Aliment Pharmacol Ther* 1999; **13**: 35-42 [PMID: 9892877 DOI: 10.1046/j.1365-2036.1999.00435.x]

4 **Kigasawa K**, Ohtani H. Decomposition and stabilization of drugs. XV. Structure and stability of aminoalkylesters. (7) (author's transl). *Yakugaku Zasshi* 1976; **96**: 6-11 [PMID: 943510]

5 **Yang JC**, Chien CT. A new approach for the prevention and treatment of Helicobacter pylori infection via upregulation of autophagy and downregulation of apoptosis. *Autophagy* 2009; **5**: 413-414 [PMID: 19197143]

6 **Malfertheiner P**, Megraud F, O'Morain CA, Atherton J, Axon AT, Bazzoli F, Gensini GF, Gisbert JP, Graham DY, Rokkas T, El-Omar EM, Kuipers EJ. Management of Helicobacter pylori infection--the Maastricht IV/ Florence Consensus Report. *Gut* 2012; **61**: 646-664 [PMID: 22491499 DOI: 10.1136/gutjnl-2012-302084]

7 **Caron TJ**, Scott KE, Fox JG, Hagen SJ. Tight junction disruption: Helicobacter pylori and dysregulation of the gastric mucosal barrier. *World J Gastroenterol* 2015; **21**: 11411-11427 [PMID: 26523106 DOI: 10.3748/wjg.v21.i40.11411]

8 **Burger O**, Ofek I, Tabak M, Weiss EI, Sharon N, Neeman I. A high molecular mass constituent of cranberry juice inhibits helicobacter pylori adhesion to human gastric mucus. *FEMS Immunol Med Microbiol* 2000; **29**: 295-301 [PMID: 11118911 DOI: 10.1111/j.1574-695X.2000.tb01537.x]

9 **Joffe M**. Epidemiology of occupational reproductive hazards: methodological aspects. *Rev Epidemiol Sante Publique* 1992; **40** Suppl 1: S17-S25 [PMID: 1626103 DOI: 10.1371/journal.ppat.0020110]

10 **Teneberg S**. The Multiple Carbohydrate Binding Specificities of Helicobacter pylori. *Top Curr Chem* 2009; **288**: 121-138 [PMID: 22328028 DOI: 10.1007/128_2008_14]

11 **Wadström T**, Hirmo S, Borén T. Biochemical aspects of Helicobacter pylori colonization of the human gastric mucosa. *Aliment Pharmacol Ther* 1996; **10** Suppl 1: 17-27 [PMID: 8730256 DOI: 10.1046/j.1365-2036.1996.22164002.x]

12 **Hirmo S**, Kelm S, Iwersen M, Hotta K, Goso Y, Ishihara K, Suguri T, Morita M, Wadström T, Schauer R. Inhibition of Helicobacter pylori sialic acid-specific haemagglutination by human gastrointestinal mucins and milk glycoproteins. *FEMS Immunol Med Microbiol* 1998; **20**: 275-281 [PMID: 9626932 DOI: 10.1111/j.1574-695X.1998.tb01137.x]

13 **Falk P**, Roth KA, Borén T, Westblom TU, Gordon JI, Normark S. An in vitro adherence assay reveals that Helicobacter pylori exhibits cell lineage-specific tropism in the human gastric epithelium. *Proc Natl Acad Sci USA* 1993; **90**: 2035-2039 [PMID: 8383333]

14 **Sakarya S**, Gunay N. Saccharomyces boulardii expresses neuraminidase activity selective for α2,3-linked sialic acid that decreases Helicobacter pylori adhesion to host cells. *APMIS* 2014; **122**: 941-950 [PMID: 24628732 DOI: 10.1111/apm.12237]

15 **Narod S**. Measles vaccination in Haiti. *N Engl J Med* 1986; **314**: 581-582 [PMID: 3945300 DOI: 10.1074/jbc.M113.513135]

16 **Salcedo J**, Barbera R, Matencio E, Alegría A, Lagarda MJ. Gangliosides and sialic acid effects upon newborn pathogenic bacteria adhesion: an in vitro study. *Food Chem* 2013; **136**: 726-734 [PMID: 23122120 DOI: 10.1016/j.foodchem.2012.08.078]

17 **Valkonen KH**, Wadström T, Moran AP. Identification of the N-acetylneuraminyllactose-specific laminin-binding protein of Helicobacter pylori. *Infect Immun* 1997; **65**: 916-923 [PMID: 9038297]

P- Reviewer: Ierardi E, Pellicano R **S- Editor**: Qi Y
L- Editor: Wang TQ **E- Editor**: Wu HL

World Journal of Gastrointestinal Pathophysiology

Submit a Manuscript: http://www.wjgnet.com/esps/
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.4291/wjgp.v7.i4.307

World J Gastrointest Pathophysiol 2016 November 15; 7(4): 307-313
ISSN 2150-5330 (online)

ORIGINAL ARTICLE

Retrospective Study

Microscopic colitis in patients with mild duodenal damage: A new clinical and pathological entity ("lymphocytic enterocolitis")?

Gabriele Antonio Bonagura, Davide Giuseppe Ribaldone, Sharmila Fagoonee, Nicoletta Sapone, Gian Paolo Caviglia, Giorgio Maria Saracco, Marco Astegiano, Rinaldo Pellicano

Gabriele Antonio Bonagura, Davide Giuseppe Ribaldone, Nicoletta Sapone, Giorgio Maria Saracco, Marco Astegiano, Rinaldo Pellicano, Unit of Gastroenterology and Hepatology, Molinette Hospital, 10126 Torino, Italy

Sharmila Fagoonee, Institute for Biostructures and Bioimages-CNR c/o Molecular Biotechnology Center, University of Torino, 10126 Turin, Italy

Gian Paolo Caviglia, Department of Medical Sciences, University of Turin, 10123 Turin, Italy

Giorgio Maria Saracco, Department of Oncology, University of Torino, 10126 Torino, Italy

Author contributions: Bonagura GA and Ribaldone DG equally contributed to this paper; Bonagura GA, Ribaldone DG, Saracco GM, Astegiano M and Pellicano R designed the research; Bonagura GA, Ribaldone DG, Fagoonee S and Sapone N performed the research; Fagoonee S and Caviglia GP analyzed the data; Bonagura GA, Ribaldone DG, Astegiano M and Pellicano R wrote the paper.

Institutional review board statement: This study was reviewed and approved by the Ethics Committee of Molinette Hospital.

Informed consent statement: Patients were not required to give informed consent to the study because the analysis used anonymous data and it was performed several years after the consultation (retrospective).

Conflict-of-interest statement: None to declare.

Data sharing statement: No additional data are available.

Manuscript source: Invited manuscript

Correspondence to: Davide Giuseppe Ribaldone, MD, Unit of Gastroenterology and Hepatology, Molinette Hospital, S.G.A.S., Via Cavour 31, 10126 Torino, Italy. davrib_1998@yahoo.com
Telephone: +39-01-16335208
Fax: +39-01-16336752

Received: June 22, 2016
Peer-review started: June 22, 2016
First decision: September 1, 2016
Revised: September 3, 2016
Accepted: September 21, 2016
Article in press: September 22, 2016
Published online: November 15, 2016

Abstract

AIM

To evaluate the potential association between mild duodenal damage and microscopic colitis (MC).

METHODS

We retrospectively included 105 consecutive patients with type I Marsh-Oberhuber duodenal damage and negativity for immunoglobulin A anti-endomysium and anti-tissue transglutaminase. The following parameters were analyzed: Sex, age at execution of esophago-gastroduodenoscopy, duodenal damage, and number of intraepithelial lymphocytes at biopsies, prevalence

of *Helicobacter pylori* infection, age at execution of colonoscopy, macroscopic and microscopic features of colonoscopy, family history of gastrointestinal and autoimmune diseases, smoking habits, biochemical parameters of inflammation and autoimmunity, use of proton pump inhibitors or nonsteroidal anti-inflammatory drugs, adverse reactions to drugs or foods, pathologies known to be associated with celiac disease or MC, living on a gluten-free diet or on a gluten-low diet for at least 1 mo.

RESULTS

Colonoscopy was performed in 59 patients, but only in 48 of them biopsies were taken in the entire colon. Considering the latter cohort, the diagnosis of MC was met in 25 (52.1%) patients while in 18 patients other pathologic findings were reported: 13 (27%) cases of nonspecific inflammatory bowel disease, 2 (4.2%) cases of Crohn's disease, 2 (4.2%) cases of eosinophilic gastroenteritis, and 1 (2.1%) case of autoimmune enteritis. Five (10.4%) patients had a normal colonoscopic result. Matching the groups by age, and considering only patients who underwent colonoscopy (42.7 ± 15.5 years) *vs* those who did not undergo colonoscopy (36.9 ± 10.6 years), a statistical difference was found (P = 0.039). Focusing on symptoms, diarrhea was statistically more prevalent in MC group than in patients who did not undergo colonoscopy (P = 0.03).

CONCLUSION

Mild duodenal damage is associated with MC in more than half of the cases. This association supports the hypothesis of a link between these two entities.

Key words: Autoimmune diseases; Celiac disease; *Helicobacter pylori*; Intraepithelial lymphocytes; Lymphocytic colitis; Lymphocytic enterocolitis; Microscopic colitis

Core tip: Scarce information is available on patients with symptoms suggestive for celiac disease but with negative serologic tests and mild duodenal damage (type I Marsh-Oberhuber classification). Our data show that mild duodenal damage is associated with microscopic colitis in more than the half of the investigated cases. This association may support the hypothesis of a new clinical and pathological entity, the "lymphocytic enterocolitis".

Bonagura GA, Ribaldone DG, Fagoonee S, Sapone N, Caviglia GP, Saracco GM, Astegiano M, Pellicano R. Microscopic colitis in patients with mild duodenal damage: A new clinical and pathological entity ("lymphocytic enterocolitis")? *World J Gastrointest Pathophysiol* 2016; 7(4): 307-313 Available from: URL: http://www.wjgnet.com/2150-5330/full/v7/i4/307.htm DOI: http://dx.doi.org/10.4291/wjgp.v7.i4.307

INTRODUCTION

Celiac disease (CD) is a chronic inflammatory disease characterized by a pathological reaction against gluten proteins[1]. Currently, the prevalence of CD in the general population is proximally 1%, with a ratio between diagnosed and undiagnosed cases of about 1:7[2,3]. CD presents often signs and symptoms such as chronic diarrhea, bloating, abdominal pain and malabsorption[4]. However, in a substantial number of cases, CD can manifest only extra-intestinal symptoms or signs, and it can be associated with autoimmune pathologies, as autoimmune thyroiditis, type I diabetes mellitus and rheumatoid arthritis[5,6]. The diagnosis of CD is based on the finding of positive antibody tests (anti-endomysium and anti-tissue transglutaminase), confirmed by biopsies taken during esophagogastroduodenoscopy (EGD) that reveal the characteristic duodenal damage. The Marsh-Oberhuber classification is usually used to grade the severity of duodenal lesions, with the type III representative of CD[7]. The search for human leukocyte antigen (HLA) haplotypes DQ2 and DQ8, due to its high negative predictive value, is used to exclude CD[8]. Nevertheless, there are patients with suggestive symptoms of CD, mild duodenal damage [*i.e.*, an increase of intraepithelial lymphocytes (IEL)] defined type I, according to Marsh-Oberhuber classification, and negative antibody tests. This clinical condition, that does not conform with the diagnosis of CD, needs to be investigated for other causes[9].

Microscopic colitis (MC) is a chronic inflammatory bowel disease, distinct in lymphocytic colitis (LC)[10] and collagenous colitis (CC)[11]. The diagnosis of MC is obtained by multiple colonic mucosal biopsies taken during colonoscopy[12]. Typically, in CC, the histological feature is a thickening of the subepithelial collagen layer beneath the basal membrane, of more than 7-10 μm (0-3 μm in the normal colon)[13]. The histological feature of LC is the presence of more than 20 IEL/100 surface epithelial cells (< 5 IEL/100 in the normal colon)[14]. Paucicellular LC is a term used when the number of IEL is comprised between 5 IEL/100 and 20/100 surface epithelial cells. In MC, IEL are T-Lymphocyte $CD3^+$ and $CD8^+$, similar to those described in case of type I Marsh-Oberhuber lesions. Previously considered rare, MC is now a relatively common cause of chronic watery nonbloody diarrhea, especially in the elderly[15]. Both LC and CC are associated with autoimmune diseases and allergy[16]. Finally, it has been shown that patients with MC have an increased rate of HLA-DQ2 and HLA-DQ8 positivity[17], even if this association is less strict than with CD.

Although some authors reported an association between MC and type I Marsh-Oberhuber duodenal damage[18-23], the interpretation of this finding is poorly described. Nevertheless, there are few studies[24] that searched for the inverse association.

The aim of this study was to evaluate, for the first time, the association between type I Marsh-Oberhuber

duodenal damage and MC, arguing for the existence of a possible "microscopic enterocolitis"[25,26].

MATERIALS AND METHODS

We retrospectively included 105 (86 females, mean age 40.1 ± 13.7) consecutive patients with type Ⅰ Marsh-Oberhuber duodenal damage and negativity for anti-endomysium (EmA) and anti-tissue transglutaminase (tTG) immunoglobulin (Ig)A antibodies. No sign of Whipple disease were reported in duodenal biopsies. Patients affected by small bowel bacterial overgrowth were excluded from the analysis. The analysis included patients observed in the period 1 January 2003-31 December 2013 in the outpatients clinic of the Unit of Gastroenterology and Hepatology, Molinette Hospital, Turin, Italy.

In 5 cases of IgA deficiency, the genetic assessment (HLA-DQ2/DQ8) was performed: In 3, the result was negative while in the remaining 2 HLA-DQ2 positivity was found.

The following parameters were analyzed: Sex, age at execution of EGD, duodenal damage with number of IEL at biopsies, age at execution of colonoscopy, macroscopic and microscopic features of colonoscopy, family history of gastrointestinal and autoimmune diseases, smoking habits, dosage of erythrocyte sedimentation rate (ESR), C-reactive protein (CRP) and anti-nuclear antibody (ANA), use of proton pump inhibitors (PPIs) or nonsteroidal anti-inflammatory drugs (NSAIDs)[27], adverse reactions to drugs or foods, pathologies associated with CD or MC, living on a gluten-free diet or on a gluten-low diet for at least 1 mo. Data on the prevalence of watery diarrhea, constipation, epigastric pain, abdominal pain, weight loss, nausea and/or vomiting, bloating, and asthenia were collected. Malabsorption was defined as the presence of at least one of these elements: Hemoglobin (Hb) < 12 g/L and low levels of serum iron or folate or vitamin B12; hypoalbuminemia; weight loss > 10% without hypocaloric diet. *Helicobacter pylori* (*H. pylori*) infection was investigated by urea breath test and gastric biopsies. The previous eradication treatment of this infection, if any, was reported.

The pharmacological anamnesis for assumption of prednisone, mesalamine, salazopyrin, budesonide, and antibiotics (rifaximin, ciprofloxacin, metronidazole) was conducted. Patients who took serotonin reuptake inhibitors or antiplatelets were excluded from the analysis.

Statistical analysis

For parametric data, we initially used the "normal probability plot", to value a normal data distribution; in case of positive return, the Student T test to match the two subgroups was used.

For non-parametric data, the subgroups were matched with the Yates' χ^2 test or with the Fisher's exact test if data were $\leqslant$ 5.

Confidence interval (CI) was set at 95%, with the statistical significance set at P value < 0.05. All statistical analyses were performed using MedCalc software (MedCalc Software, version 9.2.1.0).

RESULTS

Overall, colonoscopy was performed in 59 patients, but in only 48 cases biopsies were taken along the entire colon. In the remaining 11 cases, biopsies were not taken or taken only in the left colon. The histological findings permitted to divide the cohort into two groups: That including 25 patients with diagnosis of MC and that including 23 patients without MC (Figure 1).

Matching by age, patients who underwent colonoscopy (42.7 ± 15.5 years) *vs* those who did not undergo colonoscopy (36.9 ± 10.6 years), a statistical difference was found (P = 0.039). On the contrary, there was no significant difference between the group of patients who did not undergo colonoscopy (36.9 ± 10.6 years) *vs* the group who underwent colonoscopy and executed multiple biopsies (40.4 ± 13.7 years) (P = 0.186). Considering the symptoms, there were no statistical differences between patients who did not undergo colonoscopy *vs* those who underwent colonoscopy and executed multiple biopsies (P = 0.09 and P = 0.14 for epigastric pain and diarrhea, respectively). Diarrhea was statistically more prevalent in MC group than in patients who did not undergo colonoscopy (P = 0.03). Patients who did not undergo colonoscopy *vs* those who underwent colonoscopy and executed multiple biopsies had not statistical differences when comparing the heterodimers HLA-DQ2 and HLA-DQ8 (P = 0.19).

Among patients who underwent colonoscopy, an inflammatory pattern was found in 89.6% of cases. Focusing on the 25 patients with MC, the females to males ratio resulted 5:1 and the mean age was 40 ± 16.3 years. The diagnosis was LC in 13 cases, paucicellular LC in 9, CC in 2, and undefined MC in the remaining patient. The average duodenal IEL were 41.6/100 epithelial cells and colonic IELs were 25.4/100 epithelial cells. Watery diarrhea was present in 17/25 (68%) patients, abdominal pain in 16/25 (64%), weight loss in 11/25 (44%), nausea or vomiting in 7/25 (28%), epigastric pain in 6/25 (24%), asthenia in 5/25 (20%), bloating in 4/25 (16%), and gastroesophageal reflux disease (GERD) in 2/25 (8%). A family history of Crohn's disease, thyroiditis, rheumatoid arthritis or spondylitis, was present in 1/25 (4%) patient for each one. Regarding smoking habits, 19/25 (76%) patients were non-smokers while the remaining 6 (24%) were smokers. ANA test resulted positive in 4/25 (16%) patients, ESR increased in 2/25 (8%), and CRP in 2/25 (8%). Four out of twenty-five (16%) patients had a positive history of PPIs use, and 1/25 (4%) of NSAIDs use. Autoimmune thyroiditis was diagnosed in 4/25 (16%) patients, asthma in 3/25 (12%), rheumatoid arthritis in 3/25 (12%). Anamnesis of adverse reactions to drugs or foods resulted in 10/25 (40%) patients.

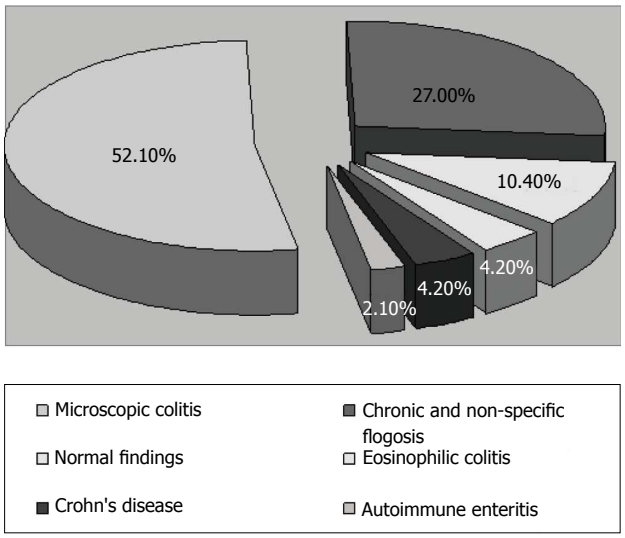


Figure 1 Microscopic findings at colonoscopy (48 cases).

Table 1 Main clinical and laboratory parameters of enrolled patients

	Patients with MC	Patients without MC	*P* value
Watery diarrhea	17/25 (68%)	10/23 (43%)	0.08
Abdominal pain	16/25 (64%)	10/23 (43%)	0.15
Weight loss	11/25 (44%)	3/23 (13%)	0.01
Nausea/vomiting	7/25 (28%)	4/23 (17%)	0.29
Epigastric pain	6/25 (24%)	5/23 (21%)	0.85
Asthenia	5/25 (20%)	4/23 (17%)	0.55
Bloating	4/25 (16%)	5/23 (21%)	0.44
GERD	2/25 (8%)	4/23 (17%)	0.33
Constipation	0/25 (0%)	4/23 (17%)	0.04
History of allergy	10/25 (40%)	10/23 (43%)	0.40
PPIs use	4/25 (16%)	6/23 (26%)	0.44
NSAIDs use	1/25 (4%)	0/23 (0%)	0.34
ANA positivity	4/25 (16%)	8/23 (34%)	0.46
ESR increased	2/25 (8%)	6/23 (26%)	0.13
CRP increased	2/25 (8%)	5/23 (21%)	0.21
Helicobacter pylori infection	6/25 (24%)	8/23 (34%)	0.61

MC: Microscopic colitis; GERD: Gastroesophageal reflux disease; PPIs: Proton pump inhibitors; NSAIDs: Nonsteroidal anti-inflammatory drugs; ANA: Anti-nuclear antibody; ESR: Erythrocyte sedimentation rate; CRP: C-reactive protein.

Thirteen patients (52%) had HLA-DQ2 positivity, 8 (32%) HLA-DQ2/DQ8 negativity, and 4 (16%) HLA-DQ8 positivity. Regarding *H. pylori* infection, 19/25 (76%) had negativity *ab initio* while 4 out of 6 with positivity (66.6%), eradicated the infection after antibiotic treatment. None of the tested patients had positivity at coproculture or at parasitological fecal test (6 and 2 cases, respectively). Fourteen (56%) patients undertook a gluten-free diet for at least 1 mo with a clinical improvement in 3/14 (21.4%). Malabsorption was observed in 12 (48%) patients.

Among patients without MC, the female to male ratio resulted 3.8:1 and the mean age was 40.5 ± 13.7 years. The diagnosis was of chronic and non-specific inflammation in 13 (56.5%) cases, there was a normal finding in 5 (21.7%), eosinophilic colitis in 2 (8.6%), Crohn's disease in 2 (8.6%), and autoimmune enteritis in the last one (4.3%). The average duodenal IEL resulted 42.1/100 epithelial cells. Based on the available data, a family history of Crohn's disease, rheumatoid arthritis or spondylitis was reported in one out of 23 (4.3%) patients for each disease. Regarding smoking habits, 15/23 (65.2%) patients were non-smokers while the remaining 8 (34.8%) were smokers. Abdominal pain was present in 10/23 (43.4%) patients, watery diarrhea in 10/23 (43.4%), epigastric pain in 5/23 (21.7%), bloating in 5/23 (21.7%), asthenia in 4/23 (17.3%), nausea or vomiting in 4/23 (17.3%), GERD in 4/23 (17.3%), constipation in 4/23 (17.3%), and weight loss in 3/23 (13.0%). ANA test resulted positive in 8/23 (34.8%) patients, ESR and CPR was increased in 6/23 (26.1%) and 5/23 (21.7%), respectively. A history of PPIs or NSAIDs use was reported in 6/23 (26.1%) and no patient, respectively. Autoimmune thyroiditis was reported in 3/23 (13%) of the patients, asthma in 2/23 (8.6%), rheumatoid arthritis in 2/23 (8.6%), systemic erythematosus lupus (SLE), autoimmune hepatitis and multiple autoimmune diseases in 1/23 (4.3%) for each one. Adverse reactions to drugs or foods resulted in 10/23 (43.4%) patients. Ten (43.4%) patients had HLA-DQ2/DQ8 negativity, 9 (39.1%) had HLA-DQ2 positivity, 3 (13%) HLA-DQ8 positivity, and one (4.3%) had HLA-DQ2/DQ8 positivity. Regarding *H. pylori* infection, 15/23 (65.2%) of the tested patients were negative *ab initio*, while 8/23 (34.8%) were positive. Of the latter group, 5 out of 8 (62.5%) eradicated the infection after antibiotic treatment. None of the tested patients had positivity at coproculture or at parasitological fecal test (4 and 1 case, respectively).

Comparing patients with MC *vs* those without MC (Table 1), the only variables that had a statistical difference were weight loss ($P = 0.01$), more frequent in case of MC, and constipation ($P = 0.04$) more frequent in absence of MC. Diarrhea ($P = 0.08$), abdominal pain ($P = 0.15$), epigastric pain ($P = 0.85$), GERD ($P = 0.33$), autoimmune thyroiditis ($P = 0.79$), smoking habits ($P = 0.66$), asthma ($P = 0.72$), rheumatoid arthritis ($P = 0.72$), autoimmune hepatitis ($P = 0.31$), multiple autoimmune diseases ($P = 0.35$), HLA-DQ2 positivity ($P = 0.51$), HLA-DQ8 positivity ($P = 0.79$) did not reach statistical significance.

Among patients suffering from MC, budesonide was used in 14 patients, of whom 13 (92.9%) responded to therapy; 8 patients used mesalamine, of them 4 (50%) responded to therapy; 4 patients used salazopyrin, with response in 2 (50%); 1 patient used prednisone, with response. No difference about the response to therapy resulted from the comparison between budesonide and mesalamine ($P = 0.27$), budesonide and salazopyrin ($P = 0.41$), budesonide and prednisone ($P = 0.94$). Among patients without MC, 7 used budesonide and 6/7 (85.7%) responded to therapy; 4 patients used prednisone without response; 6 patients used mesalamine with response in 3 (50%).

DISCUSSION

In this study, we found a strong association between type Ⅰ Marsh-Oberhuber duodenal damage and MC, mainly LC. More than half (52.1%) of the patients who underwent colonoscopy with multiple biopsies had MC. This percentage is significantly higher than the historical prevalence of MC in the general population (0.5%)[28]. An intriguing data was that LC and paucicellular LC, considered together, were diagnosed in much more cases than CC (22 *vs* 2, respectively). Usually, literature considered incidence and prevalence of CC higher than LC; however, more recent studies, according to our data, report that the incidence of LC is significantly rising[29].

Patients who underwent colonoscopy were significantly older than those who did not undergo colonoscopy. This could be partially explained considering the age as a parameter associated to augmented risk of malignancy. Hence, clinicians recurred to endoscopy in case of unexplained symptoms and increasing age. However, there was no difference in the median age between patients who did not undergo colonoscopy *vs* those who underwent colonoscopy with multiple biopsies.

Considering biochemical results and symptoms among various groups, only chronic diarrhea was significantly higher in patients with MC than in those who did not undergo colonoscopy (P = 0.03). Thus, in patients with mild duodenal damage only this symptom could predict MC. However, due to its multifactorial pathogenesis, the presence of diarrhea cannot be the only element to decide whether this type of patients should undergo colonoscopy with multiple biopsies. On the other hand, the absence of diarrhea cannot exclude the indication for colonoscopy with multiple biopsies, because only 5 out of 48 (10.4%) patients who had a colonoscopy with biopsies had normal microscopic findings, despite suffering also from abdominal pain, weight loss, constipation, positive fecal occult blood. The search for HLA-DQ2/DQ8 haplotypes seems to be useful, although the data in our retrospective study are not broad enough to provide definitive conclusions. Since this test has a very high negative predictive value in the diagnosis of CD, in patients with mild duodenal damage, negative serological tests for CD and the above reported symptoms, the negativity of HLA-DQ2/DQ8 haplotypes can definitively exclude this disease and propel to search for other etiologies, as MC.

The median age at which the diagnosis of MC was made in our patients (40 years) is lower than literature reports. Such finding may contrast with the idea of MC as disease of the elderly pointing out to a possible underestimation of this condition.

Another element that emerged from this study was the low prevalence of *H. pylori*-infection both in patients with MC (24%) than without MC (34%). The literature reports that *H. pylori* infection is related to duodenal lymphocytosis[9], which disappears after bacterial eradication. At the same time, we have recently found an inverse association between MC and *H. pylori* infection[29]. The results of the present study agree with the fact that in case of mild duodenal damage and MC the prevalence of *H. pylori* infection is lower than the general population (in our case 24% *vs* 47%)[30]. Moreover, the rate of *H. pylori* eradication in this context, is similar to that obtained in the general population[31].

In our study, the role of pharmacological therapy in the pathogenesis of MC is not fully clear. In fact, only 4 patients used PPIs and 1 patient used NSAIDs before the diagnosis of MC. This differs from the well-known data reporting that this type of medications are often implicated as a cause of MC[32], and could be explained by a β error (*i.e.,* the failure to detect an effect that is present) due to the small sample size. Considering the outcome of therapy used to treat MC, budesonide emerged as the best treatment, due to a clinical improvement, in more than 90% of patients. Mesalamine seemed to be a valid therapeutic approach for less severe cases. According to some reports, our results confirm the appropriateness of this management[32].

Although in literature an association between MC and malabsorption is not reported[33], in our study 12 (48%) patients presented signs of it. A potential disease of the small intestine, beyond the duodenum, could explain these features. More efforts are thus needed to understand this clinical condition.

This retrospective analysis shows inadequate habits of clinicians to search for a coproculture or a parasitological test; also the search for *Giardia Lamblia* was out of routine. Such investigations should play an important role in the attempt to identify the cause of duodenal damage. In fact, literature reports that the search for *Giardia Lamblia* or other pathogens should be included in the diagnostic work up of type Ⅰ Marsh-Oberhuber duodenal damage[34].

A potential limitation of our study is its retrospective design with a theoretical loss of balance on parameters analyzed. Nevertheless, we noticed uniform diagnostic and follow-up criteria.

In conclusion, MC is frequently associated with mild duodenal damage. This association may suggest the existence of a "microscopic enterocolitis", and specifically of a "lymphocytic enterocolitis", that involves the entire gastrointestinal tract. It is advisable to perform a colonoscopy with biopsies in all patients with type Ⅰ Marsh-Oberhuber duodenal damage and symptoms as chronic diarrhea, abdominal or epigastric pain, loss of weight, after exclusion of standard causes.

COMMENTS

Background

The diagnosis of celiac disease (CD) is based on the finding of positive antibody tests (anti-endomysium and anti-tissue transglutaminase), confirmed by biopsies that reveal the characteristic duodenal damage. The Marsh-Oberhuber classification is usually used to grade the severity of duodenal lesions, with the type Ⅲ representative of CD. Nevertheless, there are patients with suggestive symptoms of CD, mild duodenal damage [*i.e.,* an increase of intraepithelial lymphocytes (IEL)] defined type Ⅰ, according to Marsh-Oberhuber classification,

and negative antibody tests. This clinical condition, that does not conform with the diagnosis of CD, needs to be investigated for other causes as well as for comorbidities. Microscopic colitis (MC), previously considered rare, was demonstrated as a relatively common cause of chronic, watery, diarrhoea. While some isolated studies reported some association between MC and Marsh I duodenal damage, the interpretation of this finding is poorly described.

Research frontiers

To date, scarce information is available on the association between mild duodenal damage and MC.

Innovations and breakthroughs

This study is the first showing that type I Marsh-Oberhuber duodenal damage is strongly associated with MC, mainly lymphocytic colitis (LC). More than half (52.1%) of the patients who underwent colonoscopy with multiple biopsies had MC. This percentage is significantly higher than prevalence of MC in the general population (0.5%). This association supports the hypothesis of a link between these two entities.

Applications

These findings, of association between type I Marsh-Oberhuber duodenal damage and MC, may suggest the existence of a "microscopic enterocolitis", and specifically of a "lymphocytic enterocolitis", that involves the entire gastrointestinal tract. It is advisable to perform a colonoscopy with biopsies in all patients with type I Marsh-Oberhuber duodenal damage and symptoms as chronic diarrhea, abdominal or epigastric pain, loss of weight, after exclusion of standard causes.

Terminology

The Marsh-Oberhuber classification is usually used to grade the severity of duodenal lesions, with the type III representative of CD. There are patients with suggestive symptoms of CD, mild duodenal damage (*i.e.*, an increase of IEL) defined type I, according to Marsh-Oberhuber classification, and negative antibody tests, that do not conform with the diagnosis of CD. MC is a chronic inflammatory bowel disease, distinct in LC and collagenous colitis. The histological feature of LC is the presence of more than 20 IEL/100 surface epithelial cells (< 5 IEL/100 in the normal colon). Paucicellular LC is a term used when the number of IEL is comprised between 5 IEL/100 and 20/100 surface epithelial cells. In MC, IEL are T-Lymphocyte $CD3^+$ and $CD8^+$, similar to those described in case of type I Marsh-Oberhuber lesions. Here we report for the first time the association between type I Marsh-Oberhuber duodenal damage and MC, arguing for the existence of a possible "microscopic enterocolitis".

Peer-review

This is an interesting manuscript.

REFERENCES

1 **Kagnoff MF**. Celiac disease: pathogenesis of a model immunogenetic disease. *J Clin Invest* 2007; **117**: 41-49 [PMID: 17200705 DOI: 10.1172/jci30253]

2 **Fasano A**, Berti I, Gerarduzzi T, Not T, Colletti RB, Drago S, Elitsur Y, Green PH, Guandalini S, Hill ID, Pietzak M, Ventura A, Thorpe M, Kryszak D, Fornaroli F, Wasserman SS, Murray JA, Horvath K. Prevalence of celiac disease in at-risk and not-at-risk groups in the United States: a large multicenter study. *Arch Intern Med* 2003; **163**: 286-292 [PMID: 12578508 DOI: 10.1001/archinte.163.3.286]

3 **Corazza GR**, Frisoni M, Treggiari EA, Valentini RA, Filipponi C, Volta U, Gasbarrini G. Subclinical celiac sprue. Increasing occurrence and clues to its diagnosis. *J Clin Gastroenterol* 1993; **16**: 16-21 [PMID: 8421137 DOI: 10.1097/00004836-199301000-00006]

4 **Casella G**, Di Bella C, Salemme M, Villanacci V, Antonelli E, Baldini V, Bassotti G. Celiac disease, non-celiac gluten sensitivity and inflammatory bowel disease. *Minerva Gastroenterol Dietol* 2015; **61**: 267-271 [PMID: 26006779]

5 **Pellicano R**, De Angelis C, Ribaldone DG, Fagoonee S, Astegiano M. 2013 update on celiac disease and eosinophilic esophagitis. *Nutrients* 2013; **5**: 3329-3336 [PMID: 23974065 DOI: 10.3390/nu5093329]

6 **Ribaldone DG**, Astegiano M, Fagoonee S, Rizzetto M, Pellicano R. Epilepsy and celiac disease: review of literature. *Panminerva Med* 2011; **53**: 213-216 [PMID: 22146418]

7 **Oberhuber G**, Granditsch G, Vogelsang H. The histopathology of coeliac disease: time for a standardized report scheme for pathologists. *Eur J Gastroenterol Hepatol* 1999; **11**: 1185-1194 [PMID: 10524652 DOI: 10.1097/00042737-199910000-00019]

8 **Green PH**, Jabri B. Coeliac disease. *Lancet* 2003; **362**: 383-391 [PMID: 12907013]

9 **Simondi D**, Ribaldone DG, Bonagura GA, Foi S, Sapone N, Garavagno M, Villanacci V, Bernardi D, Pellicano R, Rizzetto M, Astegiano M. Helicobacter pylori in celiac disease and in duodenal intraepithelial lymphocytosis: Active protagonist or innocent bystander? *Clin Res Hepatol Gastroenterol* 2015; **39**: 740-745 [PMID: 25956489 DOI: 10.1016/j.clinre.2015.03.005]

10 **Lindström CG**. 'Collagenous colitis' with watery diarrhoea--a new entity? *Pathol Eur* 1976; **11**: 87-89 [PMID: 934705]

11 **Lazenby AJ**, Yardley JH, Giardiello FM, Jessurun J, Bayless TM. Lymphocytic ("microscopic") colitis: a comparative histopathologic study with particular reference to collagenous colitis. *Hum Pathol* 1989; **20**: 18-28 [PMID: 2912870 DOI: 10.1016/0046-8177(89)90198-6]

12 **Carpenter HA**, Tremaine WJ, Batts KP, Czaja AJ. Sequential histologic evaluations in collagenous colitis. Correlations with disease behavior and sampling strategy. *Dig Dis Sci* 1992; **37**: 1903-1909 [PMID: 1361906 DOI: 10.1007/bf01308086]

13 **Tanaka M**, Mazzoleni G, Riddell RH. Distribution of collagenous colitis: utility of flexible sigmoidoscopy. *Gut* 1992; **33**: 65-70 [PMID: 1740280 DOI: 10.1136/gut.33.1.65]

14 **Fasoli R**, Talbot I, Reid M, Prince C, Jewell DP. Microscopic colitis: can it be qualitatively and quantitatively characterized? *Ital J Gastroenterol* 1992; **24**: 393-396 [PMID: 1392021]

15 **Olesen M**, Eriksson S, Bohr J, Järnerot G, Tysk C. Microscopic colitis: a common diarrhoeal disease. An epidemiological study in Orebro, Sweden, 1993-1998. *Gut* 2004; **53**: 346-350 [PMID: 14960513 DOI: 10.1136/gut.37.3.394]

16 **Roth B**, Manjer J, Ohlsson B. Microscopic Colitis is Associated with Several Concomitant Diseases. *Drug Target Insights* 2013; **7**: 19-25 [PMID: 24003301 DOI: 10.4137/DTI.S12109]

17 **Fernández-Bañares F**, Esteve M, Farré C, Salas A, Alsina M, Casalots J, Espinós J, Forné M, Viver JM. Predisposing HLA-DQ2 and HLA-DQ8 haplotypes of coeliac disease and associated enteropathy in microscopic colitis. *Eur J Gastroenterol Hepatol* 2005; **17**: 1333-1338 [PMID: 16292086 DOI: 10.1097/00042737-200512000-00011]

18 **Matteoni CA**, Goldblum JR, Wang N, Brzezinski A, Achkar E, Soffer EE. Celiac disease is highly prevalent in lymphocytic colitis. *J Clin Gastroenterol* 2001; **32**: 225-227 [PMID: 11246349 DOI: 10.1097/00004836-200103000-00009]

19 **Thijs WJ**, van Baarlen J, Kleibeuker JH, Kolkman JJ. Microscopic colitis: prevalence and distribution throughout the colon in patients with chronic diarrhoea. *Neth J Med* 2005; **63**: 137-140 [PMID: 15869041]

20 **Geboes K**. Lymphocytic, collagenous and other microscopic colitides: pathology and the relationship with idiopathic inflammatory bowel diseases. *Gastroenterol Clin Biol* 2008; **32**: 689-694 [PMID: 18538968 DOI: 10.1016/j.gcb.2008.04.021]

21 **Aziz I**, Evans KE, Hopper AD, Smillie DM, Sanders DS. A prospective study into the aetiology of lymphocytic duodenosis. *Aliment Pharmacol Ther* 2010; **32**: 1392-1397 [PMID: 21050242 DOI: 10.1111/j.1365-2036.2010.04477.x]

22 **Shmidt E**, Smyrk TC, Boswell CL, Enders FT, Oxentenko AS. Increasing duodenal intraepithelial lymphocytosis found at upper endoscopy: time trends and associations. *Gastrointest Endosc* 2014; **80**: 105-111 [PMID: 24565068 DOI: 10.1016/j.gie.2014.01.008]

23 **Losurdo G**, Piscitelli D, Giangaspero A, Principi M, Buffelli F,

Giorgio F, Montenegro L, Sorrentino C, Amoruso A, Ierardi E, Di Leo A. Evolution of nonspecific duodenal lymphocytosis over 2 years of follow-up. *World J Gastroenterol* 2015; **21**: 7545-7552 [PMID: 26140001 DOI: 10.3748/wjg.v21.i24.7545]

24 **Astegiano M**, Pellicano R, Verme G, Rizzetto M. High rate of microscopic colitis in patients with Marsh I-II duodenal damage. *Scand J Gastroenterol* 2009; **44**: 1266-1267 [PMID: 19658019 DOI: 10.1080/00365520903144414]

25 **Rostami K**, Villanacci V. Microscopic enteritis: novel prospect in coeliac disease clinical and immuno-histogenesis. Evolution in diagnostic and treatment strategies. *Dig Liver Dis* 2009; **41**: 245-252 [PMID: 18657490 DOI: 10.1016/j.dld.2008.06.008]

26 **Rostami K**, Aldulaimi D, Holmes G, Johnson MW, Robert M, Srivastava A, Fléjou JF, Sanders DS, Volta U, Derakhshan MH, Going JJ, Becheanu G, Catassi C, Danciu M, Materacki L, Ghafarzadegan K, Ishaq S, Rostami-Nejad M, Peña AS, Bassotti G, Marsh MN, Villanacci V. Microscopic enteritis: Bucharest consensus. *World J Gastroenterol* 2015; **21**: 2593-2604 [PMID: 25759526 DOI: 10.3748/wjg.v21.i9.2593]

27 **Beaugerie L**, Pardi DS. Review article: drug-induced microscopic colitis - proposal for a scoring system and review of the literature. *Aliment Pharmacol Ther* 2005; **22**: 277-284 [PMID: 16097993 DOI: 10.1111/j.1365-2036.2005.02561.x]

28 **Tong J**, Zheng Q, Zhang C, Lo R, Shen J, Ran Z. Incidence, prevalence, and temporal trends of microscopic colitis: a systematic review and meta-analysis. *Am J Gastroenterol* 2015; **110**: 265-276; quiz 277 [PMID: 25623658 DOI: 10.1038/ajg.2014.431]

29 **Ribaldone DG**, Simondi D, Astegiano M, Pellicano R. On Inverse Association Between Helicobacter pylori Gastritis and Microscopic Colitis: The European Data. *Inflamm Bowel Dis* 2016; **22**: E11-E12 [PMID: 26871398 DOI: 10.1097/MIB.0000000000000704]

30 **Ponzetto A**, Pellicano R, Morgando A, Cirillo D, Marchiaro G, Curti F, Rizzetto M. Seroprevalence of Helicobacter pylori infection among blood donors in Torino, Italy. *Minerva Gastroenterol Dietol* 2001; **47**: 3-7 [PMID: 16491063]

31 **Ribaldone DG**, Fagoonee S, Astegiano M, Saracco G, Pellicano R. Efficacy of amoxycillin and clarithromycin-based triple therapy for Helicobacter pylori eradication: a 10-year trend in Turin, Italy. *Panminerva Med* 2015; **57**: 145-146 [PMID: 25971330]

32 **Park T**, Cave D, Marshall C. Microscopic colitis: A review of etiology, treatment and refractory disease. *World J Gastroenterol* 2015; **21**: 8804-8810 [PMID: 26269669 DOI: 10.3748/wjg.v21.i29.8804]

33 **Mellander MR**, Ekbom A, Hultcrantz R, Löfberg R, Öst Å, Björk J. Microscopic colitis: a descriptive clinical cohort study of 795 patients with collagenous and lymphocytic colitis. *Scand J Gastroenterol* 2016; **51**: 556-562 [PMID: 26679722 DOI: 10.3109/00365521.2015.1124283]

34 **Patterson ER**, Shmidt E, Oxentenko AS, Enders FT, Smyrk TC. Normal villous architecture with increased intraepithelial lymphocytes: a duodenal manifestation of Crohn disease. *Am J Clin Pathol* 2015; **143**: 445-450 [PMID: 25696804 DOI: 10.1309/AJCPBKQND4SHVX9Q]

P- Reviewer: Bonaz B **S- Editor**: Ji FF **L- Editor**: A
E- Editor: Wu HL

World Journal of Gastrointestinal Pathophysiology

Submit a Manuscript: http://www.wjgnet.com/esps/
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.4291/wjgp.v7.i4.314

World J Gastrointest Pathophysiol 2016 November 15; 7(4): 314-319
ISSN 2150-5330 (online)

META-ANALYSIS

Hepatitis C infection and renal cell carcinoma: A systematic review and meta-analysis

Karn Wijarnpreecha, Pitchaphon Nissaisorakarn, Suthanya Sornprom, Charat Thongprayoon, Natanong Thamcharoen, Kunlatida Maneenil, Alexander J Podboy, Wisit Cheungpasitporn

Karn Wijarnpreecha, Suthanya Sornprom, Charat Thongprayoon, Natanong Thamcharoen, Department of Internal Medicine, Bassett Medical Center, Cooperstown, NY 13326, United States

Pitchaphon Nissaisorakarn, Department of Internal Medicine, Jacobi Medical Center, Albert Einstein College of Medicine, Bronx, NY 10461, United States

Kunlatida Maneenil, Division of Medical Oncology, Mayo Clinic, Rochester, MN 55905, United States

Alexander J Podboy, Department of Internal Medicine, Mayo Clinic, Rochester, MN 55905, United States

Wisit Cheungpasitporn, Division of Nephrology and Hypertension, Department of Internal Medicine, Mayo Clinic, Rochester, MN 55905, United States

Author contributions: All authors had access to the data and a role in writing the manuscript.

Conflict-of-interest statement: The authors deny any conflict of interest.

Data sharing statement: No additional data are available.

Manuscript source: Invited manuscript

Correspondence to: Wisit Cheungpasitporn, MD, Division of Nephrology and Hypertension, Department of Internal Medicine, Mayo Clinic, 200 First Street SW, Rochester, MN 55905, United States. wcheungpasitporn@gmail.com
Telephone: +1-507-2848450
Fax: +1-507-2667891

Received: April 6, 2016
Peer-review started: April 8, 2016
First decision: May 19, 2016
Revised: July 30, 2016
Accepted: August 17, 2016
Article in press: August 18, 2016
Published online: November 15, 2016

Abstract

AIM

To investigate the association between hepatitis C virus (HCV) infection and risk of renal cell carcinoma (RCC).

METHODS

A literature search was performed from inception until February 2016. Studies that reported relative risks, odd ratios, hazard ratios or standardized incidence ratio comparing the risk of RCC among HCV-infected participants *vs* those without HCV infection were included. Participants without HCV infection were used as comparators. Pooled odds ratios and 95%CI were calculated using a random-effect, generic inverse variance method.

RESULTS

Seven observational studies were with 196826 patients were included in the analysis to assess the risk of RCC in patients with HCV. A significantly increased risk of RCC among participants with HCV infection was found with a pooled RR of 1.86 (95%CI: 1.11-3.11). The association between RCC and HCV was marginally insignificant after a sensitivity analysis limited only to studies with adjusted analysis, with a pooled RR of 1.50 (95%CI: 0.93-2.42).

CONCLUSION

Our study demonstrated a potential association between HCV infection and RCC. Further studies of RCC

surveillance in patients with HCV are required.

Key words: Hepatitis C virus; Renal cancer; Kidney cancer; Systematic review; Meta-analysis

Core tip: Hepatitis C virus (HCV) is a leading cause of cirrhosis in the United States with a steadily increasing prevalence over the past two decades. Interestingly, HCV infection may also be associated with an increased risk of renal cell carcinoma (RCC) as observed in several epidemiologic studies. To further investigate this possible association, we conducted this systematic review and meta-analysis of observational studies reporting the risk of RCC among HCV-infected patients. We found a significantly increased risk of RCC among participants with HCV infection with the pooled risk ratio of 1.86 (95%CI: 1.11-3.11).

Wijarnpreecha K, Nissaisorakarn P, Sornprom S, Thongprayoon C, Thamcharoen N, Maneenil K, Podboy AJ, Cheungpasitporn W. Hepatitis C infection and renal cell carcinoma: A systematic review and meta-analysis. *World J Gastrointest Pathophysiol* 2016; 7(4): 314-319 Available from: URL: http://www.wjgnet.com/2150-5330/full/v7/i4/314.htm DOI: http://dx.doi.org/10.4291/wjgp.v7.i4.314

INTRODUCTION

Hepatitis C virus (HCV) remains the most common cause of chronic liver disease and cirrhosis worldwide and is one of the leading causes of chronic hepatitis and cirrhosis in the United States with a steadily increasing prevalence over the past two decades[1,2]. While commonly associated with hepatocellular carcinoma[2], hepatitis C infection is associated with extrahepatic malignancies including cholangiocarcinoma, non-Hodgkin's lymphoma and possibly myeloma[3-5]. The oncogenic properties of hepatitis C are hypothesized to be secondary to chronic antigenic stimulation of the immune system, promotion of a chronic inflammatory state or direct oxidative stress[6]. Besides, chronic HCV infection has also been linked to a myriad of extrahepatic diseases including increasing the risk of renal disease and increasing the prevalence of chronic kidney disease up to 40% higher compared to non-infected patients[7,8].

Renal cell carcinoma (RCC), arising from the renal cortex is responsible for 80% of all renal malignancies and accounts for approximately 14000 deaths each year in the United States[9]. The risk factors for RCC such as acquired cystic kidney disease, smoking, kidney stones, obesity, hereditary factors have been described[10-14]. The epidemiological studies have demonstrated an increasing incidence of RCC, particularly in African Americans[15].

Secondary to the oncogenic nature of Hepatitis C, several studies have linked chronic infection with an increased risk for development of RCC[16-22]. However, the findings from these studies were contradictory. Thus, we performed this meta-analysis to examine the risk of RCC in HCV-infected patients.

MATERIALS AND METHODS

Literature search

Two investigators (Karn Wijarnpreecha and Wisit Cheungpasitporn) independently reviewed published studies indexed in MEDLINE, EMBASE, and the Cochrane database from their inception to February 2016 using the search strategy that included the terms for "hepatitis" and "renal cancer" as described in Item S1 in online supplementary data. No limitation on language was applied. A manual search for additional studies using references of selected retrieved articles was also performed. Three investigators (Karn Wijarnpreecha, Charat Thongprayoon and Wisit Cheungpasitporn) independently reviewed the titles and abstracts of the studies identified in the search based on inclusion and exclusion criteria. The full text of the included studies from the first phase was reviewed independently to ascertain whether or not they matched the inclusion criteria. We also performed a manual search of conference proceedings from major gastroenterology and hepatology meetings for additional abstracts on the topic. When additional information was needed, we contacted the corresponding investigators of eligible studies.

Study selection

The inclusion criteria were as follows: (1) observational studies assessing the association between hepatitis C and RCC; (2) odds ratios, relative risks or hazard ratios with 95%CI were provided; and (3) individuals without HCV infection were used as comparators in cohort studies while individuals without RCC were applied as comparators in the cross-sectional and case-control studies.

Study acceptability was individually defined by the three investigators mentioned above. Disagreements in the ascertainment of study eligibility were settled by joint agreement. Also, the quality of each study was individually appraised by each investigator. We used the validated Newcastle-Ottawa quality assessment scale for cohort and case-control studies[23] and modified Newcastle-Ottawa scale[24] for the cross-sectional study.

Data extraction

A data collection report was utilized to derive the information from each study including name of title and the first author, year of study and publication, country, demographic data of the participants, number of participants, method used to diagnose the HCV infection and RCC, effect estimates (odds ratios, relative risks or hazard ratios) with 95%CI, and factors adjusted in the multivariate analysis. To assure the certainty, this data

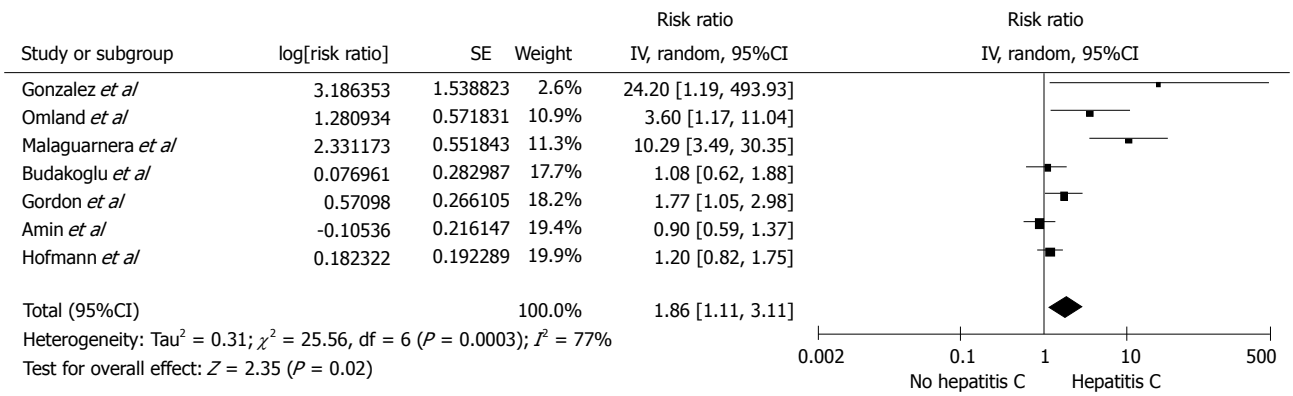


Figure 1 Forest plot of all included studies of the association between hepatitis C infection and renal cell carcinoma. Square data markers represent risk ratios (RRs); horizontal lines, the 95%CI with marker size reflecting the statistical weight of the study using random-effects meta-analysis. A diamond data marker represents the overall RR and 95%CI for the outcome of interest.

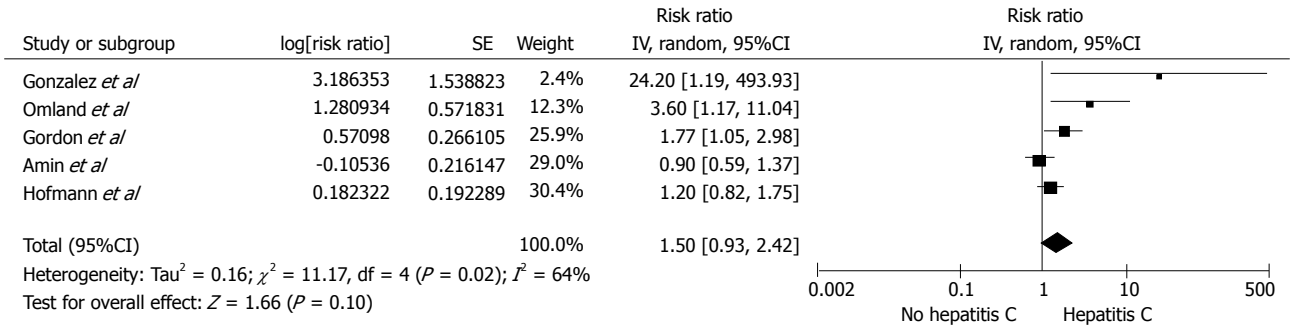


Figure 2 Forrest plot of all included studies in sensitivity analysis of the association between hepatitis C infection and renal cell carcinoma. Square data markers represent risk ratios (RRs); horizontal lines, the 95%CIs with marker size reflecting the statistical weight of the study using random-effects meta-analysis. A diamond data marker represents the overall RR and 95%CI for the outcome of interest.

extraction process was reviewed by all investigators.

Statistical analysis

Review Manager (RevMan) 5.3 software from the Cochrane Collaboration was utilized for meta-analysis. Generic inverse variance (DerSimonian and Laird) method[25] was employed to combined adjusted point estimates and standard errors from each study. We used a random-effect model due to the high likelihood of between-study variance from different study designs and populations. Cochran's *Q* test and I^2 statistic were used to determine the between-study heterogeneity. A value of I^2 of 0%-25%, 25%-50%, 50%-75%, and greater than 75% embodied insignificant, low, moderate and high heterogeneity, respectively[26].

RESULTS

Of 5778 potentially relevant articles, 5582 articles were excluded by the title and abstract not fulfilling inclusion criteria due to the type of article, study design, population, or outcome of interest. Additionally, 189 articles were excluded (35 articles were not observational studies, and 154 articles did not describe the outcomes of interest, Macleod *et al*[27]'s study did not contain data on specific viral hepatitis{Macleod, 2013 #83}). Finally, seven observational studies (4 cohort and 3 case-controlled studies) with 196826 patients were included in the meta-analysis[16-22]. Item S2 describes the study selection flow. The characteristics and quality appraisal of the included studies of HCV and RCC are shown in Table 1. Four studies were conducted in Europe, 2 in the United States, and 1 in Australia.

The risk of renal cell carcinoma in patients with hepatitis C virus infection

Among individuals with HCV infection, there was a significantly increased risk of RCC with the pooled risk ratio (RR) of 1.86 (95%CI: 1.11-3.11, I^2 =77%), as demonstrated in Figure 1. The statistical heterogeneity was high with an I^2 of 77%. The association between RCC and HCV was marginally insignificant after the sensitivity analysis including only studies with confounder adjustment[16,18-20,22] with a pooled RR of 1.50 (95%CI: 0.93-2.42, I^2 =64%), as shown in Figure 2.

Evaluation for publication bias

Two authors (Karn Wijarnpreecha and Wisit Cheungpasitporn) independently performed the assessment of the risk of bias of the included studies. Only minor disagreements between 2 reviewers were present and were resolved by discussion and consensus. A funnel plot was constructed to assess publication bias for the risk of RCC in HCV-infected patients (Figure S1). The

Table 1 Main characteristics of the studies included in this meta-analysis

Ref.	Country	Study design	Year	Total number	Study sample	Exposure definition	Exposure measurement	Outcome definition	Outcome ascertainment	Adjusted OR	Confounder adjustment	Quality assessment (Newcastle-Ottawa scale)
Amin *et al*[16]	Australia	Cohort study	2006	75834 in HCV patients	People notified with HCV infection to the New South Wales Health Department's Notifiable Diseases Database between 1 January 1990 and 31 December 2002 (HCV group) and NSW population (control group)	HCV infection	Detection of anti-HCV antibody or HCV RNA	Renal cancer or kidney cancer	The NSW Central Cancer Registry with ICD-10 code C64 for kidney cancer and C65 for renal cancer	Kidney cancer 0.9 (0.6–1.4)	Age, sex and calendar year	Selection: 4 Comparability: 1 Outcome: 3
Malagu arnera *et al*[21]	Italy	Case control study	2006	315 (15 case and 300 control)	Elderly kidney cancer patients attending geriatric department (case) and elderly volunteers (control)	Positive Anti-HCV	Detection of Anti-HCV antibody using enzyme-linked immunosorbent assay. Assay positive samples were confirmed by immunoblotting	Kidney cancer	N/A	10.29 (3.49-30.36)	None	Selection: 3 Comparability: 0 Outcome: 2
Omland *et al*[22]	Denmark	Cohort study	2010	4204 in HCV patients	Acute or chronic HCV infected patients listed in the Danish National Hospital Registry between 1994 and 2003 without previous diagnosis of cancer (HCV) and general population (control)	Acute or chronic HCV infection	ICD-10 code (B17.1 and B18.2) from the Danish National Hospital Registry	Kidney cancer	The Danish Cancer Registry with ICD-7 code 180	3.60 (0.98–9.22)	Age, sex and year of diagnosis	Selection: 4 Comparability: 1 Outcome: 2
Gordon *et al*[19]	United States	Cohort study	2010	67063	HCV-tested patients (both positive and negative) between 1997 and 2006 from administrative data from Henry Ford hospital	HCV infection	Positive anti-HCV test using enzyme-linked immunosorbent assay, confirmed by a documented positive molecular assay for HCV RNA	Renal cell carcinoma	Health system cancer registry, confirmed by medical record and pathology report review	1.77 (1.05-2.98)	Age, race, gender, chronic kidney disease	Selection: 4 Comparability: 1 Outcome: 3
Budakoğlu *et al*[17]	Turkey	Case-control study	2011	6170 (903 case and 5267 control)	Patients who had histologically proven renal cell carcinoma diagnosis between 2005 to 2010 from six tertiary cancer centers (case) and healthy people who were living in the same geographic regions (control)	Positive anti-HCV	Positive anti-HCV test using enzyme-linked immunosorbent assay	Renal cell carcinoma	Histopathology report	1.08 (0.62-1.88)	None	Selection: 3 Comparability: 0 Outcome: 3
Hofmann *et al*[20]	Sweden	Cohort study	2011	43000 in HCV patients	All Swedish residents diagnosed with HCV infection between 1990 and 2006 (HCV group) and general population (control)	Chronic HCV infection	The national surveillance database at the Swedish Institute for Infectious Disease Control	Kidney cancer	The national Cancer register with ICD-7 code 180.0 and 180.9 and histologic confirmation	1.2 (0.8–1.7)	Age, sex and calendar year	Selection: 4 Comparability: 1 Outcome: 3
Gonzalez *et al*[18]	United States	Case-control study	2015	240 (140 case and 100 control)	Newly diagnosed renal cell carcinoma (case) and newly diagnosed colon cancer patients (control)	Chronic HCV infection	Detection of anti-HCV antibody or HCV RNA	Renal cell carcinoma	Histopathology report	Positive HCV RNA 24.20 (2.4 - > 999.9)	Sex, age, race, BMI, smoking, alcohol abuse, hypertension, diabetes mellitus, dyslipidemia, coronary artery disease, congestive heart failure, chronic kidney disease, cirrhosis	Selection: 3 Comparability: 2 Outcome: 3

BMI: Body mass index; DNHR: Danish National Hospital Registry; ELISA: Enzyme-linked immunosorbent assay; HCV: Hepatitis C virus; N/A: Not available; NSW: New South Wales; RCC: Renal cell carcinoma; SIR: Standardized incidence ratio.

funnel plot was suggestive of a small publication bias toward studies with a positive correlation between HCV infection and RCC.

DISCUSSION

This meta-analysis was conducted to summarize all presently available data on the association between HCV infection and RCC. Our study demonstrated a 1.86-fold increased risk of RCC among participants who had HCV infection compared to those without HCV infection. Our analysis also illustrates that that RCC patients with HCV were significantly younger than RCC patients without HCV. The results of our study reinforce the hypothesis that HCV may accelerate the risk of developing RCC.

Although the nature in which HCV induces RCC is not entirely understood, several hypotheses exist. A recent bioinformatics study demonstrated a plausible biological relationship between HCV infection and the development of RCC *via* the NY-REN-54 protein. The NY-REN-54 protein which is an altered ubiquitin-related protein that plays a role in the disturbance impairs the autophagic response *via* to the ubiquitin-protein ligase-related self-regulatory mechanism, which in turn promotes oncogenesis[28]. Additionally, inhibition of cytotoxic T-lymphocyte-dependent apoptosis by the hepatitis virus secondarily leads to a disturbance in host immunity and normal tissue homeostasis leading to carcinoma formation[29]. Lukkonen *et al*[30] demonstrated an increased expression of serine protease inhibitor Kazal (SPIK), a cellular protein that inhibits serine protease-related apoptosis, in RCC tissue samples as an additional mechanism for HCV-induced RCC.

There are several limitations in our study. Firstly, there was a high amount of statistical heterogeneity present in the completed analysis. The potential source of this heterogeneity includes variation in confounder-adjusted methods (*e.g.,* age, sex, ethnicity, and chronic kidney disease), exposure measurement, outcome ascertainments, and follow-up duration. Secondly, it should be noted that there was a potential small publication bias with a positive association between HCV infection and RCC. The possibility of selection bias could play a role in chart-reviewed population base study. Finally, this meta-analysis of observational studies could only show an association. It cannot establish causality as an unknown number of confounders could play a role in the association between HCV and RCC.

In summary, this study demonstrates a potential association between HCV infection and RCC. In the new era of treatment of HCV infection, direct-acting antiviral agents have been demonstrated to be effective therapy and increasingly used[31]. These agents may potentially decrease the incidence of RCC in HCV patients in the long term.

COMMENTS

Background

Hepatitis C virus (HCV) is one of the leading causes of cirrhosis as the prevalence of HCV has been steadily increasing over the past two decades in the United States. Extrahepatic manifestations of HCV are common. Interestingly, HCV infection could also increase the risk of renal cell carcinoma (RCC) as observed in several epidemiologic studies.

Research frontiers

The results of those epidemiologic studies were inconsistent. To further investigate this possible association of HCV and RCC, the authors conducted this systematic review and meta-analysis of observational studies reporting the risk of RCC among HCV-infected patients.

Innovations and breakthroughs

The authors found a significantly increased risk of RCC among participants with HCV infection with the pooled risk ratio (RR) of 1.86 (95%CI: 1.11-3.11). The sensitivity analysis including only studies with confounder adjustment also demonstrated increased risk of RCC among participants with HCV infection with the pooled RR of 1.50 (95%CI: 0.93-2.42) even though without reaching statistical significance.

Applications

This study demonstrated a potential association between HCV infection and RCC. This finding suggests that a history of HCV is potentially associated with RCC and may impact clinical management and cancer surveillance.

Peer-review

The manuscript is an interesting meta-analysis about the correlation of HCV infection and RCC development. The aim of the meta-analysis is clearly stated and methods are well-described.

REFERENCES

1 **Udompap P**, Mannalithara A, Heo NY, Kim D, Kim WR. Increasing prevalence of cirrhosis among U.S. adults aware or unaware of their chronic hepatitis C virus infection. *J Hepatol* 2016; **64**: 1027-1032 [PMID: 26809112 DOI: 10.1016/j.jhep.2016.01.009]

2 **de Oliveria Andrade LJ**, D'Oliveira A, Melo RC, De Souza EC, Costa Silva CA, Paraná R. Association between hepatitis C and hepatocellular carcinoma. *J Glob Infect Dis* 2009; **1**: 33-37 [PMID: 20300384 DOI: 10.4103/0974-777x.52979]

3 **Davila JA**, Morgan RO, Shaib Y, McGlynn KA, El-Serag HB. Hepatitis C infection and the increasing incidence of hepatocellular carcinoma: a population-based study. *Gastroenterology* 2004; **127**: 1372-1380 [PMID: 15521006]

4 **Giordano TP**, Henderson L, Landgren O, Chiao EY, Kramer JR, El-Serag H, Engels EA. Risk of non-Hodgkin lymphoma and lymphoproliferative precursor diseases in US veterans with hepatitis C virus. *JAMA* 2007; **297**: 2010-2017 [PMID: 17488966 DOI: 10.1001/jama.297.18.2010]

5 **Hartridge-Lambert SK**, Stein EM, Markowitz AJ, Portlock CS. Hepatitis C and non-Hodgkin lymphoma: the clinical perspective. *Hepatology* 2012; **55**: 634-641 [PMID: 22120959 DOI: 10.1002/hep.25499]

6 **Vlaar AP**, Rietdijk ST, Zeerleder SS, Boerman T, Beuers U. Malignancies associated with chronic hepatitis C: case report and review of the literature. *Neth J Med* 2011; **69**: 211-215 [PMID: 21646667]

7 **Cacoub P**, Renou C, Rosenthal E, Cohen P, Loury I, Loustaud-Ratti V, Yamamoto AM, Camproux AC, Hausfater P, Musset L, Veyssier P, Raguin G, Piette JC. Extrahepatic manifestations associated with hepatitis C virus infection. A prospective multicenter study of 321 patients. The GERMIVIC. Groupe d'Etude et de Recherche en Medecine Interne et Maladies Infectieuses sur le Virus de l'Hepatite C. *Medicine* (Baltimore) 2000; **79**: 47-56 [PMID: 10670409]

8 **Fabrizi F**, Verdesca S, Messa P, Martin P. Hepatitis C Virus Infection Increases the Risk of Developing Chronic Kidney Disease: A Systematic Review and Meta-Analysis. *Dig Dis Sci* 2015; **60**: 3801-3813 [PMID: 26195311 DOI: 10.1007/s10620-015-3801-y]

9 **Siegel RL**, Miller KD, Jemal A. Cancer statistics, 2016. *CA Cancer J*

Clin 2016; **66**: 7-30 [PMID: 26742998 DOI: 10.3322/caac.21332]
10 **Adams KF**, Leitzmann MF, Albanes D, Kipnis V, Moore SC, Schatzkin A, Chow WH. Body size and renal cell cancer incidence in a large US cohort study. *Am J Epidemiol* 2008; **168**: 268-277 [PMID: 18544571 DOI: 10.1093/aje/kwn122]
11 **Cheungpasitporn W**, Thongprayoon C, O'Corragain OA, Edmonds PJ, Ungprasert P, Kittanamongkolchai W, Erickson SB. The risk of kidney cancer in patients with kidney stones: a systematic review and meta-analysis. *QJM* 2015; **108**: 205-212 [PMID: 25208892 DOI: 10.1093/qjmed/hcu195]
12 **Cumberbatch MG**, Rota M, Catto JW, La Vecchia C. The Role of Tobacco Smoke in Bladder and Kidney Carcinogenesis: A Comparison of Exposures and Meta-analysis of Incidence and Mortality Risks. *Eur Urol* 2016; **70**: 458-466 [PMID: 26149669 DOI: 10.1016/j.eururo.2015.06.042]
13 **Ljungberg B**, Campbell SC, Choi HY, Jacqmin D, Lee JE, Weikert S, Kiemeney LA. The epidemiology of renal cell carcinoma. *Eur Urol* 2011; **60**: 615-621 [PMID: 21741761 DOI: 10.1016/j.eururo.2011.06.049]
14 **Wiklund F**, Tretli S, Choueiri TK, Signoretti S, Fall K, Adami HO. Risk of bilateral renal cell cancer. *J Clin Oncol* 2009; **27**: 3737-3741 [PMID: 19597028 DOI: 10.1200/JCO.2008.20.6524]
15 **Lipworth L**, Tarone RE, McLaughlin JK. The epidemiology of renal cell carcinoma. *J Urol* 2006; **176**: 2353-2358 [PMID: 17085101 DOI: 10.1016/j.juro.2006.07.130]
16 **Amin J**, Dore GJ, O'Connell DL, Bartlett M, Tracey E, Kaldor JM, Law MG. Cancer incidence in people with hepatitis B or C infection: a large community-based linkage study. *J Hepatol* 2006; **45**: 197-203 [PMID: 16684579 DOI: 10.1016/j.jhep.2006.02.014]
17 **Budakoğlu B**, Aksoy S, Arslan Ç, Üyetürk Ü, Babacan NA, Özcan MF, Yıldız R, Öven BB, Özdemir NY, Dizdar Ö, Büyükberber S, Akıncı MB, Türker I, Öksüzoğlu B, Altundag K, Zengin N. Frequency of HCV infection in renal cell carcinoma patients. *Med Oncol* 2012; **29**: 1892-1895 [PMID: 21461964 DOI: 10.1007/s12032-011-9928-6]
18 **Gonzalez HC**, Lamerato L, Rogers CG, Gordon SC. Chronic hepatitis C infection as a risk factor for renal cell carcinoma. *Dig Dis Sci* 2015; **60**: 1820-1824 [PMID: 25592719 DOI: 10.1007/s10620-015-3521-3]
19 **Gordon SC**, Moonka D, Brown KA, Rogers C, Huang MA, Bhatt N, Lamerato L. Risk for renal cell carcinoma in chronic hepatitis C infection. *Cancer Epidemiol Biomarkers Prev* 2010; **19**: 1066-1073 [PMID: 20332260 DOI: 10.1158/1055-9965.EPI-09-1275]
20 **Hofmann JN**, Törner A, Chow WH, Ye W, Purdue MP, Duberg AS. Risk of kidney cancer and chronic kidney disease in relation to hepatitis C virus infection: a nationwide register-based cohort study in Sweden. *Eur J Cancer Prev* 2011; **20**: 326-330 [PMID: 21386707 DOI: 10.1097/CEJ.0b013e32834572fa]
21 **Malaguarnera M**, Gargante MP, Risino C, Ranno S, Berretta M, Cannizzaro MA, Costanzo M, Fricia T, Rampello E, Romano M. Hepatitis C virus in elderly cancer patients. *Eur J Intern Med* 2006; **17**: 325-329 [PMID: 16864006 DOI: 10.1016/j.ejim.2006.02.004]
22 **Omland LH**, Farkas DK, Jepsen P, Obel N, Pedersen L. Hepatitis C virus infection and risk of cancer: a population-based cohort study. *Clin Epidemiol* 2010; **2**: 179-186 [PMID: 20865115]
23 **Stang A**. Critical evaluation of the Newcastle-Ottawa scale for the assessment of the quality of nonrandomized studies in meta-analyses. *Eur J Epidemiol* 2010; **25**: 603-605 [PMID: 20652370 DOI: 10.1007/s10654-010-9491-z]
24 **Herzog R**, Álvarez-Pasquin MJ, Díaz C, Del Barrio JL, Estrada JM, Gil Á. Are healthcare workers' intentions to vaccinate related to their knowledge, beliefs and attitudes? A systematic review. *BMC Public Health* 2013; **13**: 154 [PMID: 23421987 DOI: 10.1186/1471-2458-13-154]
25 **DerSimonian R**, Laird N. Meta-analysis in clinical trials. *Control Clin Trials* 1986; **7**: 177-188 [PMID: 3802833]
26 **Higgins JP**, Thompson SG, Deeks JJ, Altman DG. Measuring inconsistency in meta-analyses. *BMJ* 2003; **327**: 557-560 [PMID: 12958120 DOI: 10.1136/bmj.327.7414.557]
27 **Macleod LC**, Hotaling JM, Wright JL, Davenport MT, Gore JL, Harper J, White E. Risk factors for renal cell carcinoma in the VITAL study. *J Urol* 2013; **190**: 1657-1661 [PMID: 23665301 DOI: 10.1016/j.juro.2013.04.130]
28 **Wiwanitkit V**. Renal cell carcinoma and hepatitis C virus infection: is there any cause-outcome relationship? *J Cancer Res Ther* 2011; **7**: 226-227 [PMID: 21768723 DOI: 10.4103/0973-1482.82931]
29 **Chisari FV**. Cytotoxic T cells and viral hepatitis. *J Clin Invest* 1997; **99**: 1472-1477 [PMID: 9119989 DOI: 10.1172/JCI119308]
30 **Lukkonen A**, Lintula S, von Boguslawski K, Carpén O, Ljungberg B, Landberg G, Stenman UH. Tumor-associated trypsin inhibitor in normal and malignant renal tissue and in serum of renal-cell carcinoma patients. *Int J Cancer* 1999; **83**: 486-490 [PMID: 10508484]
31 **Kalaghatgi P**, Sikorski AM, Knops E, Rupp D, Sierra S, Heger E, Neumann-Fraune M, Beggel B, Walker A, Timm J, Walter H, Obermeier M, Kaiser R, Bartenschlager R, Lengauer T. Geno2pheno[HCV] - A Web-based Interpretation System to Support Hepatitis C Treatment Decisions in the Era of Direct-Acting Antiviral Agents. *PLoS One* 2016; **11**: e0155869 [PMID: 27196673 DOI: 10.1371/journal.pone.0155869]

P- Reviewer: Levinson A, Malnick S, Procopio G **S- Editor**: Kong JX
L- Editor: A **E- Editor**: Wu HL

Published by **Baishideng Publishing Group Inc**
8226 Regency Drive, Pleasanton, CA 94588, USA
Telephone: +1-925-223-8242
Fax: +1-925-223-8243
E-mail: bpgoffice@wjgnet.com
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
http://www.wjgnet.com