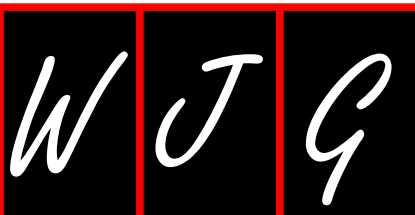


World Journal of *Gastroenterology*

World J Gastroenterol 2016 October 21; 22(39): 8641-8852





Editorial Board

2014-2017

The *World Journal of Gastroenterology* Editorial Board consists of 1375 members, representing a team of worldwide experts in gastroenterology and hepatology. They are from 68 countries, including Algeria (2), Argentina (7), Australia (31), Austria (9), Belgium (11), Brazil (20), Brunei Darussalam (1), Bulgaria (2), Cambodia (1), Canada (25), Chile (4), China (165), Croatia (2), Cuba (1), Czech (6), Denmark (2), Egypt (9), Estonia (2), Finland (6), France (20), Germany (58), Greece (31), Guatemala (1), Hungary (14), Iceland (1), India (33), Indonesia (2), Iran (10), Ireland (9), Israel (18), Italy (194), Japan (149), Jordan (1), Kuwait (1), Lebanon (7), Lithuania (1), Malaysia (1), Mexico (11), Morocco (1), Netherlands (5), New Zealand (4), Nigeria (3), Norway (6), Pakistan (6), Poland (12), Portugal (8), Puerto Rico (1), Qatar (1), Romania (10), Russia (3), Saudi Arabia (2), Singapore (7), Slovenia (2), South Africa (1), South Korea (69), Spain (51), Sri Lanka (1), Sudan (1), Sweden (12), Switzerland (5), Thailand (7), Trinidad and Tobago (1), Tunisia (2), Turkey (55), United Kingdom (49), United States (180), Venezuela (1), and Vietnam (1).

EDITORS-IN-CHIEF

Stephen C Strom, *Stockholm*
Andrzej S Tarnawski, *Long Beach*
Damian Garcia-Olmo, *Madrid*

ASSOCIATE EDITORS

Yung-Jue Bang, *Seoul*
Vincent Di Martino, *Besancon*
Daniel T Farkas, *Bronx*
Roberto J Firpi, *Gainesville*
Maria Gazouli, *Athens*
Chung-Feng Huang, *Kaohsiung*
Namir Katkhouda, *Los Angeles*
Anna Kramvis, *Johannesburg*
Wolfgang Kruis, *Cologne*
Peter L Lakatos, *Budapest*
Han Chu Lee, *Seoul*
Christine McDonald, *Cleveland*
Nahum Mendez-Sanchez, *Mexico City*
George K Michalopoulos, *Pittsburgh*
Suk Woo Nam, *Seoul*
Shu-You Peng, *Hangzhou*
Daniel von Renteln, *Montreal*
Angelo Sangiovanni, *Milan*
Hildegard M Schuller, *Knoxville*
Dong-Wan Seo, *Seoul*
Adrian John Stanley, *Glasgow*
Jurgen Stein, *Frankfurt*
Bei-Cheng Sun, *Nanjing*
Yoshio Yamaoka, *Yufu*

GUEST EDITORIAL BOARD MEMBERS

Jia-Ming Chang, *Taipei*
Jane CJ Chao, *Taipei*

Kuen-Feng Chen, *Taipei*
Tai-An Chiang, *Tainan*
Yi-You Chiou, *Taipei*
Seng-Kee Chuah, *Kaohsiung*
Wan-Long Chuang, *Kaohsiung*
How-Ran Guo, *Tainan*
Ming-Chih Hou, *Taipei*
Po-Shiuan Hsieh, *Taipei*
Ching-Chuan Hsieh, *Chiayi county*
Jun-Te Hsu, *Taoyuan*
Chung-Ping Hsu, *Taichung*
Chien-Ching Hung, *Taipei*
Chao-Hung Hung, *Kaohsiung*
Chen-Guo Ker, *Kaohsiung*
Yung-Chih Lai, *Taipei*
Teng-Yu Lee, *Taichung City*
Wei-Jei Lee, *Taoyuan*
Jin-Ching Lee, *Kaohsiung*
Jen-Kou Lin, *Taipei*
Ya-Wen Lin, *Taipei*
Hui-kang Liu, *Taipei*
Min-Hsiung Pan, *Taipei*
Bor-Shyang Sheu, *Tainan*
Hon-Yi Shi, *Kaohsiung*
Fung-Chang Sung, *Taichung*
Dar-In Tai, *Taipei*
Jung-Fa Tsai, *Kaohsiung*
Yao-Chou Tsai, *New Taipei City*
Chih-Chi Wang, *Kaohsiung*
Liang-Shun Wang, *New Taipei City*
Hsiu-Po Wang, *Taipei*
Jaw-Yuan Wang, *Kaohsiung*
Yuan-Huang Wang, *Taipei*
Yuan-Chuen Wang, *Taichung*

Deng-Chyang Wu, *Kaohsiung*
Shun-Fa Yang, *Taichung*
Hsu-Heng Yen, *Changhua*

MEMBERS OF THE EDITORIAL BOARD



Algeria

Saadi Berkane, *Algiers*
Samir Rouabhia, *Batna*



Argentina

N Tolosa de Talamoni, *Córdoba*
Eduardo de Santibanes, *Buenos Aires*
Bernardo Frider, *Capital Federal*
Guillermo Mazzolini, *Pilar*
Carlos Jose Pirola, *Buenos Aires*
Bernabé Matías Quesada, *Buenos Aires*
María Fernanda Troncoso, *Buenos Aires*



Australia

Golo Ahlenstiel, *Westmead*
Minoti V Apte, *Sydney*
Jacqueline S Barrett, *Melbourne*
Michael Beard, *Adelaide*
Filip Braet, *Sydney*
Guy D Eslick, *Sydney*
Christine Feinle-Bisset, *Adelaide*
Mark D Gorrell, *Sydney*
Michael Horowitz, *Adelaide*

Gordon Stanley Howarth, *Roseworthy*
 Seungha Kang, *Brisbane*
 Alfred King Lam, *Gold Coast*
 Ian C Lawrance, *Perth/Fremantle*
 Barbara Anne Leggett, *Brisbane*
 Daniel A Lemberg, *Sydney*
 Rupert W Leong, *Sydney*
 Finlay A Macrae, *Victoria*
 Vance Matthews, *Melbourne*
 David L Morris, *Sydney*
 Reme Mountifield, *Bedford Park*
 Hans J Netter, *Melbourne*
 Nam Q Nguyen, *Adelaide*
 Liang Qiao, *Westmead*
 Rajvinder Singh, *Adelaide*
 Ross Cyril Smith, *St Leonards*
 Kevin J Spring, *Sydney*
 Debbie Trinder, *Fremantle*
 Daniel R van Langenberg, *Box Hill*
 David Ian Watson, *Adelaide*
 Desmond Yip, *Garran*
 Li Zhang, *Sydney*



Austria

Felix Aigner, *Innsbruck*
 Gabriela A Berlakovich, *Vienna*
 Herwig R Cerwenka, *Graz*
 Peter Ferenci, *Wien*
 Alfred Gangl, *Vienna*
 Kurt Lenz, *Linz*
 Markus Peck-Radosavljevic, *Vienna*
 Markus Raderer, *Vienna*
 Stefan Riss, *Vienna*



Belgium

Michael George Adler, *Brussels*
 Benedicte Y De Winter, *Antwerp*
 Mark De Ridder, *Jette*
 Olivier Detry, *Liege*
 Denis Dufrane Dufrane, *Brussels*
 Sven M Francque, *Edegem*
 Nikos Kotzampassakis, *Liège*
 Geert KMM Robaey, *Genk*
 Xavier Sagaert, *Leuven*
 Peter Starkel, *Brussels*
 Eddie Wisse, *Keerbergen*



Brazil

SMP Balzan, *Santa Cruz do Sul*
 JLF Caboclo, *Sao Jose do Rio Preto*
 Fábio Guilherme Campos, *Sao Paulo*
 Claudia RL Cardoso, *Rio de Janeiro*
 Roberto J Carvalho-Filho, *Sao Paulo*
 Carla Daltro, *Salvador*
 José Sebastiao dos Santos, *Ribeirão Preto*
 Eduardo LR Mello, *Rio de Janeiro*
 Stihela Maria Murad-Regadas, *Fortaleza*
 Claudia PMS Oliveira, *Sao Paulo*
 Júlio C Pereira-Lima, *Porto Alegre*
 Marcos V Perini, *Sao Paulo*
 Vietla Satyanarayana Rao, *Fortaleza*

Raquel Rocha, *Salvador*
 AC Simoes e Silva, *Belo Horizonte*
 Mauricio F Silva, *Porto Alegre*
 Aytan Miranda Sipahi, *Sao Paulo*
 Rosa Leonôra Salerno Soares, *Niterói*
 Cristiane Valle Tovo, *Porto Alegre*
 Eduardo Garcia Vilela, *Belo Horizonte*



Brunei Darussalam

Vui Heng Chong, *Bandar Seri Begawan*



Bulgaria

Tanya Kirilova Kadiyska, *Sofia*
 Mihaela Petrova, *Sofia*



Cambodia

Francois Rouet, *Phnom Penh*



Canada

Brian Bressler, *Vancouver*
 Frank J Burczynski, *Winnipeg*
 Wangxue Chen, *Ottawa*
 Francesco Crea, *Vancouver*
 Jane A Foster, *Hamilton*
 Hugh J Freeman, *Vancouver*
 Shahrokh M Ghobadloo, *Ottawa*
 Yuewen Gong, *Winnipeg*
 Philip H Gordon, *Quebec*
 Rakesh Kumar, *Edmonton*
 Wolfgang A Kunze, *Hamilton*
 Patrick Labonte, *Laval*
 Zhikang Peng, *Winnipeg*
 Jayadev Raju, *Ottawa*
 Maitreyi Raman, *Calgary*
 Giada Sebastiani, *Montreal*
 Maida J Sewitch, *Montreal*
 Eldon A Shaffer, *Alberta*
 Christopher W Teshima, *Edmonton*
 Jean Sévigny, *Québec*
 Pingchang Yang, *Hamilton*
 Pingchang Yang, *Hamilton*
 Eric M Yoshida, *Vancouver*
 Bin Zheng, *Edmonton*



Chile

Marcelo A Beltran, *La Serena*
 Flavio Nervi, *Santiago*
 Adolfo Parra-Blanco, *Santiago*
 Alejandro Soza, *Santiago*



China

Zhao-Xiang Bian, *Hong Kong*
 San-Jun Cai, *Shanghai*
 Guang-Wen Cao, *Shanghai*
 Long Chen, *Nanjing*
 Ru-Fu Chen, *Guangzhou*
 George G Chen, *Hong Kong*

Li-Bo Chen, *Wuhan*
 Jia-Xu Chen, *Beijing*
 Hong-Song Chen, *Beijing*
 Lin Chen, *Beijing*
 Yang-Chao Chen, *Hong Kong*
 Zhen Chen, *Shanghai*
 Ying-Sheng Cheng, *Shanghai*
 Kent-Man Chu, *Hong Kong*
 Zhi-Jun Dai, *Xi'an*
 Jing-Yu Deng, *Tianjin*
 Yi-Qi Du, *Shanghai*
 Zhi Du, *Tianjin*
 Hani El-Nezami, *Hong Kong*
 Bao-Ying Fei, *Hangzhou*
 Chang-Ming Gao, *Nanjing*
 Jian-Ping Gong, *Chongqing*
 Zuo-Jiong Gong, *Wuhan*
 Jing-Shan Gong, *Shenzhen*
 Guo-Li Gu, *Beijing*
 Yong-Song Guan, *Chengdu*
 Mao-Lin Guo, *Luoyang*
 Jun-Ming Guo, *Ningbo*
 Yan-Mei Guo, *Shanghai*
 Xiao-Zhong Guo, *Shenyang*
 Guo-Hong Han, *Xi'an*
 Ming-Liang He, *Hong Kong*
 Peng Hou, *Xi'an*
 Zhao-Hui Huang, *Wuxi*
 Feng Ji, *Hangzhou*
 Simon Law, *Hong Kong*
 Yan-Chang Lei, *Hangzhou*
 Yu-Yuan Li, *Guangzhou*
 Meng-Sen Li, *Haikou*
 Shu-De Li, *Shanghai*
 Zong-Fang Li, *Xi'an*
 Qing-Quan Li, *Shanghai*
 Kang Li, *Lasa*
 Han Liang, *Tianjin*
 Xing'e Liu, *Hangzhou*
 Zheng-Wen Liu, *Xi'an*
 Xiao-Fang Liu, *Yantai*
 Bin Liu, *Tianjin*
 Quan-Da Liu, *Beijing*
 Hai-Feng Liu, *Beijing*
 Fei Liu, *Shanghai*
 Ai-Guo Lu, *Shanghai*
 He-Sheng Luo, *Wuhan*
 Xiao-Peng Ma, *Shanghai*
 Yong Meng, *Shantou*
 Ke-Jun Nan, *Xi'an*
 Siew Chien Ng, *Hong Kong*
 Simon SM Ng, *Hong Kong*
 Zhao-Shan Niu, *Qingdao*
 Di Qu, *Shanghai*
 Ju-Wei Mu, *Beijing*
 Rui-Hua Shi, *Nanjing*
 Bao-Min Shi, *Shanghai*
 Xiao-Dong Sun, *Hangzhou*
 Si-Yu Sun, *Shenyang*
 Guang-Hong Tan, *Haikou*
 Wen-Fu Tang, *Chengdu*
 Anthony YB Teoh, *Hong Kong*
 Wei-Dong Tong, *Chongqing*
 Eric Tse, *Hong Kong*
 Hong Tu, *Shanghai*

Rong Tu, *Haikou*
 Jian-She Wang, *Shanghai*
 Kai Wang, *Jinan*
 Xiao-Ping Wang, *Xianyang*
 Xiu-Yan Wang, *Shanghai*
 Dao-Rong Wang, *Yangzhou*
 De-Sheng Wang, *Xi'an*
 Chun-You Wang, *Wuhan*
 Ge Wang, *Chongqing*
 Xi-Shan Wang, *Harbin*
 Wei-hong Wang, *Beijing*
 Zhen-Ning Wang, *Shenyang*
 Wai Man Raymond Wong, *Hong Kong*
 Chun-Ming Wong, *Hong Kong*
 Jian Wu, *Shanghai*
 Sheng-Li Wu, *Xi'an*
 Wu-Jun Wu, *Xi'an*
 Qing Xia, *Chengdu*
 Yan Xin, *Shenyang*
 Dong-Ping Xu, *Beijing*
 Jian-Min Xu, *Shanghai*
 Wei Xu, *Changchun*
 Ming Yan, *Jinan*
 Xin-Min Yan, *Kunming*
 Yi-Qun Yan, *Shanghai*
 Feng Yang, *Shanghai*
 Yong-Ping Yang, *Beijing*
 He-Rui Yao, *Guangzhou*
 Thomas Yau, *Hong Kong*
 Winnie Yeo, *Hong Kong*
 Jing You, *Kunming*
 Jian-Qing Yu, *Wuhan*
 Ying-Yan Yu, *Shanghai*
 Wei-Zheng Yang, *Chengdu*
 Zong-Ming Zhang, *Beijing*
 Dian-Liang Zhang, *Qingdao*
 Ya-Ping Zhang, *Shijiazhuang*
 You-Cheng Zhang, *Lanzhou*
 Jian-Zhong Zhang, *Beijing*
 Ji-Yuan Zhang, *Beijing*
 Hai-Tao Zhao, *Beijing*
 Jian Zhao, *Shanghai*
 Jian-Hong Zhong, *Nanning*
 Ying-Qiang Zhong, *Guangzhou*
 Ping-Hong Zhou, *Shanghai*
 Yan-Ming Zhou, *Xiamen*
 Tong Zhou, *Nanchong*
 Li-Ming Zhou, *Chengdu*
 Guo-Xiong Zhou, *Nantong*
 Feng-Shang Zhu, *Shanghai*
 Jiang-Fan Zhu, *Shanghai*
 Zhao-Hui Zhu, *Beijing*



Croatia

Tajana Filipec Kanizaj, *Zagreb*
 Mario Tadic, *Zagreb*



Cuba

Damian Casadesus, *Havana*



Czech

Jan Bures, *Hradec Kralove*
 Marcela Kopacova, *Hradec Kralove*

Otto Kucera, *Hradec Kralove*
 Marek Minarik, *Prague*
 Pavel Soucek, *Prague*
 Miroslav Zavoral, *Prague*



Denmark

Vibeke Andersen, *Odense*
 E Michael Danielsen, *Copenhagen*



Egypt

Mohamed MM Abdel-Latif, *Assiut*
 Hussein Atta, *Cairo*
 Ashraf Elbahrawy, *Cairo*
 Mortada Hassan El-Shabrawi, *Cairo*
 Mona El Said El-Raziky, *Cairo*
 Elrashdy M Redwan, *New Borg Alrab*
 Zeinab Nabil Ahmed Said, *Cairo*
 Ragaa HM Salama, *Assiut*
 Maha Maher Shehata, *Mansoura*



Estonia

Margus Lember, *Tartu*
 Tamara Vorobjova, *Tartu*



Finland

Marko Kalliomäki, *Turku*
 Thomas Kietzmann, *Oulu*
 Kaija-Leena Kolho, *Helsinki*
 Eija Korkeila, *Turku*
 Heikki Makisalo, *Helsinki*
 Tanja Pessi, *Tampere*



France

Armando Abergel Clermont, *Ferrand*
 Elie K Chouillard, *Polssy*
 Pierre Cordelier, *Toulouse*
 Pascal P Crenn, *Garches*
 Catherine Daniel, *Lille*
 Fanny Daniel, *Paris*
 Cedric Dray, *Toulouse*
 Benoit Foligne, *Lille*
 Jean-Noel Freund, *Strasbourg*
 Hervé Guillou, *Toulouse*
 Nathalie Janel, *Paris*
 Majid Khatib, *Bordeaux*
 Jacques Marescaux, *Strasbourg*
 Jean-Claude Marie, *Paris*
 Driffa Moussata, *Pierre Benite*
 Hang Nguyen, *Clermont-Ferrand*
 Hugo Perazzo, *Paris*
 Alain L Servin, *Chatenay-Malabry*
 Chang Xian Zhang, *Lyon*



Germany

Stavros A Antoniou, *Monchengladbach*
 Erwin Biecker, *Siegburg*
 Hubert E Blum, *Freiburg*

Thomas Bock, *Berlin*
 Katja Breitkopf-Heinlein, *Mannheim*
 Elke Cario, *Essen*
 Güralp Onur Ceyhan, *Munich*
 Angel Cid-Arregui, *Heidelberg*
 Michael Clemens Roggendorf, *München*
 Christoph F Dietrich, *Bad Mergentheim*
 Valentin Fuhrmann, *Hamburg*
 Nikolaus Gassler, *Aachen*
 Andreas Geier, *Wuerzburg*
 Markus Gerhard, *Munich*
 Anton Gillissen, *Muenster*
 Thorsten Oliver Goetze, *Offenbach*
 Daniel Nils Gotthardt, *Heidelberg*
 Robert Grützmann, *Dresden*
 Thilo Hackert, *Heidelberg*
 Claus Hellerbrand, *Regensburg*
 Harald Peter Hoensch, *Darmstadt*
 Jens Hoeppner, *Freiburg*
 Richard Hummel, *Muenster*
 Jakob Robert Izbicki, *Hamburg*
 Gernot Maximilian Kaiser, *Essen*
 Matthias Kapischke, *Hamburg*
 Michael Keese, *Frankfurt*
 Andrej Khandoga, *Munich*
 Jorg Kleeff, *Munich*
 Alfred Koenigsrainer, *Tuebingen*
 Peter Christopher Konturek, *Saalfeld*
 Michael Linnebacher, *Rostock*
 Stefan Maier, *Kaufbeuren*
 Oliver Mann, *Hamburg*
 Marc E Martignoni, *Munic*
 Thomas Minor, *Bonn*
 Oliver Moeschler, *Osnabrueck*
 Jonas Mudter, *Eutin*
 Sebastian Mueller, *Heidelberg*
 Matthias Ocker, *Berlin*
 Andreas Ommer, *Essen*
 Albrecht Piiper, *Frankfurt*
 Esther Raskopf, *Bonn*
 Christoph Reichel, *Bad Brückenau*
 Elke Roeb, *Giessen*
 Udo Rolle, *Frankfurt*
 Karl-Herbert Schafer, *Zweibrücken*
 Peter Schemmer, *Heidelberg*
 Andreas G Schreyer, *Regensburg*
 Manuel A Silva, *Penzberg*
 Georgios C Sotiropoulos, *Essen*
 Ulrike S Stein, *Berlin*
 Dirk Uhlmann, *Leipzig*
 Michael Weiss, *Halle*
 Hong-Lei Weng, *Mannheim*
 Karsten Wursthorn, *Hamburg*



Greece

Alexandra Alexopoulou, *Athens*
 Nikolaos Antonakopoulos, *Athens*
 Stelios F Assimakopoulos, *Patras*
 Grigoris Chatzimavroudis, *Thessaloniki*
 Evangelos Cholongitas, *Thessaloniki*
 Gregory Christodoulidis, *Larisa*
 George N Dalekos, *Larisa*
 Urania Georgopoulou, *Athens*
 Eleni Gigi, *Thessaloniki*

Stavros Gourgiotis, *Athens*
 Leontios J Hadjileontiadis, *Thessaloniki*
 Thomas Hyphantis, *Ioannina*
 Ioannis Kanellos, *Thessaloniki*
 Stylianos Karatapanis, *Rhodes*
 Michael Koutsilieris, *Athens*
 Spiros D Ladas, *Athens*
 Theodoros K Liakakos, *Athens*
 Emanuel K Manesis, *Athens*
 Spiliot Manolakopoulos, *Athens*
 Gerassimos John Mantzaris, *Athens*
 Athanasios D Marinis, *Piraeus*
 Nikolaos Ioannis Nikiteas, *Athens*
 Konstantinos X Papamichael, *Athens*
 George Sgourakis, *Athens*
 Konstantinos C Thomopoulos, *Patras*
 Konstantinos Triantafyllou, *Athens*
 Christos Triantos, *Patras*
 Georgios Zacharakis, *Athens*
 Petros Zezos, *Alexandroupolis*
 Demosthenes E Ziogas, *Ioannina*



Guatemala

Carlos Maria Parellada, *Guatemala*



Hungary

Mihaly Boros, *Szeged*
 Tamás Decsi, *Pécs*
 Gyula Farkas, *Szeged*
 Andrea Furka, *Debrecen*
 Y vette Mandi, *Szeged*
 Peter L Lakatos, *Budapest*
 Pal Miheller, *Budapest*
 Tamás Molnar, *Szeged*
 Attila Olah, *Gyor*
 Maria Papp, *Debrecen*
 Ferenc Sipos, *Budapest*
 Miklós Tanyi, *Debrecen*
 Tibor Wittmann, *Szeged*



Iceland

Tryggvi Bjorn Stefánsson, *Reykjavík*



India

Brij B Agarwal, *New Delhi*
 Deepak N Amarapurkar, *Mumbai*
 Shams ul Bari, *Srinagar*
 Sriparna Basu, *Varanasi*
 Runu Chakravarty, *Kolkata*
 Devendra C Desai, *Mumbai*
 Nutan D Desai, *Mumbai*
 Suneela Sunil Dhaneshwar, *Pune*
 Radha K Dhiman, *Chandigarh*
 Pankaj Garg, *Mohali*
 Uday C Ghoshal, *Lucknow*
 Kalpesh Jani, *Vadodara*
 Premashis Kar, *New Delhi*
 Jyotdeep Kaur, *Chandigarh*
 Rakesh Kochhar, *Chandigarh*
 Pradyumna K Mishra, *Mumbai*

Asish K Mukhopadhyay, *Kolkata*
 Imtiyaz Murtaza, *Srinagar*
 P Nagarajan, *New Delhi*
 Samiran Nundy, *Delhi*
 Gopal Pande, *Hyderabad*
 Benjamin Perakath, *Vellore*
 Arun Prasad, *New Delhi*
 D Nageshwar Reddy, *Hyderabad*
 Lekha Saha, *Chandigarh*
 Sundeep Singh Saluja, *New Delhi*
 Mahesh Prakash Sharma, *New Delhi*
 Sadiq Saleem Sikora, *Bangalore*
 Sarman Singh, *New Delhi*
 Rajeev Sinha, *Jhansi*
 Rupjyoti Talukdar, *Hyderabad*
 Rakesh Kumar Tandon, *New Delhi*
 Narayanan Thirumoorthy, *Coimbatore*



Indonesia

David Handojo Muljono, *Jakarta*
 Andi Utama, *Jakarta*



Iran

Arezoo Aghakhani, *Tehran*
 Seyed Mohsen Dehghani, *Shiraz*
 Ahad Eshraghian, *Shiraz*
 Hossein Khedmat, *Tehran*
 Sadegh Massarrat, *Tehran*
 Marjan Mohammadi, *Tehran*
 Roja Rahimi, *Tehran*
 Farzaneh Sabahi, *Tehran*
 Majid Sadeghizadeh, *Tehran*
 Farideh Siavoshi, *Tehran*



Ireland

Gary Alan Bass, *Dublin*
 David J Brayden, *Dublin*
 Ronan A Cahill, *Dublin*
 Glen A Doherty, *Dublin*
 Liam J Fanning, *Cork*
 Barry Philip McMahon, *Dublin*
 RossMcManus, *Dublin*
 Dervla O'Malley, *Cork*
 Sinead M Smith, *Dublin*



Israel

Dan Carter, *Ramat Gan*
 Jorge-Shmuel Delgado, *Metar*
 Eli Magen, *Ashdod*
 Nitsan Maharshak, *Tel Aviv*
 Shaul Mordechai, *Beer Sheva*
 Menachem Moshkowitz, *Tel Aviv*
 William Bahij Nseir, *Nazareth*
 Shimon Reif, *Jerusalem*
 Ram Reifen, *Rehovot*
 Ariella Bar-Gil Shitrit, *Jerusalem*
 Noam Shussman, *Jerusalem*
 Igor Sukhotnik, *Haifa*
 Nir Wasserberg, *Petach Tikva*
 Jacob Yahav, *Rehovot*

Doron Levi Zamir, *Gedera*
 Shira Zelber-Sagi, *Haifa*
 Romy Zemel, *Petach-Tikva*



Italy

Ludovico Abenavoli, *Catanzaro*
 Luigi Elio Adinolfi, *Naples*
 Carlo Virginio Agostoni, *Milan*
 Anna Alisi, *Rome*
 Piero Luigi Almasio, *Palermo*
 Donato Francesco Altomare, *Bari*
 Amedeo Amedei, *Florence*
 Pietro Andreone, *Bologna*
 Imerio Angriman, *Padova*
 Vito Annese, *Florence*
 Paolo Aurello, *Rome*
 Salvatore Auricchio, *Naples*
 Gian Luca Baiocchi, *Brescia*
 Gianpaolo Balzano, *Milan*
 Antonio Basoli, *Rome*
 Gabrio Bassotti, *San Sisto*
 Mauro Bernardi, *Bologna*
 Alberto Biondi, *Rome*
 Ennio Biscaldi, *Genova*
 Massimo Bolognesi, *Padua*
 Luigi Bonavina, *Milano*
 Aldo Bove, *Chieti*
 Raffaele Bruno, *Pavia*
 Luigi Bruscianno, *Napoli*
 Giuseppe Cabibbo, *Palermo*
 Carlo Calabrese, *Bologna*
 Daniele Calistri, *Meldola*
 Vincenza Calvaruso, *Palermo*
 Lorenzo Camellini, *Reggio Emilia*
 Marco Candela, *Bologna*
 Raffaele Capasso, *Naples*
 Lucia Carulli, *Modena*
 Renato David Caviglia, *Rome*
 Luigina Cellini, *Chieti*
 Giuseppe Chiarioni, *Verona*
 Claudio Chiesa, *Rome*
 Michele Cicala, *Roma*
 Rachele Ciccocioppo, *Pavia*
 Sandro Contini, *Parma*
 Gaetano Corso, *Foggia*
 Renato Costi, *Parma*
 Alessandro Cucchetti, *Bologna*
 Rosario Cuomo, *Napoli*
 Giuseppe Currò, *Messina*
 Paola De Nardi, *Milano*
 Giovanni D De Palma, *Naples*
 Raffaele De Palma, *Napoli*
 Giuseppina De Petro, *Brescia*
 Valli De Re, *Aviano*
 Paolo De Simone, *Pisa*
 Giuliana Decorti, *Trieste*
 Emanuele Miraglia del Giudice, *Napoli*
 Isidoro Di Carlo, *Catania*
 Matteo Nicola Dario Di Minno, *Naples*
 Massimo Donadelli, *Verona*
 Mirko D'Onofrio, *Verona*
 Maria Pina Dore, *Sassari*
 Luca Elli, *Milano*
 Massimiliano Fabozzi, *Aosta*
 Massimo Falconi, *Ancona*

Ezio Falletto, *Turin*
 Silvia Fargion, *Milan*
 Matteo Fassan, *Verona*
 Gianfranco Delle Fave, *Roma*
 Alessandro Federico, *Naples*
 Francesco Feo, *Sassari*
 Davide Festi, *Bologna*
 Natale Figura, *Siena*
 Vincenzo Formica, *Rome*
 Mirella Fraquelli, *Milan*
 Marzio Frazzoni, *Modena*
 Walter Fries, *Messina*
 Gennaro Galizia, *Naples*
 Andrea Galli, *Florence*
 Matteo Garcovich, *Rome*
 Eugenio Gaudio, *Rome*
 Paola Ghorzo, *Genoa*
 Edoardo G Giannini, *Genova*
 Luca Gianotti, *Monza*
 Maria Cecilia Giron, *Padova*
 Alberto Grassi, *Rimini*
 Gabriele Grassi, *Trieste*
 Francesco Greco, *Bergamo*
 Luigi Greco, *Naples*
 Antonio Grieco, *Rome*
 Fabio Grizzi, *Rozzano*
 Laurino Grossi, *Pescara*
 Simone Guglielmetti, *Milan*
 Tiberiu Hershcovici, *Jerusalem*
 Calogero Iacono, *Verona*
 Enzo Ierardi, *Bari*
 Amedeo Indriolo, *Bergamo*
 Raffaele Iorio, *Naples*
 Paola Iovino, *Salerno*
 Angelo A Izzo, *Naples*
 Loretta Kondili, *Rome*
 Filippo La Torre, *Rome*
 Giuseppe La Torre, *Rome*
 Giovanni Latella, *L'Aquila*
 Salvatore Leonardi, *Catania*
 Massimo Libra, *Catania*
 Anna Licata, *Palermo*
 Carmela Loguercio, *Naples*
 Amedeo Lonardo, *Modena*
 Carmelo Luigiano, *Catania*
 Francesco Luzzo, *Catanzaro*
 Giovanni Maconi, *Milano*
 Antonio Macrì, *Messina*
 Mariano Malaguarnera, *Catania*
 Francesco Manguso, *Napoli*
 Tommaso Maria Manzia, *Rome*
 Daniele Marrelli, *Siena*
 Gabriele Masselli, *Rome*
 Sara Massironi, *Milan*
 Giuseppe Mazzarella, *Avellino*
 Michele Milella, *Rome*
 Giovanni Milito, *Rome*
 Antonella d' Arminio Monforte, *Milan*
 Fabrizio Montecucco, *Genoa*
 Giovanni Monteleone, *Rome*
 Mario Morino, *Torino*
 Vincenzo La Mura, *Milan*
 Gerardo Nardone, *Naples*
 Riccardo Nascimbeni, *Brescia*
 Gabriella Nesi, *Florence*
 Giuseppe Nigri, *Rome*

Erica Novo, *Turin*
 Veronica Ojetti, *Rome*
 Michele Orditura, *Naples*
 Fabio Pace, *Seriate*
 Lucia Pacifico, *Rome*
 Omero Alessandro Paoluzi, *Rome*
 Valerio Pazienza, *San Giovanni Rotondo*
 Rinaldo Pellicano, *Turin*
 Adriano M Pellicelli, *Rome*
 Nadia Peparini, *Ciampino*
 Mario Pescatori, *Rome*
 Antonio Picardi, *Rome*
 Alberto Pilotto, *Padova*
 Alberto Piperno, *Monza*
 Anna Chiara Piscaglia, *Rome*
 Maurizio Pompili, *Rome*
 Francesca Romana Ponziani, *Rome*
 Cosimo Prantero, *Rome*
 Girolamo Ranieri, *Bari*
 Carlo Ratto, *Tome*
 Barbara Renga, *Perugia*
 Alessandro Repici, *Rozzano*
 Maria Elena Riccioni, *Rome*
 Lucia Ricci-Vitiani, *Rome*
 Luciana Rigoli, *Messina*
 Mario Rizzetto, *Torino*
 Ballarin Roberto, *Modena*
 Roberto G Romanelli, *Florence*
 Claudio Romano, *Messina*
 Luca Roncucci, *Modena*
 Cesare Ruffolo, *Treviso*
 Lucia Sacchetti, *Napoli*
 Rodolfo Sacco, *Pisa*
 Lapo Sali, *Florence*
 Romina Salpini, *Rome*
 Giulio Aniello, *Santoro Treviso*
 Armando Santoro, *Rozzano*
 Edoardo Savarino, *Padua*
 Marco Senzolo, *Padua*
 Annalucia Serafino, *Rome*
 Giuseppe S Sica, *Rome*
 Pierpaolo Sileri, *Rome*
 Cosimo Sperti, *Padua*
 Vincenzo Stanghellini, *Bologna*
 Cristina Stasi, *Florence*
 Gabriele Stocco, *Trieste*
 Roberto Tarquini, *Florence*
 Mario Testini, *Bari*
 Guido Torzilli, *Milan*
 Guido Alberto Massimo, *Tiberio Brescia*
 Giuseppe Toffoli, *Aviano*
 Alberto Tommasini, *Trieste*
 Francesco Tonelli, *Florence*
 Cesare Tosetti Porretta, *Terme*
 Lucio Trevisani, *Cona*
 Guglielmo M Trovato, *Catania*
 Mariapia Vairetti, *Pavia*
 Luca Vittorio Valenti, *Milano*
 Mariateresa T Ventura, *Bari*
 Giuseppe Verlato, *Verona*
 Marco Vivarelli, *Ancona*
 Giovanni Li Volti, *Catania*
 Giuseppe Zanotti, *Padua*
 Vincenzo Zara, *Lecce*
 Gianguglielmo Zehender, *Milan*
 Anna Linda Zignego, *Florence*
 Rocco Antonio Zoccali, *Messina*

Angelo Zullo, *Rome*



Japan

Yasushi Adachi, *Sapporo*
 Takafumi Ando, *Nagoya*
 Masahiro Arai, *Tokyo*
 Makoto Arai, *Chiba*
 Takaaki Arigami, *Kagoshima*
 Itaru Endo, *Yokohama*
 Munechika Enjoji, *Fukuoka*
 Shunji Fujimori, *Tokyo*
 Yasuhiro Fujino, *Akashi*
 Toshiyoshi Fujiwara, *Okayama*
 Yosuke Fukunaga, *Tokyo*
 Toshio Fukusato, *Tokyo*
 Takahisa Furuta, *Hamamatsu*
 Osamu Handa, *Kyoto*
 Naoki Hashimoto, *Osaka*
 Yoichi Hiasa, *Toon*
 Masatsugu Hiraki, *Saga*
 Satoshi Hirano, *Sapporo*
 Keiji Hirata, *Fukuoka*
 Toru Hiyama, *Higashihiroshima*
 Akira Hokama, *Nishihara*
 Shu Hoteya, *Tokyo*
 Masao Ichinose, *Wakayama*
 Tatsuya Ide, *Kurume*
 Masahiro Iizuka, *Akita*
 Toshiro Iizuka, *Tokyo*
 Kenichi Ikejima, *Tokyo*
 Tetsuya Ikemoto, *Tokushima*
 Hiroyuki Imaeda, *Saitama*
 Atsushi Imagawa, *Kan-onji*
 Hiroo Imazu, *Tokyo*
 Shuji Isaji, *Tsu*
 Toru Ishikawa, *Niigata*
 Toshiyuki Ishiwata, *Tokyo*
 Soichi Itaba, *Kitakyushu*
 Yoshiaki Iwasaki, *Okayama*
 Tatehiro Kagawa, *Isehara*
 Satoru Kakizaki, *Maebashi*
 Naomi Kakushima, *Shizuoka*
 Terumi Kamisawa, *Tokyo*
 Akihide Kamiya, *Isehara*
 Osamu Kanauchi, *Tokyo*
 Tatsuo Kanda, *Chiba*
 Shin Kariya, *Okayama*
 Shigeyuki Kawa, *Matsumoto*
 Takumi Kawaguchi, *Kurume*
 Takashi Kawai, *Tokyo*
 Soo Ryang Kim, *Kobe*
 Shinsuke Kiriya, *Gunma*
 Tsuneo Kitamura, *Urayasu*
 Masayuki Kitano, *Osakasayama*
 Hirotoshi Kobayashi, *Tokyo*
 Hironori Koga, *Kurume*
 Takashi Kojima, *Sapporo*
 Satoshi Kokura, *Kyoto*
 Shuhei Komatsu, *Kyoto*
 Tadashi Kondo, *Tokyo*
 Yasuteru Kondo, *Sendai*
 Yasuhiro Kuramitsu, *Yamaguchi*
 Yukinori Kurokawa, *Osaka*
 Shin Maeda, *Yokohama*
 Koutarou Maeda, *Toyoake*

Hitoshi Maruyama, *Chiba*
 Atsushi Masamune, *Sendai*
 Hiroyuki Matsubayashi, *Suntogun*
 Akihisa Matsuda, *Inzai*
 Hirofumi Matsui, *Tsukuba*
 Akira Matsumori, *Kyoto*
 Yoichi Matsuo, *Nagoya*
 Y Matsuzaki, *Ami*
 Toshihiro Mitaka, *Sapporo*
 Kouichi Miura, *Akita*
 Shinichi Miyagawa, *Matumoto*
 Eiji Miyoshi, *Suita*
 Toru Mizuguchi, *Sapporo*
 Nobumasa Mizuno, *Nagoya*
 Zenichi Morise, *Nagoya*
 Tomohiko Moriyama, *Fukuoka*
 Kunihiko Murase, *Tusima*
 Michihiro Mutoh, *Tsukiji*
 Akihito Nagahara, *Tokyo*
 Hikaru Nagahara, *Tokyo*
 Hidenari Nagai, *Tokyo*
 Koichi Nagata, *Shimotsuke-shi*
 Masaki Nagaya, *Kawasaki*
 Hisato Nakajima, *Nishi-Shinbashi*
 Toshifusa Nakajima, *Tokyo*
 Hiroshi Nakano, *Kawasaki*
 Hiroshi Nakase, *Kyoto*
 Toshiyuki Nakayama, *Nagasaki*
 Takahiro Nakazawa, *Nagoya*
 Shoji Natsugoe, *Kagoshima City*
 Tsutomu Nishida, *Suita*
 Shuji Nomoto, *Naogya*
 Sachiyo Nomura, *Tokyo*
 Takeshi Ogura, *Takatsukishi*
 Nobuhiro Ohkohchi, *Tsukuba*
 Toshifumi Ohkusa, *Kashiwa*
 Hirohide Ohnishi, *Akita*
 Teruo Okano, *Tokyo*
 Satoshi Osawa, *Hamamatsu*
 Motoyuki Otsuka, *Tokyo*
 Michitaka Ozaki, *Sapporo*
 Satoru Saito, *Yokohama*
 Naoaki Sakata, *Sendai*
 Ken Sato, *Maebashi*
 Toshiro Sato, *Tokyo*
 Tomoyuki Shibata, *Toyoake*
 Tomohiko Shimatani, *Kure*
 Yukihiro Shimizu, *Nanto*
 Tadashi Shimoyama, *Hirosaki*
 Masayuki Sho, *Nara*
 Ikuo Shoji, *Kobe*
 Atsushi Sofuni, *Tokyo*
 Takeshi Suda, *Niigata*
 M Sugimoto, *Hamamatsu*
 Ken Sugimoto, *Hamamatsu*
 Haruhiko Sugimura, *Hamamatsu*
 Shoichiro Sumi, *Kyoto*
 Hidekazu Suzuki, *Tokyo*
 Masahiro Tajika, *Nagoya*
 Hitoshi Takagi, *Takasaki*
 Toru Takahashi, *Niigata*
 Yoshihisa Takahashi, *Tokyo*
 Shinsuke Takeno, *Fukuoka*
 Akihiro Tamori, *Osaka*
 Kyosuke Tanaka, *Tsu*
 Shinji Tanaka, *Hiroshima*

Atsushi Tanaka, *Tokyo*
 Yasuhito Tanaka, *Nagoya*
 Shinji Tanaka, *Tokyo*
 Minoru Tomizawa, *Yotsukaido City*
 Kyoko Tsukiyama-Kohara, *Kagoshima*
 Takuya Watanabe, *Niigata*
 Kazuhiro Watanabe, *Sendai*
 Satoshi Yamagiwa, *Niigata*
 Takayuki Yamamoto, *Yokkaichi*
 Hiroshi Yamamoto, *Otsu*
 Kosho Yamanouchi, *Nagasaki*
 Ichiro Yasuda, *Gifu*
 Yutaka Yata, *Maebashi-city*
 Shin-ichi Yokota, *Sapporo*
 Norimasa Yoshida, *Kyoto*
 Hiroshi Yoshida, *Tama-City*
 Hitoshi Yoshiji, *Kashihara*
 Kazuhiko Yoshimatsu, *Tokyo*
 Kentaro Yoshioka, *Toyoake*
 Nobuhiro Zaima, *Nara*



Jordan

Khaled Ali Jadallah, *Irbid*



Kuwait

Islam Khan, *Kuwait*



Lebanon

Bassam N Abboud, *Beirut*
 Kassem A Barada, *Beirut*
 Marwan Ghosn, *Beirut*
 Iyad A Issa, *Beirut*
 Fadi H Mourad, *Beirut*
 AIA Sharara, *Beirut*
 Rita Slim, *Beirut*



Lithuania

Antanas Mickevicius, *Kaunas*



Malaysia

Huck Joo Tan, *Petaling Jaya*



Mexico

Richard A Awad, *Mexico City*
 Carlos R Camara-Lemarroy, *Monterrey*
 Norberto C Chavez-Tapia, *Mexico City*
 Wolfgang Gaertner, *Mexico City*
 Diego Garcia-Compean, *Monterrey*
 Arturo Panduro, *Guadalajara*
 OT Teramoto-Matsubara, *Mexico City*
 Felix Tellez-Avila, *Mexico City*
 Omar Vergara-Fernandez, *Mexico City*
 Saúl Villa-Trevino, *Cuidad de México*



Morocco

Samir Ahboucha, *Khouribga*



Netherlands

Robert J de Knegt, *Rotterdam*
 Tom Johannes Gerardus Gevers, *Nijmegen*
 Menno Hoekstra, *Leiden*
 BW Marcel Spanier, *Arnhem*
 Karel van Erpecum, *Utrecht*



New Zealand

Leo K Cheng, *Auckland*
 Andrew Stewart Day, *Christchurch*
 Jonathan Barnes Koea, *Auckland*
 Max Petrov, *Auckland*



Nigeria

Olufunmilayo Adenike Lesi, *Lagos*
 Jesse Abiodun Otegbayo, *Ibadan*
 Stella Ifeanyi Smith, *Lagos*



Norway

Trond Berg, *Oslo*
 Trond Arnulf Buanes, *Krokkleiva*
 Thomas de Lange, *Rud*
 Magdy El-Salhy, *Stord*
 Rasmus Goll, *Tromso*
 Dag Arne Lihaug Hoff, *Aalesund*



Pakistan

Zaigham Abbas, *Karachi*
 Usman A Ashfaq, *Faisalabad*
 Muhammad Adnan Bawany, *Hyderabad*
 Muhammad Idrees, *Lahore*
 Saeed Sadiq Hamid, *Karachi*
 Yasir Waheed, *Islamabad*



Poland

Thomas Brzozowski, *Cracow*
 Magdalena Chmiela, *Lodz*
 Krzysztof Jonderko, *Sosnowiec*
 Anna Kasicka-Jonderko, *Sosnowiec*
 Michal Kukla, *Katowice*
 Tomasz Hubert Mach, *Krakow*
 Agata Mulak, *Wroclaw*
 Danuta Owczarek, *Kraków*
 Piotr Socha, *Warsaw*
 Piotr Stalke, *Gdansk*
 Julian Teodor Swierczynski, *Gdansk*
 Anna M Zawilak-Pawlik, *Wroclaw*



Portugal

Marie Isabelle Cremers, *Setubal*
 Ceu Figueiredo, *Porto*
 Ana Isabel Lopes, *Lisbon*
 M Paula Macedo, *Lisboa*
 Ricardo Marcos, *Porto*
 Rui T Marinho, *Lisboa*
 Guida Portela-Gomes, *Estoril*

Filipa F Vale, *Lisbon*



Puerto Rico

Caroline B Appleyard, *Ponce*



Qatar

Abdulbari Bener, *Doha*



Romania

Mihai Ciocirlan, *Bucharest*

Dan Lucian Dumitrascu, *Cluj-Napoca*

Carmen Fierbinteanu-Braticevici, *Bucharest*

Romeo G Mihaila, *Sibiu*

Lucian Negreanu, *Bucharest*

Adrian Saftoiu, *Craiova*

Andrada Seicean, *Cluj-Napoca*

Ioan Sporea, *Timisoara*

Letitia Adela Maria Streba, *Craiova*

Anca Trifan, *Iasi*



Russia

Victor Pasechnikov, *Stavropol*

Vasiliy Ivanovich Reshetnyak, *Moscow*

Vitaly Skoropad, *Obninsk*



Saudi Arabia

Abdul-Wahed N Meshikhes, *Dammam*

M Ezzedien Rabie, *Khamis Mushait*



Singapore

Brian KP Goh, *Singapore*

Richie Soong, *Singapore*

Ker-Kan Tan, *Singapore*

Kok-Yang Tan, *Singapore*

Yee-Joo Tan, *Singapore*

Mark Wong, *Singapore*

Hong Ping Xia, *Singapore*



Slovenia

Matjaz Homan, *Ljubljana*

Martina Perse, *Ljubljana*



South Korea

Sang Hoon Ahn, *Seoul*

Seung Hyuk Baik, *Seoul*

Soon Koo Baik, *Wonju*

Soo-Cheon Chae, *Iksan*

Byung-Ho Choe, *Daegu*

Suck Chei Choi, *Iksan*

Hoon Jai Chun, *Seoul*

Yeun-Jun Chung, *Seoul*

Young-Hwa Chung, *Seoul*

Ki-Baik Hahm, *Seongnam*

Sang Young Han, *Busan*

Seok Joo Han, *Seoul*

Seung-Heon Hong, *Iksan*

Jin-Hyeok Hwang, *Seoungnam*

Jeong Won Jang, *Seoul*

Jin-Young Jang, *Seoul*

Dae-Won Jun, *Seoul*

Young Do Jung, *Kwangju*

Gyeong Hoon Kang, *Seoul*

Sung-Bum Kang, *Seoul*

Koo Jeong Kang, *Daegu*

Ki Mun Kang, *Jinju*

Chang Moo Kang, *Seodaemun-gu*

Gwang Ha Kim, *Busan*

Sang Soo Kim, *Goyang-si*

Jin Cheon Kim, *Seoul*

Tae Il Kim, *Seoul*

Jin Hong Kim, *Suwon*

Kyung Mo Kim, *Seoul*

Kyongmin Kim, *Suwon*

Hyung-Ho Kim, *Seongnam*

Seoung Hoon Kim, *Goyang*

Sang Il Kim, *Seoul*

Hyun-Soo Kim, *Wonju*

Jung Mogg Kim, *Seoul*

Dong Yi Kim, *Gwangju*

Kyun-Hwan Kim, *Seoul*

Jong-Han Kim, *Ansan*

Sang Wun Kim, *Seoul*

Ja-Lok Ku, *Seoul*

Kyu Taek Lee, *Seoul*

Hae-Wan Lee, *Chuncheon*

Inchul Lee, *Seoul*

Jung Eun Lee, *Seoul*

Sang Chul Lee, *Daejeon*

Song Woo Lee, *Ansan-si*

Hyuk-Joon Lee, *Seoul*

Seong-Wook Lee, *Yongin*

Kil Yeon Lee, *Seoul*

Jong-Inn Lee, *Seoul*

Kyung A Lee, *Seoul*

Jong-Baeck Lim, *Seoul*

Eun-Yi Moon, *Seoul*

SH Noh, *Seoul*

Seung Woon Paik, *Seoul*

Won Sang Park, *Seoul*

Sung-Joo Park, *Iksan*

Kyung Sik Park, *Daegu*

Se Hoon Park, *Seoul*

Yoonkyung Park, *Gwangju*

Seung-Wan Ryu, *Daegu*

Il Han Song, *Cheonan*

Myeong Jun Song, *Daejeon*

Yun Kyoung Yim, *Daejeon*

Dae-Yeul Yu, *Daejeon*



Spain

Mariam Aguas, *Valencia*

Raul J Andrade, *Málaga*

Antonio Arroyo, *Elche*

Josep M Bordas, *Barcelona*

Lisardo Boscá, *Madrid*

Ricardo Robles Campos, *Murcia*

Jordi Camps, *Reus*

Carlos Cervera, *Barcelona*

Alfonso Clemente, *Granada*

Pilar Codoner-Franch, *Valencia*

Fernando J Corrales, *Pamplona*

Fermin Sánchez de Medina, *Granada*

Alberto Herreros de Tejada, *Majadahonda*

Enrique de-Madaria, *Alicante*

JE Dominguez-Munoz, *Santiago de Compostela*

Vicente Felipo, *Valencia*

CM Fernandez-Rodriguez, *Madrid*

Carmen Frontela-Saseta, *Murcia*

Julio Galvez, *Granada*

Maria Teresa García, *Vigo*

MI Garcia-Fernandez, *Málaga*

Emilio Gonzalez-Reimers, *La Laguna*

Marcel Jimenez, *Bellaterra*

Angel Lanas, *Zaragoza*

Juan Ramón Larrubia, *Guadalajara*

Antonio Lopez-Sanroman, *Madrid*

Vicente Lorenzo-Zuniga, *Badalona*

Alfredo J Lucendo, *Tomelloso*

Vicenta Soledad Martinez-Zorzano, *Vigo*

José Manuel Martin-Villa, *Madrid*

Julio Mayol, *Madrid*

Manuel Morales-Ruiz, *Barcelona*

Alfredo Moreno-Egea, *Murcia*

Albert Pares, *Barcelona*

Maria Pellise, *Barcelona*

José Perea, *Madrid*

Miguel Angel Plaza, *Zaragoza*

María J Pozo, *Cáceres*

Enrique Quintero, *La Laguna*

Jose M Ramia, *Madrid*

Francisco Rodriguez-Frias, *Barcelona*

Silvia Ruiz-Gaspa, *Barcelona*

Xavier Serra-Aracil, *Barcelona*

Vincent Soriano, *Madrid*

Javier Suarez, *Pamplona*

Carlos Taxonera, *Madrid*

M Isabel Torres, *Jaén*

Manuel Vazquez-Carrera, *Barcelona*

Benito Velayos, *Valladolid*

Silvia Vidal, *Barcelona*



Sri Lanka

Arjuna Priyadarsin De Silva, *Colombo*



Sudan

Ishag Adam, *Khartoum*



Sweden

Roland G Andersson, *Lund*

Bergthor Björnsson, *Linköping*

Johan Christopher Bohr, *Örebro*

Mauro D'Amato, *Stockholm*

Thomas Franzen, *Norrköping*

Evangelos Kalaitzakis, *Lund*

Riadh Sadik, *Gothenburg*

Per Anders Sandstrom, *Linköping*

Ervin Toth, *Malmö*

Konstantinos Tsimogiannis, *Vasteras*

Apostolos V Tsolakis, *Uppsala*

**Switzerland**

Gieri Cathomas, *Liestal*
 Jean Louis Frossard, *Geneve*
 Christian Toso, *Geneva*
 Stephan Robert Vavricksa, *Zurich*
 Dominique Velin, *Lausanne*

**Thailand**

Thawatchai Akaraviputh, *Bangkok*
 P Yoysungnoen Chintana, *Pathumthani*
 Veerapol Kukongviriyapan, *Muang*
 Vijitra Leardkamolkarn, *Bangkok*
 Varut Lohsiriwat, *Bangkok*
 Somchai Pinlaor, *Khaon Kaen*
 D Wattanasirichaigoon, *Bangkok*

**Trinidad and Tobago**

B Shivananda Nayak, *Mount Hope*

**Tunisia**

Ibtissem Ghedira, *Sousse*
 Lilia Zouiten-Mekki, *Tunis*

**Turkey**

Inci Alican, *Istanbul*
 Mustafa Altindis, *Sakarya*
 Mutay Aslan, *Antalya*
 Oktar Asoglu, *Istanbul*
 Yasemin Hatice Balaban, *Istanbul*
 Metin Basaranoglu, *Ankara*
 Yusuf Bayraktar, *Ankara*
 Süleyman Bayram, *Adiyaman*
 Ahmet Bilici, *Istanbul*
 Ahmet Sedat Boyacioglu, *Ankara*
 Züleyha Akkan Cetinkaya, *Kocaeli*
 Cavit Col, *Bolu*
 Yasar Colak, *Istanbul*
 Cagatay Erden Daphan, *Kirikkale*
 Mehmet Demir, *Hatay*
 Ahmet Merih Dobrucali, *Istanbul*
 Gülsüm Ozlem Elpek, *Antalya*
 Ayse Basak Engin, *Ankara*
 Eren Ersoy, *Ankara*
 Osman Ersoy, *Ankara*
 Yusuf Ziya Erzin, *Istanbul*
 Mukaddes Esrefoglu, *Istanbul*
 Levent Filik, *Ankara*
 Ozgur Harmanaci, *Ankara*
 Koray Hekimoglu, *Ankara*
 Abdurrahman Kadayifci, *Gaziantep*
 Cem Kalayci, *Istanbul*
 Selin Kapan, *Istanbul*
 Huseyin Kayadibi, *Adana*
 Sabahattin Kaymakoglu, *Istanbul*
 Metin Kement, *Istanbul*
 Mevlut Kurt, *Bolu*
 Resat Ozaras, *Istanbul*
 Elvan Ozbek, *Adapazari*

Cengiz Ozcan, *Mersin*
 Hasan Ozen, *Ankara*
 Halil Ozguc, *Bursa*
 Mehmet Ozturk, *Izmir*
 Orhan V Ozkan, *Sakarya*
 Semra Paydas, *Adana*
 Ozlem Durmaz Suoglu, *Istanbul*
 Ilker Tasci, *Ankara*
 Müge Tecder-ünal, *Ankara*
 Mesut Tez, *Ankara*
 Serdar Topaloglu, *Trabzon*
 Murat Toruner, *Ankara*
 Gokhan Tumgor, *Adana*
 Oguz Uskudar, *Adana*
 Mehmet Yalniz, *Elazig*
 Mehmet Yaman, *Elazig*
 Veli Yazisiz, *Antalya*
 Yusuf Yilmaz, *Istanbul*
 Ozlem Yilmaz, *Izmir*
 Oya Yucel, *Istanbul*
 Ilhami Yuksel, *Ankara*

**United Kingdom**

Nadeem Ahmad Afzal, *Southampton*
 Navneet K Ahluwalia, *Stockport*
 Yeng S Ang, *Lancashire*
 Ramesh P Arasaradnam, *Coventry*
 Ian Leonard Phillip Beales, *Norwich*
 John Beynon, *Swansea*
 Barbara Braden, *Oxford*
 Simon Bramhall, *Birmingham*
 Geoffrey Burnstock, *London*
 Ian Chau, *Sutton*
 Thean Soon Chew, *London*
 Helen G Coleman, *Belfast*
 Anil Dhawan, *London*
 Sunil Dolwani, *Cardiff*
 Piers Gatenby, *London*
 Anil T George, *London*
 Pasquale Giordano, *London*
 Paul Henderson, *Edinburgh*
 Georgina Louise Hold, *Aberdeen*
 Stefan Hubscher, *Birmingham*
 Robin D Hughes, *London*
 Nusrat Husain, *Manchester*
 Matt W Johnson, *Luton*
 Konrad Koss, *Macclesfield*
 Anastasios Koulaouzidis, *Edinburgh*
 Simon Lal, *Salford*
 John S Leeds, *Aberdeen*
 JK K Limdi, *Manchester*
 Hongxiang Liu, *Cambridge*
 Michael Joseph McGarvey, *London*
 Michael Anthony Mendall, *London*
 Alexander H Mirnezami, *Southampton*
 J Bernadette Moore, *Guildford*
 Claudio Nicoletti, *Norwich*
 Savvas Papagrigoriadis, *London*
 Sylvia LF Pender, *Southampton*
 David Mark Pritchard, *Liverpool*
 James A Ross, *Edinburgh*
 Kamran Rostami, *Worcester*
 Xiong Z Ruan, *London*
 Frank I Tovey, *London*
 Dhiraj Tripathi, *Birmingham*

Vamsi R Velchuru, *Great Yarmouth*
 Nicholas T Ventham, *Edinburgh*
 Diego Vergani, *London*
 Jack Westwood Winter, *Glasgow*
 Terence Wong, *London*
 Ling Yang, *Oxford*

**United States**

Daniel E Abbott, *Cincinnati*
 Ghassan K Abou-Alfa, *New York*
 Julian Abrams, *New York*
 David William Adelson, *Los Angeles*
 Jonathan Steven Alexander, *Shreveport*
 Tauseef Ali, *Oklahoma City*
 Mohamed R Ali, *Sacramento*
 Rajagopal N Aravalli, *Minneapolis*
 Hassan Ashktorab, *Washington*
 Shashi Bala, *Worcester*
 Charles F Barish, *Raleigh*
 P Patrick Basu, *New York*
 Robert L Bell, *Berkeley Heights*
 David Bentrem, *Chicago*
 Henry J Binder, *New Haven*
 Joshua Bleier, *Philadelphia*
 Wojciech Blonski, *Johnson City*
 Kenneth Boorum, *Corvallis*
 Brian Boulay, *Chicago*
 Carla W Brady, *Durham*
 Kyle E Brown, *Iowa City*
 Adeel A Butt, *Pittsburgh*
 Weibiao Cao, *Providence*
 Andrea Castillo, *Cheney*
 Fernando J Castro, *Weston*
 Adam S Cheifetz, *Boston*
 Xiaoxin Luke Chen, *Durham*
 Ramsey Cheung, *Palo Alto*
 Parimal Chowdhury, *Little Rock*
 Edward John Ciccio, *New York*
 Dahn L Clemens, *Omaha*
 Yingzi Cong, *Galveston*
 Laura Iris Cosen-Binker, *Boston*
 Joseph John Cullen, *Iowa*
 Mark J Czaja, *Bronx*
 Mariana D Dabeva, *Bronx*
 Christopher James Damman, *Seattle*
 Isabelle G De Plaen, *Chicago*
 Punita Dhawan, *Nashville*
 Hui Dong, *La Jolla*
 Wael El-Rifai, *Nashville*
 Sukru H Emre, *New Haven*
 Paul Feuerstadt, *Hamden*
 Josef E Fischer, *Boston*
 Laurie N Fishman, *Boston*
 Joseph Che Forbi, *Atlanta*
 Temitope Foster, *Atlanta*
 Amy E Foxx-Orenstein, *Scottsdale*
 Daniel E Freedberg, *New York*
 Shai Friedland, *Palo Alto*
 Virgilio George, *Indianapolis*
 Ajay Goel, *Dallas*
 Oliver Grundmann, *Gainesville*
 Stefano Guandalini, *Chicago*
 Chakshu Gupta, *St. Joseph*
 Grigoriy E Gurvits, *New York*

Xiaonan Han, *Cincinnati*
 Mohamed Hassan, *Jackson*
 Martin Hauer-Jensen, *Little Rock*
 Koichi Hayano, *Boston*
 Yingli Hee, *Atlanta*
 Samuel B Ho, *San Diego*
 Jason Ken Hou, *Houston*
 Lifang Hou, *Chicago*
 K-Qin Hu, *Orange*
 Jamal A Ibdah, *Columbia*
 Robert Thomas Jensen, *Bethesda*
 Huanguang "Charlie" Jia, *Gainesville*
 Rome Jutabha, *Los Angeles*
 Andreas M Kaiser, *Los Angeles*
 Avinash Kambadakone, *Boston*
 David Edward Kaplan, *Philadelphia*
 Randeep Kashyap, *Rochester*
 Rashmi Kaul, *Tulsa*
 Ali Keshavarzian, *Chicago*
 Amir Maqbul Khan, *Marshall*
 Nabeel Hasan Khan, *New Orleans*
 Sahil Khanna, *Rochester*
 Kusum K Kharbanda, *Omaha*
 Hyun Sik Kim, *Pittsburgh*
 Joseph Kim, *Duarte*
 Jae S Kim, *Gainesville*
 Miran Kim, *Providence*
 Timothy R Koch, *Washington*
 Burton I Korelitz, *New York*
 Betsy Kren, *Minneapolis*
 Shiu-Ming Kuo, *Buffalo*
 Michelle Lai, *Boston*
 Andreas Larentzakis, *Boston*
 Edward Wolfgang Lee, *Los Angeles*
 Daniel A Leffler, *Boston*
 Michael Leitman, *New York*
 Suthat Liangpunsakul, *Indianapolis*
 Joseph K Lim, *New Haven*
 Elaine Y Lin, *Bronx*
 Henry C Lin, *Albuquerque*
 Rohit Loomba, *La Jolla*
 James David Luketich, *Pittsburgh*

Li Ma, *Stanford*
 Mohammad F Madhoun, *Oklahoma City*
 Thomas C Mahl, *Buffalo*
 Ashish Malhotra, *Bettendorf*
 Pranoti Mandrekar, *Worcester*
 John Marks, *Wynnewood*
 Wendy M Mars, *Pittsburgh*
 Julien Vahe Matricon, *San Antonio*
 Craig J McClain, *Louisville*
 Tamir Miloh, *Phoenix*
 Ayse Leyla Mindikoglu, *Baltimore*
 Huanbiao Mo, *Denton*
 Klaus Monkemuller, *Birmingham*
 John Morton, *Stanford*
 Adnan Muhammad, *Tampa*
 Michael J Nowicki, *Jackson*
 Patrick I Okolo, *Baltimore*
 Giusepp Orlando, *Winston Salem*
 Natalia A Osona, *Omaha*
 Virendra N Pandey, *Newark*
 Mansour A Parsi, *Cleveland*
 Michael F Picco, *Jacksonville*
 Daniel S Pratt, *Boston*
 Xiaofa Qin, *Newark*
 Janardan K Reddy, *Chicago*
 Victor E Reyes, *Galveston*
 Jon Marc Rhoads, *Houston*
 Giulia Roda, *New York*
 Jean-Francois Armand Rossignol, *Tampa*
 Paul A Rufo, *Boston*
 Madhusudana Girija Sanal, *New York*
 Miguel Saps, *Chicago*
 Sushil Sarna, *Galveston*
 Ann O Scheimann, *Baltimore*
 Bernd Schnabl, *La Jolla*
 Matthew J Schuchert, *Pittsburgh*
 Ekihiro Seki, *La Jolla*
 Chanjuan Shi, *Nashville*
 David Quan Shih, *Los Angeles*
 Shadab A Siddiqi, *Orlando*
 William B Silverman, *Iowa City*
 Shashideep Singhal, *New York*

Bronislaw L Slomiany, *Newark*
 Steven F Solga, *Bethlehem*
 Byoung-Joon Song, *Bethesda*
 Dario Sorrentino, *Roanoke*
 Scott R Steele, *Fort Lewis*
 Branko Stefanovic, *Tallahassee*
 Arun Swaminath, *New York*
 Kazuaki Takabe, *Richmond*
 Naoki Tanaka, *Bethesda*
 Hans Ludger Tillmann, *Durham*
 George Triadafilopoulos, *Stanford*
 John Richardson Thompson, *Nashville*
 Andrew Ukleja, *Weston*
 Miranda AL van Tilburg, *Chapel Hill*
 Gilberto Vaughan, *Atlanta*
 Vijayakumar Velu, *Atlanta*
 Gebhard Wagener, *New York*
 Kasper Saonun Wang, *Los Angeles*
 Xiangbing Wang, *New Brunswick*
 Daoyan Wei, *Houston*
 Theodore H Welling, *Ann Arbor*
 C Mel Wilcox, *Birmingham*
 Jacqueline Lee Wolf, *Boston*
 Laura Ann Woollett, *Cincinnati*
 Harry Hua-Xiang Xia, *East Hanover*
 Wen Xie, *Pittsburgh*
 Guang Yu Yang, *Chicago*
 Michele T Yip-Schneider, *Indianapolis*
 Sam Zakhari, *Bethesda*
 Kezhong Zhang, *Detroit*
 Huiping Zhou, *Richmond*
 Xiao-Jian Zhou, *Cambridge*
 Richard Zubarik, *Burlington*



Venezuela

Miguel Angel Chiurillo, *Barquisimeto*



Vietnam

Van Bang Nguyen, *Hanoi*

**DIAGNOSTIC ADVANCES**

- 8641** Potential of hybrid adaptive filtering in inflammatory lesion detection from capsule endoscopy images
Charisis VS, Hadjileontiadis LJ

REVIEW

- 8658** Endoscopic ultrasound-guided techniques for diagnosing pancreatic mass lesions: Can we do better?
Storm AC, Lee LS
- 8670** Update on the endoscopic treatments for achalasia
Uppal DS, Wang AY
- 8684** Human bocavirus: Current knowledge and future challenges
Guido M, Tumolo MR, Verri T, Romano A, Serio F, De Giorgi M, De Donno A, Bagordo F, Zizza A
- 8698** Improved glucose metabolism following bariatric surgery is associated with increased circulating bile acid concentrations and remodeling of the gut microbiome
Kaska L, Sledzinski T, Chomiczewska A, Dettlaff-Pokora A, Swierczynski J
- 8720** Update on occult hepatitis B virus infection
Makvandi M
- 8735** Metastasis-associated long noncoding RNAs in gastrointestinal cancer: Implications for novel biomarkers and therapeutic targets
Zhang FF, Luo YH, Wang H, Zhao L

MINIREVIEWS

- 8750** Preoperative therapy in locally advanced esophageal cancer
Garg PK, Sharma J, Jakhetiya A, Goel A, Gaur MK

ORIGINAL ARTICLE**Basic Study**

- 8760** Oral administration of a non-absorbable plant cell-expressed recombinant anti-TNF fusion protein induces immunomodulatory effects and alleviates nonalcoholic steatohepatitis
Ilan Y, Ben Ya'acov A, Shabbat Y, Gingis-Velitski S, Almon E, Shaaltiel Y
- 8770** Predicting malignant transformation of esophageal squamous cell lesions by combined biomarkers in an endoscopic screening program
Zhang H, Li H, Ma Q, Yang FY, Diao TY

Retrospective Study

- 8779** Macrophage colony-stimulating factor expressed in non-cancer tissues provides predictive powers for recurrence in hepatocellular carcinoma
Kono H, Fujii H, Furuya S, Hara M, Hirayama K, Akazawa Y, Nakata Y, Tsuchiya M, Hosomura N, Sun C
- 8790** Slow-pull and different conventional suction techniques in endoscopic ultrasound-guided fine-needle aspiration of pancreatic solid lesions using 22-gauge needles
Chen JY, Ding QY, Lv Y, Guo W, Zhi FC, Liu SD, Cheng TM
- 8798** Cyclooxygenase-2 expression is associated with initiation of hepatocellular carcinoma, while prostaglandin receptor-1 expression predicts survival
Yang HJ, Jiang JH, Yang YT, Yang XD, Guo Z, Qi YP, Zeng FH, Zhang KL, Chen NZ, Xiang BD, Li LQ

Observational Study

- 8806** Epidemiological study: Correlation between diet habits and constipation among elderly in Beijing region
Yang XJ, Zhang M, Zhu HM, Tang Z, Zhao DD, Li BY, Gabriel A
- 8812** Comparison of magnetic resonance spectroscopy, proton density fat fraction and histological analysis in the quantification of liver steatosis in children and adolescents
Di Martino M, Pacifico L, Bezzi M, Di Miscio R, Sacconi B, Chiesa C, Catalano C

Randomized Clinical Trial

- 8820** 22-gauge core vs 22-gauge aspiration needle for endoscopic ultrasound-guided sampling of abdominal masses
Sterlacci W, Sioulas AD, Veits L, Gönüllü P, Schachschal G, Groth S, Anders M, Kontos CK, Topalidis T, Hinsch A, Vieth M, Rösch T, Denzer UW

META-ANALYSIS

- 8831** Progression from low-grade dysplasia to malignancy in patients with Barrett's esophagus diagnosed by two or more pathologists
Moole H, Patel J, Ahmed Z, Duvvuri A, Vennelaganti S, Moole V, Dharmapuri S, Boddireddy R, Yedama P, Bondalapati N, Uppu A, Vennelaganti P, Puli S

CASE REPORT

- 8844** Cyclophosphamide-associated enteritis: A rare association with severe enteritis
Yang LS, Cameron K, Papaluca T, Basnayake C, Jackett L, McKelvie P, Goodman D, Demediuk B, Bell SJ, Thompson AJ
- 8849** Crowned dens syndrome developed after an endoscopic retrograde cholangiopancreatography procedure
Nakano H, Nakahara K, Michikawa Y, Suetani K, Morita R, Matsumoto N, Itoh F

Contents

World Journal of Gastroenterology
Volume 22 Number 39 October 21, 2016

ABOUT COVER

Editorial board member of *World Journal of Gastroenterology*, Il Han Song, MD, PhD, Professor, Division of Hepatology, Department of Internal Medicine, Dankook University College of Medicine, Dankook University Hospital, Cheonan 330-715, South Korea

AIMS AND SCOPE

World Journal of Gastroenterology (*World J Gastroenterol*, *WJG*, print ISSN 1007-9327, online ISSN 2219-2840, DOI: 10.3748) is a peer-reviewed open access journal. *WJG* was established on October 1, 1995. It is published weekly on the 7th, 14th, 21st, and 28th each month. The *WJG* Editorial Board consists of 1375 experts in gastroenterology and hepatology from 68 countries.

The primary task of *WJG* is to rapidly publish high-quality original articles, reviews, and commentaries in the fields of gastroenterology, hepatology, gastrointestinal endoscopy, gastrointestinal surgery, hepatobiliary surgery, gastrointestinal oncology, gastrointestinal radiation oncology, gastrointestinal imaging, gastrointestinal interventional therapy, gastrointestinal infectious diseases, gastrointestinal pharmacology, gastrointestinal pathophysiology, gastrointestinal pathology, evidence-based medicine in gastroenterology, pancreatology, gastrointestinal laboratory medicine, gastrointestinal molecular biology, gastrointestinal immunology, gastrointestinal microbiology, gastrointestinal genetics, gastrointestinal translational medicine, gastrointestinal diagnostics, and gastrointestinal therapeutics. *WJG* is dedicated to become an influential and prestigious journal in gastroenterology and hepatology, to promote the development of above disciplines, and to improve the diagnostic and therapeutic skill and expertise of clinicians.

INDEXING/ABSTRACTING

World Journal of Gastroenterology (*WJG*) is now indexed in Current Contents[®]/Clinical Medicine, Science Citation Index Expanded (also known as SciSearch[®]), Journal Citation Reports[®], Index Medicus, MEDLINE, PubMed, PubMed Central, Digital Object Identifier, and Directory of Open Access Journals. The 2015 edition of Journal Citation Reports[®] released by Thomson Reuters (ISI) cites the 2015 impact factor for *WJG* as 2.787 (5-year impact factor: 2.848), ranking *WJG* as 38 among 78 journals in gastroenterology and hepatology (quartile in category Q2).

FLYLEAF

I-IX Editorial Board

EDITORS FOR THIS ISSUE

Responsible Assistant Editor: *Xiang Li*
Responsible Electronic Editor: *Cai-Hong Wang*
Proofing Editor-in-Chief: *Lian-Sheng Ma*

Responsible Science Editor: *Yuan Qi*
Proofing Editorial Office Director: *Jin-Lei Wang*

NAME OF JOURNAL
World Journal of Gastroenterology

ISSN
ISSN 1007-9327 (print)
ISSN 2219-2840 (online)

LAUNCH DATE
October 1, 1995

FREQUENCY
Weekly

EDITORS-IN-CHIEF
Damian Garcia-Olmo, MD, PhD, Doctor, Professor, Surgeon, Department of Surgery, Universidad Autonoma de Madrid; Department of General Surgery, Fundacion Jimenez Diaz University Hospital, Madrid 28040, Spain

Stephen C Strom, PhD, Professor, Department of Laboratory Medicine, Division of Pathology, Karolinska Institutet, Stockholm 141-86, Sweden

Andrzej S Tarnawski, MD, PhD, DSc (Med), Professor of Medicine, Chief Gastroenterology, VA Long Beach Health Care System, University of California, Irvine, Irvine, CA, 92615, United States

fornia, Irvine, CA, 92615, United States

EDITORIAL BOARD MEMBERS
All editorial board members resources online at <http://www.wjgnet.com/1007-9327/editorialboard.htm>

EDITORIAL OFFICE
Jin-Lei Wang, Director
Yuan Qi, Vice Director
World Journal of Gastroenterology
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: +1-925-2238242
Fax: +1-925-2238243
E-mail: bpgoffice@wjgnet.com
Help Desk: <http://www.wjgnet.com/esps/helpdesk.aspx>

<http://www.wjgnet.com>

PUBLICATION DATE
October 21, 2016

COPYRIGHT
© 2016 Baishideng Publishing Group Inc. Articles published by this Open-Access journal are distributed under the terms of the Creative Commons Attribution Non-commercial License, which permits use, distribution, and reproduction in any medium, provided the original work is properly cited, the use is non commercial and is otherwise in compliance with the license.

SPECIAL STATEMENT
All articles published in journals owned by the Baishideng Publishing Group (BPG) represent the views and opinions of their authors, and not the views, opinions or policies of the BPG, except where otherwise explicitly indicated.

INSTRUCTIONS TO AUTHORS
Full instructions are available online at <http://www.wjgnet.com/bpg/gerinfo/204>

ONLINE SUBMISSION
<http://www.wjgnet.com/esps/>

Potential of hybrid adaptive filtering in inflammatory lesion detection from capsule endoscopy images

Vasileios S Charisis, Leontios J Hadjileontiadis

Vasileios S Charisis, Leontios J Hadjileontiadis, Department of Electrical and Computer Engineering, Aristotle University of Thessaloniki, GR54124 Thessaloniki, Greece

Leontios J Hadjileontiadis, Department of Electrical and Computer Engineering, Khalifa University, Abu Dhabi, PO Box 127788, United Arab Emirates

Author contributions: Charisis VS designed the texture feature extraction procedure, namely DLac, and wrote the manuscript; Hadjileontiadis LJ designed the hybrid adaptive filtering procedure and made revisions to the manuscript.

Conflict-of-interest statement: No potential conflicts of interest relevant to this article were reported.

Open-Access: This article is an open-access article which was selected by an in-house editor and fully peer-reviewed by external reviewers. It is distributed in accordance with the Creative Commons Attribution Non Commercial (CC BY-NC 4.0) license, which permits others to distribute, remix, adapt, build upon this work non-commercially, and license their derivative works on different terms, provided the original work is properly cited and the use is non-commercial. See: <http://creativecommons.org/licenses/by-nc/4.0/>

Manuscript source: Invited manuscript

Correspondence to: Leontios J Hadjileontiadis, PhD, Professor, Composer/Musicologist, Director of the Signal Processing and Biomedical Technology Unit (SPBTU), Department of Electrical and Computer Engineering, Aristotle University of Thessaloniki, University Campus, Build. D, 6th floor, GR54124 Thessaloniki, Greece. leontios@auth.gr
 Telephone: +30-231-0996340
 Fax: +30-231-0996312

Received: July 13, 2016
 Peer-review started: July 16, 2016
 First decision: August 19, 2016
 Revised: September 2, 2016
 Accepted: September 14, 2016
 Article in press: September 14, 2016
 Published online: October 21, 2016

Abstract

A new feature extraction technique for the detection of lesions created from mucosal inflammations in Crohn's disease, based on wireless capsule endoscopy (WCE) images processing is presented here. More specifically, a novel filtering process, namely Hybrid Adaptive Filtering (HAF), was developed for efficient extraction of lesion-related structural/textural characteristics from WCE images, by employing Genetic Algorithms to the Curvelet-based representation of images. Additionally, Differential Lacunarity (DLac) analysis was applied for feature extraction from the HAF-filtered images. The resulted scheme, namely HAF-DLac, incorporates support vector machines for robust lesion recognition performance. For the training and testing of HAF-DLac, an 800-image database was used, acquired from 13 patients who undertook WCE examinations, where the abnormal cases were grouped into mild and severe, according to the severity of the depicted lesion, for a more extensive evaluation of the performance. Experimental results, along with comparison with other related efforts, have shown that the HAF-DLac approach evidently outperforms them in the field of WCE image analysis for automated lesion detection, providing higher classification results, up to 93.8% (accuracy), 95.2% (sensitivity), 92.4% (specificity) and 92.6% (precision). The promising performance of HAF-DLac paves the way for a complete computer-aided diagnosis system that could support physicians' clinical practice.

Key words: Capsule endoscopy; Curvelet; Ulcer; Genetic algorithms; Crohn's disease

© **The Author(s) 2016.** Published by Baishideng Publishing Group Inc. All rights reserved.

Core tip: This paper presents a novel procedure to analyze wireless capsule endoscopy (WCE) images and extract features towards the automatic detection of Crohn's disease-based lesions. In this direction, a

hybrid adaptive filtering process is proposed that aims to refine the WCE images, prior to feature extraction, by selecting *via* a genetic algorithm approach the most informative curvelet-based components of the images. Then, differential lacunarity is employed for extracting color-texture features in YCbCr color space. The experimental results showed that the proposed WCE image analysis scheme is robust and outperforms related approaches of the literature, mainly in the case of mild lesions detection.

Charisis VS, Hadjileontiadis LJ. Potential of hybrid adaptive filtering in inflammatory lesion detection from capsule endoscopy images. *World J Gastroenterol* 2016; 22(39): 8641-8657 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v22/i39/8641.htm> DOI: <http://dx.doi.org/10.3748/wjg.v22.i39.8641>

INTRODUCTION

Wireless capsule endoscopy (WCE)^[1] is a novel medical procedure which has revolutionized gastrointestinal (GI) diagnostics by turning into reality the concept of painless and effective visual inspection of the entire length of small bowel (SB). In recent years, the validity of SB WCE in clinical practice has been systematically reviewed^[2]. Out of this evidence base, it clearly emerges that WCE is invaluable in evaluating various disorders, such as Crohn's disease (CD), and mucosal ulcers. CD is a chronic disorder of the GI tract (GT) that may affect the deepest layers of the intestinal walls. In 45% of cases, CD lesions are located in small intestine. One of the main characteristics of inflammatory bowel diseases, such as CD, is the evolution of extended internal inflammations to ulcers, or open sores, in the GT. CD is not lethal by itself, but serious complications are of high risk, rendering early diagnosis and treatment essential.

Despite the great advantages of WCE and the revolution that has brought, there are challenging issues to deal with. A WCE system produces more than 55000 images per examination that are reviewed in a form of a video, that requires more than one hour of intense labor for the expert, in order to be examined^[3]. This time consuming task is a burden, since the clinician has to stay focused and undistracted in front of a monitor for such a long period. Moreover, it is not guaranteed that all findings will be detected. It is not rare that abnormal findings are visible in only one or two frames and easily missed by the physician. Thus, automatic inspection and analysis of WCE images is of immediate need, in order to reduce the labor of the clinician and eliminate the possibility of omitting a lesion due to the clinician's non-concentration. Motivated by the latter, a number of automatic GI content interpretation research efforts have been proposed in the literature (see Section "Related

Work").

In this work, we introduce a novel WCE image analysis system for the recognition of lesions created by mucosal inflammation in CD. The main contributions of this paper are in: (1) extending the relatively limited research efforts on CD lesions and ulcers detection; (2) developing the novel Hybrid Adaptive Filtering (HAF) for efficiently isolating the lesion-related WCE image characteristics, by applying genetic algorithms (GA)^[4] to the representation of the WCE images on the Curvelet Transform^[5] domain; and (3) examining the performance of the proposed approach, namely, HAF-DLac, based on the severity of lesions. Additionally, this work extends the effectiveness of Differential Lacunarity (DLac)-based feature vector, presented in^[6] and further examines the potential of the YCbCr space for efficient lesion detection.

RELATED WORK

In the recent literature, the principal research interest (more than 75% of the WCE-related published works^[7]) towards the reduction of the examination time of WCE data deals with detection of certain disorders in the internal mucous membrane. The major types of pathologies targeted are polyps, bleeding, ulcerations, celiac disease, and CD. As far as inflammatory tissue (*i.e.*, ulcer and CD lesions) detection is concerned, only a small proportion of research efforts (7% for ulcers and 2% for CD^[7]) are targeted towards this direction, in spite of the great importance and wide-spreading of such disorders. Detecting such kind of eroded tissue is very challenging, since it is characterized by huge diversity in appearance. For ulcer detection, a feature vector that consists of curvelet-based rotation invariant uniform local binary patterns (riuLBP) classified by multilayer perceptron was proposed^[8]. Detection rates are heartening, but the performance is affected by the downsides of LBP. Although riuLBP perform well in illumination variations, they are based on the assumption that the local differences of the central pixel and its neighbors are independent of the central pixel itself, which is not always guaranteed, as the value of the central pixel may also be significant. Moreover, there is lack of between-scale texture information that is highly important for medical image analysis. In^[9], the authors present a segmentation scheme, utilizing log Gabor filters, color texture features, and support vector machines (SVM) classifier, based on Hue-Saturation-Value (HSV) space. Classification results are promising, but the dataset is rather limited (50 images) and includes perforated ulcerations that are quite easily detected due to clear appearance. Additionally, the HSV model suffers some shortcomings, as the RGB model^[10]. The authors in^[11] propose bag-of-words-based local texture features (LBP and scale-invariant feature transform-SIFT) extracted in RGB space and SVM classifier, whereas in^[12], a saliency map is used along with contour and LBP data.

Both approaches are affected by the weaknesses of LBP and SIFT features, which are of narrow use, since such features are often limited in relatively small regions of interest, are susceptible to noise, and exhibit insufficient sensitivity results. In the same direction, the works^[6,13,14] investigate the potential of Empirical Mode Decomposition-based structural features extracted from various color spaces, introduce texture features based on color rotation, and perform preliminary research on Curvelet-based lacunarity texture features. The proposed results are promising, but the dataset used for validation is rather small. To the best of our knowledge, the main research efforts reported in the literature dealing with the detection of CD lesions are^[15-17]. In^[15,16], Color Histogram statistics, MPEG-7 features along with a Haralick features and a Mean-Shift algorithm are used, whereas in^[17] a fusion of MPEG-7 descriptors and SVM classifiers are employed. Even though the classification performance is promising, MPEG-7 standards were not particularly designed to describe medical images; thus, there are several problems behind applying them in medical image analysis. They were developed for multimedia content description; hence, in case of images, they describe the overall content of the image, not allowing efficient characterization of local properties or arbitrary shaped regions of interest. Besides, they compute descriptors within relatively big rectangular regions that are inadequate for description of local medical image properties.

Aside from schemes developed to detect a single abnormality, there are efforts towards broad frameworks that detect multiple abnormalities, such as blood, erythema, polyps, ulcers and villous edema^[18-24]. Nevertheless, none of them deals with less straightforward lesions created by CD inflammations. It is unquestionable that detecting multiple abnormalities is important for an overall computer-assisted diagnosis tool, but it is crucial that all abnormalities are equally detected properly. This is extremely challenging and not achieved in any of the aforementioned techniques, where ulcer detection results are rather low. There is no one-size-fits-all approach, particularly for CD lesions and ulcerations that exhibit huge diversity in appearance, with attributes (color, texture, size) varying significantly over severity and position.

From a methodological validation point of view, a serious limitation of the preceding efforts is the employment of quite small databases (< 250 images instead of > 500^[7]), which are often unbalanced and not described in detail (severity, incorporation of confusing tissue). Moreover, the inclusion of multiple instances from the lesion taken in the very same region within the GT that exhibit high similarity is a possible source of overfitting and virtual optimistic results. Last but not least, none of the published approaches validates the performance against the severity of lesions, apart from^[17], where lesion severity classification takes place.

In the direction of reducing the reading time of WCE images, apart from the research efforts reported in the literature, there are some existing software solutions that have been implemented within the WCE image reviewing software by the manufacturing companies of the wireless capsules. The first software module designed towards reading time reduction was the Suspected Blood Indicator^[25]. This software module analyses WCE images with respect to color and selects the frames that contain a large number of red pixels, detecting, in this way, blood or other lesions characterized by the red color. The notion behind this software module is the same as the ones presented above (and the one proposed in this work), *i.e.*, automatic detection of a specific disorder. However, the performance is substandard in terms of sensitivity (40.9%) and specificity (70.7%)^[25] and, thus, cannot be reliably used in clinical practice. Another software tool aiming at reading time reduction is Automatic Mode^[26], that groups images with similar semantics based on color, shape and texture features and projects only one representative frame. No automatic detection of disorders, however, takes place. In this context, the physician saves time (up to 47%^[26]) by reviewing less images. Nevertheless, lesions that only appear in few images and small-sized lesions that do not cause significant shifts of the image features are often missed. Consequently, this tool is suggested to be used when diffuse or large lesions are expected to be found^[26]. Last but not least, two software tools targeting data enhancement have been incorporated in WCE images reading software, namely Blue Mode^[27] and Fuji Intelligent Colour Enhancement (FICE)^[27]. Blue Mode enhances the images by applying color shifting in the short wavelength range of visible light (around the wavelength of blue color). On the other hand, FICE, based on Spectral Estimation Technology^[28], analyses an image, estimates spectra at various wavelengths and produces an enhanced image of a given wavelength of light (most often to narrowed blue and green). Both techniques do not provide direct automatic lesion detection, but reduce WCE reading time in an indirect way. By improving image quality and intestinal surface structure representation, the doctors, theoretically, can more easily identify pathologic changes and, thus, review the whole sequence faster. Although such tools seem to improve the detection accuracy of lesions^[27], the significance of WCE data reading time reduction in clinical practice has not been studied yet.

PROPOSED HAD-DLAC APPROACH

Overview

The objective of HAF-DLac scheme is as follows. Given a region of interest (ROI) I within a WCE image, identify if I corresponds to normal tissue or CD lesion. The overall structure of HAF-DLac scheme, along with a working example, is depicted in Figure 1. After a pre-

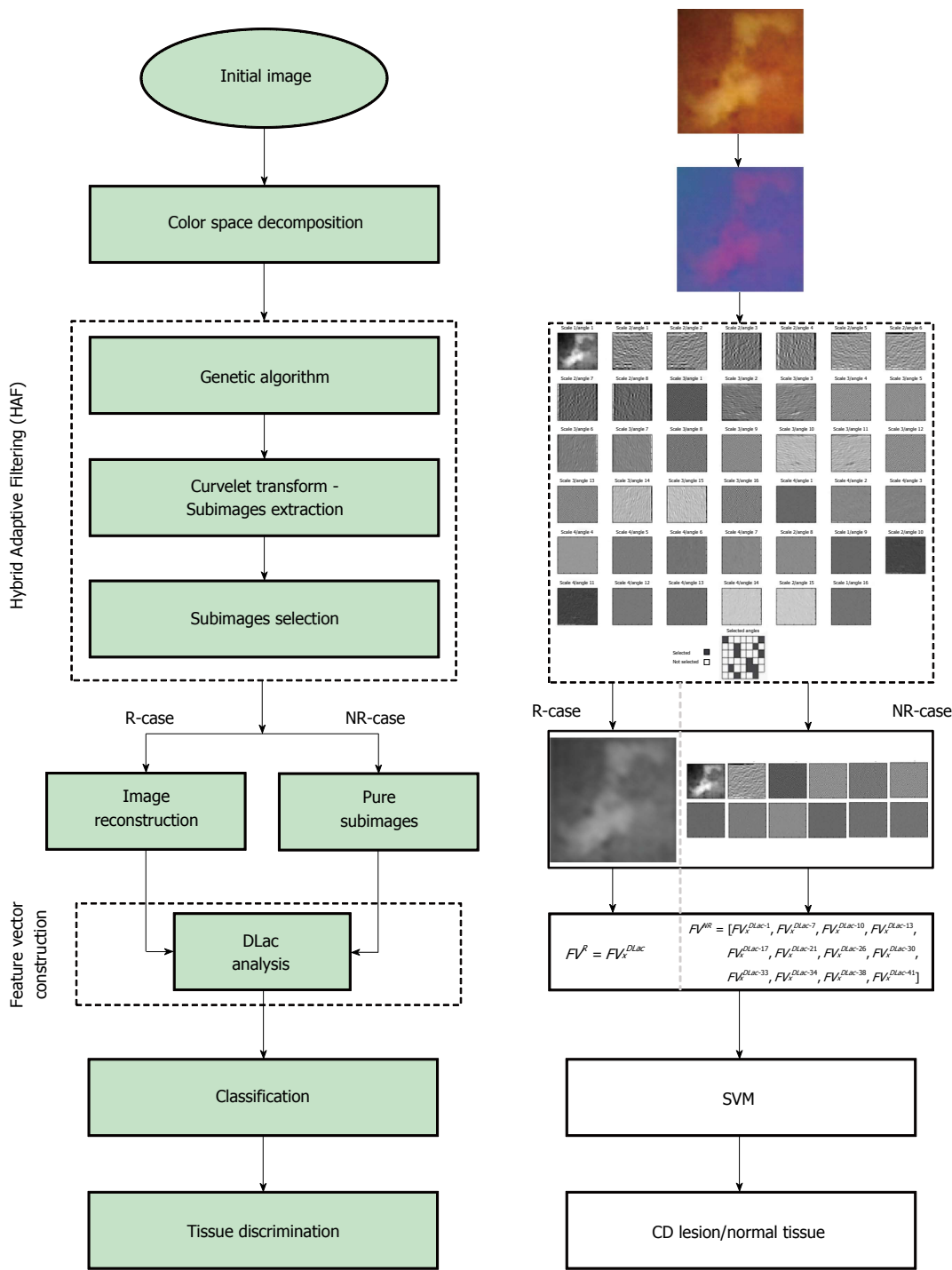


Figure 1 Proposed HAF-DLac scheme along with a working example.

processing stage, where the RGB image is converted to YCbCr space and the chromatic channels are extracted, the WCE image is inputted to HAF section of HAF-DLac scheme. YCbCr space was selected because it is a perceptually uniform color space that separates color from brightness information and overcomes the disadvantage of high correlation between the RGB channels^[29]. The role of HAF is to isolate the CD lesion-related WCE image characteristics, facilitating the task of feature vector extraction that follows. To achieve this, HAF incorporates GA that acts upon the

representation of WCE images on the Curvelet space. In the latter, the image is decomposed into a series of Curvelet-based sub-images of various scales and orientations. Then, GA is employed and, by using energy- or Lacunarity curve gradient-based fitness function, selects the optimum sub-images that relate the most with the CD lesion-related characteristics. The HAF output consists of the selected sub-images that could be either combined through a reconstruction process to produce a reconstructed image (R-case), or used directly with no reconstruction (NR-case).

Under both scenarios, the HAF output is used as input to the DLac section of the HAF-DLac scheme. There, DLac-based analysis is performed, resulting in efficient extraction of feature vector (FV^{DLac}), corresponding to the R- and NR-case (FV^R and FV^{NR} , respectively). The latter is forwarded to SVM-based classification.

Hybrid adaptive filtering

In order to follow the WCE image characteristics and focus upon the ones that mostly relate to the CD lesion information, a hybrid adaptive filtering (HAF) approach was developed. As declared by the term “hybrid”, HAF entails two processing tools, *i.e.*, CT and a simple GA optimization concept, so as to construct a filtering process adapted to specific characteristics of the filtered signal. CT is qualified as a filter bank due to its functionality to decompose an image into sub-images at various scales and orientations that can be interpreted as a pseudo-spectral-spatial representation^[30]. In order to exploit the aforementioned capability of CT, a new GA-based approach was introduced for the optimized selection of sub-images that correspond to specific features of an image. The concept of decomposing a WCE image in curvelet domain and selecting specific informative sub-images was implanted by a previous preliminary study^[6], where it was evidenced that sub-images at certain scales and angles exhibit high discrimination capabilities. One of the most important modules of the filtering procedure described above is the fitness function (FF) of the GA, as this is a pivotal criterion according to which the filtering is implemented. Energy-based fitness function (EFF) and Lacunarity curve gradient-based (LFF) FFs were employed in this approach.

EFF

The aim of using EFF was to conduct a filtering procedure by selecting the sub-images which embed the minority of the energy of the image. Based on the results of^[6], we observed that the sub-images with better performance exhibited lower mean energy compared to the others that achieved worse results. This may be explained by the fact that the sub-images with high mean energy contain abrupt and steep structures that do not convey valuable information about the texture of normal and eroded mucosa. On the contrary, the low energy sub-images are free from misleading content and are more likely to contain CD lesion-based information. This potential is evidenced in Figure 2A, where we can see the decomposition of an ulcer image (Y channel) at scale 3 and 8 angles. It is clear that sub-images at angles 1, 4, 5 and 8, which contain less mean energy than the rest (Figure 2B), are more likely to exhibit informative texture content, since they display informative, apparently, distribution of non-zero pixels and they do not contain sharp changes. On the contrary, the sub-images at angles 2, 3, 6 and 7 exhibit intense variations at their

borders, highlighted by the grater intensity range, that may conceal the delicate textural patterns and hinder efficient features extraction. The formula used for the EFF is:

$$f(S) = \sum_{\{S|S_r=1\}} E\{C_r\}^2 / \sum_{i=1}^M E\{C_i^2\}, \quad (1)$$

where S is the string of $1/0$ s, $S_r = 1$ is the set of the elements of S with value 1, C_i represents the sub-image at angle i .

LFF

Apart from the EFF, a second approach was attempted, by employing the gradient of the DLac-w curve estimation in the FF structure. As mentioned before, DLac is a measure of heterogeneity and a scale-dependent measure to characterize and discriminate textures and patterns^[31]. In this way, an image with uniform patterns delivers lower DLac values than an image with arbitrary and irregular patterns. The DLac analysis scale is determined by the size w of the gliding window (see Section “DLac-Based Feature Vector”). The DLac-w curve can be considered as a multi-scale description of structural patterns and its gradient can reveal the existence of specific textures and structures. For example, if an image contains microstructures with moderate differentiation for a variety of observation scales, the gradient of the DLac-w curve is expected to be lower than the one that corresponds to more abrupt and irregular structures that can be described rather diversely from various scale perspectives.

The aim of using LFF was to capture the variations in structural and textural characteristics of WCE images. Images with small DLac-w curve gradient may correspond to structures of normal and eroded mucosa, while DLac-w curves with steeper slope may account for distracting content. To this end, by considering the capability of DLac-w curve to monitor the existence of valuable or meaningless content, DLac-based filtering would act as a boosting procedure of the information of the initial WCE images related to the CD lesion structures in it. This concept is validated by the observations made, based on the results of^[6], where the sub-images that provided better performance exhibited divergent DLac-w curve gradient compared to those that granted worse results. In Figure 3, the boxplot of the local gradient of DLac-w curves vs the analysis scale is depicted for efficient (black) and non-efficient (gray) sub-images at the Curvelet domain, coming from 30 randomly selected WCE images depicting CD lesion or normal tissue. From Figure 3 it is clear that non-efficient sub-images tend to expose higher slope at smaller scales and lower slope at bigger scales. The gradient of DLac curve at scale i ($Gr(i)$) is calculated as the difference $\Delta(i+1) - \Delta(i-1)$. The formula used for the LFF is expressed by

$$f(S) = [Gr(4) + Gr(5)] / \sum_{i=1}^{13} Gr(i), \quad (2)$$

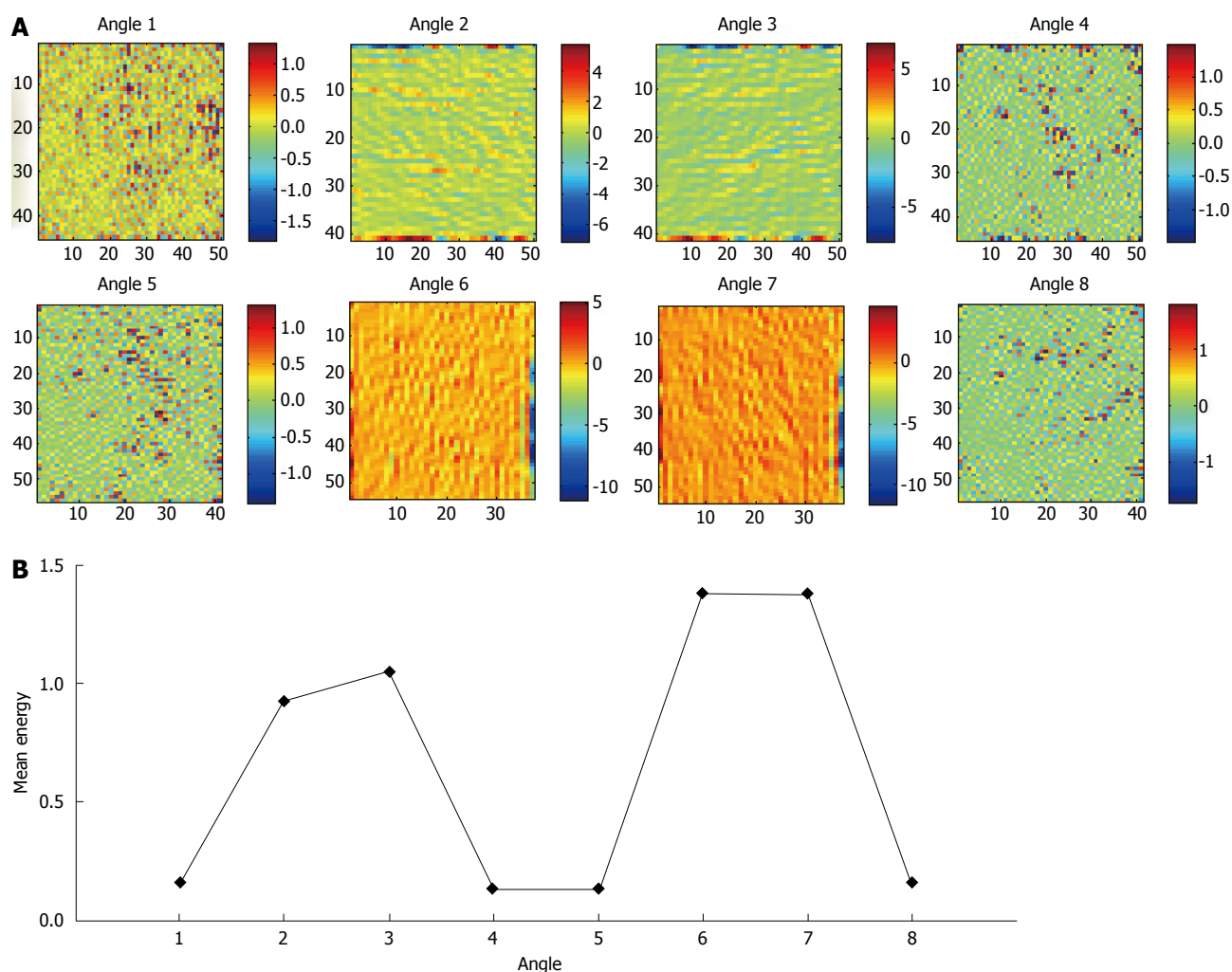


Figure 2 Curvelet-based decomposition of a wireless capsule endoscopy image. A: Decomposition of the Y channel of an ulcer image at scale three and eight angles; B: Mean energy of each angle depicted in (A).

since the gradient at the first two scales has to be high and the gradient of the rest scales has to be low (based on Figure 3).

DLac-based feature vector

The second part of the proposed HAF-DLac scheme is DLac analysis that aims to efficiently extract FV^{DLac} . As noted before, CD lesions exhibit widely diverse appearance; thus, a robust tool is required to be able to perform multi-scale, translation invariant texture analysis. DLac is such an attractive tool due to its simple calculation and precision that has been previously used successfully for WCE image analysis^[6,14]. The rationale for using DLac^[32] is its capability of revealing either sharp or slight changes in neighboring pixels (that characterize CD lesion texture), since it does not use thresholding, as does a very common feature extraction tool, namely riulBP, that conceals the magnitude of changes. The downsides of riulBP and other feature extraction approaches are presented in Section "Related Work". Moreover, DLac is tolerant to: (1) non-uniform illumination (very common in WCE images), due

to the differential calculation; and (2) rotational translation, since the pixel arrangement in the gliding box is irrelevant. In general, DLac surpasses the simple statistical (e.g., Haralick features, co-occurrence matrix, etc.) as well as the more advanced structural approaches (such as riulBP, textons, texture spectrum, etc.) of texture because it is based on, neither plain non-scale statistical analysis of the raw pixel intensities, nor predefined structural patterns. On the contrary, it relies on the statistical analysis of pseudo-patterns (box mass), defined by the data itself, at multiple scales while providing between-scale information. For the above reasons, DLac is expected to produce powerful features from the HAF-enhanced WCE images and achieve advanced classification results that is evidenced by the experimental results. In order to exploit the multi-scale analysis advantage of DLac, the value $\Lambda(w,r)$ [see (1)] is not calculated for a single set of parameters. In this work, we calculate Λ vs w , with r being a constant, despite the fact that initial approaches suggested the opposite^[32]. This technique^[33,34] is adopted because w is the primary feature that affects the scale of the analysis, since it

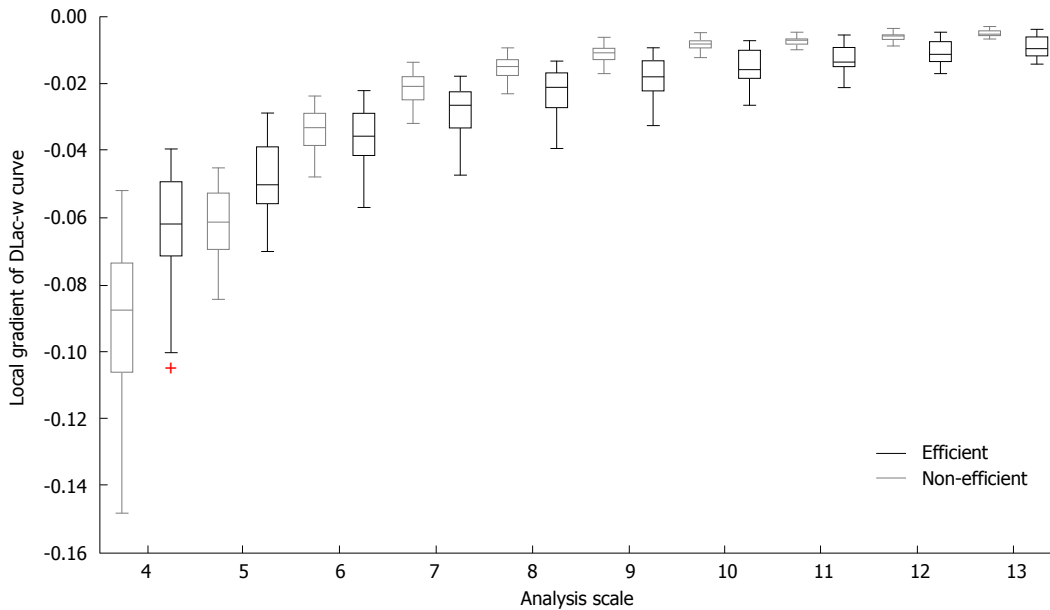


Figure 3 Boxplot of local gradient of DLac curves vs analysis scale (parameter w) for efficient (black) and non-efficient (gray) sub-images at curvelet space based on [6]. +: Extreme value.

determines the size of the image region on which the box mass will be calculated. According to [31,33], the larger the area on which the box mass is calculated, the coarser the scale of Lac analysis becomes. On the contrary, r value affects the scale of DLac analysis only to a certain degree, by determining the size of the neighborhood on which the differential height is calculated and, consequently, the sensitivity to recognize intensity variations. Thus, in our approach, we achieve to identify slight variations in neighboring pixels (by selecting a small value for r) and to analyze structure patterns at different scales. Moreover, $\Lambda(w)|_{w_{min}}$ curve is normalized ($\Lambda^N(w)$) to the value $\Lambda(w)$, in order to secure an identical reference level and extract more efficient information [35].

The decay of $\Lambda^N(w)$ as a function of window size follows characteristic patterns for random, self-similar, and structured spatial arrangements, and lacunarity functions can provide a framework for identifying such diversities. Thus, the $\Lambda^N(w)$ curve may form the FV. The concept of reducing the feature space dimension introduces the essence of modelling $\Lambda^N(w)$ with another function $L(w)$. The normalized DLac- w curves bear resemblance to hyperbola. On this ground, the function

$$L(w) = b/w^a + c, w = [w_{min}, w_{max}] \quad (3)$$

was chosen to model the $\Lambda^N(w)$ curves [35]. Parameter a portrays the convergence of $L(w)$, b represents the concavity of hyperbola and c is the translational term. The best interpretation of $\Lambda^N(w)$ by the model $L(w)$ is computed as the solution of a least squares problem, where parameters a , b , c are the independent variables [36]. Parameters a , b , c embody the global

behaviour of the $\Lambda^N(w)$ curve, i.e., the DLac-based texture features of a WCE image. Another way to reduce the feature space dimension established by the DLac curve is to use six statistical measures that are calculated on the $\Lambda^N(w)$ curve [8,37]. The six common statistical features extracted from $\Lambda^N(w)$ curve are: mean ($MN = E[X]$), standard deviation ($STD = (E[(X-\mu)/\sigma^2])^{1/2}$), entropy ($ENT = -\sum(p_i \cdot \log(p_i))$), energy ($ENG = E[X^2]$), skewness ($r_3 = E[(X-\mu)/\sigma^3]$, measure of the asymmetry of the probability distribution), and kurtosis ($r_4 = E[(X-\mu)/\sigma^4]$, descriptor of the shape of probability distribution), where p_i is the probability of value x_i , X is a random variable with mean value μ and standard deviation σ .

In order to draw more conclusive results about the efficiency of DLac-based FV, five different types of FVs are constructed:

$$FV_1^{DLac} = [\Lambda^N(w_{min} + 1), \dots, \Lambda^N(w_{min} + 5)], \quad (4)$$

$$FV_2^{DLac} = [a, b, c], \quad (5)$$

$$FV_3^{DLac} = [a, b, c, \Lambda^N(w_{min} + 1), \Lambda^N(w_{min} + 2), \Lambda^N(w_{min} + 3)], \quad (6)$$

$$FV_4^{DLac} = [MN, STD, ENT, ENG, r_3, r_4], \quad (7)$$

$$FV_5^{DLac} = [FV_3^{DLac}, FV_4^{DLac}]. \quad (8)$$

In FV_1^{DLac} , the entire DLac curve values are not used, in order to avoid the "curse of dimensionality" effect and because the length of the curve depends on the size of the input image/sub-image (for more details see Section "Parameter Setting, HAF Realization and FV Construction). FV_3^{DLac} aims to express the *global*,

i.e., both global (parameters a , b , c) and local (values $\Lambda^N(w)$), behavior of the curve. As a previous study^[6] has shown, this is quite an efficient approach to replace the lengthy DLac-w curve, without omitting crucial information. At last, FV_5^{DLac} constitutes an augmented version of FV_3^{DLac} , in terms of global DLac-w curve behavior representation.

EXPERIMENTAL AND IMPLEMENTATION ISSUES

Dataset

A fundamental part to develop a robust and efficient algorithm for WCE-based lesion detection, in general, is the existence of a sufficiently rich database, on which the algorithm is going to be tested. Unfortunately, the majority of related approaches (59%) are based on databases consisting of less than 500 images^[7]. Using a limited number of images, or even highly correlated images can doubtlessly lead to overfitting that may produce a virtual, unrealistic, fruitful performance.

The WCE image database used in this study contains 400 frames depicting CD-related lesions and 400 lesion-free frames acquired from 13 patients who undertook a WCE examination. The exams were rated twice by two clinicians. Then, we selected only the images that have been classified all four times into the same class. This procedure allowed to assess the inter-/intra-rater variability and acquire a highly confident dataset. Moreover, the physicians, upon mutual agreement, manually computed a ROI in each image. Some characteristic examples are given in Figure 4. The CD lesion images were manually annotated into mild (152 samples) and severe (248 samples) cases, based upon the size and severity of the lesion. The mild case includes lesions at an early stage with vague boundaries that are difficult to recognize (Figure 4 bottom), whereas the severe case contains lesions that are clearly shaped (Figure 4 middle). This discrimination was performed in order to extensively assess the performance of the proposed scheme on the basis of the lesion detection difficulty. Additionally, a "total" scenario that contains all lesion images is examined, so as to assess the performance from a spherical perspective. The 400 abnormal images were taken from 400 different lesion events for achieving the lowest possible similarity. The normal part of the dataset contains frames that depict both simple and confusing tissue (folds, villus, bubbles, intestinal juices/debris) for creating realistic conditions and avoiding virtual optimistic results.

In order to further validate the efficacy of the proposed scheme, two open WCE databases are engaged, namely CapsuleEndoscopy.org (CaEn)^[38] and KID^[39-41]. The CaEn database contains 6 normal and 22 CD-related lesion images (collected using the Pillcam SB from Given Imaging, Israel) while the KID database contains 60 normal (30 with confusing

intestinal content) and 14 CD-related lesion images (collected using the MiroCam system, IntroMedic Co, South Korea).

Parameter setting, HAF realization and FV construction

As far as the CT is concerned, two parameters have to be determined, *i.e.*, the number of analysis scales and the number of analysis angles at the second scale. It is prevalent in related applications to use three to four scales for the analysis^[8]. One of the main factors that determine the number of scales is the input data to be processed. As the number of scales increases, the size of the computed sub-images decreases, which may lead to negative effects. In our approach, after exhaustive trials we opted for four analysis scales. Each scale employs a certain number of angles that differ from scale to scale. It has been shown^[6] that, for avoiding data redundancy and complexity, the optimum number of angles at the second scale is 8.

Considering the implementation of DLac analysis, $\Lambda(w)$ is calculated for gliding box size $r = 3$ pixels and gliding window size $w = 4$ to w_{max} , where w_{max} is the minimum dimension of the input data. We did not choose a fixed value for w_{max} because the curvelet sub-images vary a lot in size, and we needed as longer DLac curves as possible, so as to acquire more efficient FVs.

Regarding the gliding box, its size has to be small in order to be capable to recognize slight local spatial variations that characterize lesion tissue. The value $r = 3$ pixels was selected after exhaustive experiments. As far as the gliding window is concerned, its size has to range from small to large values so as to capture both micro- and macro-structures and achieve multiscale information extraction. The minimum size of gliding window adopted here is the smallest feasible value, *i.e.*, $r + 1$, in order not to miss information from the tightest possible analysis scale.

In order to implement the curvelet sub-image selection *via* HAF, the 25% of the dataset was used. From the 800 images in total, we randomly selected 100 normal and 100 abnormal samples without considering the severity class they belong. For each generation of GA, the FF value was calculated accordingly to the whole dataset of the 200 images per chromatic channel. The selected sub-images per chromatic channel and FF method were found to be (scale/angle):

{[2/(5, 6, 8), 3/(4, 5, 8, 9, 12, 13), 4/(1, 4, 5, 10, 13)]|Y; [2/(2, 6), 3/(4, 5, 9, 12, 13, 16), 4/(4, 8, 13, 16)]|Cb; [2/(1, 5, 6), 3/(1, 4, 9, 13), 4/(1, 5, 9, 13, 16)]|Cr}|EFF, and

{[1/(1), 2/(6, 7, 8), 3/(8, 9, 12, 13), 4/(1, 2, 3, 7, 9, 13)]|Y; [1/(1), 2/(2, 6), 3/(9, 12), 4/(1, 4, 8, 9, 13, 16)]|Cb; [1/(1), 2/(6), 3/(1, 4, 8, 12), 4/(1, 5, 8, 9, 13, 16)]|Cr}|LFF.

The FV_x^{DLac} , ($x = [1,5]$), was calculated for each

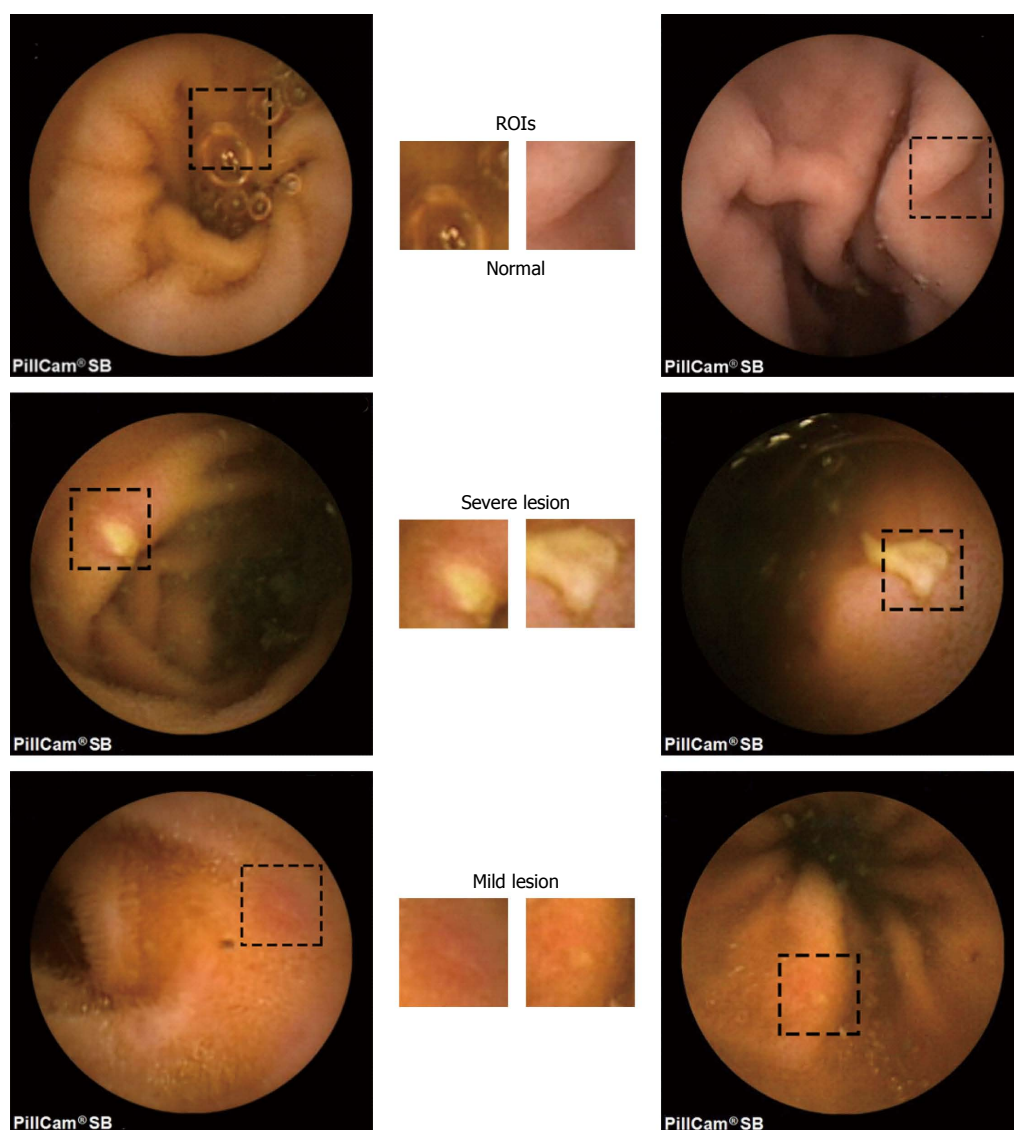


Figure 4 Six Wireless Capsule Endoscopy images of the adopted dataset and corresponding regions of interest: (from top to bottom) normal case, severe Crohn's disease lesion and mild Crohn's disease lesion.

individual chromatic channel, for the combination of all channels and for each feature extraction approach (R-/NR-case). For the combined channel scenario (Section "Hybrid Adaptive Filtering"), the NR-case was not taken into consideration, as it would lead to a lengthy FV and the classification procedure would suffer from the "curse of dimensionality" effect. For example, for the FV_5^{DLac} and EFF case the resulted FV would contain 456 features (12 features/sub-images \times 38 sub-images).

Classification setup

The classification phase of the HAF-DLac scheme is performed by a SVM classifier with radial basis kernel function^[42]. SVM have been used extensively in pattern recognition applications related to WCE image analysis^[6,9,11,17], showing superior performance. The data from the database that did not contribute to the sub-image selection, were used for the classification

procedure. In order to achieve as much generalization as possible, 3-fold cross validation was applied 100 times and the average accuracy (ACC), sensitivity (SENS), specificity (SPEC), and precision (PREC) values were estimated.

RESULTS

The performance of the proposed scheme is evaluated through the experimental results derived from the application of the CD lesion detection technique to the experimental dataset. To this end, results from every individual channel (Y, Cb, Cr) and the combination of them, under both HAF-DLac implementation scenarios (R/NR-case) and all severity cases (mild, severe, total) are presented.

Individual channel case

For the individual channel case, ACC values were

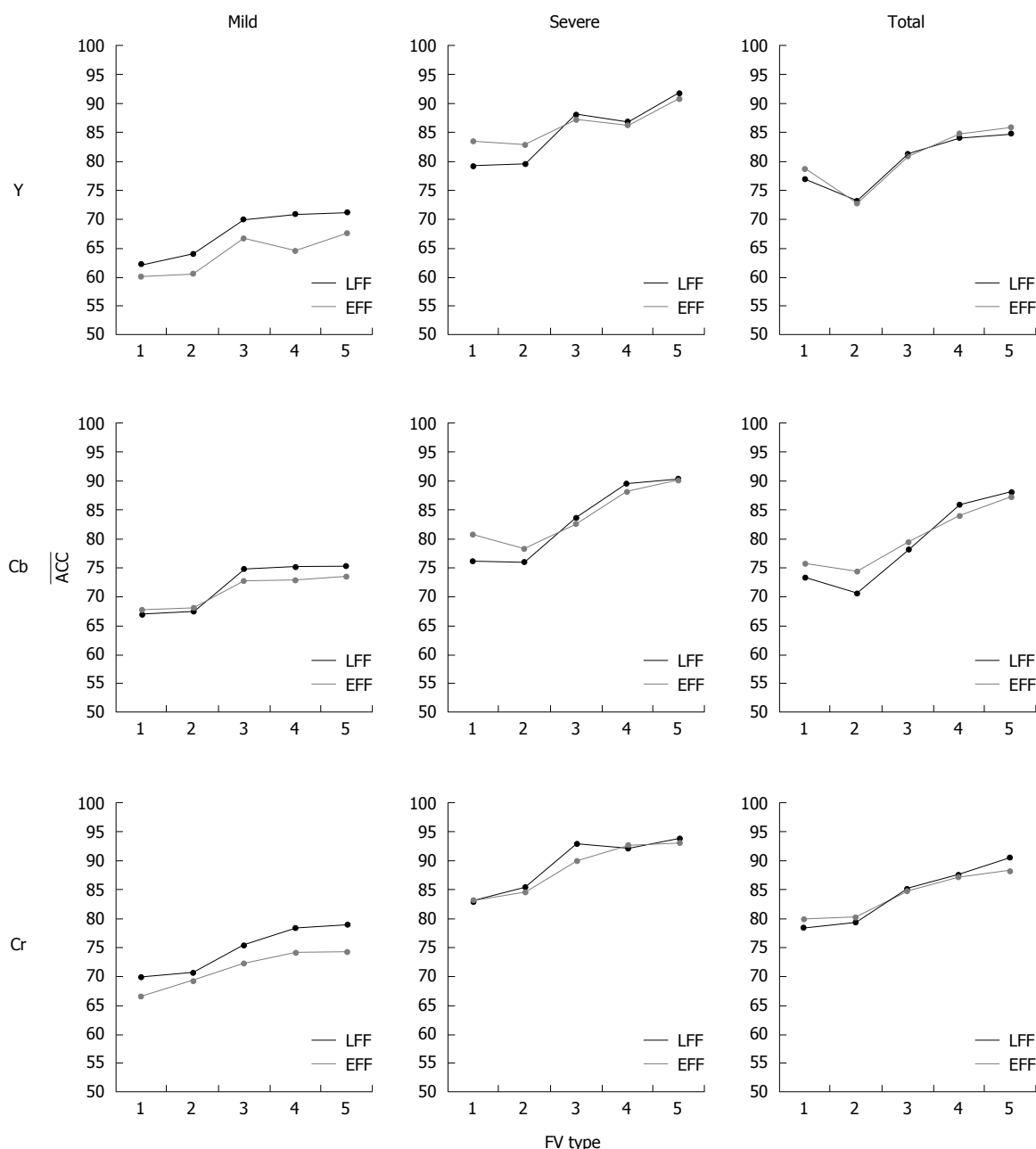


Figure 5 Classification ACC values using both fitness functions LFF (lacunarity curve gradient-based fitness function) and EFF (energy-based fitness function), for the R-individual channel case, all severity cases and all feature vector (FV) types [see (2) - (6)].

calculated for R and NR cases, for all severity scenarios and FVs.

Reconstruction case: For the R-case, the ACC values for all individual channels and all CD lesion cases are depicted in Figure 5 for the two FFs used and for the five types of FV. For the mild lesion case, it is clear that the augmented FV (FV_5^{DLac}) extracted from Cr channel provides with the best performance (78.8% ACC). Channel Cb achieves 3.7 percentage points (pp) lower ACC than Cr, whereas channel Y delivers the worst detection accuracy (71.2%) for the same FV. These results refer to the LFF case. On the contrary, the EFF scenario evidently exhibits deteriorated performance

for all channels. This is explained by the fact that LFF-based filtering, due to the intuitive characteristics of DLac, is able to discern and boost more efficiently the textural structures of mucosa that slightly differ in case of mild lesions. In case of severe lesions, the detection accuracy of the HAF-DLac is significantly higher, as expected, for all channels compared to mild lesion scenario. ACC is 91.5%, 90.3% and 93.8% for Y, Cb and Cr channels, respectively, for the LFF case and FV_5^{DLac} . Given the easier task of discriminating severe lesions, the EFF-based filtering, provides with results that slightly differ (-0.2 to -0.9 pp) from the LFF ones, as opposed to the mild lesion case, where the difference is -1.6 to -4.8 pp. Finally, at the total

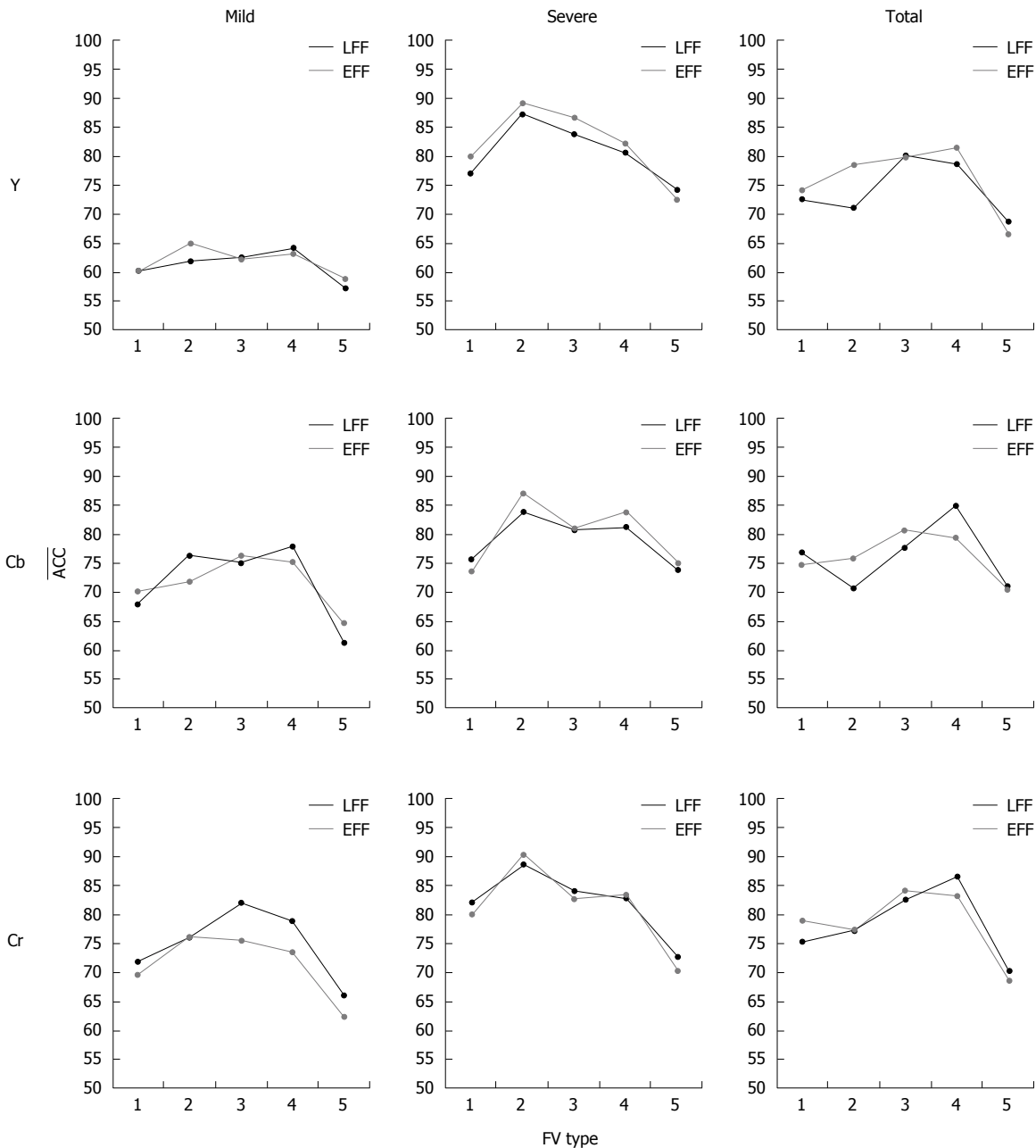


Figure 6 Classification ACC values using both fitness functions LFF (lacunarity curve gradient-based fitness function) and EFF (energy-based fitness function), for the NR-individual channel case, all severity cases and all feature vector (FV) types [see (2) - (6)].

scenario, Cr channel also provides with the best performance (90.5% ACC), followed by Cb (88.0% ACC) for FV_5^{DLac} and LFF. Y channel achieves 85.7% ACC or the same FV but for EFF case.

No-reconstruction case: The procedure followed in the R-case was also adopted in NR-case. More specifically, Figure 6 shows the ACC values for all individual channels. It is clear that, as in R-case, channel Cr and LFF approach exhibit the best performance regarding the value of ACC for the majority of cases. Considering mild lesions, the highest ACC values achieved are 64.8% for {EFF, FV_2^{DLac} , Y}, 77.8% for {LFF, FV_4^{DLac} , Cb} and 81.2% for {LFF, FV_3^{DLac} ,

Cr}. For severe lesions, the best ACC values are 89.1%, 87.0% and 90.2% for {EFF, FV_2^{DLac} , Y/Cb/Cr (respectively)}. Last but not least, for the total CD lesion case, the highest ACC value is 86.3% for Cr channel, followed by Cb channel with 84.8% ACC value for {LFF, FV_4^{DLac} }. The worst performance is delivered by Y channel, achieving 81.5% ACC for {EFF, FV_4^{DLac} }.

Combined channel case (R-case only): The evaluation of HAF-DLac for the combined channel data followed the same practice as in individual channel data. The ACC values for the combination of Y, Cb and Cr channels and all CD lesion scenarios are depicted in Figure 7 for the two FFs used [LFF (black line) and

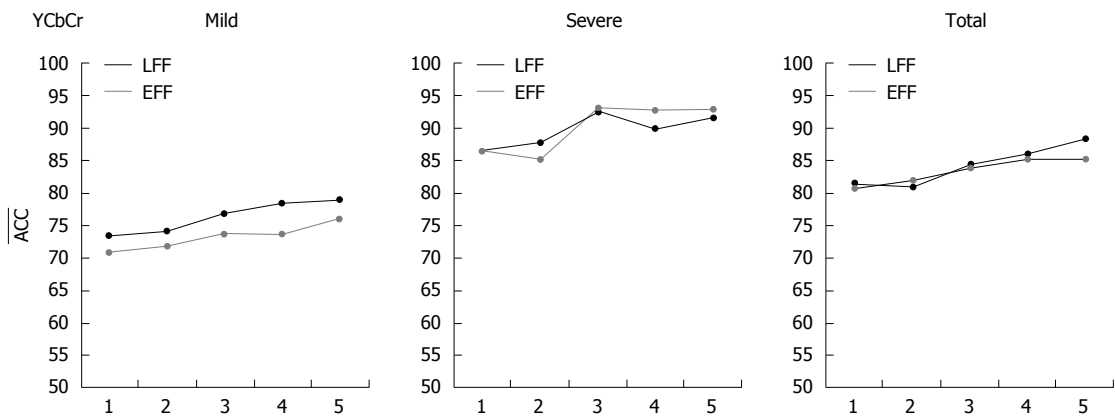


Figure 7 Classification ACC values using both fitness functions LFF (lacunarity curve gradient-based fitness function) and EFF (energy-based fitness function), for the R-combined channel case, all severity cases and all feature vector (FV) types [see (2) - (6)].

Table 1 Best ACC values for both individual and combined channel cases

Channel	Severity scenario		
	Mild	Severe	Total
Y	71.3% (R-LFF-FV ₅)	91.5% (R-LFF-FV ₅)	85.7% (R-EFF-FV ₅)
Cb	77.8% (NR-LFF-FV ₄)	90.3 % (R-LFF-FV ₅)	88.0% (R-LFF-FV ₅)
Cr	81.2% (NR-LFF-FV ₅)	93.8% (R-LFF-FV ₅)	90.5% (R-LFF-FV ₅)
YCbCr	79.0% (R-LFF-FV ₅)	93.2% (R-EFF-FV ₅)	88.3% (R-LFF-FV ₅)

The format (X-Y-Z) corresponds to (R/NR-case - FF - FV type). The best ACC value for each severity scenario is formatted in bold.

EFF (gray line)] and for the five types of FV. For mild lesions, the highest classification ACC value for LFF approach is 79% and for EFF approach is 75.9% for FV_5^{DLac} . In case of severe lesions, the ACC values are increased by 13.7 pp and 17.3 pp (*i.e.*, 92.7% and 93.2%) for LFF and EFF, respectively, for FV_3^{DLac} . At last, in the total case, LFF achieves 88.3% ACC value for FV_5^{DLac} , whereas EFF provides with 85.2% ACC value for the same FV.

Overall performance

Table 1 presents the best ACC values in the format of “percent (R/NR-case - FF - FV type)”, both for individual and combined channel cases and all three severity scenarios from a spherical perspective. The best mean results for each severity scenario are formatted in bold. The *SENS-SPEC* values for Cr-mild, Cr-severe and Cr-total are 76.6%-85.8%, 95.2%-92.4%, and 91.8%-89.2%, respectively. Moreover, for comparison purposes, the best classification results of the proposed scheme for all severity scenarios, and the classification results when using some of the most promising schemes in literature, proposed in^[6] (CurvLac)^[8], (CurvLBP), and^[17] (ECT), are presented in Table 2. In^[6], the authors engaged curvelet-based Lac features extracted from single or combined sub-images in the curvelet domain, whereas in^[8], curvelet-based LBP is applied for ulcer recognition, and in^[17], MPEG-7-based edge, color and texture features are used in order

to detect CD lesions. At last, Table 3 presents the classification results acquired from applying the above approaches to the open databases CaEn and KID.

Statistical analysis

To examine the robustness of the proposed HAF-DLac approach, sensitivity analysis with regard to image noise, the parameter *r* of DLac, and some GA parameters (initial population (IP), generations, $P_{0 \rightarrow 1}$, and $P_{1 \rightarrow 0}$) was performed. In particular, the sensitivity of the quantities *SENS* and *SPEC*, defined as $\delta(X) = (|X_{new} - X_{base}|/X_{base}) \times 100\%$, where *X* is *SENS* or *SPEC*, *Xbase* is the base value that is achieved with the current settings and *Xnew* is the new value acquired after changing one parameter of the system, was estimated. Given that the δ calculation with respect to each examined parameter requires full analysis, we performed it only for the total scenario. The *SENSbase* and *SPECbase* used in this study are the highest values achieved for the total scenario R-case LFF approach and FV_5^{DLac} , *i.e.*, 91.8% and 89.2%, respectively (see Section “Overall Performance”).

As far as the resiliency to noise is concerned, zero mean Gaussian noise was added to the images. The variance of the added noise ranged from 0.005 to 0.05 in increments of 0.001 up to 0.01 and 0.005 from 0.01 to 0.05. In Figure 8A, the *SENS* and *SPEC* values are depicted, whereas in Figure 8B the index δ for these metrics is shown. It is observed that the proposed system is rather robust to noise, as the sensitivities of *SENS* and *SPEC* are < 2% for noise variance 0.005 and do not exceed 6% and 2.5%, respectively, for noise variance up to 0.01. When more intense noise is added, the performance notably drops; however, even in such a case, 83% *SENS* and 74.4% *SPEC* for 0.02 variance noise are quite acceptable.

Another significant parameter of the proposed system is the size *r* of the gliding box of DLac analysis. Figure 8C presents the *SENS* and *SPEC* values when *r* ranges from 2 to 15 pixels, whereas Figure 8D depicts the corresponding sensitivity values of *SENS* and *SPEC*. The base values correspond to *r* = 3. It is

Table 2 Best *ACC/SENS/SPEC/PRES* for HAF-DLac vs other approaches^[6,8,17]

Methodology	Severity scenario		
	Mild	Severe	Total
HAF-DLac	81.2%/76.6%/85.8%/84.3%78.8/73.2/84.4/82.4 ¹	93.8%/95.2%/92.4%/92.6%	90.5%/91.8%/89.2%/89.5%
CurvLac	69.8%/64.3%/75.3%/72.2%	90.4%/92.5%/88.3%/88.8%	84.5%/87.1%/81.9%/82.8%
CurvLBP	73.4%/67.2%/79.6%/76.7%	89.6%/91.9%/87.3%/87.9%	81.7%/83.2%/80.2%/80.8%
ECT	71.8%/65.9%/77.7%/74.7%	91.2%/92.8%/89.6%/89.9%	85.6%/87.5%/83.7%/84.3%

¹These values correspond to R-case FV5^{DLac}.

Table 3 Classification results on CaEn^[38] and KID^[39-41] open wireless capsule endoscopy databases

Classification measures	CaEn database				KID database			
	HAF-DLac	CurvLac	CurvLBP	ECT	HAF-DLac	CurvLac	CurvLBP	ECT
ACC	89.3%	75.0%	75.0%	82.1%	85.1%	74.3%	78.4%	75.7%
SENS	90.9%	77.3%	77.3%	81.8%	85.7%	57.1%	71.4%	64.3%
SPEC	83.3%	66.7%	66.7%	83.3%	85.0%	78.3%	80.0%	78.3%
PREC	95.2%	89.5%	89.5%	94.7%	57.1%	38.1%	45.5%	40.9%

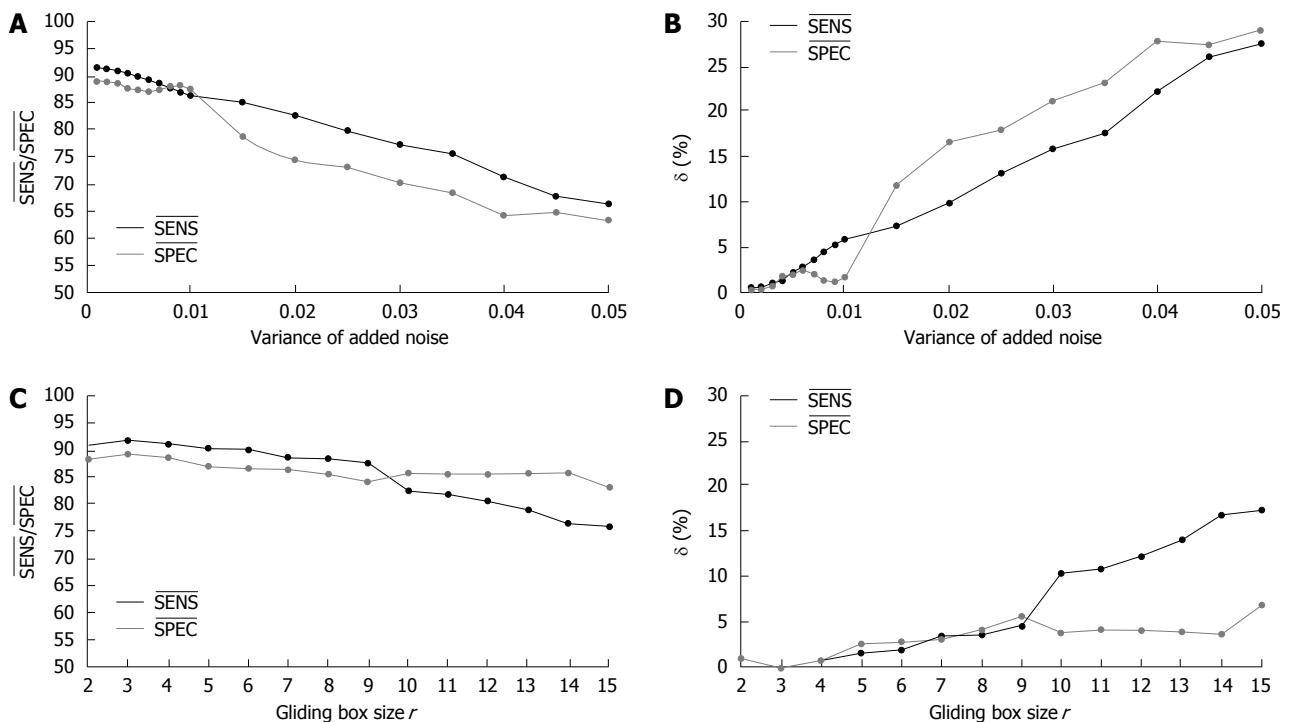


Figure 8 Robustness study for HAF-DLac scheme. A: *SENS*, *SPEC* values when zero-mean Gaussian noise of various variances is added; B: Sensitivity of *SENS*, *SPEC* for (A); C: *SENS*, *SPEC* values for various sizes of the gliding box r of DLac analysis; D: Sensitivity of *SENS*, *SPEC* for (C).

evident that the bigger the gliding window, the lower the performance. However, the HAF-DLac scheme exhibits remarkable robustness, since the sensitivities of *SENS* and *SPEC* are 4.5% and 5.5%, respectively, even for tripling the size of the gliding window. At the extreme case of $r = 15$, the *SENS* value is more than 75% and *SPEC* value is more than 83%, indicating an efficient performance.

Finally, Table 4 tabulates the results of the sensitivity calculations for +20% and -20% shift of four GA parameters. It is clear that the proposed approach is very robust with respect to all parameters, as the

sensitivity of *SENS* and *SPEC* is less than 1% for all tested cases.

Validation of DLac-based features

In order to validate the choice of DLac analysis for the extraction of texture features a comparative study took place of the classification performance of various widely used statistical features that include: nine histogram-based features (Hist), five gradient-based features (Grad), five feature based on the autoregressive model (AR), 11 features based on the co-occurrence matrix for four directions (44 features

Table 4 Robustness study for HAF-DLac with respect to genetic algorithms parameters

Parameter (%) change	IP		Generations		P ₀₋₁		P ₁₋₀	
	20	-20	20	-20	20	-20	20	-20
δ (SENS)	0.27%	0.64%	0.54%	0.68%	0.14%	0.08%	0.21%	0.17%
δ (SPEC)	0.41%	0.87%	0.81%	0.96%	0.17%	0.11%	0.11%	0.09%

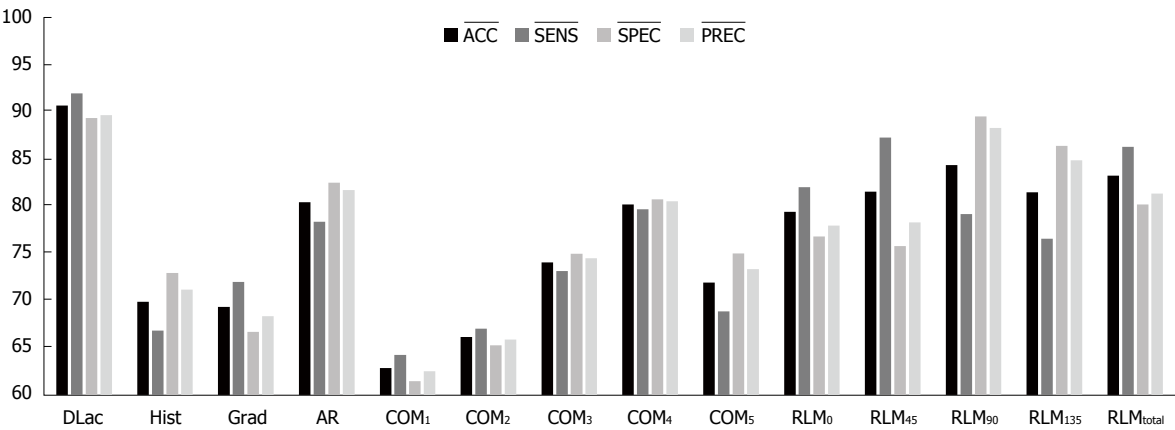


Figure 9 Classification results for various texture feature extraction techniques.

in total) for five inter-pixel distances (COM₁-COM₅) and five run-length-matrix-based features for four different directions (RLM₀, RLM₄₅, RLM₉₀ and RLM₁₃₅) and a combination of them (RLM_t)^[43]. These feature were calculated from the HAF-outputted images (R-case and LFF approach) in Cr channel (most efficient setup) and only the total scenario of data was considered. Figure 9 presents the classification results (*ACC*, *SENS*, *SPEC*, *PREC*) achieved by each of the latter FVs along with the DLac-based FV (FV_5^{DLac}).

DISCUSSION

In this paper we developed the HAF-DLac scheme for the detection of CD-based lesions. HAF-DLac was thoroughly tested on a rich dataset to ensure its good performance.

As the experimental results have shown at the individual channel R-case, engaging specific DLac values alone, *i.e.*, FV_1^{DLac} , is incapable to potently describe the characteristics of CD lesions. In the same way, the mere representation of the DLac curve with the hyperbola parameters does not provide fruitful effects. Moreover, in some cases (Y and Cb channels, severe and total scenario) the performance decreases, implying the loss of critical information. On the other hand, the utilization of FV_5^{DLac} that incorporates information in various formats from the entire DLac curve significantly improves the performance of the proposed scheme. FV_5^{DLac} provides the highest classification results, however, in many cases (mild-Y/Cb/Cr, severe-Cb/Cr, total-Y) FV_3^{DLac} and/or FV_4^{DLac} that are half in size compared to FV_5^{DLac} , achieve similar or slightly lower results (less than one pp lower

ACC value), while reducing the complexity. It is also evidenced that the Cr component is the most efficient for CD lesion detection, implying that the majority of CD lesion-related information is more thoroughly expressed by the amount of blue-greenish or fuchsia-reddish hues. This might be explained by the fact that the reflected reddish-greenish light is closely related to blood volume. Intestine walls are packed with a blood-vessels grid that is locally deformed by the mucosal erosion. On the contrary, the luminance plane Y, is the least competent for such an approach, specifically when the lesions are mild. Luminance varies significantly within GT, even for normal regions. Consequently, Y plane cannot adequately capture the modest lighting variations caused by eroded intestine walls.

As far as the NR-case is concerned, from an overall point of view and considering the FV types performance, it becomes apparent that the most powerful FVs are the second and the forth with an exception of the mild-Cr case, where FV_3^{DLac} is the most efficient. In contrast to the R-case, the lengthiest FV (FV_5^{DLac}) is unable to correctly classify abnormal regions, maybe owing to the over-length of FV resulting in the “curse of dimensionality” effect. Moreover, in most cases, FV_2^{DLac} performs better than FV_1^{DLac} , denoting that the synopsis of the DLac curve information in just three parameters, although it omits important information (as shown in R-case), tends to improve the detection potential, because multiple sub-images are simultaneously employed. We should also highlight the low results presented by Y channel during the mild lesion scenario, implying that combining information from multiple sub-images in the

low-potential luminance plane results in deteriorated performance. Finally, as far as the two selection approaches are concerned (EFF and LFF) in NR-case, it is apparent that there is no clear winner. The utilization of features extracted from multiple structural components of the WCE images (sub-images) permits to camouflage, to a certain extent, the shortcomings introduced by each approach.

Considering the combined channel case, the differences in terms of classification accuracy between the two FFs is marginal, with an exception of mild lesion detection. Similarly to the R-individual channel case, the FVs that include augmented information from the entire DLac-w curve (FV_3^{DLac} , FV_4^{DLac} and FV_5^{DLac}) achieve better performance, compared to the more simple FVs (FV_1^{DLac} and FV_2^{DLac}).

From an overall perspective, it is evident that Cr channel delivers the highest classification accuracy for all severity scenarios. The LFF approach and FV_5^{DLac} are also proven to provide better results for the majority of cases. However, this notion does not apply for the NR-case, where FV_3^{DLac} and FV_4^{DLac} are more efficient. It is also noteworthy that, although the highest ACC value (81.2%) for the Cr-mild case is achieved for NR-case and FV_3^{DLac} , the corresponding result for the R-case and FV_5^{DLac} (which are the most efficient settings for severe and total scenarios) is only 2.4 pp lower, implying a rather satisfactory behavior. Comparing the results between R- and NR-cases of individual channels we can conclude that NR-case results in decreased classification performance for the majority of cases. The utilization of features from individual curvelet-based sub-images results in lengthy overall FVs and the classification process suffers from the increased complexity. The detection of mild CD lesions in Cb and Cr channels is an exception, where NR yields better results, engaging, however, smaller FVs that have half the size of the most efficient FV at the R-case. The abundance of information caused by the individual sub-images leads to better discrimination of slightly eroded tissue. By considering the results of the combined channel case we observe that the use of information simultaneously captured from all planes leads to slightly diminished performance, compared to the behavior of the individual Cr channel, although it surpasses the performance of the other two individual channels. This might be explained by the inclusion of misleading data from Y channel and the tripling is size of the FVs.

Compared to other approaches, HAF-DLac scheme demonstrates superior performance, exhibiting 4.9, 4.3, 5.5 and 5.2 pp higher accuracy, sensitivity, specificity and precision, respectively, than the second most efficient method^[17], when considering the total case. Even more, significant is the progress (7.8, 9.4, 6.2 and 7.6 pp in terms of accuracy, sensitivity, specificity and precision, respectively, compared to^[8]) in successfully detecting mild erosions, an examination that none of the other approaches has

conducted. The detection rates (accuracy, sensitivity, specificity and precision) of severe, clearly defined lesions reach the considerable values of 93.8%, 95.2%, 92.4% and 92.6%, respectively, surpassing the other efforts^[6,8,17] by at least 2.6, 2.4, 2.8 and 2.7 pp, respectively. Moreover, the advanced behavior of the proposed scheme is evidenced by the classification results obtained by CaEn and KID database. The performance of HAF-DLac is rather solid in recognizing efficiently both kinds of tissue [20/22-5/6 (CaEn) and 12/14-51/60 (KID) lesion-normal images]. On the contrary, the other approaches exhibited inferior discrimination capability as they failed to identify, mainly, the lesion cases, and, specifically, from the KID database. The above results highlight that the HAF process combined with DLac-based features surpass two of the most promising methodologies in the literature that use LBP and MPEG-7 features.

Regarding the performance of the various feature extraction techniques, it becomes apparent (Figure 9) that the proposed FV is by far the most efficient, validating our hypothesis about the superiority of DLac analysis in extracting texture features compared to widely used statistical approaches.

Furthermore, it should be noted that the proposed scheme is quite efficient in terms of computational cost; that is < 0.5 s for a ROI of 140 × 140 pixels (average size) on a 4-core, 2.67 GHz desktop computer given the unoptimized Matlab implementation. It should be stressed that the time-consuming training phase is not included in the computational cost since it is performed only once during the development of the application. Focusing on even more efficient realizations, other programming languages (such as C++), and multithreading programming should be considered.

CONCLUSION

A new method, namely HAF-DLac, for CD inflammatory tissue detection using WCE images in YCbCr color space was presented. The proposed scheme combined HAF with DLac analysis to initially process the WCE data for enhancing the underlying lesion information, by incorporating Curvelet transform and GA-based techniques and, then, applying feature extraction analysis that resulted in five different DLac-based feature vectors with increased classification potential. The dataset (800 images with normal, mild and severe CD lesion cases) was subjected to HAF-DLac analysis for individual-channel and combined channel cases. Extensive classification tests were implemented concerning all severity scenarios showing the effectiveness of the proposed method. The comparison of the introduced HAF-DLac scheme with other relevant WCE-based lesion recognition methods evidenced its efficiency, consistency and robustness to more competent detection of CD lesions. The promising performance sets the ball rolling for an integrated

computer-aided diagnosis system on the service of gastroenterologists.

ACKNOWLEDGMENTS

The authors would like to thank the anonymous reviewers for the constructive comments that contributed to improving the final version of the paper.

REFERENCES

- 1 **Iddan G**, Meron G, Glukhovskiy A, Swain P. Wireless capsule endoscopy. *Nature* 2000; **405**: 417 [PMID: 10839527 DOI: 10.1038/35013140]
- 2 **Rondonotti E**, Soncini M, Girelli C, Ballardini G, Bianchi G, Brunati S, Centenara L, Cesari P, Cortelezzi C, Curioni S, Gozzini C, Gullotta R, Lazzaroni M, Maino M, Mandelli G, Mantovani N, Morandi E, Pansoni C, Piubello W, Putignano R, Schalling R, Tatarella M, Villa F, Vitagliano P, Russo A, Conte D, Masci E, de Franchis R. Small bowel capsule endoscopy in clinical practice: a multicenter 7-year survey. *Eur J Gastroenterol Hepatol* 2010; **22**: 1380-1386 [PMID: 20173646 DOI: 10.1097/MEG.0b013e3283352ced]
- 3 **Maieron A**, Hubner D, Blaha B, Deutsch C, Schickmair T, Ziachehabi A, Kerstan E, Knoflach P, Schoefl R. Multicenter retrospective evaluation of capsule endoscopy in clinical routine. *Endoscopy* 2004; **36**: 864-868 [PMID: 15452781 DOI: 10.1055/s-2004-825852]
- 4 **Goldberg DE**. Genetic algorithms in search, optimization and machine learning. Boston, MA: Addison-Wesley Longman Publishing, 1989: 1-432
- 5 **Candes J**, Demanet L, Donoho D, Ying L. Fast discrete curvelet transforms. *Multiscale Model Simul* 2006; **5**: 861-899 [DOI: 10.1137/05064182X]
- 6 **Eid A**, Charisis VS, Hadjileontiadis LJ, Sergiadis GD. A curvelet-based lacunarity approach for ulcer detection from wireless capsule endoscopy images. Proceedings of the 26th IEEE International Symposium on Computer-Based Medical Systems; 2013 Jun 20-22. Porto, Portugal: IEEE, 2013: 273-278 [DOI: 10.1109/CBMS.2013.6627801]
- 7 **Liedlgruber M**, Uhl A. Computer-aided decision support systems for endoscopy in the gastrointestinal tract: a review. *IEEE Rev Biomed Eng* 2011; **4**: 73-88 [PMID: 22273792 DOI: 10.1109/RBME.2011.2175445]
- 8 **Li B**, Meng M. Texture analysis for ulcer detection in capsule endoscopy images. *Image Vis Comput* 2009; **27**: 1336-1342 [DOI: 10.1016/J.IMAVIS.2008.12.003]
- 9 **Karargyris A**, Bourbakis N. Identification of ulcers in wireless capsule endoscopy videos. Proceedings of the 6th IEEE International Symposium on Biomedical Imaging: From Nano to Macro; 2009 Jun 28-Jul 01. Boston, USA: IEEE, 2009: 554-557 [DOI: 10.1109/isbi.2009.5193107]
- 10 **Gevers T**, Weijer J, Stokman H. Color feature detection. In: Lukac R, Plataniotis K. Color image processing: Methods and applications. Boca Raton: CRC Press, 2006: 203-226
- 11 **Yu L**, Yuen PC, Lai J. Ulcer detection in wireless capsule endoscopy images. Proceedings of the 21st IEEE International Conference on Pattern Recognition. 2012 Nov 11-15. Tsukuba, Japan: IEEE, 2012: 45-48
- 12 **Chen Y**, Lee J. Ulcer detection in wireless capsule endoscopy videos. Proceedings of the 20th ACM International Conference on Multimedia. 2012 Oct 29-Nov 2. Nara, Japan, New York: ACM, 2012: 1181-1184 [DOI: 10.1145/2393347.2396413]
- 13 **Charisis VS**, Katsimerou C, Hadjileontiadis LJ, Liatsos CN, Sergiadis GD. Computer-aided capsule endoscopy images evaluation based on color rotation and texture features: An educational tool to physicians. In: Rodrigues PP, Pechenizkiy M, Gama J, Correia RC, Liu J, Traina A, Lucas P, Soda P, editors. Proceedings of the 26th IEEE International Symposium On Computer-Based Medical Systems. 2013 Jun 20-22; Porto, Portugal. Red Hook: IEEE, 2013: 203-208 [DOI: 10.1109/CBMS.2013.6627789]
- 14 **Charisis VS**, Hadjileontiadis LJ, Liatsos CN, Mavrogiannis CC, Sergiadis GD. Capsule endoscopy image analysis using texture information from various colour models. *Comput Methods Programs Biomed* 2012; **107**: 61-74 [PMID: 22056811 DOI: 10.1016/J.CMPB.2011.10.004]
- 15 **Girgis H**, Mitchell B, Dassopoulos T, Mullin G, Hager G. An intelligent system to detect Crohn's disease inflammation in wireless capsule endoscopy videos. Proceedings of the 7th IEEE International Symposium on Biomedical Imaging: From Nano to Macro. 2010 Apr 14-17. Rotterdam, The Netherlands: IEEE, 2010: 1373-1376 [DOI: 10.1109/ISBI.2010.5490253]
- 16 **Jebarani W**, Daisy VJ. Assessment of Crohn's disease lesions in wireless capsule endoscopy images using SVM based classification. In: Proceedings of the 2013 IEEE International Conference on Signal Processing, Image Processing, and Pattern Recognition. 2013 Feb 7-8. Coimbatore, India: IEEE, 2013: 303-307 [DOI: 10.1109/ICSPR.2013.6497945]
- 17 **Kumar R**, Zhao Q, Seshamani S, Mullin G, Hager G, Dassopoulos T. Assessment of Crohn's disease lesions in wireless capsule endoscopy images. *IEEE Trans Biomed Eng* 2012; **59**: 355-362 [PMID: 22020661 DOI: 10.1109/TBME.2011.2172438]
- 18 **Coimbra M**, Cunha J. MPEG-7 visual descriptors-contributions for automated feature extraction in capsule endoscopy. *IEEE Trans Circuits Syst Video Technol* 2006; **16**: 628-637 [DOI: 10.1109/TCSVT.2006.873158]
- 19 **Li B**, Meng MQ. Computer-based detection of bleeding and ulcer in wireless capsule endoscopy images by chromaticity moments. *Comput Biol Med* 2009; **39**: 141-147 [PMID: 19147126 DOI: 10.1016/J.COMPBIOMED.2008.11.007]
- 20 **Hwang S**. Bag-of-visual-words approach to abnormal image detection in wireless capsule endoscopy videos. In: Bebis G, Richard B, Parvin B, Koracin D, Wang S, Kyungnam K, Benes B, Moreland K, Borst C, DiVerdi S, Yi-Jen C, Ming J, editors. Advances in visual computing. Lecture Notes in Computer Science 6939. Proceedings of the 7th International Symposium on Visual Computing, Part II; 2011 Sept 26-28. Las Vegas, NV, USA, New York: Springer, 2011: 320-327 [DOI: 10.1007/978-3-642-24031-7_32]
- 21 **Karargyris A**, Bourbakis N. Detection of small bowel polyps and ulcers in wireless capsule endoscopy videos. *IEEE Trans Biomed Eng* 2011; **58**: 2777-2786 [PMID: 21592915 DOI: 10.1109/TBME.2011.2155064]
- 22 **Nawarathna R**, Oh J, Muthukudage J, Tavanapong W, Wong J, de Groen PC, Tang SJ. Abnormal Image Detection in Endoscopy Videos Using a Filter Bank and Local Binary Patterns. *Neurocomputing* 2014; **144**: 70-91 [PMID: 25132723 DOI: 10.1016/J.NEUCOM.2014.02.064]
- 23 **Iakovidis DK**, Koulaouzidis A. Automatic lesion detection in capsule endoscopy based on color saliency: closer to an essential adjunct for reviewing software. *Gastrointest Endosc* 2014; **80**: 877-883 [PMID: 25088924 DOI: 10.1016/J.GIE.2014.06.026]
- 24 **Szczypiński P**, Klepaczko A, Pazurek M, Daniel P. Texture and color based image segmentation and pathology detection in capsule endoscopy videos. *Comput Methods Programs Biomed* 2014; **113**: 396-411 [PMID: 23164524 DOI: 10.1016/j.cmpb.2012.09.004]
- 25 **Signorelli C**, Villa F, Rondonotti E, Abbiati C, Beccari G, de Franchis R. Sensitivity and specificity of the suspected blood identification system in video capsule enteroscopy. *Endoscopy* 2005; **37**: 1170-1173 [PMID: 16329012 DOI: 10.1055/s-2005-870410]
- 26 **Kyriakos N**, Karagiannis S, Galanis P, Liatsos C, Zouboulis-Vafiadis I, Georgiou E, Mavrogiannis C. Evaluation of four time-saving methods of reading capsule endoscopy videos. *Eur J Gastroenterol Hepatol* 2012; **24**: 1276-1280 [PMID: 22825645 DOI: 10.1097/MEG.0b013e32835718d2]
- 27 **Krystallis C**, Koulaouzidis A, Douglas S, Plevris JN. Chromoendoscopy in small bowel capsule endoscopy: Blue mode or Fuji

- Intelligent Colour Enhancement? *Dig Liver Dis* 2011; **43**: 953-957 [PMID: 21893436 DOI: 10.1016/j.dld.2011.07.018]
- 28 **Haneishi H**, Hasegawa T, Hosoi A, Yokoyama Y, Tsumura N, Miyake Y. System design for accurately estimating the spectral reflectance of art paintings. *Appl Opt* 2000; **39**: 6621-6632 [PMID: 18354676 DOI: 10.1364/AO.39.006621]
 - 29 **Tkalcic M**, Tasic JF. Colour spaces: perceptual historical and applicational background. In: Zajc B, Tkalcic M, editors. Proceedings of the IEEE Region 8 EUROCON 2003; 2003 Jul 1-4. Ljubljana, Slovenia: IEEE, 2003: 304-308 [DOI: 10.1109/EURCON.2003.1248032]
 - 30 **Starck JL**, Candès EJ, Donoho DL. The curvelet transform for image denoising. *IEEE Trans Image Process* 2002; **11**: 670-684 [PMID: 18244665 DOI: 10.1109/TIP.2002.1014998]
 - 31 **Plotnick RE**, Gardner RH, Hargrove WW, Prestegard K, Perlmutter M. Lacunarity analysis: A general technique for the analysis of spatial patterns. *Phys Rev E Stat Phys Plasmas Fluids Relat Interdiscip Topics* 1996; **53**: 5461-5468 [PMID: 9964879 DOI: 10.1103/PhysRevE.53.5461]
 - 32 **Dong P**. Test of a new lacunarity estimation method for image texture analysis. *Int J Remote Sens* 2000; **21**: 3369-3373 [DOI: 10.1080/014311600750019985]
 - 33 **Plotnick RE**, Gardner RH, O'Neill RV. Lacunarity indices as measures of landscape texture. *Landscape Ecol* 1993; **8**: 201-211 [DOI: 10.1007/BF00125351]
 - 34 **Zaia A**, Eleonori R, Maponi P, Rossi R, Murri R. MR imaging and osteoporosis: fractal lacunarity analysis of trabecular bone. *IEEE Trans Inf Technol Biomed* 2006; **10**: 484-489 [PMID: 16871715 DOI: 10.1109/TITB.2006.872078]
 - 35 **Hadjileontiadis LJ**. A texture-based classification of crackles and squawks using lacunarity. *IEEE Trans Biomed Eng* 2009; **56**: 718-732 [PMID: 19174342 DOI: 10.1109/TBME.2008.2011747]
 - 36 **Marquardt D**. An algorithm for least squares estimation of nonlinear parameters. *J Soc Indust Appl Math* 1963; **11**: 431-441 [DOI: 10.1137/111030]
 - 37 **Haralick RM**. Statistical and structural approaches to texture. *Proc IEEE* 1979; **67**: 786-804 [DOI: 10.1109/PROC.1979.11328]
 - 38 **Given Imaging**. Capsule endoscopy. 2014. Available from: URL: <http://www.capsuleendoscopy.org>
 - 39 **Iakovidis DK**, Koulaouzidis A. Automatic lesion detection in wireless capsule endoscopy - A simple solution for a complex problem. Proceedings of the 2014 IEEE International Conference on Image Processing; 2014 Oct 27-30. Paris, France: IEEE, 2014: 2236-2240
 - 40 **Iakovidis DK**, Koulaouzidis A. Software for enhanced video capsule endoscopy: challenges for essential progress. *Nat Rev Gastroenterol Hepatol* 2015; **12**: 172-186 [PMID: 25688052 DOI: 10.1038/NRGASTRO.2015.13]
 - 41 **Koulaouzidis A**, Iakovidis DK. KID: Koulaouzidis-Iakovidis Database for Capsule Endoscopy. Available from: URL: <http://is-innovation.eu/kid>
 - 42 **Cristianini N**, Shawe-Taylor J. An introduction to support vector machines and other kernel-based learning methods. Cambridge: Cambridge University Press, 2000: 93-124
 - 43 **Szczypiński PM**, Strzelecki M, Materka A, Klepaczko A. MaZda - a software package for image texture analysis. *Comput Methods Programs Biomed* 2009; **94**: 66-76 [PMID: 18922598 DOI: 10.1016/J.CMPB.2008.08.005]

P- Reviewer: Hosoe N, Sakin YS, Lakatos L **S- Editor:** Qi Y
L- Editor: A **E- Editor:** Wang CH



Endoscopic ultrasound-guided techniques for diagnosing pancreatic mass lesions: Can we do better?

Andrew C Storm, Linda S Lee

Andrew C Storm, Linda S Lee, Division of Gastroenterology, Hepatology and Endoscopy, Brigham and Women's Hospital, Harvard Medical School, Boston, MA 02115, United States

Author contributions: All authors contributed to the manuscript.

Conflict-of-interest statement: Both authors have no conflicts of interest to disclose including no pharmaceutical or industry support.

Open-Access: This article is an open-access article which was selected by an in-house editor and fully peer-reviewed by external reviewers. It is distributed in accordance with the Creative Commons Attribution Non Commercial (CC BY-NC 4.0) license, which permits others to distribute, remix, adapt, build upon this work non-commercially, and license their derivative works on different terms, provided the original work is properly cited and the use is non-commercial. See: <http://creativecommons.org/licenses/by-nc/4.0/>

Manuscript source: Invited manuscript

Correspondence to: Linda S Lee, Assistant Professor, Division of Gastroenterology, Hepatology and Endoscopy, Brigham and Women's Hospital, Harvard Medical School, 75 Francis St., Boston, MA 02115, United States. lslee@partners.org
Telephone: +1-617-2780359
Fax: +1-617-2645132

Received: July 1, 2016
Peer-review started: July 4, 2016
First decision: August 8, 2016
Revised: August 24, 2016
Accepted: September 14, 2016
Article in press: September 14, 2016
Published online: October 21, 2016

Abstract

The diagnostic approach to a possible pancreatic mass

lesion relies first upon various non-invasive imaging modalities, including computed tomography, ultrasound, and magnetic resonance imaging techniques. Once a suspect lesion has been identified, tissue acquisition for characterization of the lesion is often paramount in developing an individualized therapeutic approach. Given the high prevalence and mortality associated with pancreatic cancer, an ideal approach to diagnosing pancreatic mass lesions would be safe, highly sensitive, and reproducible across various practice settings. Tools, in addition to radiologic imaging, currently employed in the initial evaluation of a patient with a pancreatic mass lesion include serum tumor markers, endoscopic retrograde cholangiopancreatography, and endoscopic ultrasound-guided fine needle aspiration (EUS-FNA). EUS-FNA has grown to become the gold standard in tissue diagnosis of pancreatic lesions.

Key words: Endoscopic ultrasound; Fine needle aspiration; Pancreatic cancer; Pancreatic mass; Endoscopy

© The Author(s) 2016. Published by Baishideng Publishing Group Inc. All rights reserved.

Core tip: Evidence-based techniques to increase the diagnostic yield during endoscopic ultrasound-guided fine needle aspiration (FNA) of pancreatic masses include: (1) use of general anesthesia; (2) use smaller (22 or 25G) needles for transduodenal FNA; (3) use If histology is desired, use 19G or core biopsy needles; (4) use suction; (5) use the "fanning technique"; and (6) use on-site cytopathologist or perform 7 needle passes.

Storm AC, Lee LS. Endoscopic ultrasound-guided techniques for diagnosing pancreatic mass lesions: Can we do better? *World J Gastroenterol* 2016; 22(39): 8658-8669 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v22/i39/8658.htm> DOI: <http://dx.doi.org/10.3748/wjg.v22.i39.8658>

INTRODUCTION

Peripheral blood tumor markers, among the least invasive diagnostic tests, are not yet useful in the initial evaluation of a patient with a pancreatic mass. Cancer antigen (CA) 19-9, the leading tumor marker used to monitor pancreatic adenocarcinoma, has sensitivity and specificity as low as 70% and 68% respectively in diagnosing pancreatic adenocarcinoma, which has led to recommendations that it not be used routinely for diagnosis of this condition^[1,2]. The CA 19-9 marker is, however, useful for post-surgical cancer surveillance^[3]. Because of this, a CA 19-9 level may be checked prior to any surgical intervention with curative intent and serum concentrations of the marker followed thereafter to detect disease recurrence.

Prior to the introduction of the EUS-FNA technique in the early 1990's, pancreatic masses were diagnosed using ERCP and percutaneous biopsy techniques (Figure 1). ERCP is limited by a sensitivity of 49%-66% with pancreatic duct brushing, and a reported complication rate of pancreatitis up to 6%^[4,5]. Use of CT or ultrasound guided biopsy carries a sensitivity of 62%-90% and specificity up to 100% with a randomized study demonstrating higher sensitivity (84%) for EUS-FNA compared to CT or ultrasound-guided biopsy (62%)^[6-10]. In addition, the risk of tumor seeding into the peritoneum or along the percutaneous needle tract has led to avoidance of the percutaneous approach to tissue diagnosis, and studies have suggested a significantly lower risk of peritoneal carcinomatosis using EUS-FNA^[11].

STANDARD OF CARE: EUS-FNA

EUS-FNA is a safe, effective and efficient diagnostic tool in the evaluation of pancreatic mass lesions (Figure 2). Cytopathological specimens, and more recently core biopsies, may be obtained with high sensitivity (75%-98%), specificity (71%-100%), positive predictive value (96%-100%), negative predictive value (33%-85%) and accuracy (79%-98%) in the diagnosis of pancreatic cancer as compared to other modalities (Table 1)^[12-16]. The one caveat to the high diagnostic yield of EUS-FNA is in the presence of chronic pancreatitis where sensitivity decreases to 74% compared to 91% with normal surrounding pancreatic parenchyma^[17]. Studies have shown that repeating EUS-FNA does improve diagnostic yield by enabling definitive diagnosis in about 63%-84% of patients^[18-20]. Thus, EUS-FNA is the standard of care approach with repeat procedure recommended when the initial procedure is nondiagnostic.

EUS-FNA TECHNIQUE

Numerous studies have aimed at determining the ideal EUS-FNA equipment and techniques to obtain a diagnosis when evaluating a pancreatic mass. In

Table 1 Sensitivity and specificity of various diagnostic approaches to a pancreatic mass lesion

Modality	Sensitivity	Specificity
CA 19-9	70%-92%	68%-92%
CT	77%-97%	56%-89%
Transabdominal ultrasound	89%	99%
Percutaneous FNA	62%-90%	98%-100%
ERCP	49%-66%	96%
EUS-FNA	75%-98%	71%-100%
EUS-FNB	85%-95%	86%-100%

EUS-FNA: Endoscopic ultrasound-guided fine needle aspiration; EUS-FNB: Endoscopic ultrasound-guided fine needle biopsy; ERCP: Endoscopic retrograde cholangiopancreatography.

Table 2 Techniques to increase diagnostic yield and decrease complications during endoscopic ultrasound-guided fine needle aspiration of a pancreatic mass

Pre-procedural considerations	General anesthesia may increase yield Goal platelet count greater than 50000 and INR less than 1.5 to reduce risk of bleeding Hold antiplatelet and antithrombotic agents except aspirin or NSAIDs
Procedural Considerations	Take caution when duodenal diverticulum is present to reduce risk of perforation Use Doppler to identify vasculature prior to needle advancement to avoid bleeding Use smaller (22 or 25) gauge needles for transduodenal FNA of the pancreatic head and uncinate If core histology samples needed, use 19G (in body or tail) or core biopsy needles Use suction Use the "fanning technique" during FNA Traverse the least amount of normal pancreatic tissue to reduce pancreatitis
Specimen Processing	Use on-site cytopathology or perform 7 needle passes

EUS-FNA: Endoscopic ultrasound-guided fine needle aspiration.

basic principal, the target lesion is visualized by EUS, the most ideal lesion puncture approach is located, a chosen needle is advanced to puncture the lesion, the stylet is removed (if used), suction is applied (or not), the needle is advanced and withdrawn through the lesion to obtain cellular material, and finally the needle is removed and the tissue is collected for cytopathological examination.

Within this basic technique, more complex issues of scope positioning, selection of the puncture site, selection of the FNA needle, use of a stylet and suction, the technique of needle puncture, the number of needle punctures and use of an on-site cytopathologist have been studied (Table 2).

Scope positioning and puncture site

The first task in performing high quality EUS-FNA involves locating the target tissue and determining the ideal needle approach. Perhaps for this reason, use of general anesthesia has shown to be associated with increased diagnostic yield (83% with vs 73%



Figure 1 Endoscopic ultrasound-guided fine needle aspiration of a mass in the pancreatic head. Arrows show the needle course to the tip of the needle within the hypoechoic mass (bottommost arrow).

without) during EUS-FNA of pancreatic mass lesions^[21]. However this was a single center retrospective study and further study is required to confirm these results.

Limitations in approaching a pancreatic mass include difficult location, small size, necrosis and vascularity. Ideally the mass should be located in the six o'clock position with the ultrasound transducer firmly applied to the luminal wall with suction. When possible, a transgastric approach is usually simplest as this avoids angulation of the scope permitting the needle to more easily pass through the biopsy channel. It may be difficult to advance the needle through the thicker gastric wall, which may be countered by suctioning the gastric wall, increasing the angle at which the needle passes through the gastric wall, and briskly advancing the needle. Acute angulation of the scope is often required when performing transduodenal FNA. Thus in these cases advancing the needle out of the biopsy channel may be more challenging, which makes smaller gauge needles (22 or 25 gauge) preferred with a transduodenal approach^[22]. The needle should not be forced out of the echoendoscope, which may need to be withdrawn to reduce any loops in the instrument to advance the needle forward. A site with minimal intervening vasculature should be chosen through use of Doppler imaging to avoid bleeding complications, discussed later in this review.

Selection of FNA needle

There are a wide variety of EUS-FNA needles on the market, with the main differentiating factor being gauge (G) (Figure 3). These range from highly flexible and smaller 25G needles, to commonly used 22G and even larger 19G needles. Contrary to the mantra "bigger is better" studies have repeatedly shown that larger gauge needles may not provide more adequate diagnostic samples of target tissue within the pancreas^[23-25]. In fact in one study, biopsy of lesions located within the pancreatic head and uncinate process showed a trend towards better diagnostic success with 25G needles over 22G

needles^[24]. Additionally, a meta-analysis comparing 22G and 25G needles for FNA of pancreatic masses found that sensitivity was significantly higher (93% vs 85%, $P = 0.0003$) with a 25G needle^[25]. Numerous other studies have suggested that the diagnostic yield is not statistically different between 22G and 25G FNA needles^[24,26-29]. One theme that rings true throughout the literature is that smaller gauge needles (22G and 25G) should be chosen when performing transduodenal FNA of the head and uncinate process of the pancreas given the significant bend and tension on the distal scope limiting movement of the needle. Larger needles, particularly 19G, carry higher technical failure rates in this situation, though without increase in rate of complications^[23,30]. A prospective study evaluated the use of an algorithmic approach to choosing needle size in EUS-FNA, which recommended using 25G needles for transduodenal approach, 22G or 25G for transgastric approach and 19G or core needles when more tissue is required^[31]. Following this algorithmic approach led to improved technical outcomes and cost savings without negatively impacting diagnostic accuracy. In general, for a transduodenal approach, a 22G or 25G FNA needle should be used while for a transgastric route, a 19G FNA needle may also be used especially if more tissue is desired. Core biopsies will be discussed further below, however, if the oncologists desire more tissue for molecular marker testing, the corresponding gauge core biopsy needle can be used.

Different commercially available FNA needles have different echogenicity and appearance under EUS guidance. Readily visualizing the needle tip is critical to performing FNA. To improve visibility, needle tips are tailored by different techniques including laser etching, mechanical dimpling, or sandblasting. One large multicenter study involved multiple experienced endosonographers internationally who evaluated and ranked 10 different EUS needles in a bench top model based on their echogenicity and sharpness of distinction from the surrounding phantom. A prototype needle with polymeric coating had significantly higher overall ranking, which suggested that this coating to the needle tip and shaft may improve visualization^[32].

Use of stylet and suction

A stylet is pre-loaded within all EUS-FNA needles with the intent of preventing sample contamination from the needle passing through other tissue prior to penetrating the target lesion. However, studies suggest that there is no difference in diagnostic success with or without use of the stylet^[33-35]. Some EUS centers perform no-stylet FNA procedures, which means that the stylet is completely removed and not replaced during FNA. The stylet may be useful to the nurse assistant for advancing a tissue sample out of the needle after removal from the echoendoscope especially if air flushing fails. A randomized study found no difference in diagnostic samples or accuracy

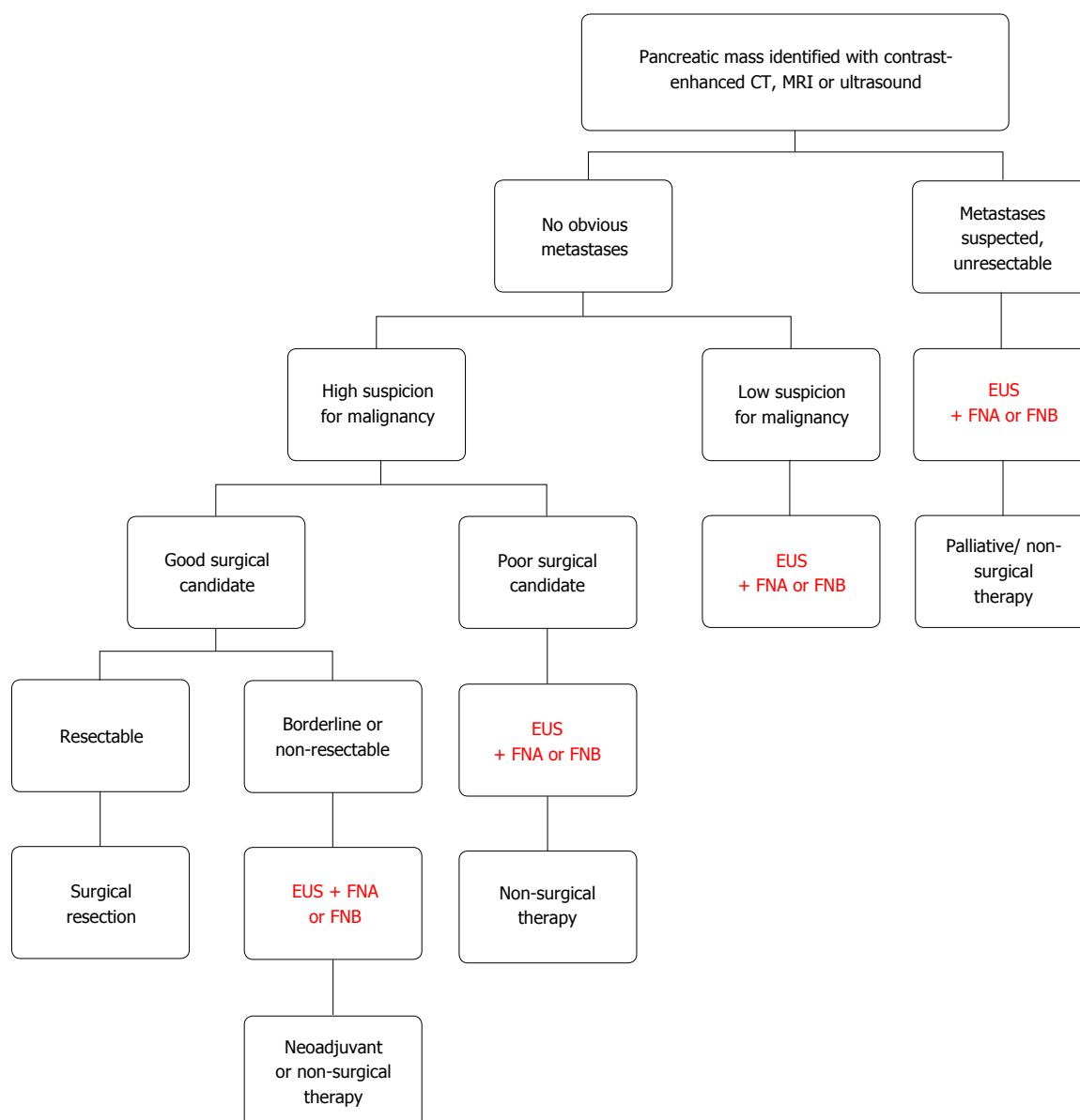


Figure 2 Algorithmic approach to a pancreatic mass.

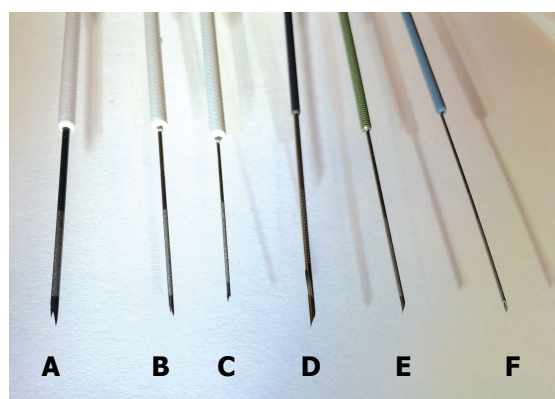


Figure 3 Representative endoscopic ultrasound biopsy and fine needle aspiration needles. A: 19G core biopsy needle; B: 22G core biopsy needle; C: 25G core biopsy needle; D: 19G FNA needle; E: 22G FNA needle; F: 25G FNA needle.

between air flushing or using the stylet to express the aspirate from the needle^[36]. Other centers replace the stylet in between passes, withdraw it a little to sharpen the needle before puncture, and after entering the lesion, push the stylet in completely to expel any tissue collected at the tip of the needle.

Use of suction is also variable, though multiple studies agree that suction will increase the amount of target cellular material at the expense of a bloodier specimen^[37,38]. If the cytology samples prove bloody, subsequent FNA passes should be performed with minimal or no suction. Also, the syringe vacuum suction must be turned off before withdrawing the needle from the lesion. One randomized study found that during EUS-FNA of solid lesions including pancreatic masses, sensitivity was significantly

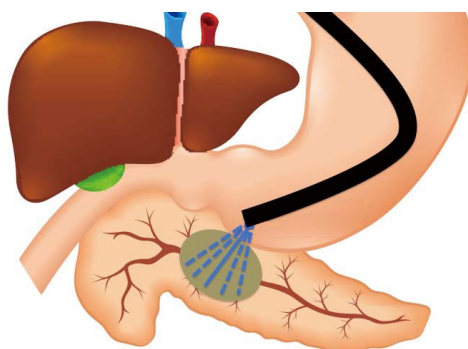


Figure 4 Schematization of the “fanning” technique for endoscopic ultrasound-guided fine needle aspiration. Dashed lines represent the change in course of the aspiration needle during each needle pass.

improved from 67% without suction to 86% with the use of 10 mL of suction^[37]. Another prospective study of only pancreatic masses confirmed higher diagnostic samples with the use of suction during EUS-FNA^[36]. Higher negative pressure suction (50 mL negative pressure) showed a trend toward increased diagnostic yield as compared to lower negative pressure (10 mL negative pressure) that did not reach significance^[39]. A “wet suction” technique, where the stylet is removed, the FNA needle is preloaded with saline and then 10 mL of syringe vacuum applied during FNA has been proposed. In a prospective randomized trial of solid lesions with the majority being pancreatic masses, this wet suction technique significantly improved specimen adequacy compared with standard 10 mL syringe vacuum suction (86% vs 75%, $P < 0.035$)^[40]. The “slow pull” technique, whereby the stylet is slowly withdrawn as the needle passes through the lesion to provide gentle capillary suction, has not proven superior when compared to standard syringe vacuum suction during FNA^[41-43]. Suction does seem to improve diagnostic yield although whether different methods of suction application (simple syringe, “wet suction,” “slow pull”) makes a difference remains unclear.

Technique of needle puncture

The methods used during FNA are also hotly debated. Given that inner portions of a pancreatic tumor may be necrotic, targeting the peripheral areas of the mass may improve diagnostic yield. However, dense desmoplastic reaction at the periphery may also pose challenges to obtaining adequate tissue for diagnosis. Therefore, endosonographers advocate for the “fanning” technique, which involves adjusting the trajectory of the needle typically using the elevator and/or dials on the head of the echoendoscope (Figure 4). Thus, instead of advancing the needle back and forth through the same portion of the mass, it samples different areas. A randomized trial comparing fanning with standard tissue acquisition during EUS-FNA reported superiority of the fanning technique after a single pass (86% diagnostic yield) as compared

to standard technique (58% diagnostic yield)^[44]. Therefore, after puncturing the lesion, the needle should be advanced back and forth through as much of the lesion as possible about 12-15 times using the fanning technique, if possible. Other methods include the “door knocking method” where after puncturing the mass, the needle stopper is locked at a distance just short of the length from the tip of the needle to the most distal extent of the lesion, the needle quickly advanced through the mass until it hits the stopper, and slowly withdrawn to the opposite side of the mass. This sequence of rapid insertion and slow pullback is repeated until that needle pass is completed. A multicenter prospective assessment of the role of this technique in diagnostic yield found no difference in reaching a histologic diagnosis between the door knocking and conventional needle puncture methods^[45].

Role of on-site cytopathology

The diagnostic accuracy of EUS-FNA is reported to be over 90% in most studies when rapid on-site evaluation (ROSE) for cytopathology samples is employed^[46-48]. With ROSE, the endosonographer typically makes one to two passes and then allows the pathologist to evaluate the sample smears for diagnostic yield. Further passes may be made as needed in order to achieve diagnostic success. If bloody aspirates are consistently seen by an onsite cytologist, switch to a smaller gauge needle without using suction. Older retrospective studies suggested decrease in nondiagnostic samples as well as need for repeat EUS with ROSE. Limitations of ROSE include increased cost due to cytopathologist time commitment, as well as limited access to cytopathologists and low reimbursements for ROSE^[49].

In the absence of ROSE, several studies have concluded that 5-7 needle passes are ideal in order to achieve high diagnostic success^[50-54]. In one large study, 5-6 passes achieved ROSE-level yields of 90%^[50]. Another study using only 22G needles found that the sensitivity increased from 17% after the first pass to nearly 90% after the seventh pass, thus suggesting that 7 passes with a 22G needle may be required^[51]. Yet another study using 25G needles suggested that four needle passes are sufficient^[52]. Recently 2 randomized studies evaluated the diagnostic yield of performing 7 passes using a 22G or 25G FNA needle without ROSE to cytologist-guided FNA in pancreatic masses. Both studies found no significant difference in diagnostic yield. Therefore, if onsite cytology review is not available, 7 FNA passes into the pancreatic mass should be performed^[53,54].

FINE NEEDLE BIOPSY

In theory a fine needle biopsy (FNB), or core needle biopsy, contains a superior tissue sample with preserved cellular architecture as compared to that

from FNA. It has been hypothesized that this will yield increased diagnostic accuracy with tissue processing and testing more easily accomplished through routine histology specimen processing. Three randomized studies of 22G FNA and 22G core biopsy needle (EchoTip ProCore, Cook Medical, Bloomington, IN) produced 3 different conclusions^[55-57]. One study reported comparable diagnostic yield (89%-100%), number of passes needed for diagnosis, and complications while another suggested significantly worse diagnostic capability (94% FNA vs 28% core needle) and ease of use with the core biopsy needle. The most recent study found significantly higher diagnostic yield with the core biopsy needle (90%) compared to the FNA needle (67%)^[57]. A metaanalysis of ProCore compared with FNA needles found no difference in diagnostic yield although the ProCore needle obtained diagnosis with fewer passes^[58]. Another prospective randomized comparison of 22G fenestrated core biopsy needle to standard 22G FNA needle in solid pancreatic lesions showed similar accuracy between the two needle types, though the fenestrated needle required, on average, one less pass (two instead of three) to achieve a diagnosis^[59].

A retrospective study of a newer FNB needle (SharkCore, Medtronic, Minneapolis, MN) compared with FNA needles reported higher yield of tissue sufficient for histology with the core needle (95% SharkCore vs 59% FNA needle) with fewer median passes to achieve this (2 passes FNA vs 4 passes for SharkCore)^[60]. Comparison of 2 different 19G core biopsy needles (ProCore and Quick-Core, both Cook Medical, Bloomington, IN) in a randomized study found the ProCore had significantly higher diagnostic histology (85% vs 57%)^[61,62]. Another study compared 22G and 25G core biopsy needles and found no statistical difference between diagnostic accuracy of one needle size over another^[63]. Given the increased use of molecular studies on tissue samples required for gene-specific oncologic therapy, obtaining histologic sized specimens, rather than cytopathology, will be of importance in the future. FNB may also play a critical role in rescue procedures when EUS-FNA is nondiagnostic. It may also change our practice if proven more efficacious than FNA needles whereby only FNB needles may be necessary without ROSE to obtain adequate specimens. Ongoing study of EUS-FNB regarding the clinical effectiveness as compared to FNA and cost analyses are required.

POTENTIAL ADVERSE EVENTS

Compared to other interventional procedures like ERCP, EUS-FNA procedures are very safe with reported overall complication rates ranging from 0.3% to 2.2%^[64,65]. Smaller (≤ 20 mm) and pancreatic neuroendocrine lesions were associated with increased risk of complications including pancreatitis, abdominal

pain, and bleeding in a retrospective single center study^[66]. Several complications of EUS-FNA and best-practice tips to avoid them are discussed.

Pancreatitis

The most common serious complication of EUS-FNA of pancreatic mass lesions is pancreatitis, which can occur in anywhere from 0.29% to 2% of cases^[66-68]. Needle gauge has no impact on the development of pancreatitis, which is thought to occur when the needle traverses normal pancreatic parenchyma and ducts to reach the target lesion. When discussing this risk with patients, it may be helpful to note that the risk of pancreatitis reported during percutaneous FNA is slightly higher at 3%^[69]. To avoid pancreatitis after EUS-FNA, it is recommended to select a needle path that will traverse the least amount of normal pancreas as possible. Whether administration of rectal indomethacin in potentially higher risk EUS-FNA procedures reduces the risk of post-EUS-FNA pancreatitis requires further study^[70].

Hemorrhage

Bleeding during and after EUS-FNA procedures has been reported to occur from 1.0%-4.4% of cases^[62-71]. Bleeding may be intraluminal or extraluminal and in most cases is self-limited. Steps to avoid procedure-related hemorrhage include avoidance of antithrombotic medications when possible, though generally aspirin and NSAIDs may be continued. A minimum platelet count of 50000 and INR less than 1.5 is also recommended as with many endoscopic procedures^[72]. Additionally, use of Doppler ultrasonography to avoid intervening bleed vessels during needle puncture is advised. If blood is seen filling the suction syringe during EUS-FNA, FNA should be stopped.

Perforation

Perforation of the esophagus is reported to occur in 0.009% to 0.15% of procedures^[73,74]. This is likely related to the larger diameter (generally 12-14 mm) of the echoendoscope, oblique position of the endoscopic camera limiting visualization of esophageal intubation, and blunt tip of some echoendoscopes. Duodenal perforation is more common, occurring in 0.02% to 0.86% in different series, and is often attributed to the presence of duodenal diverticula^[65,75]. Avoidance of perforation is achieved through scope lubrication, careful intubation, avoidance of undue pressure and awareness of any risky anatomic features including a diverticulum.

Infection

Infection is rare, though several studies have shown that there may be a small risk of bacteremia comparable to the risk associated with routine endoscopic procedures at about 2%^[76-78]. Clinically

significant infection is exceedingly rare, therefore, routine prophylactic antibiotics are not advised^[79]. If the solid lesion has a significant cystic component, prophylactic antibiotics should be administered as recommended by the American Society for Gastrointestinal Endoscopy^[80].

Tumor seeding

Tumor seeding is perhaps the most feared complication, however there are only limited single case reports of EUS-FNA associated tumor seeding and thus the risk is thought to be extremely low^[81]. To avoid this complication, it is important to ensure the echoendoscope is as close to the suspected malignancy as possible to limit the amount of tissue traversed. It is also important to perform EUS-FNA only when the results of the procedure will impact management of the patient, and to send patients straight to exploratory or curative surgery when appropriate. A retrospective study evaluating the impact of preoperative EUS-FNA found no difference in postoperative complications and overall or recurrence-free survival between patients who had and had not undergone preoperative EUS-FNA^[82].

STAGING OF PANCREATIC MASSES

Staging pancreatic cancer is of paramount importance in determining the resectability of any given cancer. Only approximately 10%-15% of patients with a pancreatic cancer will be candidates for surgical resection; therefore, an evaluation for distant metastases, vascular invasion, and lymphatic spread are considered. While pancreatic protocol CT scan of the abdomen is generally recommended as first line for this purpose, other modalities have been evaluated and play a role in staging. In addition to providing diagnostic information about the pancreatic mass, EUS is also important in detecting metastatic disease not seen on ultrasound or CT imaging. An older study found that 12% of patients with pancreatic masses had metastatic disease involving lymph nodes, liver, ascites, and the retroperitoneum identified by EUS-FNA that were not visualized by abdominal ultrasound or CT^[83]. Whether this would still hold true with improved abdominal imaging technology today is unclear.

With advancements in cross-sectional imaging, CT and MRI are now comparable to EUS for T-staging with accuracy ranging from 62% to 94%^[84]. A systematic review of the literature suggested that nodal staging also has similar accuracy between EUS (62%) and CT scan (63%)^[85]. Presence of malignant celiac lymph nodes may preclude resection, therefore, this area should be examined carefully by EUS. EUS also seems comparable to CT scan for detecting vascular invasion. For determination of vascular invasion, sensitivity of EUS varies depending on the vessels involved. EUS is superior to CT for assessing vascular

invasion of the portal vein (60%-100% sensitive) while inferior for judging involvement of the SMV, SMA, and celiac artery (17%-83% sensitive)^[86-89]. There is no consensus regarding the EUS criteria used to assess vascular invasion. Complete vascular obstruction, venous collaterals and visible tumor in the vessel have the highest specificity for vascular invasion and therefore, are the best criteria to use^[90]. Regarding resectability, a systematic review inclusive of 678 patients demonstrated that EUS was 63%-93% accurate in identifying surgically curable cases, which was generally similar to or better than CT scan (60%-83% sensitive)^[85]. Routine cross-sectional imaging is still recommended in order to evaluate for other intraperitoneal and hepatic metastases that may not be well evaluated with EUS.

ANCILLARY EUS TECHNIQUES

In an effort to further push the diagnostic accuracy of EUS to 100%, several complementary technologies have been developed including elastography, contrast-harmonic EUS and fluorescence in situ hybridization (FISH). Elastography during EUS may be used to calculate tissue stiffness, which may be of utility given that the properties of normal pancreatic vs cancerous tissue differ. Most cancerous lesions will be "harder" showing less elasticity, while benign lesions are generally "soft." Meta-analyses of elastography have reported sensitivity in detecting pancreatic cancer of 95%, though use of the technology for this indication is not yet mainstream and only available on certain ultrasound processors^[91-93].

Contrast harmonic ultrasonography involves use of intravenous microbubble contrast to enhance visualization of the microvasculature during EUS, theoretically improving the ability to detect malignancies. Lesions may be differentiated based on their enhancement with this microbubble contrast, whereby most carcinomas show hypoenhancement and normal tissue is non-enhancing. A systematic review of 82 reports using contrast harmonic EUS for solid pancreatic lesions found that the heterogeneous hypoenhancement pattern was 89%-96% sensitive and 69%-94% specific compared to a hyperenhancing pattern in diagnosing pancreatic adenocarcinoma^[94]. The accuracy of this technique was comparable to EUS-FNA, and whether the concomitant use of contrast harmonic EUS with EUS-FNA significantly improves overall diagnostic sensitivity compared to using each technique alone requires further study. In addition, interobserver agreement ranges from fair to good, which may improve with the advent of quantitative contrast harmonic EUS^[94].

Several tissue-based techniques may improve diagnosis of pancreatic masses. FISH uses pre-specified fluorescently labeled DNA probes and has been shown to improve the diagnostic yield of



Figure 5 Confocal laser endomicroscopy miniprobe through a 19G FNA needle. Photo provided with permissions by Mauna Kea, Paris, France.

indeterminate cytology from EUS-FNA samples of pancreatic masses, but is not readily available^[95,96]. For inconclusive EUS-FNA specimens from pancreatic solid masses, a metaanalysis of 931 patients found that the addition of K-ras mutation analysis significantly increased sensitivity from 81% to 89% and reduced the false-negative rate by 56%^[97]. This was associated with a concomitant reduction in specificity from 97% to 92% and an 11% increase in false-positive rate. RNA sequencing of EUS-FNA samples has also been recently reported in a proof-of-principle study with 87% sensitivity and 75% specificity in diagnosing pancreatic adenocarcinoma^[98]. A 5 microRNA panel was found to augment cytologic diagnosis of pancreatic ductal adenocarcinoma from 79% to 91% and out of 39 cytologically benign, indeterminate, or nondiagnostic samples, 22 were correctly diagnosed as malignant by the microRNA classifier^[99]. This requires further study and is not yet available clinically.

OTHER TOOLS ON THE HORIZON

Probe based confocal laser endomicroscopy

As probe based confocal endomicroscopy has been further miniaturized, needle confocal endomicroscopy, or nCLE (AQ-Flex 19 miniprobe, Mauna Kea, Paris, France), has become available for clinical use (Figure 5). The nCLE miniprobe has 0.85 mm diameter and may be inserted through a 19G EUS-FNA needle to provide real-time cellular level imaging. The probe can be preloaded into the FNA needle before performing EUS-FNA or loaded after the mass has been punctured with the FNA needle and stylet removed. After administering 2.5-5 mL of 10% fluorescein sodium intravenously, the probe is advanced about 3-5 mm beyond the tip of the needle to image the mass. A pilot study of nCLE for diagnosis of pancreatic mass lesions has reported findings of dark clumps measuring greater than 40 μ m associated with malignancy, no complications, and good interobserver agreement amongst three endosonographers blinded to all clinical data. However, this technology will require

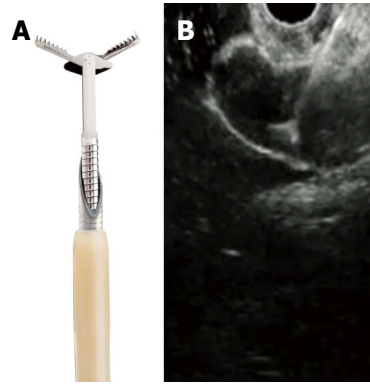


Figure 6 Miniature biopsy forceps. A: In open position, passing through a 19G FNA needle; B: EUS view of open biopsy forceps through the FNA needle. Photo provided with permissions by US Endoscopy, Mentor, OH.

further evaluation to determine its place in diagnosis of solid pancreatic masses^[100].

Through-the-needle biopsy forceps

A new miniaturized 0.75 mm biopsy forceps is available that can be advanced through a 19G EUS-FNA needle to obtain histology (Figure 6). The stylet is removed from the FNA needle and the biopsy forceps preloaded into the needle with the end positioned about 2-3 mm proximal to the needle tip. After puncturing the lesion with the FNA needle, the biopsy forceps is advanced out of the needle and 2-3 bites obtained before removing it. FNA can then be performed in the usual manner. The forceps can also be advanced through the needle after puncturing the mass. If difficulty is encountered in pushing out the forceps, it should be opened and closed by the assistant while the endoscopist continues advancing it forward. Using the mini-forceps through an FNA needle has been proven feasible and safe for pancreatic tissue acquisition^[101]. While only a pilot study has been completed, this initial report suggested high diagnostic sensitivity with no device failures or complications. This may offer an attractive alternative for the future.

CONCLUSION

EUS-FNA has overtaken all other technologies in the diagnosis of unknown pancreatic mass lesions. While it is clearly the single best test for elucidation of a pancreatic mass, cross-sectional imaging plays an important role in the initial evaluation and staging of pancreatic cancer. EUS-FNA is minimally invasive, safe, and highly effective in tissue acquisition. Diagnostic accuracy is enhanced with attention to the ideal technique through the choice of needle, biopsy technique and number of passes. When EUS-FNA does fail to provide a diagnosis, there are several adjunctive technologies currently under study which may assist in obtaining necessary diagnostic information including

novel core biopsy needles, elastography, contrast harmonic EUS, through the needle confocal imaging probes and biopsy forceps, and tissue-based technology including FISH, DNA and RNA analysis.

REFERENCES

- 1 **Pleskow DK**, Berger HJ, Gyves J, Allen E, McLean A, Podolsky DK. Evaluation of a serologic marker, CA19-9, in the diagnosis of pancreatic cancer. *Ann Intern Med* 1989; **110**: 704-709 [PMID: 2930108 DOI: 10.7326/0003-4819-110-9-704]
- 2 **Cwik G**, Wallner G, Skoczylas T, Ciechanski A, Zinkiewicz K. Cancer antigens 19-9 and 125 in the differential diagnosis of pancreatic mass lesions. *Arch Surg* 2006; **141**: 968-973; discussion 974 [PMID: 17043274 DOI: 10.1001/archsurg.141.10.968]
- 3 **van den Bosch RP**, van Eijck CH, Mulder PG, Jeekel J. Serum CA19-9 determination in the management of pancreatic cancer. *Hepatogastroenterology* 1996; **43**: 710-713 [PMID: 8799418]
- 4 **Uchida N**, Kamada H, Tsutsui K, Ono M, Aritomo Y, Masaki T, Kushida Y, Haba R, Nakatsu T, Kuriyama S. Utility of pancreatic duct brushing for diagnosis of pancreatic carcinoma. *J Gastroenterol* 2007; **42**: 657-662 [PMID: 17701129 DOI: 10.1007/s00535-007-2071-7]
- 5 **Yamaguchi T**, Shirai Y, Nakamura N, Sudo K, Nakamura K, Hironaka S, Hara T, Denda T. Usefulness of brush cytology combined with pancreatic juice cytology in the diagnosis of pancreatic cancer: significance of pancreatic juice cytology after brushing. *Pancreas* 2012; **41**: 1225-1229 [PMID: 23086246 DOI: 10.1097/MPA.0b013e31825d60fc]
- 6 **DelMaschio A**, Vanzulli A, Sironi S, Castrucci M, Mellone R, Staudacher C, Carlucci M, Zerbi A, Parolini D, Faravelli A. Pancreatic cancer versus chronic pancreatitis: diagnosis with CA 19-9 assessment, US, CT, and CT-guided fine-needle biopsy. *Radiology* 1991; **178**: 95-99 [PMID: 1984331 DOI: 10.1148/radiology.178.1.1984331]
- 7 **Johnson DE**, Pendurthi TK, Balshem AM, Ross E, Litwin S, Eisenberg BL, Hoffman JP. Implications of fine-needle aspiration in patients with resectable pancreatic cancer. *Am Surg* 1997; **63**: 675-679; discussion 679-680 [PMID: 9247432]
- 8 **Erturk SM**, Mortelé KJ, Tuncali K, Saltzman JR, Lao R, Silverman SG. Fine-needle aspiration biopsy of solid pancreatic masses: comparison of CT and endoscopic sonography guidance. *AJR Am J Roentgenol* 2006; **187**: 1531-1535 [PMID: 17114547 DOI: 10.2214/AJR.05.1657]
- 9 **Horwhat JD**, Paulson EK, McGrath K, Branch MS, Baillie J, Tyler D, Pappas T, Enns R, Robuck G, Stiffler H, Jowell P. A randomized comparison of EUS-guided FNA versus CT or US-guided FNA for the evaluation of pancreatic mass lesions. *Gastrointest Endosc* 2006; **63**: 966-975 [PMID: 16733111 DOI: 10.1016/j.gie.2005.09.028]
- 10 **Volmar KE**, Vollmer RT, Jowell PS, Nelson RC, Xie HB. Pancreatic FNA in 1000 cases: a comparison of imaging modalities. *Gastrointest Endosc* 2005; **61**: 854-861 [PMID: 15933687 DOI: 10.1016/S0016-5107(05)00364-0]
- 11 **Micames C**, Jowell PS, White R, Paulson E, Nelson R, Morse M, Hurwitz H, Pappas T, Tyler D, McGrath K. Lower frequency of peritoneal carcinomatosis in patients with pancreatic cancer diagnosed by EUS-guided FNA vs. percutaneous FNA. *Gastrointest Endosc* 2003; **58**: 690-695 [PMID: 14595302 DOI: 10.1016/S0016-5107(03)02009-1]
- 12 **Puli SR**, Bechtold ML, Buxbaum JL, Eloubeidi MA. How good is endoscopic ultrasound-guided fine-needle aspiration in diagnosing the correct etiology for a solid pancreatic mass?: A meta-analysis and systematic review. *Pancreas* 2013; **42**: 20-26 [PMID: 23254913 DOI: 10.1097/MPA.0b013e3182546e79]
- 13 **Hartwig W**, Schneider L, Diener MK, Bergmann F, Büchler MW, Werner J. Preoperative tissue diagnosis for tumours of the pancreas. *Br J Surg* 2009; **96**: 5-20 [PMID: 19016272 DOI: 10.1002/bjs.6407]
- 14 **Hewitt MJ**, McPhail MJ, Possamai L, Dhar A, Vlavianos P, Monahan KJ. EUS-guided FNA for diagnosis of solid pancreatic neoplasms: a meta-analysis. *Gastrointest Endosc* 2012; **75**: 319-331 [PMID: 22248600 DOI: 10.1016/j.gie.2011.08.049]
- 15 **Harewood GC**, Wiersma MJ. Endosonography-guided fine needle aspiration biopsy in the evaluation of pancreatic masses. *Am J Gastroenterol* 2002; **97**: 1386-1391 [PMID: 12094855 DOI: 10.1111/j.1572-0241.2002.05777.x]
- 16 **Ardengh JC**, Lopes CV, de Lima LF, de Oliveira JR, Venco F, Santo GC, Modena JL. Diagnosis of pancreatic tumors by endoscopic ultrasound-guided fine-needle aspiration. *World J Gastroenterol* 2007; **13**: 3112-3116 [PMID: 17589929 DOI: 10.3748/wjg.v13.i22.3112]
- 17 **Varadarajulu S**, Tamhane A, Eloubeidi MA. Yield of EUS-guided FNA of pancreatic masses in the presence or the absence of chronic pancreatitis. *Gastrointest Endosc* 2005; **62**: 728-736; quiz 51, 53
- 18 **Eloubeidi MA**, Varadarajulu S, Desai S, Wilcox CM. Value of repeat endoscopic ultrasound-guided fine needle aspiration for suspected pancreatic cancer. *J Gastroenterol Hepatol* 2008; **23**: 567-570 [PMID: 18397485 DOI: 10.1111/j.1440-1746.2007.05119.x]
- 19 **Suzuki R**, Lee JH, Krishna SG, Ramireddy S, Qiao W, Weston B, Ross WA, Bhutani MS. Repeat endoscopic ultrasound-guided fine needle aspiration for solid pancreatic lesions at a tertiary referral center will alter the initial inconclusive result. *J Gastrointest Liver Dis* 2013; **22**: 183-187 [PMID: 23799217]
- 20 **DeWitt J**, McGreevy K, Sherman S, LeBlanc J. Utility of a repeated EUS at a tertiary-referral center. *Gastrointest Endosc* 2008; **67**: 610-619 [PMID: 18279866 DOI: 10.1016/j.gie.2007.09.037]
- 21 **Ootaki C**, Stevens T, Vargo J, You J, Shiba A, Foss J, Borkowski R, Maurer W. Does general anesthesia increase the diagnostic yield of endoscopic ultrasound-guided fine needle aspiration of pancreatic masses? *Anesthesiology* 2012; **117**: 1044-1050 [PMID: 23042221 DOI: 10.1097/ALN.0b013e31826e0590]
- 22 **Itoi T**, Itokawa F, Kurihara T, Sofuni A, Tsuchiya T, Ishii K, Tsuji S, Ikeuchi N, Kawai T, Moriyasu F. Experimental endoscopy: objective evaluation of EUS needles. *Gastrointest Endosc* 2009; **69**: 509-516 [PMID: 19231491 DOI: 10.1016/j.gie.2008.07.017]
- 23 **Song TJ**, Kim JH, Lee SS, Eum JB, Moon SH, Park DY, Seo DW, Lee SK, Jang SJ, Yun SC, Kim MH. The prospective randomized, controlled trial of endoscopic ultrasound-guided fine-needle aspiration using 22G and 19G aspiration needles for solid pancreatic or peripancreatic masses. *Am J Gastroenterol* 2010; **105**: 1739-1745 [PMID: 20216532 DOI: 10.1038/ajg.2010.108]
- 24 **Siddiqui UD**, Rossi F, Rosenthal LS, Padda MS, Murali-Dharan V, Aslanian HR. EUS-guided FNA of solid pancreatic masses: a prospective, randomized trial comparing 22-gauge and 25-gauge needles. *Gastrointest Endosc* 2009; **70**: 1093-1097 [PMID: 19640524 DOI: 10.1016/j.gie.2009.05.037]
- 25 **Madhoun MF**, Wani SB, Rastogi A, Early D, Gaddam S, Tierney WM, Maple JT. The diagnostic accuracy of 22-gauge and 25-gauge needles in endoscopic ultrasound-guided fine needle aspiration of solid pancreatic lesions: a meta-analysis. *Endoscopy* 2013; **45**: 86-92 [PMID: 23307148 DOI: 10.1055/s-0032-1325992]
- 26 **Bang JY**, Varadarajulu S. Procore and flexible 19 gauge needle can replace trucut biopsy needle? *Clin Endosc* 2013; **46**: 503-505 [PMID: 24143312 DOI: 10.5946/ce.2013.46.5.503]
- 27 **Lee JH**, Stewart J, Ross WA, Anandasabapathy S, Xiao L, Staerckel G. Blinded prospective comparison of the performance of 22-gauge and 25-gauge needles in endoscopic ultrasound-guided fine needle aspiration of the pancreas and peri-pancreatic lesions. *Dig Dis Sci* 2009; **54**: 2274-2281 [PMID: 19669880 DOI: 10.1007/s10620-009-0906-1]
- 28 **Affolter KE**, Schmidt RL, Matynia AP, Adler DG, Factor RE. Needle size has only a limited effect on outcomes in EUS-guided fine needle aspiration: a systematic review and meta-analysis. *Dig Dis Sci* 2013; **58**: 1026-1034 [PMID: 23086117 DOI: 10.1007/s10620-012-2439-2]
- 29 **Brugge WR**. EUS. *Gastrointest Endosc* 2013; **78**: 414-420 [PMID: 23948189 DOI: 10.1016/j.gie.2013.07.002]
- 30 **Larghi A**, Verna EC, Stavropoulos SN, Rotterdam H, Lightdale

- CJ, Stevens PD. EUS-guided trucut needle biopsies in patients with solid pancreatic masses: a prospective study. *Gastrointest Endosc* 2004; **59**: 185-190 [PMID: 14745390 DOI: 10.1016/S0016-5107(03)02538-0]
- 31 **Bang JY**, Hawes RH, Varadarajulu S. Objective evaluation of a new endoscopic ultrasound processor. *Dig Endosc* 2013; **25**: 554-555 [PMID: 23889517 DOI: 10.1111/den.12148]
 - 32 **Tang SJ**, Vilmann AS, Saftoiu A, Wang W, Streba CT, Fink PP, Griswold M, Wu R, Dietrich CF, Jenssen C, Hocke M, Kantowski M, Pohl J, Fockens P, Annema JT, van der Heijden EH, Havre RF, Pham KD, Kunda R, Deprez PH, Mariana J, Vazquez-Sequeiros E, Larghi A, Buscarini E, Fusaroli P, Lahav M, Puri R, Garg PK, Sharma M, Maluf-Filho F, Sahai A, Brugge WR, Lee LS, Aslanian HR, Wang AY, Shami VM, Markowitz A, Siddiqui AA, Mishra G, Scheiman JM, Isenberg G, Siddiqui UD, Shah RJ, Buxbaum J, Watson RR, Willingham FF, Bhutani MS, Levy MJ, Harris C, Wallace MB, Nolsøe CP, Lorentzen T, Bang N, Sørensen SM, Gilja OH, D'Onofrio M, Piscaglia F, Gritzmman N, Radzina M, Sparchez ZA, Sidhu PS, Freeman S, McCowan TC, de Araujo CR, Patel A, Ali MA, Campbell G, Chen E, Vilmann P. EUS Needle Identification Comparison and Evaluation study (with videos). *Gastrointest Endosc* 2016; **84**: 424-433.e2 [PMID: 26873530 DOI: 10.1016/j.gie.2016.01.068]
 - 33 **Rastogi A**, Wani S, Gupta N, Singh V, Gaddam S, Reddymasu S, Ulusarac O, Fan F, Romanas M, Dennis KL, Sharma P, Bansal A, Oropeza-Vail M, Olyae M. A prospective, single-blind, randomized, controlled trial of EUS-guided FNA with and without a stylet. *Gastrointest Endosc* 2011; **74**: 58-64 [PMID: 21514932 DOI: 10.1016/j.gie.2011.02.015]
 - 34 **Sahai AV**, Paquin SC, Gariépy G. A prospective comparison of endoscopic ultrasound-guided fine needle aspiration results obtained in the same lesion, with and without the needle stylet. *Endoscopy* 2010; **42**: 900-903 [PMID: 20725886 DOI: 10.1055/s-0030-1255676]
 - 35 **Wani S**, Early D, Kunkel J, Leathersich A, Hovis CE, Hollander TG, Kohlmeier C, Zelenka C, Azar R, Edmundowicz S, Collins B, Liu J, Hall M, Mullady D. Diagnostic yield of malignancy during EUS-guided FNA of solid lesions with and without a stylet: a prospective, single blind, randomized, controlled trial. *Gastrointest Endosc* 2012; **76**: 328-335 [PMID: 22695205 DOI: 10.1016/j.gie.2012.03.1395]
 - 36 **Lee JK**, Choi JH, Lee KH, Kim KM, Shin JU, Lee JK, Lee KT, Jang KT. A prospective, comparative trial to optimize sampling techniques in EUS-guided FNA of solid pancreatic masses. *Gastrointest Endosc* 2013; **77**: 745-751 [PMID: 23433878 DOI: 10.1016/j.gie.2012.12.009]
 - 37 **Puri R**, Vilmann P, Saftoiu A, Skov BG, Linnemann D, Hassan H, Garcia ES, Gorunescu F. Randomized controlled trial of endoscopic ultrasound-guided fine-needle sampling with or without suction for better cytological diagnosis. *Scand J Gastroenterol* 2009; **44**: 499-504 [PMID: 19117242 DOI: 10.1080/00365520802647392]
 - 38 **Bang JY**, Ramesh J, Trevino J, Eloubeidi MA, Varadarajulu S. Objective assessment of an algorithmic approach to EUS-guided FNA and interventions. *Gastrointest Endosc* 2013; **77**: 739-744 [PMID: 23369651 DOI: 10.1016/j.gie.2012.11.029]
 - 39 **Kudo T**, Kawakami H, Hayashi T, Yasuda I, Mukai T, Inoue H, Katanuma A, Kawakubo K, Ishiwatari H, Doi S, Yamada R, Maguchi H, Isayama H, Mitsuhashi T, Sakamoto N. High and low negative pressure suction techniques in EUS-guided fine-needle tissue acquisition by using 25-gauge needles: a multicenter, prospective, randomized, controlled trial. *Gastrointest Endosc* 2014; **80**: 1030-1037.e1 [PMID: 24890422 DOI: 10.1016/j.gie.2014.04.012]
 - 40 **Attam R**, Arain MA, Bloechl SJ, Trikudanathan G, Munigala S, Bakman Y, Singh M, Wallace T, Henderson JB, Catalano MF, Guda NM. "Wet suction technique (WEST)": a novel way to enhance the quality of EUS-FNA aspirate. Results of a prospective, single-blind, randomized, controlled trial using a 22-gauge needle for EUS-FNA of solid lesions. *Gastrointest Endosc* 2015; **81**: 1401-1407 [PMID: 25733127 DOI: 10.1016/j.gie.2014.11.023]
 - 41 **Kin T**, Katanuma A, Yane K, Takahashi K, Osanai M, Takaki R, Matsumoto K, Gon K, Matsumori T, Tomonari A, Maguchi H, Shinohara T, Nojima M. Diagnostic ability of EUS-FNA for pancreatic solid lesions with conventional 22-gauge needle using the slow pull technique: a prospective study. *Scand J Gastroenterol* 2015; **50**: 900-907 [PMID: 25732902 DOI: 10.3109/00365521.2014.983155]
 - 42 **Nakai Y**, Isayama H, Chang KJ, Yamamoto N, Hamada T, Uchino R, Mizuno S, Miyabayashi K, Yamamoto K, Kawakubo K, Kogure H, Sasaki T, Hirano K, Tanaka M, Tada M, Fukayama M, Koike K. Slow pull versus suction in endoscopic ultrasound-guided fine-needle aspiration of pancreatic solid masses. *Dig Dis Sci* 2014; **59**: 1578-1585 [PMID: 24429514 DOI: 10.1007/s10620-013-3019-9]
 - 43 **Tarantino I**, Di Mitri R, Fabbri C, Pagano N, Barresi L, Granata A, Liotta R, Mocciano F, Maimone A, Baccarini P, Fabio T, Curcio G, Repici A, Traina M. Is diagnostic accuracy of fine needle aspiration on solid pancreatic lesions aspiration-related? A multicentre randomised trial. *Dig Liver Dis* 2014; **46**: 523-526 [PMID: 24704290 DOI: 10.1016/j.dld.2014.02.023]
 - 44 **Bang JY**, Magee SH, Ramesh J, Trevino JM, Varadarajulu S. Randomized trial comparing fanning with standard technique for endoscopic ultrasound-guided fine-needle aspiration of solid pancreatic mass lesions. *Endoscopy* 2013; **45**: 445-450 [PMID: 23504490 DOI: 10.1055/s-0032-1326268]
 - 45 **Mukai S**, Itoi T, Ashida R, Tsuchiya T, Ikeuchi N, Kamada K, Tanaka R, Umeda J, Tonozuka R, Fukutake N, Hoshi K, Moriyasu F, Gotoda T, Irisawa A. Multicenter, prospective, crossover trial comparing the door-knocking method with the conventional method for EUS-FNA of solid pancreatic masses (with videos). *Gastrointest Endosc* 2016; **83**: 1210-1217 [PMID: 26522372 DOI: 10.1016/j.gie.2015.10.025]
 - 46 **Chen J**, Yang R, Lu Y, Xia Y, Zhou H. Diagnostic accuracy of endoscopic ultrasound-guided fine-needle aspiration for solid pancreatic lesion: a systematic review. *J Cancer Res Clin Oncol* 2012; **138**: 1433-1441 [PMID: 22752601 DOI: 10.1007/s00432-012-1268-1]
 - 47 **Iglesias-Garcia J**, Dominguez-Munoz JE, Abdulkader I, Larino-Noia J, Eugenyeva E, Lozano-Leon A, Forteza-Vila J. Influence of on-site cytopathology evaluation on the diagnostic accuracy of endoscopic ultrasound-guided fine needle aspiration (EUS-FNA) of solid pancreatic masses. *Am J Gastroenterol* 2011; **106**: 1705-1710 [PMID: 21483464 DOI: 10.1038/ajg.2011.119]
 - 48 **Hébert-Magee S**, Bae S, Varadarajulu S, Ramesh J, Frost AR, Eloubeidi MA, Eltoum IA. The presence of a cytopathologist increases the diagnostic accuracy of endoscopic ultrasound-guided fine needle aspiration cytology for pancreatic adenocarcinoma: a meta-analysis. *Cytopathology* 2013; **24**: 159-171 [PMID: 23711182 DOI: 10.1111/cyt.12071]
 - 49 **Layfield LJ**, Bentz JS, Gopez EV. Immediate on-site interpretation of fine-needle aspiration smears: a cost and compensation analysis. *Cancer* 2001; **93**: 319-322 [PMID: 11668466 DOI: 10.1002/cncr.9046]
 - 50 **Erickson RA**, Sayage-Rabie L, Beissner RS. Factors predicting the number of EUS-guided fine-needle passes for diagnosis of pancreatic malignancies. *Gastrointest Endosc* 2000; **51**: 184-190 [PMID: 10650262 DOI: 10.1016/S0016-5107(00)70416-0]
 - 51 **LeBlanc JK**, Ciaccia D, Al-Assi MT, McGrath K, Imperiale T, Tao LC, Vallery S, DeWitt J, Sherman S, Collins E. Optimal number of EUS-guided fine needle passes needed to obtain a correct diagnosis. *Gastrointest Endosc* 2004; **59**: 475-481 [PMID: 15044881 DOI: 10.1016/S0016-5107(03)02863-3]
 - 52 **Suzuki R**, Irisawa A, Bhutani MS, Hikichi T, Takagi T, Sato A, Sato M, Ikeda T, Watanabe K, Nakamura J, Tasaki K, Obara K, Ohira H. Prospective evaluation of the optimal number of 25-gauge needle passes for endoscopic ultrasound-guided fine-needle aspiration biopsy of solid pancreatic lesions in the absence of an onsite cytopathologist. *Dig Endosc* 2012; **24**: 452-456 [PMID: 23078439 DOI: 10.1111/j.1443-1661.2012.01311.x]
 - 53 **Lee LS**, Nieto J, Watson RR, Hwang AL, Muthusamy VR, Walter L, Jajoo K, Ryou MK, Saltzman JR, Saunders MD, Suleiman

- S, Kadiyala V. Randomized Noninferiority Trial Comparing Diagnostic Yield of Cytopathologist-guided versus 7 passes for EUS-FNA of Pancreatic Masses. *Dig Endosc* 2015; Epub ahead of print [PMID: 26694852 DOI: 10.1111/den.12594]
- 54 **Wani S**, Mullady D, Early DS, Rastogi A, Collins B, Wang JF, Marshall C, Sams SB, Yen R, Rizeq M, Romanas M, Ulsarac O, Brauer B, Attwell A, Gaddam S, Hollander TG, Hosford L, Johnson S, Kushnir V, Amateau SK, Kohlmeier C, Azar RR, Vargo J, Fukami N, Shah RJ, Das A, Edmundowicz SA. The clinical impact of immediate on-site cytopathology evaluation during endoscopic ultrasound-guided fine needle aspiration of pancreatic masses: a prospective multicenter randomized controlled trial. *Am J Gastroenterol* 2015; **110**: 1429-1439 [PMID: 26346868 DOI: 10.1038/ajg.2015.262]
 - 55 **Bang JY**, Hebert-Magee S, Trevino J, Ramesh J, Varadarajulu S. Randomized trial comparing the 22-gauge aspiration and 22-gauge biopsy needles for EUS-guided sampling of solid pancreatic mass lesions. *Gastrointest Endosc* 2012; **76**: 321-327 [PMID: 22658389 DOI: 10.1016/j.gie.2012.03.1392]
 - 56 **Strand DS**, Jeffus SK, Sauer BG, Wang AY, Stelow EB, Shami VM. EUS-guided 22-gauge fine-needle aspiration versus core biopsy needle in the evaluation of solid pancreatic neoplasms. *Diagn Cytopathol* 2014; **42**: 751-758 [PMID: 24550162 DOI: 10.1002/dc.23116]
 - 57 **Aadam AA**, Wani S, Amick A, Shah JN, Bhat YM, Hamerski CM, Klapman JB, Muthusamy VR, Watson RR, Rademaker AW, Keswani RN, Keefer L, Das A, Komanduri S. A randomized controlled cross-over trial and cost analysis comparing endoscopic ultrasound fine needle aspiration and fine needle biopsy. *Endosc Int Open* 2016; **4**: E497-E505 [PMID: 27227104 DOI: 10.1055/s-0042-106958]
 - 58 **Bang JY**, Hawes R, Varadarajulu S. A meta-analysis comparing ProCore and standard fine-needle aspiration needles for endoscopic ultrasound-guided tissue acquisition. *Endoscopy* 2016; **48**: 339-349 [PMID: 26561917]
 - 59 **Alatawi A**, Beuvon F, Grabar S, Leblanc S, Chaussade S, Terris B, Barret M, Prat F. Comparison of 22G reverse-beveled versus standard needle for endoscopic ultrasound-guided sampling of solid pancreatic lesions. *United European Gastroenterol J* 2015; **3**: 343-352 [PMID: 26279842 DOI: 10.1177/2050640615577533]
 - 60 **Kandel P**, Tranesh G, Nassar A, Bingham R, Raimondo M, Woodward TA, Gomez V, Wallace MB. EUS-guided fine needle biopsy sampling using a novel fork-tip needle: a case-control study. *Gastrointest Endosc* 2016; Epub ahead of print [PMID: 27018087 DOI: 10.1016/j.gie.2016.03.1405]
 - 61 **DeWitt J**, Cho CM, Lin J, Al-Haddad M, Canto MI, Salamone A, Hruban RH, Messallam AA, Khashab MA. Comparison of EUS-guided tissue acquisition using two different 19-gauge core biopsy needles: a multicenter, prospective, randomized, and blinded study. *Endosc Int Open* 2015; **3**: E471-E478 [PMID: 26528504 DOI: 10.1055/s-0034-1392222]
 - 62 **Eloubeidi MA**, Chen VK, Eltoum IA, Jhala D, Chhieng DC, Jhala N, Vickers SM, Wilcox CM. Endoscopic ultrasound-guided fine needle aspiration biopsy of patients with suspected pancreatic cancer: diagnostic accuracy and acute and 30-day complications. *Am J Gastroenterol* 2003; **98**: 2663-2668 [PMID: 14687813]
 - 63 **Park SW**, Chung MJ, Lee SH, Lee HS, Lee HJ, Park JY, Park SW, Song SY, Kim H, Chung JB, Bang S. Prospective Study for Comparison of Endoscopic Ultrasound-Guided Tissue Acquisition Using 25- and 22-Gauge Core Biopsy Needles in Solid Pancreatic Masses. *PLoS One* 2016; **11**: e0154401 [PMID: 27149404 DOI: 10.1371/journal.pone.0154401]
 - 64 **Jani BS**, Rzhouq F, Saligram S, Lim D, Rastogi A, Bonino J, Olyae M. Endoscopic Ultrasound-Guided Fine-Needle Aspiration of Pancreatic Lesions: A Systematic Review of Technical and Procedural Variables. *N Am J Med Sci* 2016; **8**: 1-11 [PMID: 27011940 DOI: 10.4103/1947-2714.175185]
 - 65 **Jenssen C**, Faiss S, Nürnberg D. Complications of endoscopic ultrasound and endoscopic ultrasound-guided interventions - results of a survey among German centers. *Z Gastroenterol* 2008; **46**: 1177-1184 [PMID: 18937186 DOI: 10.1055/s-2008-1027334]
 - 66 **Katanuma A**, Maguchi H, Yane K, Hashigo S, Kin T, Kaneko M, Kato S, Kato R, Harada R, Osanai M, Takahashi K, Nojima M. Factors predictive of adverse events associated with endoscopic ultrasound-guided fine needle aspiration of pancreatic solid lesions. *Dig Dis Sci* 2013; **58**: 2093-2099 [PMID: 23423501 DOI: 10.1007/s10620-013-2590-4]
 - 67 **Gress F**, Michael H, Gelrud D, Patel P, Gottlieb K, Singh F, Grendell J. EUS-guided fine-needle aspiration of the pancreas: evaluation of pancreatitis as a complication. *Gastrointest Endosc* 2002; **56**: 864-867 [PMID: 12447299 DOI: 10.1067/mge.2002.129602]
 - 68 **Eloubeidi MA**, Gress FG, Savides TJ, Wiersma MJ, Kochman ML, Ahmad NA, Ginsberg GG, Erickson RA, Dewitt J, Van Dam J, Nickl NJ, Levy MJ, Clain JE, Chak A, Sivak MV, Wong R, Isenberg G, Scheiman JM, Bounds B, Kimmey MB, Saunders MD, Chang KJ, Sharma A, Nguyen P, Lee JG, Edmundowicz SA, Early D, Azar R, Etemad B, Chen YK, Waxman I, Shami V, Catalano MF, Wilcox CM. Acute pancreatitis after EUS-guided FNA of solid pancreatic masses: a pooled analysis from EUS centers in the United States. *Gastrointest Endosc* 2004; **60**: 385-389 [PMID: 15332028 DOI: 10.1016/S0016-5107(04)01714-6]
 - 69 **Mueller PR**, Miketic LM, Simeone JF, Silverman SG, Saini S, Wittenberg J, Hahn PF, Steiner E, Forman BH. Severe acute pancreatitis after percutaneous biopsy of the pancreas. *AJR Am J Roentgenol* 1988; **151**: 493-494 [PMID: 3261508 DOI: 10.2214/ajr.151.3.493]
 - 70 **Elmunzer BJ**, Serrano J, Chak A, Edmundowicz SA, Papachristou GI, Scheiman JM, Singh VK, Varadarajulu S, Vargo JJ, Willingham FF, Baron TH, Coté GA, Romagnuolo J, Wood-Williams A, Depue EK, Spitzer RL, Spino C, Foster LD, Durkalski V. Rectal indomethacin alone versus indomethacin and prophylactic pancreatic stent placement for preventing pancreatitis after ERCP: study protocol for a randomized controlled trial. *Trials* 2016; **17**: 120 [PMID: 26941086 DOI: 10.1186/s13063-016-1251-2]
 - 71 **Affi A**, Vazquez-Sequeiros E, Norton ID, Clain JE, Wiersma MJ. Acute extraluminal hemorrhage associated with EUS-guided fine needle aspiration: frequency and clinical significance. *Gastrointest Endosc* 2001; **53**: 221-225 [PMID: 11174300 DOI: 10.1067/mge.2001.111391]
 - 72 **Acosta RD**, Abraham NS, Chandrasekhara V, Chathadi KV, Early DS, Eloubeidi MA, Evans JA, Faulx AL, Fisher DA, Fonkalsrud L, Hwang JH, Khashab MA, Lightdale JR, Muthusamy VR, Pasha SF, Saltzman JR, Shaikat A, Shergill AK, Wang A, Cash BD, DeWitt JM. The management of antithrombotic agents for patients undergoing GI endoscopy. *Gastrointest Endosc* 2016; **83**: 3-16 [PMID: 26621548 DOI: 10.1016/j.gie.2015.09.035]
 - 73 **Das A**, Sivak MV, Chak A. Cervical esophageal perforation during EUS: a national survey. *Gastrointest Endosc* 2001; **53**: 599-602 [PMID: 11323585 DOI: 10.1067/mge.2001.113385]
 - 74 **Mortensen MB**, Frstrup C, Holm FS, Pless T, Durup J, Ainsworth AP, Nielsen HO, Hovendal C. Prospective evaluation of patient tolerability, satisfaction with patient information, and complications in endoscopic ultrasonography. *Endoscopy* 2005; **37**: 146-153 [PMID: 15692930 DOI: 10.1055/s-2005-861142]
 - 75 **Raut CP**, Grau AM, Staerkel GA, Kaw M, Tamm EP, Wolff RA, Vauthey JN, Lee JE, Pistors PW, Evans DB. Diagnostic accuracy of endoscopic ultrasound-guided fine-needle aspiration in patients with presumed pancreatic cancer. *J Gastrointest Surg* 2003; **7**: 118-126; discussion 127-128 [PMID: 12559193 DOI: 10.1016/S1091-255X(02)00150-6]
 - 76 **Barawi M**, Gottlieb K, Cunha B, Portis M, Gress F. A prospective evaluation of the incidence of bacteremia associated with EUS-guided fine-needle aspiration. *Gastrointest Endosc* 2001; **53**: 189-192 [PMID: 11174290 DOI: 10.1067/mge.2001.108966]
 - 77 **Levy MJ**, Norton ID, Wiersma MJ, Schwartz DA, Clain JE, Vazquez-Sequeiros E, Wilson WR, Zinsmeister AR, Jondal ML. Prospective risk assessment of bacteremia and other infectious complications in patients undergoing EUS-guided FNA. *Gastrointest Endosc* 2003; **57**: 672-678 [PMID: 12709695 DOI: 10.1016/S0016-5107(03)00150-6]

- 10.1067/mge.2003.204]
- 78 **Janssen J**, König K, Knop-Hammad V, Johanns W, Greiner L. Frequency of bacteremia after linear EUS of the upper GI tract with and without FNA. *Gastrointest Endosc* 2004; **59**: 339-344 [PMID: 14997128 DOI: 10.1016/S0016-5107(03)02707-X]
- 79 **Hirota WK**, Petersen K, Baron TH, Goldstein JL, Jacobson BC, Leighton JA, Mallory JS, Waring JP, Fanelli RD, Wheeler-Harborough J, Faigel DO. Guidelines for antibiotic prophylaxis for GI endoscopy. *Gastrointest Endosc* 2003; **58**: 475-482 [PMID: 14520276 DOI: 10.1067/S0016-5107(03)01883-2]
- 80 **Khashab MA**, Chithadi KV, Acosta RD, Bruining DH, Chandrasekhara V, Eloubeidi MA, Fanelli RD, Faulx AL, Fonkalsrud L, Lightdale JR, Muthusamy VR, Pasha SF, Saltzman JR, Shaikat A, Wang A, Cash BD. Antibiotic prophylaxis for GI endoscopy. *Gastrointest Endosc* 2015; **81**: 81-89 [PMID: 25442089 DOI: 10.1016/j.gie.2014.08.008]
- 81 **Fujii LL**, Levy MJ. Basic techniques in endoscopic ultrasound-guided fine needle aspiration for solid lesions: Adverse events and avoiding them. *Endosc Ultrasound* 2014; **3**: 35-45 [PMID: 24949409 DOI: 10.4103/2303-9027.123006]
- 82 **Beane JD**, House MG, Coté GA, DeWitt JM, Al-Haddad M, LeBlanc JK, McHenry L, Sherman S, Schmidt CM, Zyromski NJ, Nakeeb A, Pitt HA, Lillemoe KD. Outcomes after preoperative endoscopic ultrasonography and biopsy in patients undergoing distal pancreatectomy. *Surgery* 2011; **150**: 844-853 [PMID: 22000199 DOI: 10.1016/j.surg.2011.07.068]
- 83 **Mortensen MB**, Pless T, Durup J, Ainsworth AP, Plagborg GJ, Hovendal C. Clinical impact of endoscopic ultrasound-guided fine needle aspiration biopsy in patients with upper gastrointestinal tract malignancies. A prospective study. *Endoscopy* 2001; **33**: 478-483 [PMID: 11437039 DOI: 10.1055/s-2001-14966]
- 84 **Varadarajulu S**, Wallace MB. Applications of endoscopic ultrasonography in pancreatic cancer. *Cancer Control* 2004; **11**: 15-22
- 85 **Dewitt J**, Devereaux BM, Lehman GA, Sherman S, Imperiale TF. Comparison of endoscopic ultrasound and computed tomography for the preoperative evaluation of pancreatic cancer: a systematic review. *Clin Gastroenterol Hepatol* 2006; **4**: 717-725; quiz 664 [PMID: 16675307 DOI: 10.1016/j.cgh.2006.02.020]
- 86 **Yasuda K**, Mukai H, Nakajima M, Kawai K. Staging of pancreatic carcinoma by endoscopic ultrasonography. *Endoscopy* 1993; **25**: 151-155 [PMID: 8491131 DOI: 10.1055/s-2007-1010274]
- 87 **Brugge WR**. Pancreatic cancer staging. Endoscopic ultrasonography criteria for vascular invasion. *Gastrointest Endosc Clin N Am* 1995; **5**: 741-753 [PMID: 8535622]
- 88 **Kala Z**, Válek V, Hlavsa J, Hana K, Vánová A. The role of CT and endoscopic ultrasound in pre-operative staging of pancreatic cancer. *Eur J Radiol* 2007; **62**: 166-169 [PMID: 17344007 DOI: 10.1016/j.ejrad.2007.01.039]
- 89 **Soriano A**, Castells A, Ayuso C, Ayuso JR, de Caralt MT, Ginès MA, Real MI, Gilabert R, Quintó L, Trilla A, Feu F, Montanyà X, Fernández-Cruz L, Navarro S. Preoperative staging and tumor resectability assessment of pancreatic cancer: prospective study comparing endoscopic ultrasonography, helical computed tomography, magnetic resonance imaging, and angiography. *Am J Gastroenterol* 2004; **99**: 492-501 [PMID: 15056091 DOI: 10.1111/j.1572-0241.2004.04087.x]
- 90 **Rösch T**, Dittler HJ, Strobel K, Meining A, Schusdziaara V, Lorenz R, Allescher HD, Kassem AM, Gerhardt P, Siewert JR, Höfler H, Classen M. Endoscopic ultrasound criteria for vascular invasion in the staging of cancer of the head of the pancreas: a blind reevaluation of videotapes. *Gastrointest Endosc* 2000; **52**: 469-477 [PMID: 11023562 DOI: 10.1067/mge.2000.106682]
- 91 **Pei Q**, Zou X, Zhang X, Chen M, Guo Y, Luo H. Diagnostic value of EUS elastography in differentiation of benign and malignant solid pancreatic masses: a meta-analysis. *Pancreatology* 2012; **12**: 402-408 [PMID: 23127527 DOI: 10.1016/j.pan.2012.07.013]
- 92 **Mei M**, Ni J, Liu D, Jin P, Sun L. EUS elastography for diagnosis of solid pancreatic masses: a meta-analysis. *Gastrointest Endosc* 2013; **77**: 578-589 [PMID: 23199646 DOI: 10.1016/j.gie.2012.09.035]
- 93 **Kongkam P**, Lakananurak N, Navicharn P, Chantarojanasiri T, Aye K, Ridditid W, Kritisin K, Angsuwatcharakon P, Aniwat S, Pittayanon R, Sampatanukul P, Treeprasertsuk S, Kullavanijaya P, Rerknimitr R. Combination of EUS-FNA and elastography (strain ratio) to exclude malignant solid pancreatic lesions: A prospective single-blinded study. *J Gastroenterol Hepatol* 2015; **30**: 1683-1689 [PMID: 26238152 DOI: 10.1111/jgh.13067]
- 94 **Fusaroli P**, Napoleon B, Gincul R, Lefort C, Palazzo L, Palazzo M, Kitano M, Minaga K, Caletti G, Lisotti A. The clinical impact of ultrasound contrast agents in EUS: a systematic review according to the levels of evidence. *Gastrointest Endosc* 2016; **84**: 587-596. e10 [PMID: 27311654 DOI: 10.1016/j.gie.2016.06.006]
- 95 **Reicher S**, Boyar FZ, Albitar M, Sulcova V, Agersborg S, Nga V, Zhou Y, Li G, Venegas R, French SW, Chung DS, Stabile BE, Eysselein VE, Anguiano A. Fluorescence in situ hybridization and K-ras analyses improve diagnostic yield of endoscopic ultrasound-guided fine-needle aspiration of solid pancreatic masses. *Pancreas* 2011; **40**: 1057-1062 [PMID: 21705950 DOI: 10.1097/MPA.0b013e3182200201]
- 96 **Kubiliun N**, Ribeiro A, Fan YS, Rocha-Lima CM, Sleeman D, Merchan J, Barkin J, Levi J. EUS-FNA with rescue fluorescence in situ hybridization for the diagnosis of pancreatic carcinoma in patients with inconclusive on-site cytopathology results. *Gastrointest Endosc* 2011; **74**: 541-547 [PMID: 21752364 DOI: 10.1016/j.gie.2011.04.043]
- 97 **Fuccio L**, Hassan C, Laterza L, Correale L, Pagano N, Bocus P, Fabbri C, Maimone A, Cennamo V, Repici A, Costamagna G, Bazzoli F, Larghi A. The role of K-ras gene mutation analysis in EUS-guided FNA cytology specimens for the differential diagnosis of pancreatic solid masses: a meta-analysis of prospective studies. *Gastrointest Endosc* 2013; **78**: 596-608 [PMID: 23660563 DOI: 10.1016/j.gie.2013.04.162]
- 98 **Rodriguez SA**, Impey SD, Pelz C, Enestvedt B, Bakis G, Owens M, Morgan TK. RNA sequencing distinguishes benign from malignant pancreatic lesions sampled by EUS-guided FNA. *Gastrointest Endosc* 2016; **84**: 252-258 [PMID: 26808815 DOI: 10.1016/j.gie.2016.01.042]
- 99 **Brand RE**, Adai AT, Centeno BA, Lee LS, Rateb G, Vignesh S, Menard C, Wiechowska-Kozłowska A, Böldys H, Hartleb M, Sanders MK, Munding JB, Tannapfel A, Hahn SA, Stefańczyk L, Tsongalis GJ, Whitcomb DC, Conwell DL, Morisset JA, Gardner TB, Gordon SR, Suriawinata AA, Lloyd MB, Wylie D, Labourier E, Andruss BF, Szafranska-Schwarzbach AE. A microRNA-based test improves endoscopic ultrasound-guided cytologic diagnosis of pancreatic cancer. *Clin Gastroenterol Hepatol* 2014; **12**: 1717-1723 [PMID: 24662333 DOI: 10.1016/j.cgh.2014.02.038]
- 100 **Kongkam P**, Pittayanon R, Sampatanukul P, Angsuwatcharakon P, Aniwat S, Prueksapanich P, Sriuranpong V, Navicharn P, Treeprasertsuk S, Kullavanijaya P, Rerknimitr R. Endoscopic ultrasound-guided needle-based confocal laser endomicroscopy for diagnosis of solid pancreatic lesions (ENES): a pilot study. *Endosc Int Open* 2016; **4**: E17-E23 [PMID: 26793780]
- 101 **Nakai Y**, Isayama H, Chang KJ, Yamamoto N, Mizuno S, Mohri D, Kogure H, Matsubara S, Tada M, Koike K. A pilot study of EUS-guided through-the-needle forceps biopsy (with video). *Gastrointest Endosc* 2016; **84**: 158-162 [PMID: 26772889 DOI: 10.1016/j.gie.2015.12.033]

P- Reviewer: Fabbri C S- Editor: Qi Y L- Editor: A
E- Editor: Wang CH



Update on the endoscopic treatments for achalasia

Dushant S Uppal, Andrew Y Wang

Dushant S Uppal, Andrew Y Wang, Division of Gastroenterology and Hepatology, University of Virginia Health System, Charlottesville, VA 22908, United States

Author contributions: Uppal DS and Wang AY contributed equally to this manuscript.

Conflict-of-interest statement: The authors have no conflicts of interest, financial or otherwise, to report with respect to this manuscript. Dr. Wang discloses that he has received research funding from Cook Medical on the topic of metal biliary stents.

Open-Access: This article is an open-access article which was selected by an in-house editor and fully peer-reviewed by external reviewers. It is distributed in accordance with the Creative Commons Attribution Non Commercial (CC BY-NC 4.0) license, which permits others to distribute, remix, adapt, build upon this work non-commercially, and license their derivative works on different terms, provided the original work is properly cited and the use is non-commercial. See: <http://creativecommons.org/licenses/by-nc/4.0/>

Manuscript source: Invited manuscript

Correspondence to: Andrew Y Wang, MD, AGAF, FACG, FASGE, Associate Professor of Medicine, Section Chief of Interventional Endoscopy, Division of Gastroenterology and Hepatology, University of Virginia Health System, Box 800708, Charlottesville, VA 22908, United States. ayw7d@virginia.edu
Telephone: +1-434-9241653
Fax: +1-434-2447590

Received: March 28, 2016
Peer-review started: March 29, 2016
First decision: May 12, 2016
Revised: August 20, 2016
Accepted: September 14, 2016
Article in press: September 14, 2016
Published online: October 21, 2016

Abstract

Achalasia is the most common primary motility disorder of the esophagus and presents as dysphagia to solids and liquids. It is characterized by impaired deglutitive

relaxation of the lower esophageal sphincter. High-resolution manometry allows for definitive diagnosis and classification of achalasia, with type II being the most responsive to therapy. Since no cure for achalasia exists, early diagnosis and treatment of the disease is critical to prevent end-stage disease. The central tenant of diagnosis is to first rule out mechanical obstruction due to stricture or malignancy, which is often accomplished by endoscopic and fluoroscopic examination. Therapeutic options include pneumatic dilation (PD), surgical myotomy, and endoscopic injection of botulinum toxin injection. Heller myotomy and PD are more efficacious than pharmacologic therapies and should be considered first-line treatment options. Per oral endoscopic myotomy (POEM) is a minimally-invasive endoscopic therapy that might be as effective as surgical myotomy when performed by a trained and experienced endoscopist, although long-term data are lacking. Overall, therapy should be individualized to each patient's clinical situation and based upon his or her risk tolerance, operative candidacy, and life expectancy. In instances of therapeutic failure or symptom recurrence re-treatment is possible and can include PD or POEM of the wall opposite the site of prior myotomy. Patients undergoing therapy for achalasia require counseling, as the goal of therapy is to improve swallowing and prevent late manifestations of the disease rather than to restore normal swallowing, which is unfortunately impossible.

Key words: Per oral endoscopic myotomy; Dilation; Achalasia; Treatment; Endoscopy; Myotomy; Per oral

© **The Author(s) 2016.** Published by Baishideng Publishing Group Inc. All rights reserved.

Core tip: Achalasia can be classified into three subtypes based on high-resolution manometry, with type 2 being the most responsive to therapy. Since no cure for achalasia exists, early diagnosis and treatment of the disease are critical. Pre-treatment counseling is paramount, as the goal of therapy is to improve swallowing and prevent late manifestations of the

disease, rather than to restore normal swallowing and function. Pneumatic dilation and surgical or endoscopic myotomy are efficacious and reasonable first-line treatment options in appropriate candidates. In instances of therapeutic failure or symptom recurrence, different treatment modalities might need to be applied.

Uppal DS, Wang AY. Update on the endoscopic treatments for achalasia. *World J Gastroenterol* 2016; 22(39): 8670-8683 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v22/i39/8670.htm> DOI: <http://dx.doi.org/10.3748/wjg.v22.i39.8670>

INTRODUCTION

Achalasia, a disease first described by Sir Thomas Willis in 1674 and formally named by Dr. Hertz in 1915^[1], is the most common primary motility disorder of the esophagus. It is characterized by impaired deglutitive relaxation of the lower esophageal sphincter (LES) due to loss of the myenteric plexus^[2]. While the inciting mechanism is not fully understood, autoimmune, viral immune, or neurodegenerative etiologies have been implicated^[3]. It has been speculated that achalasia arises from a cascade of neuronal degeneration that is predicated on a viral infection triggering an autoimmune process in a genetically susceptible host^[4]. An imbalance between excitatory and inhibitory neurotransmitters then leads to unopposed cholinergic stimulation thereby impairing LES relaxation. The disease has an estimated annual incidence of 1.6 in 100000 and a prevalence of 10.8 in 100000, with a peak age range between 30 and 60 years^[4]. There is no racial or gender disparity in those with the condition^[4].

Achalasia should be suspected in patients experiencing dysphagia to solids and liquids, and it is often accompanied by regurgitation of undigested food and saliva. The predominant symptom of progressive dysphagia to liquids and solids occurs in approximately 90% of patients with achalasia, and 76% to 91% of patients experience the next most common symptom of regurgitation^[5-7]. Other symptoms including nocturnal aspiration or cough, heartburn, odynophagia, and epigastric pain occur to a variable extent^[7]. Chest pain, seen in 25% to 64% of patients, is predominantly present in type III achalasia and generally responds less well to treatment than other symptoms, such as dysphagia and regurgitation^[8].

The symptoms associated with achalasia are non-specific, which can lead to long delays between symptom onset and diagnosis (sometimes taking up to 5 years to make the diagnosis)^[9,10]. Similarly, patients without achalasia can also present with symptoms of dysphagia, regurgitation, recurrent aspiration, and chest pain in the setting of prior lap-band surgery, fundoplication, or pseudoachalasia - a syndrome that can involve malignant infiltration of

the area encompassing the gastroesophageal junction (GEJ). Approximately 2% to 4% of patients suspected of having achalasia suffer from pseudoachalasia, although these patients tend to be older and have a shorter history of symptoms with more prominent weight loss^[11]. When pseudoachalasia is suspected, expeditious evaluation for an infiltrative malignancy should be undertaken *via* endoscopy and endoscopic ultrasonography or cross-sectional imaging. Certain paraneoplastic syndromes can give rise to pseudoachalasia, and in cases of suspected small-cell lung carcinomas checking for type-1 antineuronal nuclear autoantibody (ANNA-1, also known as "anti-Hu" antibody) can sometimes be diagnostic^[12]. Lastly, Chagas disease, which is caused by infection with *Trypanosoma cruzi*, can also result in achalasia, in addition to diffuse myenteric destruction, that can manifest as megacolon, heart disease, and neurologic disorders^[3].

While eosinophilic esophagitis (EoE) can present as dysphagia in the absence of mechanical obstruction, achalasia should be easily distinguishable from EoE by endoscopic and manometric findings. Furthermore, if esophageal biopsies are required to exclude EoE as a possible diagnosis, care should be taken to avoid deep or extensive biopsies along the anterior (1 to 2 o'clock) or posterior walls (5 to 7 o'clock) of the mid-to-distal esophagus so as to prevent the formation of submucosal fibrosis, which can make future surgical or endoscopic myotomy more difficult.

DIAGNOSIS

In patients with dysphagia and suspected achalasia, radiographic and endoscopic modalities should be utilized to exclude mechanical causes of dysphagia and to evaluate for GEJ narrowing or hypertonicity and esophageal dilation, which can support the diagnosis of achalasia. It should be noted that in early achalasia, both barium studies and endoscopy may be normal. However, in later stages, barium swallow may be characterized by the classic "bird's beak" appearance, whereby a dilated esophagus tapers significantly at the GEJ (Figure 1). An associated air-fluid level in the esophagus and an absent gastric bubble are other fluoroscopic clues of possible achalasia. Not only is a barium esophagram important in defining the morphology of the esophagus, but it can also be used as a functional test whereby an upright esophagram can be performed to measure esophageal emptying times over a 5-min period or longer^[13,14].

Endoscopy is important in the diagnostic workup of patients with achalasia, as it can rule out luminal malignancies of the esophagus and proximal stomach, which can also cause symptoms of dysphagia and weight loss. On endoscopic evaluation of patients with achalasia, static food and fluid are frequently found in an esophagus that is dilated proximal to the GEJ. Furthermore, a careful endoscopist can also evaluate for increased tone of the LES. In patients with "classic"

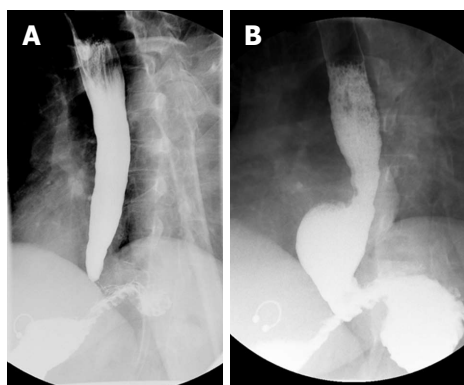


Figure 1 Barium esophagram of a patient before and several months after per oral endoscopic myotomy. A 45-year-old woman with type II achalasia underwent per oral endoscopic myotomy (POEM). The pre-procedural barium esophagram (A, left panel) demonstrated a dilated esophagus with tapering at the gastroesophageal junction. Following POEM the patient gained 32 lbs over a 9-mo period but had occasional symptoms of regurgitation, which we suspected was from eating too much too quickly. Repeat barium esophagram showed the distal tapering of the gastroesophageal junction has resolved and there was immediate and unimpeded passage contrast passage into the stomach (B, right panel). A distal esophageal diverticulum was incidentally found, which can be seen in patients following POEM with a complete myotomy of the distal esophagus that is carried across the lower esophageal sphincter and into the gastric cardia.

achalasia, a diagnostic gastroscopist can encounter moderate resistance at the LES and GEJ. With steady pressure and advancement of the gastroscopist, ultimately the scope will pass into the stomach leading to a sensation of “giving way” or a “pop.” Despite this “resistance” to passage of the gastroscopist, achalasia is characterized by the absence of any mass, stricture, or mechanical obstruction. In patients with achalasia, stasis of luminal contents in the esophagus has also been implicated in development of esophagitis and might play a role in the 7-to-140-fold increased risk of subsequent esophageal adenocarcinoma, further supporting the need for endoscopic evaluation and possible screening in these patients^[7].

Manometry remains the gold standard for diagnosis of achalasia and offers diagnostic accuracy in greater than 90% of cases^[15]. The procedure involves the placement of a flexible pressure catheter through the nose, into the esophagus, and across the GEJ. Prior conventional manometric line tracings have now been supplanted by high-resolution manometry (HRM) that presents pressure data in the context of esophageal-pressure-topography plots^[4]. HRM metrics using Clouse plots led the way to the variant descriptions of achalasia subtypes in the Chicago classification system^[16-20]. The manometric finding of aperistalsis and incomplete LES relaxation in a patient without mechanical obstruction of the esophagus supports the diagnosis of achalasia in the appropriate clinical situation^[3]. On HRM, impaired GEJ relaxation (or incomplete LES relaxation) is characterized by mean 4-second integrated relaxation pressure (IRP) of ≥ 10 to 15 mmHg^[21].

SUBTYPES OF ACHALASIA

The Chicago classification is a currently accepted system that separates “classic” achalasia into 3 clinically relevant subtypes^[21]. The manometric findings common to all types of achalasia include impaired relaxation of the LES (residual pressure or IRP of ≥ 10 mmHg) and absent peristalsis in a patient without mechanical obstruction near the LES^[3,18-20]. The variant types of achalasia are further defined as follows^[21]: Type I: has 100% absent peristalsis and no significant esophageal pressurization; Type II: has 100% absent peristalsis with $\geq 20\%$ of swallows with pan-esophageal pressurization to > 30 mmHg (Figure 2); Type III: has $\geq 20\%$ of swallows with premature spastic contractions (distal latency of < 4.5 s).

These classifications are not only descriptive, but they also have useful prognostic and therapeutic implications. Patients with type II achalasia have the best prognosis with 96% of patients reporting symptomatic improvement following treatment with myotomy or pneumatic dilation (PD). Patients with type I achalasia have response rates of around 81%, as these patients probably have late-stage disease with complete loss of inhibitory neurons. Patients with type III achalasia have the worst treatment response, estimated at around 66%, and this entity might actually represent a distinct entity unrelated to loss of inhibitory neurons^[8,21].

TREATMENT

Early diagnosis to prevent end-stage disease should be the primary goal in the management of achalasia, as no curative therapies exist for this problem. Two percent to five percent of patients with achalasia will develop manifestations of end-stage disease, characterized by massive dilation and a sigmoid-like appearance of the esophagus - the so-called “mega-esophagus,” which can necessitate esophagectomy^[22]. Encouragingly, various treatments are available to alleviate dysphagia, restore lost weight, and combat progression to later-stage disease. Traditional therapeutic options have included surgical myotomy, PD, and pharmacologic therapy. However, novel, less-invasive, endoscopic myotomy techniques have also been demonstrated to be efficacious in expert centers. These therapies, which should be individualized to each patient’s symptoms, age, and co-morbidities, are primarily aimed at disrupting the dysfunctional deep muscle of the distal esophageal and LES in order to permit the passive passage of food.

When considering an interventional procedure, it is also important to determine how clinical response can be gauged. In the case of treating achalasia, in addition to pre- and post-intervention barium and manometric studies, the Eckardt score offers a quantitative way to assess the clinical symptomatology of patients before and after achalasia therapy^[23,24].

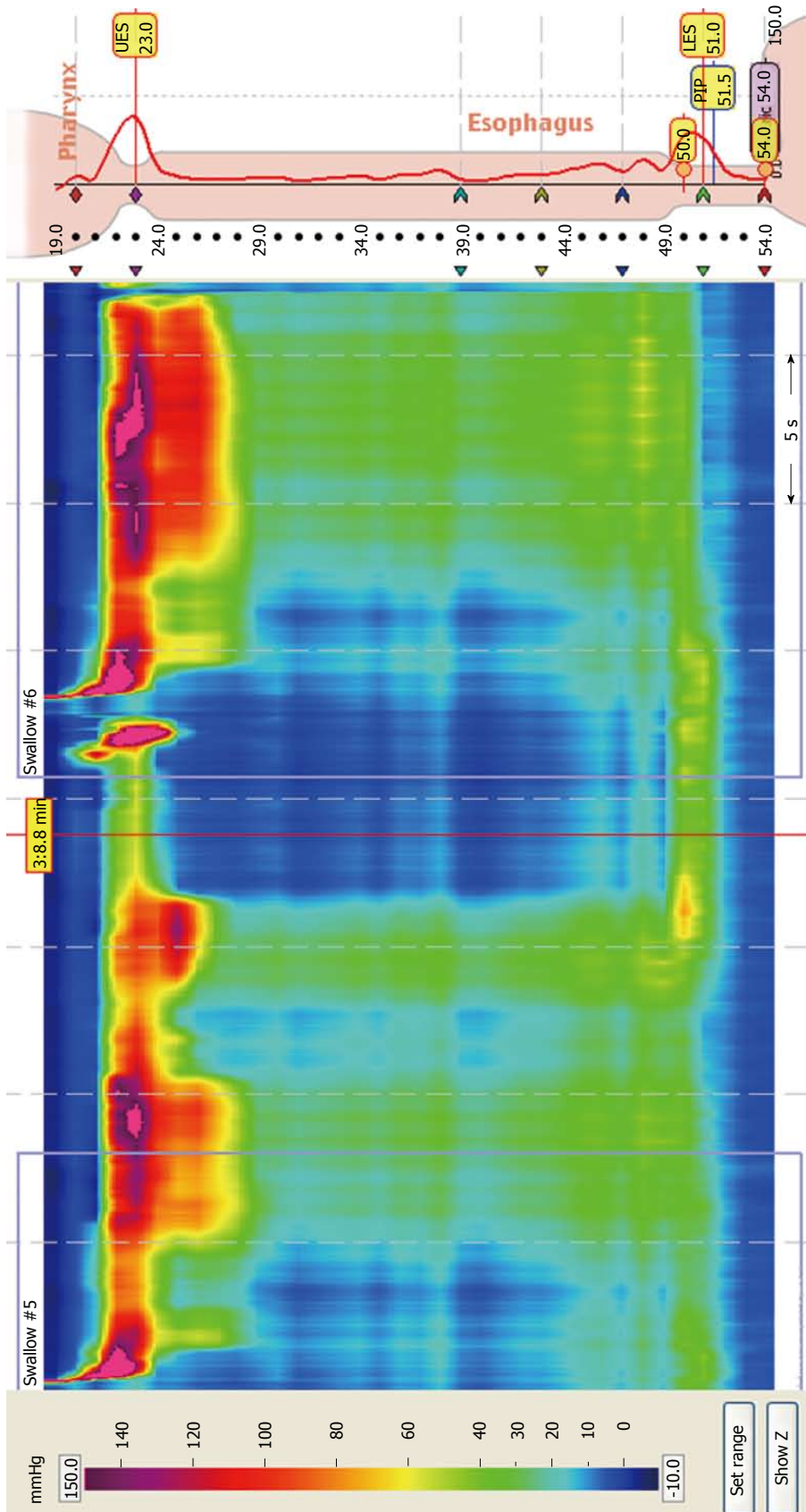


Figure 2 High-resolution esophageal manometry of a patient with achalasia. A 34-year-old man with progressive dysphagia to liquids and solids had a barium esophagram suggestive of achalasia. He presented for high-resolution manometry to confirm the diagnosis of achalasia. The manometric and topographic findings demonstrated aperistalsis with residual pressure at the lower esophageal sphincter with an elevated integrated relaxation pressure and pan-esophageal pressurization, which was consistent with type II achalasia by the Chicago classification.

Heller myotomy

Surgical myotomy was first performed and described by Heller in 1914 as a trans-abdominal extramucosal cardioplastic performed onto the anterior and posterior walls of the cardia^[1]. The procedure was widely adopted due to its success. In 1962, Dor proposed the "technique de Heller-Nissen modifiée" for the treatment of reflux esophagitis associated with cardiospasm, thereby providing relief of dysphagia while limiting gastroesophageal reflux disease (GERD)^[1]. When performed, a concomitant Dor procedure is preferred to a complete Nissen fundoplication at the time of Heller myotomy given a lower respective rate of recurrent dysphagia (2.8% vs 15%, $P <$

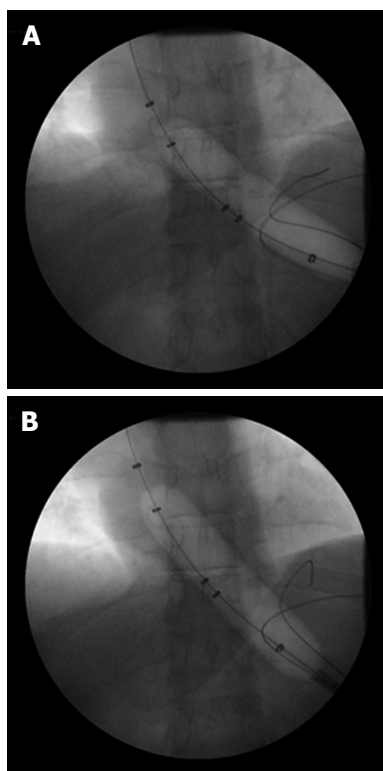


Figure 3 Pneumatic balloon dilation of the lower esophageal sphincter in a patient with achalasia. A 44-year-old man with type II achalasia underwent pneumatic dilation of the lower esophageal sphincter (LES) under fluoroscopic guidance to 30 mm in diameter. The pneumatic balloon catheter was passed across the LES over a wire and then inflated with initial evidence of a waist in the mid-portion of the balloon (A); The balloon was kept inflated until the waist in the balloon was obliterated (B). A barium esophagram done immediately following pneumatic dilation showed no evidence of perforation.

0.001)^[25]. The operation has evolved to the procedure introduced by Pellegrini in 1992, in which a minimally invasive single anterior myotomy is performed *via* a laparoscopic approach through the abdomen^[26]. The laparoscopic Heller myotomy (LHM) is currently the standard surgical approach for achalasia, due to the procedure's lower morbidity and relatively similar long-term outcomes when compared with the more invasive thoracotomy approach^[27], with rates of efficacy ranging from 88% to 95%^[4,27]. Presently, several centers, including our own, offer robot-assisted LHM with excellent clinical results.

While the reported rates of clinical success with LHM have been consistently high, persistent or recurrent dysphagia does occur after LHM, which is usually due to an incomplete myotomy. Surgical expertise is an important predictor of good clinical outcomes, as more failures and complications occur during a surgeon's first 50 operations^[28]. Complications from LHM include GERD and its sequelae (18%), need to convert to an open procedure (2%), and mucosal perforations (6.9%), although perforations are frequently identified and closed at the time of operation^[27]. Nevertheless, given the potential morbidity and the cost of the operation (\$44839 in US Dollars for professional fees

and facility charges for LHM with fundoplasty, when performed^[29]), alternative, and less invasive therapies have been pursued particularly in patients with medical co-morbidities that might preclude surgery^[30].

PD

PD disrupts the LES by forceful stretching by using large-diameter, non-compliant, dilating balloon catheters. Commercially available PD balloons include the Rigidflex II system (Boston Scientific, Natick, MA, United States) and the Achalasia Balloon Dilators (Hobbs Medical, Stafford Springs, CT, United States) that are available in 30 mm, 35 mm, and 40 mm diameters. The Achalasia Balloon (Cook Medical, Winston Salem, NC, United States) comes in 30 mm and 40 mm diameters. Other pneumatic dilating balloons are available outside the United States (Olympus Europa, Hamburg, Germany). With the aid of radio-opaque markers, the deflated balloon is positioned under fluoroscopic or endoscopic guidance across the LES^[31]. The balloon is then gradually inflated until a fluoroscopic waist is identified across the mid-portion of the balloon that is subsequently obliterated by further dilation (Figure 3). Although this procedure has been widely performed for decades^[32], no consensus exists with respect to the optimal distention protocol. Some operators only perform a single dilation^[33], although most use graded dilations starting at 30 mm and then increasing in size on subsequent sessions every 2 to 4 wk based on symptom relief that is correlated with LES pressure^[34,35]. Kadakia *et al*^[35] reported an overall success rate of 93% (in 27 of 29 patients with achalasia) undergoing graded dilation from 30 mm to 40 mm with a Rigidflex balloon. Disparate data on success rates have been reported, ranging from 35% to 85% over several years of follow-up^[36-41]. Over a mean follow-up period of 2.4 years, Dobrucali *et al*^[31] observed a success rate, defined as improvement in overall symptoms, of 56% in (24 of 43) patients with achalasia who were pneumatically dilated with a 30-mm balloon. Subsequent dilation with a 35-mm balloon resulted in improvement in 78% of the remaining patients who did not have a good initial response. However, only 54% of patients with follow-up data available at 5 years were symptom-free. Nevertheless, graded dilation appeared to be more effective in achieving symptom relief over 3 years (86% response rate) as compared to a single 30-mm balloon dilation (37% response rate)^[42].

Predictors of poor response to PD include younger age (< 40 years) at presentation^[42] and a shorter duration of symptoms prior to treatment^[43]. Two of the most important factors in predicting the need for retreatment include incomplete obliteration of the waist of the balloon and post-treatment elevated LES pressures. Incomplete balloon-waist obliteration was reported to result in 5- and 10-year symptom recurrence rates of 64% and 72%, respectively. A 3-mo post-treatment LES pressure above 10 mmHg was

associated with 5- and 10-year symptom recurrence rates of 25% and 33%, respectively^[36,43]. Success also appeared to be predicated on the treatment being provided by experienced clinicians^[38].

Overall, patients with characteristics that predict a poor outcome from PD should probably consider other options such as surgical or endoscopic myotomy. While rates of symptom improvement in a select group of patients following repeated PD for achalasia as high as 96.8% at 5 years and 93.4% at 10 years have been reported^[41], many studies demonstrate that the treatment effect and symptom remission decreases over time^[36,37,40]. Therefore, younger patients might be better managed with surgical or endoscopic myotomy, in light of possible diminishing returns with repeated PD^[40].

Botulinum toxin injection

In patients with significant comorbidities who may not be good candidates for surgical or endoscopic myotomy, or even PD, pharmacologic therapies may be offered for symptomatic management of achalasia, although the efficacy of such an approach is typically limited. Endoscopy-directed botulinum toxin (BT) injection delivers pharmacologic therapy to the LES by blocking acetylcholine release from local nerve endings, thereby reducing LES tonicity^[4]. Typically, 100 U of commercially available BT powder is dissolved in 5 mL of sterile normal saline, and this solution is injected in divided doses into the muscularis propria of the GEJ. Our practice is to perform four quadrant injections (each containing 20 U of BT in 1 mL of solution) from the distal esophagus and one final injection (20 U of BT in 1 mL of solution) from the gastric cardia using a retroflexed scope position.

Cuillière *et al*^[44] reported significant improvement in LES pressures after treatment and an improved symptom score at 2 wk, 2 mo, and 6 mo when compared with pretreatment values ($P < 0.001$). Of 31 patients treated with endoscopically-delivered BT injection to the LES, 28 improved initially, although sustained response was seen in only 20 patients beyond 3 mo with general response duration averaging 1.3 years^[45]. The response rate tended to be greater in patients older than 50 years of age (82% vs 43% in younger patients, $P = 0.03$) and in patients with vigorous (type III) achalasia (100% vs 52% with classic achalasia, $P = 0.03$). The effect of "Botox" injection tends to be temporary due to axonal regeneration, with most studies demonstrating minimal clinical efficacy after 1 year^[14,45,46]. Furthermore, repeated BT treatments can induce submucosal fibrosis of the esophagus and GEJ, which can make subsequent surgical or endoscopic myotomy more difficult, resulting in an increased risk of intraoperative perforation as well as a higher rate of procedural failure^[47]. Given the above, we do not recommend empiric BT injections as a diagnostic test for achalasia.

Comparison of traditional therapies

The efficacies of the aforementioned conventional therapeutic modalities have been evaluated against one another. When considering the efficacy of LHM vs PD, several factors must be taken into account including the patient's age, comorbid diseases that may preclude surgery or make complications (if they occur) potentially fatal, and prior therapies. Patients undergoing PD should be made aware of the possibility of perforation (overall median rate in experienced hands of 1.9% with a range of 0% to 16%)^[3], as this complication would require inpatient observation, and could necessitate emergency esophageal stenting or even emergency surgery (including possible esophagectomy).

Okike *et al*^[32] published one of the earliest and largest experiences comparing esophagomyotomy to forceful dilation of the esophagus for treatment of achalasia. Between 1949 and 1976, 431 patients underwent forceful hydrostatic or PD and 468 patients had an open transthoracic esophagomyotomy. Esophageal leaks and mediastinal sepsis occurred in 4% following dilation as compared to in 1% of patients following surgical myotomy, although no deaths resulted from these complications in either group. The 30-d mortality was 0.2% after myotomy and 0.5% after forceful dilation. The long-term results of esophagomyotomy were significantly better than those for forceful dilation ($P < 0.001$). Furthermore, 16% of patients in the dilation group were dilated twice and 2% required dilation three or more times.

Gockel *et al*^[11] followed 89 patients diagnosed with achalasia between 1998 and 2002 who underwent either PD (64 patients) or Heller myotomy (25 patients) in combination with an anterior semi-fundoplication (Dor procedure) and demonstrated more markedly improved LES resting pressures on manometry at 6 mo in patients undergoing myotomy as compared to PD (7.9 mmHg vs 14.5 mmHg, $P < 0.0001$), suggesting the surgical approach to be superior.

When patient characteristics are favorable for PD, the clinical utility and cost of this procedure appear to be beneficial relative to surgery^[26,30,39]. A cost analysis performed by Parkman in 1993 suggested myotomy to be 5 times more costly than PD^[39]. Moreover, even though the clinical benefit of repeat PD decreased over time, LHM remained at least 2.4 times greater in cost than treating with an initial PD. Using a Markov model, O'Connor *et al*^[30] also demonstrated the overall favorable cost-effectiveness of PD compared to surgical myotomy over 5 years, which was driven by the high initial cost of surgery (incremental cost of \$5376750 per quality-adjusted life-year, QALY).

In an important study published in 2011, Boeckxstaens *et al*^[48] randomized 201 patients with achalasia to PD ($n = 95$) or LHM with a Dor fundoplication ($n = 106$) and reported a mean follow-up period of 43 mo. In an intention-to-treat analysis, there was no difference between the groups in the primary outcome

of therapeutic success (as defined by a drop in the Eckardt quality-of-life score to ≤ 3) at 1 year (90% for PD vs 93% for LHM, $P = 0.46$). Similarly, no difference was found between the groups in decreased LES pressures at 1 year (10 mmHg for LHM vs 12 mmHg for PD, $P = 0.27$), which led the authors to conclude that LHM when compared to PD was not associated with superior rates of therapeutic success. Five-year follow-up data from the initial study demonstrated no significant difference in success rates between PD and LHM (82% vs 84%, respectively, $P = 0.92$), although re-dilation was necessary in 25% of patients undergoing PD^[49]. This study reported that esophageal perforation occurred in 4% of the patients during PD, and mucosal tears occurred in 12% during LHM. In a smaller randomized study published in 2015, patients with early-stage achalasia who were randomized to LHM had a 96% rate of symptom relief vs 76% in patients who underwent PD ($P = 0.04$)^[50]. This study reported comparable rates of adverse events as esophageal perforation occurred in 8% of patients during PD, whereas mucosal tears occurred in 4% of the patients during LHM.

As previously mentioned, the appropriate initial therapy for achalasia should be dictated by a patient's clinical parameters, risk tolerance, and by locally available expertise. Although the risk of perforation with PD has variably been reported between 0% to 16%^[3], Lynch *et al.*^[51] reported perforation in only 1 of 272 PD procedures (0.37%) over 12 years compared with 6 of 295 LHM procedures (2%) over a similar time period. There were no deaths in the PD group. These authors concluded that in high-volume centers PD had lower rates of complications and death compared with LHM. It should also be noted that both LHM and PD may result in post-treatment GERD, but only LHM can offer a simultaneous adjunctive procedure at the time of surgery to limit postprocedural reflux.

Studies comparing PD to BT injection have demonstrated improved clinical efficacy of PD in terms of immediate response and duration of response^[14,52,53]. PD also tends to be more effective than BT from a cost-effectiveness standpoint^[54] with an incremental cost-effectiveness ratio of \$1348 per QALY^[30]. In a study comparing LHM to BT for symptom improvement in patients with achalasia, Zaninotto *et al.*^[46] randomized 40 patients to BT injection and 40 patients to LHM. At 6-mo follow-up, symptom scores were significantly better in the patients undergoing LHM compared with those undergoing BT injection (82% vs 66%, $P < 0.05$). The probability of being symptom-free at 2 years was 87.5% after surgery vs 34% after BT ($P < 0.05$). Despite being easy to perform and possessing a good safety profile, the limited clinical efficacy of BT injection to durably treat achalasia should limit its use to elderly patients who are considered to be unfit for surgery or as a salvage therapy following failure of other therapeutic modalities.

PER ORAL ENDOSCOPIC MYOTOMY

In an effort to minimize the potential complications and invasiveness of surgery and to improve upon the clinical efficacy of non-surgical therapy for achalasia, per oral endoscopic myotomy (POEM) was conceived of by Pasricha in 2007 and performed in a porcine model^[55]. Baseline LES pressures were measured in 4 pigs who subsequently underwent upper endoscopy. A submucosal saline injection was made 5 cm above the LES followed by a small entry incision in the mucosa to facilitate introduction of a dilating balloon and creation of a submucosal tunnel. The scope was then advanced towards the LES and circular deep muscle fibers were then incised using an electrosurgical knife. The endoscope was then withdrawn back into the lumen of the esophagus and the defect was closed using endoscopically applied clips. Manometry repeated on day 5 after the procedure demonstrated the LES pressures to have decreased from an average of 16.4 mmHg prior to myotomy to an average of 6.7 mmHg following myotomy.

Based on his expertise in endoscopic submucosal dissection (ESD), Inoue *et al.*^[56] believed that this procedure could be safely performed in patients, and he performed POEM in 17 consecutive patients with achalasia (10 men and 7 women with a mean age 41.4 years). Included in this series were 3 patients who had previously received an uncomplicated balloon dilation. A dysphagia-symptoms-score was assessed for each patient prior to and several times after the procedure to objectively gauge improvement in symptoms. A barium swallow (to evaluate the degree of esophageal dilation) and a computed tomography (CT) scan (to provide information on anatomical structures adjacent to the esophagus) were performed in all patients prior to POEM. Patients with sigmoid-type achalasia (esophagus demonstrated a "U-turn" or a "double-lumen" on CT) were initially excluded, but were later included after success with the first 5 cases. A CT scan was also obtained on the day of the procedure following POEM to evaluate for mediastinal emphysema, and a barium swallow was completed the day after POEM to confirm passage of contrast through the GEJ without leakage. Esophageal manometry was also completed prior to and after the procedure.

All 17 patients underwent successful POEM for achalasia under general anesthesia and by using carbon dioxide gas for insufflation. An anterior approach was taken for 16 of these POEM procedures. A 2-cm longitudinal mucosal incision was made in the mid-esophagus to allow a 9.8-mm diagnostic gastroscope with a distal attachment cap to gain access to the submucosal space. A Triangle Tip Knife (Olympus, Tokyo, Japan) was used to dissect the submucosal layer and also to divide the circular muscle bundles. Endoscopic myotomy was begun 3 cm distal to the mucosal entry point and the submucosal tunnel averaged 12.4 cm in length. The myotomy of the deep

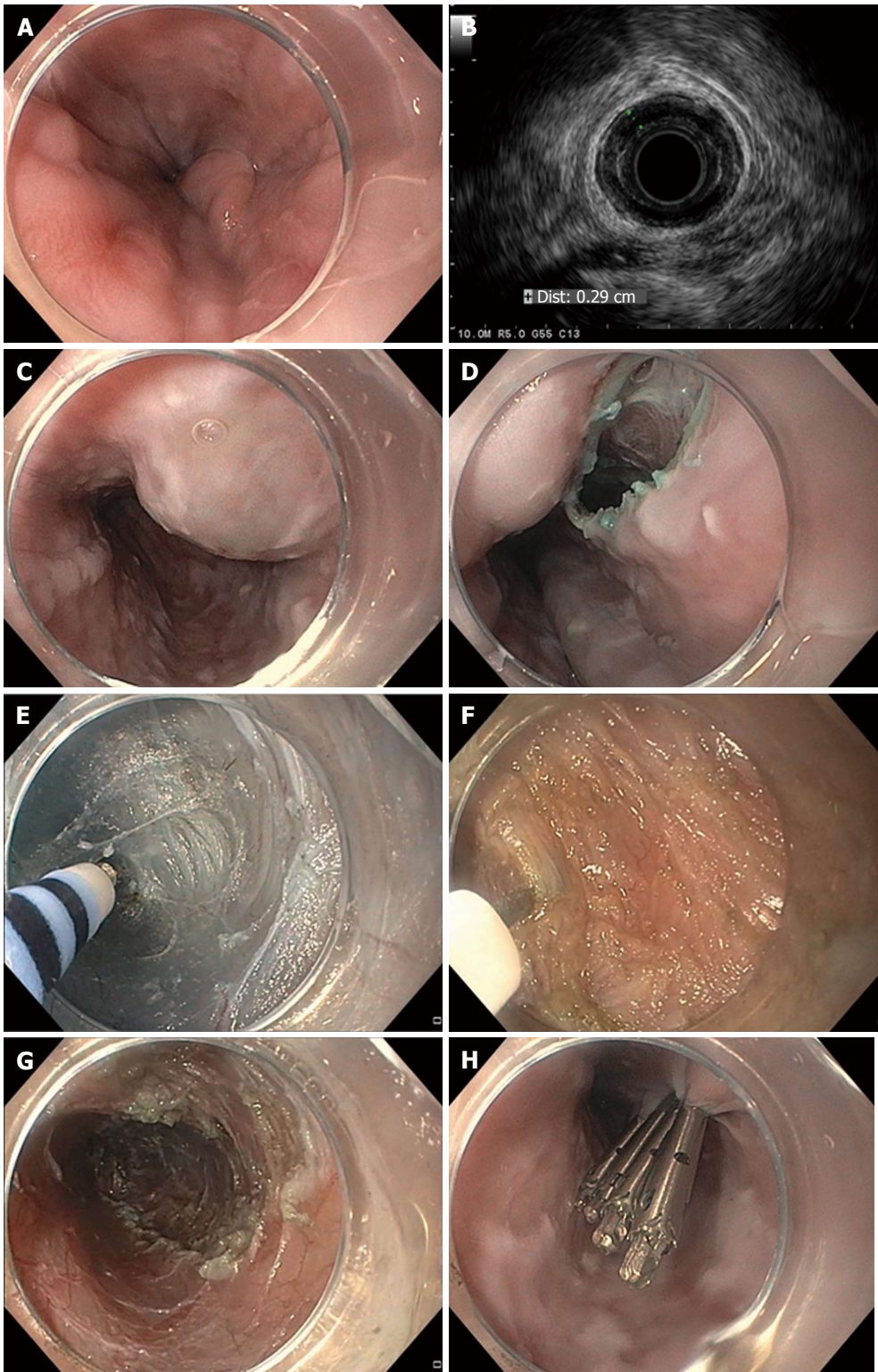


Figure 4 Per oral endoscopic myotomy in a patient with achalasia. A 56-year-old man with type II achalasia and a history of chronic alcohol use underwent attempted laparoscopic Heller myotomy. Upon retraction of the liver during surgery, large gastroesophageal varices were noted to arise and the surgery was aborted. The patient was exhorted to stop drinking alcohol. Doppler ultrasonography and magnetic resonance imaging with arterial and venous phase imaging did not show any significant gastroesophageal varices or obvious portal hypertension. Per oral endoscopic myotomy (POEM) was performed. Endoscopic views in the distal esophagus found some enlarged veins but no high-grade esophageal or gastric varices (A); Radial endosonography found a thickened deep circular muscle layer measuring 2.9 mm, which is commonly found in patients with achalasia, but no obvious esophageal varices were noted (B); A mucosal weal was created by injecting saline tinted with indigo carmine (C); and a mucosal entry incision was made (D); Submucosal dissection was carried out with sequential injection and electrosurgical dissection (E) using a T-type Hybrid knife in conjunction with an ERBEJET 2 and a VIO 300 D generator set at EndoCut Q 3-2-1 (ERBE, Marietta, GA, United States). Dissection of the circular layer of the muscularis propria was performed using the T-type Hybrid knife (F); After completion of the 7-cm-long myotomy in the distal esophagus that was carried out an additional 2 cm into the gastric cardia (G); the mucosal entry site was closed by using endoclips (H). The patient did well without any intra- or post-procedural bleeding. At clinic follow-up 2 mo later, the patient reported complete resolution of his symptoms of dysphagia and weight gain of 14 lbs.

circular muscle bundles was carried out to a distance at least 2 cm distal to the GEJ in to the proximal stomach. Minor bleeding occurred during the POEM procedures and was easily controlled by endoscopic coagulation. Once the mucosal defect was endoscopically closed with standard endoclips, substantial reduction in LES tone was confirmed by passing the endoscope through the lumen of the esophagus. Furthermore, patients' dysphagia symptom scores decreased from a mean of 10 prior to POEM to a mean of 1.3 following the procedure ($P = 0.0003$), and mean LES pressure decreased from 52.4 mmHg to 19.8 mmHg ($P = 0.0001$) after POEM^[56].

Subsequently in 2015, Inoue *et al.*^[57] published their results from a cohort of 500 patients who all underwent successful POEM. Short- and long-term follow-up was reported. These investigators found a significant reduction in Eckardt scores and LES pressures at 2 mo, 1 year, and 3 years following POEM as compared to baseline values. Adverse events were observed in 16 of 500 patients (3.2%), which included 1 pneumothorax with mediastinal emphysema, 8 mucosal injuries, 3 postoperative hematomas, 1 case of inflammation in the lesser omentum, and 2 cases of pleural effusion. GERD was the biggest delayed complication of the procedure, which was reported in 16.8% of patients at 2 mo and 21.3% at 3-year follow-up. Length of hospital stay in this series was a median of 4 d (range of 4 to 5 d) after POEM, and no perioperative mortalities occurred.

The POEM procedure, and the equipment used to perform it, continue to be refined at a number of centers of expertise around the world, with similar results and complications as those in Inoue's series having been described in subsequent series^[58-68] (Figure 4). Most endoscopists experienced with ESD no longer routinely use balloon dilation to create a submucosal tunnel, rather the tunnel is created by using electrosurgical dissection taking care to stay close to the deep muscle layer (as for a POEM, perforation is actually causing injury to the esophageal mucosa as opposed to the muscularis propria and adventitia).

The first prospective series of POEMs performed in Europe included 16 patients and achieved success in 94% of cases, with reduction in LES pressures from a mean of 27.2 mmHg prior to POEM to 11.8 mmHg following treatment. Furthermore, none of the patients subsequently developed GERD^[69]. Similar rates of overall success and complications were corroborated by the International POEM Survey (IPOEMS), which involved 16 expert centers^[70].

A meta-analysis that included 4 studies of patients with achalasia who underwent LHM vs POEM reported that POEM patients had comparable rates of complications (OR = 1.17, $P = 0.7$), postprocedural GERD (OR = 1.00, $P = 1.00$), and symptomatic recurrence by Eckardt score (OR = 0.24, $P = 0.13$), with no difference in other outcomes including pain scores, operating times, and length of hospital stays when compared to those who had LHM^[71]. In a

separate study, quality-of-life assessments based on the SF-36 instrument in patients who underwent POEM increased at 3 wk, 6 mo, and 1 year following the procedure, which was comparable to the results seen following LHM^[72]. Kumbhari *et al.*^[73] compared 49 patients with type III achalasia who underwent POEM across 8 international centers to 26 patients who underwent LHM at a single institution. Despite patients in the POEM arm receiving a longer-length myotomy (16 cm vs 8 cm, $P < 0.01$), POEM procedures were completed in a significantly shorter mean procedure time (102 min vs 264 min, $P < 0.01$), and patients who underwent POEM had significantly better clinical response (98.0% vs 80.8%, $P = 0.01$), as compared to those who underwent LHM. These authors surmised that patients with type III achalasia had a better clinical response following POEM than after LHM in this study because POEM enables a longer-length myotomy, as the endoscopic approach provides access to the esophageal body.

Furthermore, POEM was reported to have been safely performed and effective in a series of 40 consecutive patients that included 12 who had previously undergone PD or BT injection^[74]. In this study, 12 patients had undergone previous alternative therapies, while 28 patients had received no intervention prior to POEM. There was no statistical difference in the 6-mo postoperative median Eckardt scores between the two groups (1 vs 1, $P = 0.4$). Ling *et al.*^[75] also reported on 21 patients who underwent POEM following failed PD. In that series, there was no statistical difference in postprocedural Eckardt scores, LES pressures, esophageal emptying, or quality-of-life indicators in those patients who had undergone failed PD prior to POEM compared with 30 control patients who had no intervention prior to POEM. However, mean POEM operating time was longer in the failed PD group (42.4 min) compared with the control group (34.3 min, $P = 0.01$). Alternatively, in a study with 40 patients, Orenstein *et al.*^[76] reported no statistical difference in operating times, GERD metrics, and SF-12 scores between patients who had undergone no interventions prior to POEM and those who had undergone prior interventions (including BT injection, balloon dilation, or surgical myotomy). The authors did state that a different approach was necessary in patients who had failed Heller myotomy as an anterior approach would not be effective and significant scarring could be encountered. In this situation, most experts perform POEM with the mucosal entry site and subsequent myotomy along the posterior wall of the esophagus.

As of 2016, we now have relatively robust, published data that demonstrate POEM to be an effective, minimally-invasive therapy for achalasia with similar outcomes to LHM. As POEM is now routinely performed by numerous experts in referral centers worldwide, additional comparative studies with long-term follow-up should be forthcoming. At present, the technique of performing POEM (type of knife used, type of electrosurgical current used at various steps in the

procedure, isolated incision of the deep circular muscle vs full-thickness incision, etc.) and the optimal pre- and post-procedural protocol continue to be refined and can vary among high-volume centers.

For instance, in the IPOEMS survey, 88% of those surveyed reported obtaining routine early postoperative radiographic imaging^[70]. Sternbach *et al*^[77] subsequently evaluated the ability of barium esophagram obtained on postoperative day (POD) 1 in 72 patients undergoing POEM to predict symptomatic and physiologic outcomes at 1-year follow-up. Those patients found to have delayed esophageal emptying on barium esophagram performed on POD 1 had no difference at 1 year with respect to Eckhart scores, barium height at 5 min, or need for retreatment when compared with those patients without delayed emptying on barium esophagram. Despite these data, most centers still perform a barium esophagram within 1 d following POEM to rule out perforation, in addition to serving as an early indicator of efficacy following POEM.

As mentioned above, the endosurgical technique used to perform POEM can vary among centers, which is a phenomenon also seen with ESD. POEM has classically involved the use of separate devices to successively inject saline tinted with a blue dye (diluted indigo carmine or methylene blue) into the submucosal space, followed by dissection with an ESD knife (such as a DualKnife or Triangle Tip Knife, Olympus America, Center Valley, PA or an I-type or T-type Hybrid Knife, ERBE, Marietta, GA) in a sequential and repeated fashion.

However, in a recent cohort of 9 patients undergoing POEM, repeated water-jet injection of tinted saline through the dedicated channel of the gastroscope (thereby mitigating the need for an injection needle) resulted in consistent staining of the submucosal fibers, which enabled accurate, efficient, and safe dissection with a Triangle Tip Knife^[78]. Cai *et al*^[79] further demonstrated differing procedural times in 100 patients undergoing POEM who were randomized to the conventional multi-device technique (using a 23G injector needle and a TT Knife, Olympus) or to use of a HybridKnife (I-type or T-type, ERBE) with an ERBEJet2 (ERBE) system that enables atraumatic submucosal injection of saline as well as electrosurgical dissection by using a single endoscopic device. The group that underwent POEM with the HybridKnife had a significantly shorter average procedure duration (22.9 min), without any severe complications, compared with the group that underwent POEM with the conventional multi-device technique (35.2 min, $P < 0.0001$). Other commercially available knives that enable both injection and electrosurgical incision and dissection are expected to be made available in the United States in 2016-2017.

Overall, POEM is a significant addition to the armamentarium of the interventional or surgical endoscopist for the endoscopic treatment of achalasia. It is our belief that the most effective treatment for patients with achalasia is the precise visualization and

incision of the dysfunctional circular muscle of the distal esophagus, the LES, and the proximal gastric cardia. We believe that irrespective of how the myotomy is performed - whether by using a robot-assisted or laparoscopic surgical approach or by using a flexible endoscope - as long as a proper myotomy is achieved, the treatment results should be equivalent. What might differ between an endoscopic and a surgical approach might be the rates or types of complications. Furthermore, with ever changing and decreasing procedural reimbursement, particularly for flexible endoscopy codes in the United States, the relative cost of LHM vs POEM is likely to change-probably in favor of POEM. With additional comparative long-term data and multi-center reports, we believe that POEM will likely become a widely accepted and effective endoscopic therapy for achalasia.

OTHER ENDOSCOPIC INNOVATIONS

Other endoscopic advancements that might help patients with achalasia include use of the Endolumenal Functional Lumen Imaging Probe system (EndoFLIP, Crospon, Carlsbad, CA, United States) to measure LES distensibility at the time of POEM in order to improve long-term outcomes by giving an intraprocedural indicator of the adequacy of the myotomy prior to tunnel closure^[80]. Additional innovative endoscopic ideas in this field have included injection of ethanolamine at the GEJ as a cheaper but clinically equivalent alternative to BT^[81], as well as temporary use of a large caliber 30-mm self-expandable metal stent^[82] for symptomatic improvement in patients with achalasia (which is not commercially available or approved for use in the United States). Of course, as with other currently accepted techniques for treating achalasia, robust data denoting successful long-term outcomes with acceptable risk profiles will be required prior to generalized use of any new and innovative techniques.

IMPORTANCE OF COUNSELING

Regardless of whether PD, LHM, or POEM is performed, all proceduralists strive for ideal outcomes with no or few complications. However, it should be underscored that achalasia is a lifelong disease with no cure. At best, the goal of pharmacologic, radiographic, endoscopic, or surgical treatment of achalasia is to allow patients to eat better (without constant dysphagia), maintain their weight, and reduce the risk of developing a megaesophagus. Patients must understand that they will never eat "normally" as they did prior to being diagnosed with achalasia. In particular, following a complete myotomy (regardless of whether it is by LHM or POEM), patients can never lay flat because of the risk of aspiration from an incompetent LES. Also, patients must be careful to swallow only small quantities of well-chewed food and to allow gravity with adequate time to allow this

manageable food bolus to pass from the esophagus into the stomach. Patients with successful and complete myotomies who swallow too much food in too quick a manner are prone to “overflowing” the 7-to-10-cm-long myotomy in their distal esophagus, which can lead to symptoms of dysphagia and regurgitation from food that is backed-up in the non-treated mid-to-proximal esophagus, thereby mimicking an incomplete myotomy.

CONCLUSION

Endoscopy is an important tool in the diagnostic workup of achalasia. Endoscopic therapies also offer a range of minimally invasive treatments for patients with this non-malignant but incurable disease. Overall, the choice of which therapy to use to treat a patient with achalasia should be based on clinical factors including the patient’s age, medical comorbidities, risk tolerance, and the local expertise of the managing physicians. HRM should be obtained to definitively diagnose achalasia, as it also enables the diagnosis of the subtype of achalasia, which in turn might help guide treatment. We generally counsel patients on the long-term data supporting use of PD or surgical myotomy, and we also give patients the option to pursue POEM as a minimally-invasive endoscopic alternative to LHM.

Based on presently available data, we believe that both LHM and POEM, when done properly by an experienced surgeon or endoscopist, can achieve excellent clinical results, particularly in patients with type I or type II achalasia. Furthermore, we feel it reasonable to proceed with myotomy (either LHM or POEM) as first-line therapy over pharmacologic options including BT injection in younger, healthier patients. However, in patients with limited life expectancy or those who are poor operative candidates, less invasive therapies such as repeated BT injection or even percutaneous endoscopic gastrostomy tube placement are appropriate considerations. While PD is effective and can be repeated, if required, the random disruption of the LES and deep muscle layer of the distal esophagus can make subsequent LHM or POEM more challenging and more dangerous.

Ultimately, thoughtful and tailored application of various therapies for patients with achalasia can provide long-term symptomatic improvement. Despite this optimism, the data do show that recurrent symptoms can occur up to years later following successful LHM, POEM, or PD. However, re-treatment is possible typically by PD or POEM at a site that has not undergone prior myotomy. Patient counselling and behavioral modification are paramount to achieving good postprocedural outcomes, as achalasia can only be treated but not cured. Finally, endoscopic management may also extend to screening for malignancy in patients with achalasia as their risk of esophageal cancer is increased. However, with limited data and no official recommendations regarding

endoscopic screening, decisions regarding whether or not to screen and how frequently to do this falls on the judgment of the treating physician in consultation with his or her patient.

REFERENCES

- Fisichella PM**, Patti MG. From Heller to POEM (1914-2014): a 100-year history of surgery for Achalasia. *J Gastrointest Surg* 2014; **18**: 1870-1875 [PMID: 24878993 DOI: 10.1007/s11605-014-2547-8]
- Richter JE**. Oesophageal motility disorders. *Lancet* 2001; **358**: 823-828 [PMID: 11564508 DOI: 10.1016/S0140-6736(01)05973-6]
- Vaezi MF**, Pandolfino JE, Vela MF. ACG clinical guideline: diagnosis and management of achalasia. *Am J Gastroenterol* 2013; **108**: 1238-1249; quiz 1250 [PMID: 23877351 DOI: 10.1038/ajg.2013.196]
- Pandolfino JE**, Gawron AJ. Achalasia: a systematic review. *JAMA* 2015; **313**: 1841-1852 [PMID: 25965233 DOI: 10.1001/jama.2015.2996]
- Boeckxstaens GE**. Achalasia: virus-induced euthanasia of neurons? *Am J Gastroenterol* 2008; **103**: 1610-1612 [PMID: 18557706 DOI: 10.1111/j.1572-0241.2008.01967.x]
- Fisichella PM**, Raz D, Palazzo F, Niponmick I, Patti MG. Clinical, radiological, and manometric profile in 145 patients with untreated achalasia. *World J Surg* 2008; **32**: 1974-1979 [PMID: 18575930 DOI: 10.1007/s00268-008-9656-z]
- Moonen A**, Boeckxstaens G. Current diagnosis and management of achalasia. *J Clin Gastroenterol* 2014; **48**: 484-490 [PMID: 24926623 DOI: 10.1097/mcg.0000000000000137]
- Rohof WO**, Salvador R, Annese V, Bruley des Varannes S, Chaussade S, Costantini M, Elizalde JL, Gaudric M, Smout AJ, Tack J, Busch OR, Zaninotto G, Boeckxstaens GE. Outcomes of treatment for achalasia depend on manometric subtype. *Gastroenterology* 2013; **144**: 718-725; quiz e13-14 [PMID: 23277105 DOI: 10.1053/j.gastro.2012.12.027]
- Eckardt VF**. Clinical presentations and complications of achalasia. *Gastrointest Endosc Clin N Am* 2001; **11**: 281-292, vi [PMID: 11319062]
- Eckardt VF**, Köhne U, Junginger T, Westermeier T. Risk factors for diagnostic delay in achalasia. *Dig Dis Sci* 1997; **42**: 580-585 [PMID: 9073142]
- Gockel I**, Eckardt VF, Schmitt T, Junginger T. Pseudoachalasia: a case series and analysis of the literature. *Scand J Gastroenterol* 2005; **40**: 378-385 [PMID: 16028431]
- Lucchinetti CF**, Kimmel DW, Lennon VA. Paraneoplastic and oncologic profiles of patients seropositive for type 1 antineuronal nuclear autoantibodies. *Neurology* 1998; **50**: 652-657 [PMID: 9521251]
- de Oliveira JM**, Birgisson S, Doinoff C, Einstein D, Herts B, Davros W, Obuchowski N, Koehler RE, Richter J, Baker ME. Timed barium swallow: a simple technique for evaluating esophageal emptying in patients with achalasia. *AJR Am J Roentgenol* 1997; **169**: 473-479 [PMID: 9242756 DOI: 10.2214/ajr.169.2.9242756]
- Vaezi MF**, Richter JE, Wilcox CM, Schroeder PL, Birgisson S, Slaughter RL, Koehler RE, Baker ME. Botulinum toxin versus pneumatic dilatation in the treatment of achalasia: a randomised trial. *Gut* 1999; **44**: 231-239 [PMID: 9895383]
- Howard PJ**, Maher L, Pryde A, Cameron EW, Heading RC. Five year prospective study of the incidence, clinical features, and diagnosis of achalasia in Edinburgh. *Gut* 1992; **33**: 1011-1015 [PMID: 1398223]
- Ghosh SK**, Pandolfino JE, Rice J, Clarke JO, Kwiatek M, Kahrilas PJ. Impaired deglutitive EGJ relaxation in clinical esophageal manometry: a quantitative analysis of 400 patients and 75 controls. *Am J Physiol Gastrointest Liver Physiol* 2007; **293**: G878-G885 [PMID: 17690172 DOI: 10.1152/ajpgi.00252.2007]
- Ghosh SK**, Pandolfino JE, Zhang Q, Jarosz A, Shah N, Kahrilas

- PJ. Quantifying esophageal peristalsis with high-resolution manometry: a study of 75 asymptomatic volunteers. *Am J Physiol Gastrointest Liver Physiol* 2006; **290**: G988-G997 [PMID: 16410365 DOI: 10.1152/ajpgi.00510.2005]
- 18 **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]
 - 19 **Pandolfino JE**, Kahrilas PJ. American Gastroenterological Association medical position statement: Clinical use of esophageal manometry. *Gastroenterology* 2005; **128**: 207-208 [PMID: 15633137]
 - 20 **Pandolfino JE**, Kwiatek MA, Nealis T, Bulsiewicz W, Post J, Kahrilas PJ. Achalasia: a new clinically relevant classification by high-resolution manometry. *Gastroenterology* 2008; **135**: 1526-1533 [PMID: 18722376 DOI: 10.1053/j.gastro.2008.07.022]
 - 21 **Yadlapati R**, Pandolfino JE. Achalasia Update: No Longer a Tough Diagnosis to Swallow. *The New Gastroenterologist*, 2015
 - 22 **Duranceau A**, Liberman M, Martin J, Ferraro P. End-stage achalasia. *Dis Esophagus* 2012; **25**: 319-330 [PMID: 21166740 DOI: 10.1111/j.1442-2050.2010.01157.x]
 - 23 **Gockel I**, Junginger T. The value of scoring achalasia: a comparison of current systems and the impact on treatment--the surgeon's viewpoint. *Am Surg* 2007; **73**: 327-331 [PMID: 17439022]
 - 24 **Stavropoulos SN**, Friedel D, Modayil R, Iqbal S, Grendell JH. Endoscopic approaches to treatment of achalasia. *Therap Adv Gastroenterol* 2013; **6**: 115-135 [PMID: 23503707 DOI: 10.1177/1756283X12468039]
 - 25 **Rebecchi F**, Giaccone C, Farinella E, Campaci R, Morino M. Randomized controlled trial of laparoscopic Heller myotomy plus Dor fundoplication versus Nissen fundoplication for achalasia: long-term results. *Ann Surg* 2008; **248**: 1023-1030 [PMID: 19092347 DOI: 10.1097/SLA.0b013e318190a776]
 - 26 **Richter JE**, Boeckxstaens GE. Management of achalasia: surgery or pneumatic dilation. *Gut* 2011; **60**: 869-876 [PMID: 21303915 DOI: 10.1136/gut.2010.212423]
 - 27 **Campos GM**, Vittinghoff E, Rabl C, Takata M, Gadenstätter M, Lin F, Ciofica R. Endoscopic and surgical treatments for achalasia: a systematic review and meta-analysis. *Ann Surg* 2009; **249**: 45-57 [PMID: 19106675 DOI: 10.1097/SLA.0b013e31818e43ab]
 - 28 **Sharp KW**, Khaitan L, Scholz S, Holzman MD, Richards WO. 100 consecutive minimally invasive Heller myotomies: lessons learned. *Ann Surg* 2002; **235**: 631-638; discussion 638-639 [PMID: 11981208]
 - 29 **Meara MP**, Perry KA, W. HJ. Economic Impact of Per Oral Endoscopic Myotomy Versus Laparoscopic Heller Myotomy and Endoscopic Pneumatic Dilation. *Surg Endosc* 2014; **28** Suppl 1: S339
 - 30 **O'Connor JB**, Singer ME, Imperiale TF, Vaezi MF, Richter JE. The cost-effectiveness of treatment strategies for achalasia. *Dig Dis Sci* 2002; **47**: 1516-1525 [PMID: 12141811]
 - 31 **Dobrucali A**, Erzin Y, Tuncer M, Dirican A. Long-term results of graded pneumatic dilatation under endoscopic guidance in patients with primary esophageal achalasia. *World J Gastroenterol* 2004; **10**: 3322-3327 [PMID: 15484309 DOI: 10.3748/wjg.v10.i22.3322]
 - 32 **Okike N**, Payne WS, Neufeld DM, Bernatz PE, Pairolero PC, Sanderson DR. Esophagomyotomy versus forceful dilation for achalasia of the esophagus: results in 899 patients. *Ann Thorac Surg* 1979; **28**: 119-125 [PMID: 89837]
 - 33 **Eckardt VF**, Aighnerr C, Bernhard G. Predictors of outcome in patients with achalasia treated by pneumatic dilation. *Gastroenterology* 1992; **103**: 1732-1738 [PMID: 1451966]
 - 34 **Hulselmans M**, Vanuytsel T, Degreef T, Sifrim D, Coosemans W, Lerut T, Tack J. Long-term outcome of pneumatic dilation in the treatment of achalasia. *Clin Gastroenterol Hepatol* 2010; **8**: 30-35 [PMID: 19782766 DOI: 10.1016/j.cgh.2009.09.020]
 - 35 **Kadakia SC**, Wong RK. Graded pneumatic dilation using Rigidflex achalasia dilators in patients with primary esophageal achalasia. *Am J Gastroenterol* 1993; **88**: 34-38 [PMID: 8420271]
 - 36 **Eckardt VF**, Gockel I, Bernhard G. Pneumatic dilation for achalasia: late results of a prospective follow up investigation. *Gut* 2004; **53**: 629-633 [PMID: 15082578]
 - 37 **Karamanolis G**, Sgouros S, Karatzias G, Papadopoulou E, Vasiliadis K, Stefanidis G, Mantides A. Long-term outcome of pneumatic dilation in the treatment of achalasia. *Am J Gastroenterol* 2005; **100**: 270-274 [PMID: 15667481 DOI: 10.1111/j.1572-0241.2005.40093.x]
 - 38 **Katz PO**, Gilbert J, Castell DO. Pneumatic dilatation is effective long-term treatment for achalasia. *Dig Dis Sci* 1998; **43**: 1973-1977 [PMID: 9753261]
 - 39 **Parkman HP**, Reynolds JC, Ouyang A, Rosato EF, Eisenberg JM, Cohen S. Pneumatic dilatation or esophagomyotomy treatment for idiopathic achalasia: clinical outcomes and cost analysis. *Dig Dis Sci* 1993; **38**: 75-85 [PMID: 8420763]
 - 40 **West RL**, Hirsch DP, Bartelsman JF, de Borst J, Ferwerda G, Tytgat GN, Boeckxstaens GE. Long term results of pneumatic dilation in achalasia followed for more than 5 years. *Am J Gastroenterol* 2002; **97**: 1346-1351 [PMID: 12094848 DOI: 10.1111/j.1572-0241.2002.05771.x]
 - 41 **Zerbib F**, Th  tiot V, Richy F, Benajah DA, Message L, Lamouliatte H. Repeated pneumatic dilations as long-term maintenance therapy for esophageal achalasia. *Am J Gastroenterol* 2006; **101**: 692-697 [PMID: 16635216 DOI: 10.1111/j.1572-0241.2006.00385.x]
 - 42 **Farhoomand K**, Connor JT, Richter JE, Achkar E, Vaezi MF. Predictors of outcome of pneumatic dilation in achalasia. *Clin Gastroenterol Hepatol* 2004; **2**: 389-394 [PMID: 15118976]
 - 43 **Alderliesten J**, Conchillo JM, Leeuwenburgh I, Steyerberg EW, Kuipers EJ. Predictors for outcome of failure of balloon dilatation in patients with achalasia. *Gut* 2011; **60**: 10-16 [PMID: 21068135 DOI: 10.1136/gut.2010.211409]
 - 44 **Cuill  re C**, Ducrott   P, Zerbib F, Metman EH, de Looze D, Guillemot F, Hudziak H, Lamouliatte H, Grimaud JC, Ropert A, Dapoigny M, Bost R, L  mann M, Bigard MA, Denis P, Aug  t JL, Galmiche JP, Bruley des Varannes S. Achalasia: outcome of patients treated with intrasphincteric injection of botulinum toxin. *Gut* 1997; **41**: 87-92 [PMID: 9274478]
 - 45 **Pasricha PJ**, Rai R, Ravich WJ, Hendrix TR, Kalloo AN. Botulinum toxin for achalasia: long-term outcome and predictors of response. *Gastroenterology* 1996; **110**: 1410-1415 [PMID: 8613045]
 - 46 **Zaninotto G**, Annese V, Costantini M, Del Genio A, Costantino M, Epifani M, Gatto G, D'Onofrio V, Benini L, Contini S, Molena D, Battaglia G, Tardio B, Andriulli A, Ancona E. Randomized controlled trial of botulinum toxin versus laparoscopic heller myotomy for esophageal achalasia. *Ann Surg* 2004; **239**: 364-370 [PMID: 15075653 DOI: 10.1097/01.sla.0000114217.52941.c5]
 - 47 **Smith CD**, Stival A, Howell DL, Swafford V. Endoscopic therapy for achalasia before Heller myotomy results in worse outcomes than heller myotomy alone. *Ann Surg* 2006; **243**: 579-584; discussion 584-586 [PMID: 16632991 DOI: 10.1097/01.sla.0000217524.75529.2d]
 - 48 **Boeckxstaens GE**, Annese V, des Varannes SB, Chaussade S, Costantini M, Cuttitta A, Elizalde JI, Fumagalli U, Gaudric M, Rohof WO, Smout AJ, Tack J, Zwinderman AH, Zaninotto G, Busch OR. Pneumatic dilation versus laparoscopic Heller's myotomy for idiopathic achalasia. *N Engl J Med* 2011; **364**: 1807-1816 [PMID: 21561346 DOI: 10.1056/NEJMoa1010502]
 - 49 **Moonen A**, Annese V, Belmans A, Bredenoord AJ, Bruley des Varannes S, Costantini M, Douset B, Elizalde JI, Fumagalli U, Gaudric M, Merla A, Smout AJ, Tack J, Zaninotto G, Busch OR, Boeckxstaens GE. Long-term results of the European achalasia trial: a multicentre randomised controlled trial comparing pneumatic dilation versus laparoscopic Heller myotomy. *Gut* 2016; **65**: 732-739 [PMID: 26614104 DOI: 10.1136/gutjnl-2015-310602]
 - 50 **Hamdy E**, El Nakeeb A, El Hanfy E, El Hemaly M, Salah T, Hamed H, El Hak NG. Comparative Study Between Laparoscopic Heller Myotomy Versus Pneumatic Dilatation for Treatment of Early Achalasia: A Prospective Randomized Study. *J Laparoendosc Adv Surg Tech A* 2015; **25**: 460-464 [PMID: 25951417 DOI: 10.1089/lap.2014.0682]
 - 51 **Lynch KL**, Pandolfino JE, Howden CW, Kahrilas PJ. Major complications of pneumatic dilation and Heller myotomy for achalasia: single-center experience and systematic review of the

- literature. *Am J Gastroenterol* 2012; **107**: 1817-1825 [PMID: 23032978 DOI: 10.1038/ajg.2012.332]
- 52 **Jung HE**, Lee JS, Lee TH, Kim JN, Hong SJ, Kim JO, Kim HG, Jeon SR, Cho JY. Long-term outcomes of balloon dilation versus botulinum toxin injection in patients with primary achalasia. *Korean J Intern Med* 2014; **29**: 738-745 [PMID: 25378972 DOI: 10.3904/kjim.2014.29.6.738]
 - 53 **Leyden JE**, Moss AC, MacMathuna P. Endoscopic pneumatic dilation versus botulinum toxin injection in the management of primary achalasia. *Cochrane Database Syst Rev* 2014; **(2)**: CD005046 [PMID: 25485740 DOI: 10.1002/14651858.CD005046.pub3]
 - 54 **Richter JE**. Comparison and cost analysis of different treatment strategies in achalasia. *Gastrointest Endosc Clin N Am* 2001; **11**: 359-370, viii [PMID: 11319067]
 - 55 **Pasricha PJ**, Hawari R, Ahmed I, Chen J, Cotton PB, Hawes RH, Kalloo AN, Kantsevoy SV, Gostout CJ. Submucosal endoscopic esophageal myotomy: a novel experimental approach for the treatment of achalasia. *Endoscopy* 2007; **39**: 761-764 [PMID: 17703382 DOI: 10.1055/s-2007-966764]
 - 56 **Inoue H**, Minami H, Kobayashi Y, Sato Y, Kaga M, Suzuki M, Satodate H, Odaka N, Itoh H, Kudo S. Peroral endoscopic myotomy (POEM) for esophageal achalasia. *Endoscopy* 2010; **42**: 265-271 [PMID: 20354937 DOI: 10.1055/s-0029-1244080]
 - 57 **Inoue H**, Sato H, Ikeda H, Onimaru M, Sato C, Minami H, Yokomichi H, Kobayashi Y, Grimes KL, Kudo SE. Per-Oral Endoscopic Myotomy: A Series of 500 Patients. *J Am Coll Surg* 2015; **221**: 256-264 [PMID: 26206634 DOI: 10.1016/j.jamcollsurg.2015.03.057]
 - 58 **Chen X**, Li QP, Ji GZ, Ge XX, Zhang XH, Zhao XY, Miao L. Two-year follow-up for 45 patients with achalasia who underwent peroral endoscopic myotomy. *Eur J Cardiothorac Surg* 2015; **47**: 890-896 [PMID: 25193955 DOI: 10.1093/ejcts/ezu320]
 - 59 **Costamagna G**, Marchese M, Familiari P, Tringali A, Inoue H, Perri V. Peroral endoscopic myotomy (POEM) for oesophageal achalasia: preliminary results in humans. *Dig Liver Dis* 2012; **44**: 827-832 [PMID: 22609465 DOI: 10.1016/j.dld.2012.04.003]
 - 60 **Khashab MA**, Messallam AA, Onimaru M, Teitelbaum EN, Ujiki MB, Gitelis ME, Modayil RJ, Hungness ES, Stavropoulos SN, El Zein MH, Shiwaku H, Kunda R, Repici A, Minami H, Chiu PW, Ponsky J, Kumbhari V, Saxena P, Maydeo AP, Inoue H. International multicenter experience with peroral endoscopic myotomy for the treatment of spastic esophageal disorders refractory to medical therapy (with video). *Gastrointest Endosc* 2015; **81**: 1170-1177 [PMID: 25634487 DOI: 10.1016/j.gie.2014.10.011]
 - 61 **Lee BH**, Shim KY, Hong SJ, Bok GH, Cho JH, Lee TH, Cho JY. Peroral endoscopic myotomy for treatment of achalasia: initial results of a korean study. *Clin Endosc* 2013; **46**: 161-167 [PMID: 23614126 DOI: 10.5946/ce.2013.46.2.161]
 - 62 **Ling TS**, Guo HM, Yang T, Peng CY, Zou XP, Shi RH. Effectiveness of peroral endoscopic myotomy in the treatment of achalasia: a pilot trial in Chinese Han population with a minimum of one-year follow-up. *J Dig Dis* 2014; **15**: 352-358 [PMID: 24739072 DOI: 10.1111/1751-2980.12153]
 - 63 **Minami H**, Isomoto H, Yamaguchi N, Matsushima K, Akazawa Y, Ohnita K, Takeshima F, Inoue H, Nakao K. Peroral endoscopic myotomy for esophageal achalasia: clinical impact of 28 cases. *Dig Endosc* 2014; **26**: 43-51 [PMID: 23581563 DOI: 10.1111/den.12086]
 - 64 **Ren Z**, Zhong Y, Zhou P, Xu M, Cai M, Li L, Shi Q, Yao L. Peri-operative management and treatment for complications during and after peroral endoscopic myotomy (POEM) for esophageal achalasia (EA) (data from 119 cases). *Surg Endosc* 2012; **26**: 3267-3272 [PMID: 22609984 DOI: 10.1007/s00464-012-2336-y]
 - 65 **Swanstrom LL**, Kurian A, Dunst CM, Sharata A, Bhayani N, Rieder E. Long-term outcomes of an endoscopic myotomy for achalasia: the POEM procedure. *Ann Surg* 2012; **256**: 659-667 [PMID: 22982946 DOI: 10.1097/SLA.0b013e31826b5212]
 - 66 **Swanström LL**, Rieder E, Dunst CM. A stepwise approach and early clinical experience in peroral endoscopic myotomy for the treatment of achalasia and esophageal motility disorders. *J Am Coll Surg* 2011; **213**: 751-756 [PMID: 21996484 DOI: 10.1016/j.jamcollsurg.2011.09.001]
 - 67 **Verlaan T**, Rohof WO, Bredenoord AJ, Eberl S, Rösch T, Fockens P. Effect of peroral endoscopic myotomy on esophagogastric junction physiology in patients with achalasia. *Gastrointest Endosc* 2013; **78**: 39-44 [PMID: 23453184 DOI: 10.1016/j.gie.2013.01.006]
 - 68 **Von Renteln D**, Fuchs KH, Fockens P, Bauerfeind P, Vassiliou MC, Werner YB, Fried G, Breithaupt W, Heinrich H, Bredenoord AJ, Kersten JF, Verlaan T, Trevisonno M, Rösch T. Peroral endoscopic myotomy for the treatment of achalasia: an international prospective multicenter study. *Gastroenterology* 2013; **145**: 309-311.e1-3 [PMID: 23665071 DOI: 10.1053/j.gastro.2013.04.057]
 - 69 **von Renteln D**, Inoue H, Minami H, Werner YB, Pace A, Kersten JF, Much CC, Schachschal G, Mann O, Keller J, Fuchs KH, Rösch T. Peroral endoscopic myotomy for the treatment of achalasia: a prospective single center study. *Am J Gastroenterol* 2012; **107**: 411-417 [PMID: 22068665 DOI: 10.1038/ajg.2011.388]
 - 70 **Stavropoulos SN**, Modayil RJ, Friedel D, Savides T. The International Per Oral Endoscopic Myotomy Survey (IPOEMS): a snapshot of the global POEM experience. *Surg Endosc* 2013; **27**: 3322-3338 [PMID: 23549760 DOI: 10.1007/s00464-013-2913-8]
 - 71 **Wei M**, Yang T, Yang X, Wang Z, Zhou Z. Peroral esophageal myotomy versus laparoscopic Heller's myotomy for achalasia: a meta-analysis. *J Laparoendosc Adv Surg Tech A* 2015; **25**: 123-129 [PMID: 25683071 DOI: 10.1089/lap.2014.0454]
 - 72 **Vigneswaran Y**, Tanaka R, Gitelis M, Carbray J, Ujiki MB. Quality of life assessment after peroral endoscopic myotomy. *Surg Endosc* 2015; **29**: 1198-1202 [PMID: 25249144 DOI: 10.1007/s00464-014-3793-2]
 - 73 **Kumbhari V**, Tieu AH, Onimaru M, El Zein MH, Teitelbaum EN, Ujiki MB, Gitelis ME, Modayil RJ, Hungness ES, Stavropoulos SN, Shiwaku H, Kunda R, Chiu P, Saxena P, Messallam AA, Inoue H, Khashab MA. Peroral endoscopic myotomy (POEM) vs laparoscopic Heller myotomy (LHM) for the treatment of Type III achalasia in 75 patients: a multicenter comparative study. *Endosc Int Open* 2015; **3**: E195-E201 [PMID: 26171430 DOI: 10.1055/s-0034-1391668]
 - 74 **Sharata A**, Kurian AA, Dunst CM, Bhayani NH, Reavis KM, Swanström LL. Peroral endoscopic myotomy (POEM) is safe and effective in the setting of prior endoscopic intervention. *J Gastrointest Surg* 2013; **17**: 1188-1192 [PMID: 23609138 DOI: 10.1007/s11605-013-2193-6]
 - 75 **Ling T**, Guo H, Zou X. Effect of peroral endoscopic myotomy in achalasia patients with failure of prior pneumatic dilation: a prospective case-control study. *J Gastroenterol Hepatol* 2014; **29**: 1609-1613 [PMID: 24628480 DOI: 10.1111/jgh.12570]
 - 76 **Orenstein SB**, Raigani S, Wu YV, Pauli EM, Phillips MS, Ponsky JL, Marks JM. Peroral endoscopic myotomy (POEM) leads to similar results in patients with and without prior endoscopic or surgical therapy. *Surg Endosc* 2015; **29**: 1064-1070 [PMID: 25249143 DOI: 10.1007/s00464-014-3782-5]
 - 77 **Sternbach JM**, El Khoury R, Teitelbaum EN, Soper NJ, Pandolfino JE, Hungness ES. Early esophagram in per-oral endoscopic myotomy (POEM) for achalasia does not predict long-term outcomes. *Surgery* 2015; **158**: 1128-1135; discussion 1135-1136 [PMID: 26189954 DOI: 10.1016/j.surg.2015.05.023]
 - 78 **Khashab MA**, Messallam AA, Saxena P, Kumbhari V, Ricourt E, Aguila G, Roland BC, Stein E, Nandwani M, Inoue H, Clarke JO. Jet injection of dyed saline facilitates efficient peroral endoscopic myotomy. *Endoscopy* 2014; **46**: 298-301 [PMID: 24338241 DOI: 10.1055/s-0033-1359024]
 - 79 **Cai MY**, Zhou PH, Yao LQ, Xu MD, Zhong YS, Li QL, Chen WF, Hu JW, Cui Z, Zhu BQ. Peroral endoscopic myotomy for idiopathic achalasia: randomized comparison of water-jet assisted versus conventional dissection technique. *Surg Endosc* 2014; **28**: 1158-1165 [PMID: 24232052 DOI: 10.1007/s00464-013-3300-1]
 - 80 **Teitelbaum EN**, Soper NJ, Pandolfino JE, Kahrilas PJ, Hirano I, Boris L, Nicodème F, Lin Z, Hungness ES. Esophagogastric junction distensibility measurements during Heller myotomy and

POEM for achalasia predict postoperative symptomatic outcomes. *Surg Endosc* 2015; **29**: 522-528 [PMID: 25055891 DOI: 10.1007/s00464-014-3733-1]

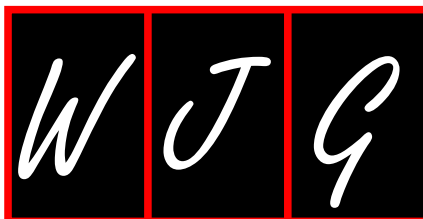
- 81 **Mikaeli J**, Veisari AK, Fazlollahi N, Mehrabi N, Soleimani HA, Shirani S, Malekzadeh R. Ethanolamine oleate versus botulinum toxin in the treatment of idiopathic achalasia. *Ann Gastroenterol*

2015; **28**: 229-235 [PMID: 25830939]

- 82 **Cheng YS**, Ma F, Li YD, Chen NW, Chen WX, Zhao JG, Wu CG. Temporary self-expanding metallic stents for achalasia: a prospective study with a long-term follow-up. *World J Gastroenterol* 2010; **16**: 5111-5117 [PMID: 20976849 DOI: 10.3748/wjg.v16.i40.5111]

P- Reviewer: Luo LS **S- Editor:** Yu J **L- Editor:** A
E- Editor: Zhang FF





Human bocavirus: Current knowledge and future challenges

Marcello Guido, Maria Rosaria Tumolo, Tiziano Verri, Alessandro Romano, Francesca Serio, Mattia De Giorgi, Antonella De Donno, Francesco Bagordo, Antonella Zizza

Marcello Guido, Francesca Serio, Mattia De Giorgi, Antonella De Donno, Francesco Bagordo, Laboratory of Hygiene, Department of Biological and Environmental Sciences and Technologies, Faculty of Sciences, University of Salento, 73100 Lecce, Italy

Maria Rosaria Tumolo, Antonella Zizza, Institute of Clinical Physiology, National Research Council, 73100 Lecce, Italy

Tiziano Verri, Laboratory of Physiology, Department of Biological and Environmental Sciences and Technologies, University of Salento, 73100 Lecce, Italy

Alessandro Romano, Neuropathology Unit, Institute of Experimental Neurology and Division of Neuroscience, IRCCS San Raffaele Scientific Institute, 20132 Milan, Italy

Author contributions: Guido M and Tumolo MR are co-first authors and equally contributed to this article; all authors contributed to the conception and design of the study, literature review and analysis, drafting, critical revision and editing of the manuscript, and approval of the final version to be published.

Conflict-of-interest statement: The authors declare no conflicts of interests for this article.

Open-Access: This article is an open-access article which was selected by an in-house editor and fully peer-reviewed by external reviewers. It is distributed in accordance with the Creative Commons Attribution Non Commercial (CC BY-NC 4.0) license, which permits others to distribute, remix, adapt, build upon this work non-commercially, and license their derivative works on different terms, provided the original work is properly cited and the use is non-commercial. See: <http://creativecommons.org/licenses/by-nc/4.0/>

Manuscript source: Invited manuscript

Correspondence to: Marcello Guido, Professor, Laboratory of Hygiene, Department of Biological and Environmental Sciences and Technologies, Faculty of Sciences, University of Salento, Via Provinciale Lecce-Monteroni, 73100 Lecce, Italy. marcello.guido@unisalento.it
Telephone: +39-832-298686
Fax: +39-832-298626

Received: March 28, 2016

Peer-review started: April 1, 2016

First decision: May 12, 2016

Revised: August 30, 2016

Accepted: September 14, 2016

Article in press: September 14, 2016

Published online: October 21, 2016

Abstract

Human bocavirus (HBoV) is a parvovirus isolated about a decade ago and found worldwide in both respiratory samples, mainly from early life and children of 6-24 mo of age with acute respiratory infection, and in stool samples, from patients with gastroenteritis. Since then, other viruses related to the first HBoV isolate (HBoV1), namely HBoV2, HBoV3 and HBoV4, have been detected principally in human faeces. HBoVs are small non-enveloped single-stranded DNA viruses of about 5300 nucleotides, consisting of three open reading frames encoding the first two the non-structural protein 1 (NS1) and nuclear phosphoprotein (NP1) and the third the viral capsid proteins 1 and 2 (VP1 and VP2). HBoV pathogenicity remains to be fully clarified mainly due to the lack of animal models for the difficulties in replicating the virus in *in vitro* cell cultures, and the fact that HBoV infection is frequently accompanied by at least another viral and/or bacterial respiratory and/or gastroenteric pathogen infection. Current diagnostic methods to support HBoV detection include polymerase chain reaction, real-time PCR, enzyme-linked immunosorbent assay and enzyme immunoassay using recombinant VP2 or virus-like particle capsid proteins, although sequence-independent amplification techniques combined with next-generation sequencing platforms promise rapid and simultaneous detection of the pathogens in the future. This review presents the current knowledge on HBoV genotypes with emphasis on taxonomy, phylogenetic relationship and genomic analysis, biology, epidemiology, pathogenesis and

diagnostic methods. The emerging discussion on HBoVs as true pathogen or innocent bystander is also emphasized.

Key words: Human bocavirus; Respiratory virus; Molecular tests; Gastrointestinal virus; Pathogenesis; Epidemiology; Immunoassay methods

© **The Author(s) 2016.** Published by Baishideng Publishing Group Inc. All rights reserved.

Core tip: Four genotypes compose the genus *Bocavirus*: Human bocavirus (HBoV) 1, predominantly found in the respiratory tract; and, HBoV2, 3 and 4, mainly detected in stool and associated with gastroenteritis. Despite worldwide occurrence, human bocavirus infection remains poorly understood, and the comprehension of many aspects of these viruses' biology (*i.e.*, taxonomy, phylogenetic relationships with other viruses, epidemiology, molecular mechanisms of interaction with human cells, association with other pathogens, *etc.*) is necessary to clarify whether they are harmless passengers or true pathogens. Development of new diagnostic tools for detection of human bocaviruses will support this type of research.

Guido M, Tumolo MR, Verri T, Romano A, Serio F, De Giorgi M, De Donno A, Bagordo F, Zizza A. Human bocavirus: Current knowledge and future challenges. *World J Gastroenterol* 2016; 22(39): 8684-8697 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v22/i39/8684.htm> DOI: <http://dx.doi.org/10.3748/wjg.v22.i39.8684>

INTRODUCTION

Human bocavirus (HBoV) is a parvovirus that was first identified in 2005 using a protocol based on DNase treatment, random PCR amplification, high-throughput sequencing and bioinformatics analysis. When this virus-screening technique was initially applied to nasopharyngeal swabs and washings from children with unresolved respiratory tract infections, it gave a positive result rate of 3.1%; hence, it was proposed that HBoV is a causative pathogen of respiratory tract diseases^[1].

Three additional HBoV subtypes were subsequently identified in human stool samples, named as HBoV2, HBoV3 and HBoV4 to differentiate them from the first isolated subtype, named HBoV1^[2-4]. Notably, studies of both respiratory and faecal samples have shown the presence of HBoV in association with other potential pathogens^[5-8], which led to the hypothesis that the virus may be a harmless passenger rather than a true pathogen^[9,10]. Moreover, the virus has been detected in other biological samples, including blood^[11], saliva^[12], faeces^[13] and urine^[14], as well as environmental samples, including river water^[15] and sewage^[16].

Conversely, recent research has raised concerns over its presence in transfusion medicine^[17].

HBoV has been found in individuals of all ages, although it mainly affects infants aged 6-24 mo with respiratory symptoms^[1,18,19]. Based upon Koch's modified postulates, however, the virus cannot yet be confirmed as a causative agent of disease due to the lack of animal models and/or for the difficulties in replicating it in *in vitro* cultured cells^[20-23]. Thus, research and discussion about the potential role of this pathogen (alone or in combination with other types of viruses) in patients with respiratory infections and gastroenteritis is on going.

In this review, we examine the current knowledge and recent findings on the taxonomy, biology, epidemiology, pathogenesis and diagnosis methods of HBoV.

CLASSIFICATION AND BIOLOGY

HBoV genotypes belong to the family *Parvoviridae*, subfamily *Parvovirinae*, genus *Bocavirus*, causing infection in vertebrates exclusively^[1,18,24]. The family *Parvoviridae* also comprises the subfamily *Densovirinae*, which infects arthropods and shares no sequence homology with the other subfamily. The current classification of the International Committee on Taxonomy of Viruses database recognizes eight genera of the subfamily *Parvovirinae*: *Amdoparvovirus*, *Aveparvovirus*, *Bocaparvovirus*, *Copiparvovirus*, *Dependoparvovirus*, *Erythroparvovirus*, *Protoparvovirus* and *Tetraparvovirus*^[24].

The name *Bocavirus* derives from the combination of the terms bovine parvovirus (BPV) and canine minute virus (CMV), and was based on the sequence similarities and genomic organization of these two close relatives^[1,25]. The parvoviruses associated with human infections are parvovirus B19 (B19V), within the genus *Erythroparvovirus*, the apathogenic adeno-associated virus, belonging to the genus *Dependoparvovirus*, and the recently discovered parvoviruses 4 (PARV4) and 5 (PARV5), affiliated with the new genus *Tetraparvovirus*^[24]. The latter has not yet been associated with any clinical significances; based on similarity, however, it has been allocated to the new genus *Hokovirus*^[26,27].

Among the human parvoviruses, B19V is particularly relevant since it is acknowledged as the etiologic agent of erythema infectiosum (also known as fifth disease) and has been characterized as a causative agent of other conditions in both children (*e.g.*, transient arthritis) and adults (*e.g.*, non-immune hydrops fetalis, several auto-immune diseases, spontaneous abortion and arthropathies)^[28-30]. Despite the close phylogenetic relationship between B19V and HBoV, they appear to be strongly divergent in nature. For example, B19V shows tropism for bone marrow and a lifelong persistence in heart tissue^[31,32], while HBoV persists in lymphatic tissue

and in tissues afflicted with chronic sinusitis^[33,34].

Parvoviruses are small, icosahedral, non-enveloped viruses of 18-26 nm in diameter that contain a single molecule of linear, negative- or positive-sense, single-stranded DNA^[1,35]. The length of the linear single-stranded HBoV genome is only about 5 kilobases, plus the terminal sequences of 32-52 nucleotides (nt) that play a key role in virus replication^[36,37] and show high similarity to the terminal sequences of BPV and CMV^[38].

The replication mechanism of HBoV has remained elusive, with two conflicting models proposed: rolling hairpin^[23,32] versus rolling-cycle^[39]. Replication of the other parvovirus occurs *via* the rolling-hairpin model, with generation of concatameric intermediates characterized by a head-to-head or tail-to-tail structure. However, while the presence of head-to-head monomers has been demonstrated in HBoV1, HBoV2 and HBoV3, concatameric intermediates have not been found yet^[23,36,37,40,41].

Conversely, recent data support the hypothesis that HBoV, during its natural infection, can become persistently established in host cells by forming extra-chromosomal closed circular episomes, instead of concatemers^[37,41]. To date, the episomal structure has been found for all the HBoV genotypes^[36,37,40,41]. In addition, head-to-tail sequences of HBoV1 have been detected in samples from patients with respiratory infections^[36]. Kapoor *et al.*^[37] identified the head-to-tail monomer in an episomal circular form (HBoV3-E1) of the HBoV3 genome from an intestinal biopsy of a child with gastrointestinal disease; the complete HBoV3-E1 genome was shown to contain 5319 nt, flanked with a 513 nt-long terminal non-coding sequence. Meanwhile, the HBoV2-C2 circular genome (5307 nt, of which 520 nt represent the non-coding terminal region) has been detected as well^[41]. A recent study demonstrated that the replicative form of the HBoV4 genotype comprises a head-to-tail nucleotide sequence in circular form^[40]. Future research efforts delving deeper into the HBoV replication mechanism are likely to improve our comprehension of the pathogenetic role of HBoV substantially^[37].

The genome of HBoV is organized in three open reading frames (ORFs): ORF1, encoding two forms of the non-structural (NS) protein NS1; ORF2, encoding an additional NS protein, the nuclear phosphoprotein NP1^[38,42]; and, ORF3, encoding the two structural viral capsid proteins VP1 and VP2, which are generated as a result of alternative splicing events^[1]. The non-coding regions contain palindromic sequences, commonly known as inverted terminal repeats, that are essential for viral replication^[37,43]. NS1 is a multifunctional protein that has various sites with differing functions in the N-terminus (binding and endonuclease), C-terminus (transactivation) and middle region (ATPase and helicase)^[44,45]. Furthermore, NS1 has a role in DNA replication, and, similar to NP1, its function is

essential for DNA replication of CMV and minute virus of mice^[46]. This non-structural protein also participates in apoptosis, cell-cycle arrest and gene transactivation in B19V^[47,48].

Recently, novel small NS proteins (NS2, NS3 and NS4) have been identified in HBoV1 through studies based upon transfection of an HBoV1 infectious proviral plasmid and viral infection of polarized human airway epithelium cells cultured at an air-liquid interface (HAE-ALI). These proteins contain the predictive domains of NS1 activities; moreover, their function is important for viral DNA replication in the human embryonic kidney 293 (HEK293) cell line. NS2 plays a critical role in HBoV1 replication in HAE-ALI cultures as well^[49]. NP1 is also a small non-structural protein, but for which the function(s) still need to be fully elucidated. Initially, NP1 from HBoV1 was shown to induce cell cycle arrest and apoptosis after transfection in HeLa cells^[50]. Recent studies, however, have shown that HBoV1 NP1 plays a critical role in the expression of viral capsid proteins^[51] and demonstrated its direct involvement in viral DNA replication at the replication origin (OriR)^[52].

VP1 and VP2 share a C-terminal region and differ only in the N-terminal region of VP1 (VP1u)^[53]. VP1u exerts phospholipase A₂ activity, which is essential for infectivity and is facilitated by release of the virus from endocytic compartments to the nucleus of the host cell^[42]. To date, the mechanism underlying the viral cell entry and *in vivo* host-range remains unclear^[36].

The complete NS1 gene sequence of HBoV1 (NC_007455.1) is 1928 nt long and encodes a polypeptide of 643 amino acid (aa) residues, much shorter than the NS1 of CMV and of BPV^[46,54]. The HBoV1 NP1 gene is 660 nt long and its encoded protein varies in length among the different strains, ranging from 214 to 219 aa residues. Moreover, the HBoV1 VP1/VP2 ORF contains the complete coding sequence of the VP1 gene (3071 nt), encoding for a protein of 671 aa residues^[4], and the VP2 gene within the VP1 sequence, encoded from nucleotide 3443 to 5071. The genomic organization of the different HBoV genotypes obtained using the Illustrator of Biological Sequences software package^[55] is shown in Figure 1, and the phylogenetic trees of RefSeq nucleotide and aa coding regions of the HBoV genomes, as constructed by the Neighbor-Joining method implemented in the program MEGA5, are shown in Figure 2^[56-58]. Remarkably, HBoV3 NS1 and NP1 sequences cluster with the homologous sequences of the HBoV1 strain, and the same holds true for HBoV2 and HBoV4. Conversely, the VP1/VP2 sequences of HBoV3 are similar to HBoV2, providing evidence that HBoV3 may have resulted from recombination between the HBoV1 and HBoV2 viruses (Figure 2)^[3,4].

A low level of polymorphisms warrants a predominant role for recombination in a genome prone to rapid evolution^[59], in a context in which both recombination

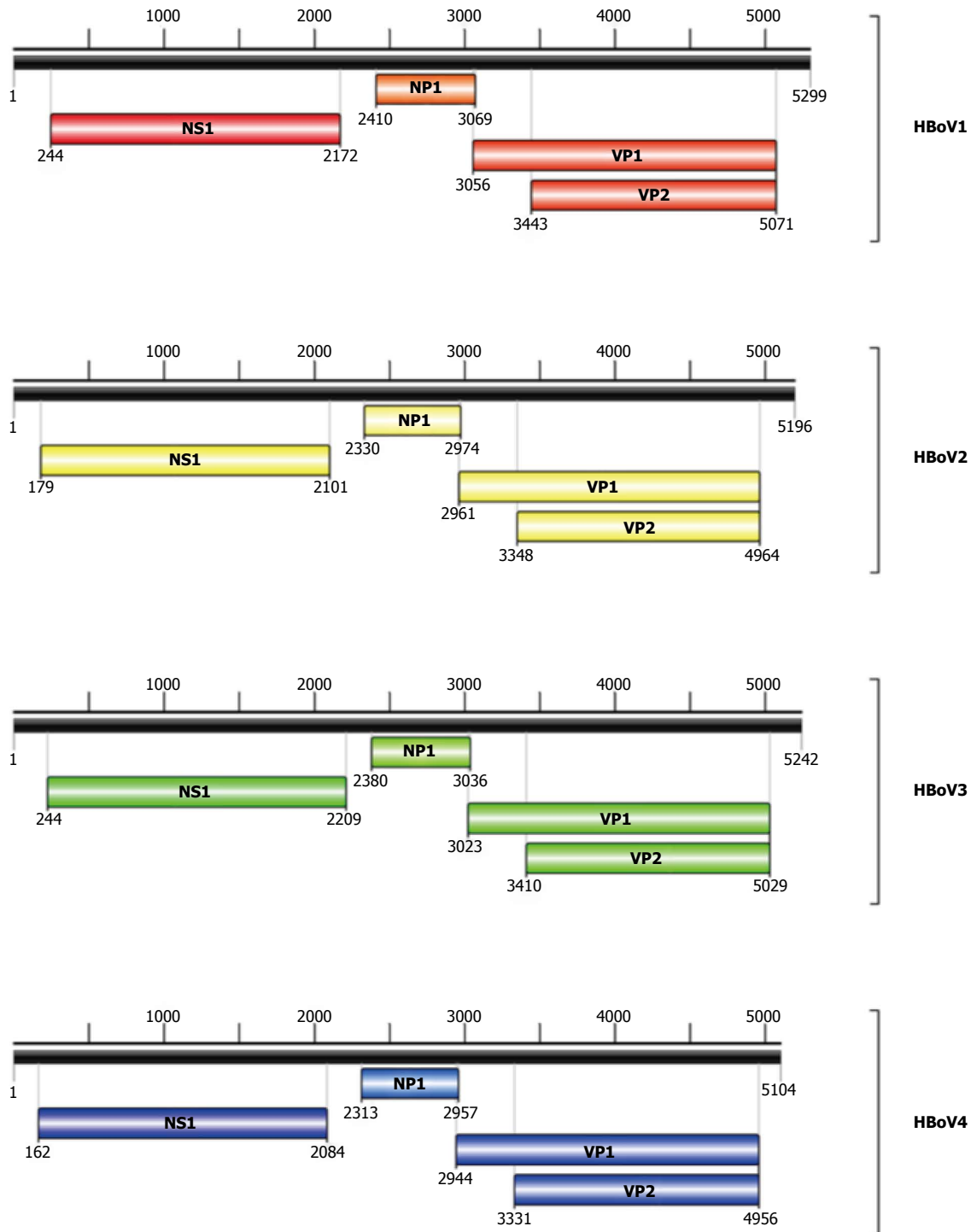


Figure 1 Genomic organization of Human Bocaviruses. Schematic maps of the Human Bocavirus genomes (HBoV1, RefSeq. NC_007455.1; HBoV2, RefSeq. NC_012042.1; HBoV3, RefSeq. NC_012564.1; HBoV4, RefSeq. NC_012729.2) were obtained using the Illustrator of Biological Sequences software package^[55]. The genes encoding the protein NS1 (non-structural protein), NP1 and VP1/VP2 (capsid proteins) and their nucleotide positions are shown.

and mutation represent major mechanisms of genetic variation in parvovirus evolution^[60]. Thus, it is not surprising that it has been proposed recently for the human bocaviruses group to be rearranged into two clusters (or species): human bocaparvovirus 1 (including the previous HBoV1 and HBoV3) and human bocaparvovirus 2 (including the previous HBoV2 and HBoV4)^[25].

PATHOGENESIS

As already stated, the pathogenesis of HBoV remains poorly characterized, mainly due to the lack of specific cell lines for virus culture or experimental animal models^[18]. The first study presenting an *in vitro* culture system for HBoV dates back to 2009, wherein pseudostratified HAE-ALI, derived from primary human

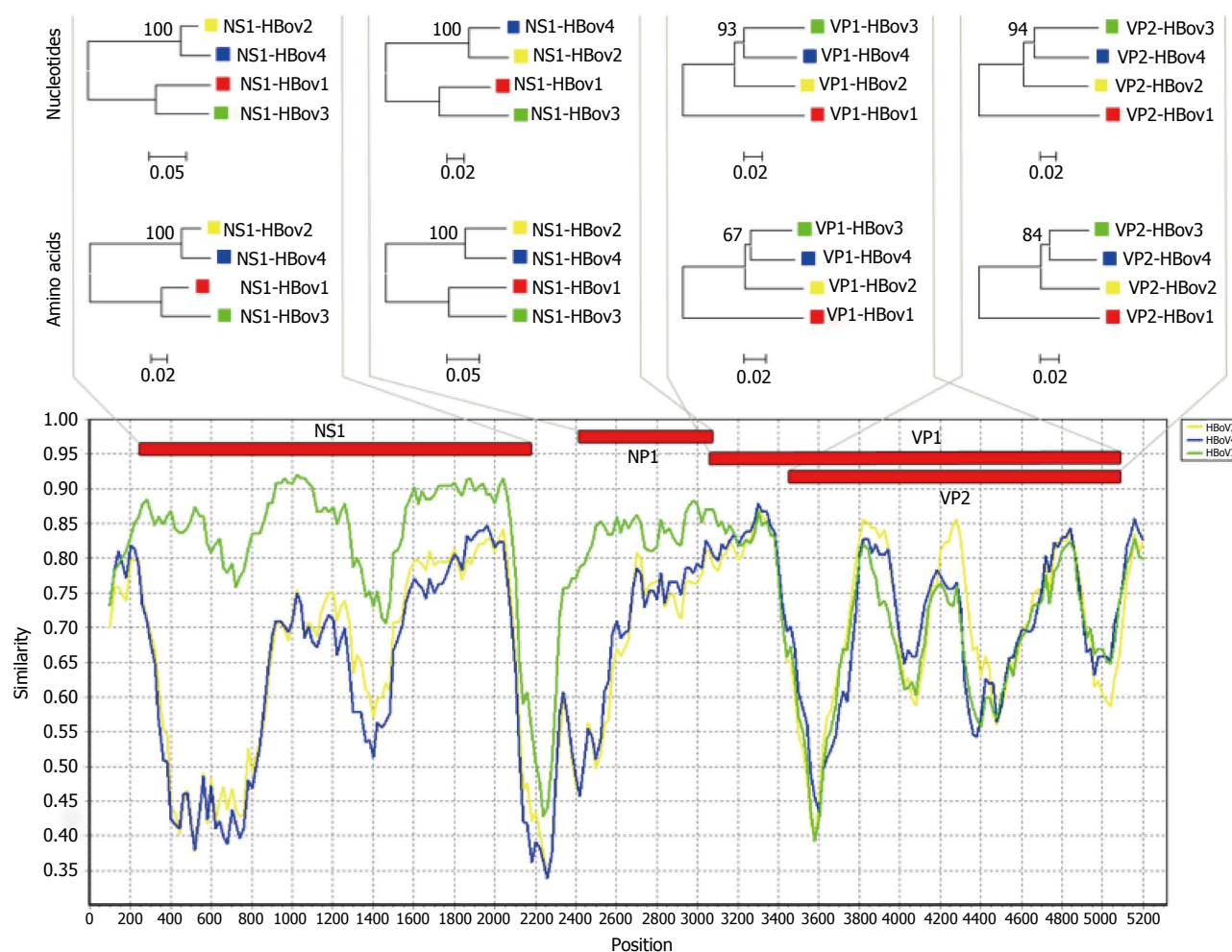


Figure 2 Similarity plot (generated by SimPlot)^[56] of Human Bocaviruses genomes. Each curve is a comparison between the HBoV1 genome (reference) and HBoV2-4 sequences. Nucleotide sequences were aligned using Clustal Omega^[57] and the plot was rendered by Simplot using a window size of 200 bp and a step size of 20 bp. The horizontal bars above the curves represent the HBoV1 genes arranged as indicated in Figure 1; Phylogenetic trees of nucleotide and amino acid sequences of the HBoV genes. Sequences were aligned by Clustal Omega and phylogenetic analysis was carried out using the Neighbor-Joining method implemented in the MEGA5 program^[58]. The numbers at the branch nodes indicate the bootstrap values calculated with 1000 replicates.

bronchial epithelial cells, was utilized as a tool for HBoV replication^[22]. Attempts had been made previously with other cell lines (HEp-2, Vero, MRC-5, *etc.*) but were unsuccessful, most likely due to the lack of expression of certain receptor(s)^[22,61,62], making these cells not susceptible to some respiratory viruses.

The HAE-ALI model was previously used to infect a wide range of respiratory RNA viruses, such as influenza viruses and human coronaviruses, among others, from the apical surface^[63,64], unlike the respiratory DNA viruses, which were accomplished only from the basolateral surface^[65]. The HBoV1 virions are capable of, both productively and persistently, infecting HAE from both the apical and basolateral surfaces; but, a consequence of this infection can be induction of airway epithelial damage, evidenced by loss of cilia, disruption of the tight junction barrier and epithelial cell hypertrophy^[23,66]. Subsequently, commercially available HAE culture systems, namely EpiAirway (MatTek, Ashland, MA, United States) and MucilAir HAE (Epithelix Sàrl, Geneva, Switzerland),

were tested for HBoV1 infection. Despite the same results as obtained by the HAE made in-house, an innovative finding was that HBoV1 infection was demonstrated to persist for as long as 50 d^[67]. More recent data have displayed, for the first time, that HBoV1 infection of HAE-ALI induces a DNA damage response that facilitates viral genome amplification^[68]. In parallel, other cell lines can be infected by HBoV; for example, it has been established that transfection of a HBoV1 infectious clone into HEK293 cells can generate high titre progeny virions^[42,52].

The virus enters the host *via* the respiratory tract and through the bloodstream or by direct ingestion, reaching the gastrointestinal tract^[69]. HBoV1 has been detected in both respiratory and gastrointestinal tract. Several studies have shown the association between HBoV1 and the upper and lower respiratory tract. In this regard, the most frequently described clinical presentation of HBoV1 infection includes cough, fever, rhinorrhea, asthma exacerbation, bronchiolitis, acute wheezing and pneumonia^[1,5,18,19,70,71]. HBoV1 DNA has

also been found in stool samples of adult patients with the gastrointestinal manifestations of nausea, vomiting and diarrhoea^[72]. However, the HBoV1-load in stool samples of paediatric patients with acute gastroenteritis was reported to be lower than the viral loads in respiratory tract samples^[73]. In fact, a viral load median of 1.88×10^4 genome/mL has been reported for stool samples, which is lower than that found in the nasopharyngeal aspirates (NPA) of patients with respiratory infections (4.9×10^3 copies/mL)^[74,75]. HBoV2, as well as the other genotypes, is found more often in stool samples^[18,40,76,77] and HBoV2, and possibly HBoV3, associates with gastroenteritis^[3,10,78]. Among these, HBoV2 has been the only species isolated from NPA of children hospitalized with acute respiratory tract infections^[79]. More recent data show that HBoV can be detected directly and specifically in tissues such as the duodenum, paranasal sinus mucosa and intestinal biopsies^[34,37,39].

HBoV mono-infections are rare, while double-infections are observed frequently^[9,80]. Cases of HBoV infection show a high rate of co-infections with other viral and bacterial respiratory and gastroenteritis pathogens, such as human rhinovirus, adenovirus, norovirus, rotavirus^[6,73,81-83]. Notably, co-infecting pathogens have been found in up to 83% of respiratory samples^[14,18,70,84]. In particular, co-infection with respiratory syncytial virus (RSV) occurs very frequently (89.5%)^[85]. HBoV1 remains detectable in NPA samples of immune-competent subjects for up to 6 mo after infection^[86,87]. Consequently, HBoV1 is often detectable with other viruses in asymptomatic patients, facilitating the reactivation of a latent virus by a super-infection^[88]. Despite a low tropism of the virus being demonstrated in the human body^[31,89], HBoV shows a high persistence in some sites, in particular the lymphatic tissue^[33]. The persistence and reactivation of HBoV may explain the high prevalence of co-infections^[90], although its effects and mechanisms are still unclear and its contribution to active disease remains to be accurately established^[88]. In addition, individuals with low viral load are more likely to be co-infected with other pathogens compared to those with high viral load (57% vs 38.9%)^[84]. Clinically relevant infections, requiring hospitalization of the subject, are associated with co-infections of up to six different pathogens in a single patient^[91,92]. Furthermore, high viral load ($> 10^4$ copies/mL) has been shown in multiple studies to be statistically associated with severe clinical manifestations and longer hospitalization^[35,84,93]. In contrast to these findings, however, Ghietto *et al.*^[85], who screened 1135 respiratory samples from children and adults, both symptomatic and asymptomatic, found no association between high viral load and illness; yet, they did demonstrate that all asymptomatic subjects had a low viral load ($P < 0.005$). High viral load ($> 10^6$ copies/mL) in NPA was reported by Christensen *et al.*^[94]; their study also demonstrated that viremia

was more frequent in the subjects with high viral load (70%) than in those with a moderate or low virus load (10%). Hence, on the basis of the data reported in the literature to date, the role of this virus as a harmless passenger, rather than a true infecting agent, is still debatable, and, therefore, it remains to be established.

In the host, T-helper (Th) cells are essential for antiviral immunity since they participate in the antiviral responses both directly and indirectly. Their direct activities are exerted *via* their production of antiviral cytokines, whereas their indirect activities are mediated *via* the Th patterns that promote B cells and cytotoxic T cells^[95]. In a study of NPA from children with acute bronchiolitis, Chung *et al.*^[96] demonstrated higher concentrations of interferon-gamma (IFN- γ), interleukin (IL)-2 and IL-4 in the HBoV-positive subjects, compared to the asymptomatic controls; however, the cytokine levels of IL-10 and tumour necrosis factor-alpha were lower than those found in RSV-positive children. Furthermore, other studies demonstrated that HBoV1 induces IFN- γ against HBoV VP2 VLPs, IL-10 and IL-13 (Th2 cells) in CD4+ T cells^[97,98]. These findings suggest that HBoV infection can induce production of Th1 and Th2 cytokines. To date, however, the precise mechanisms underlying HBoV-specific T cell immunity have not been defined.

EPIDEMIOLOGY

HBoV has a worldwide distribution; its transmission and infection occurs throughout the year but is predominant during winter and spring months^[19,98]. The worldwide distribution of HBoV involves infections of the respiratory tract and gastrointestinal tract (as evidenced in stool samples) of children as well as adults in Europe^[2,83], Asia^[6,76,77], the Americas^[37,74,78,82], Africa^[4] and Australia^[3].

We estimated the global prevalence of infection based upon a search of articles published in the Medline database from September 6, 2005 (the year of HBoV discovery) to March 15, 2016 and including studies evaluating respiratory and gastrointestinal HBoV infection (Tables 1 and 2 respectively). For each country, we calculated prevalence estimates, 95% confidence intervals (CIs) and per cent of co-infections based on pooled data from all eligible studies and extracted data in a customised database. In total, we used 357 reports on the prevalence of HBoV correlated to respiratory illness and to gastrointestinal infections (Appendix A).

The average prevalence of HBoV in respiratory tract samples ranged from 1.0% (CI: 0.0-2.0) to 56.8% (CI: 46.9-66.8) (Table 1) and in stool specimens from 1.3% (CI: 0.0-3.9) to 63% (CI: 55.0-71.1), depending on the country (Table 2). Furthermore, the worldwide HBoV total prevalence estimates in respiratory infections is 6.3% (CI: 6.2-6.4) and in gastrointestinal infections is 5.9% (CI: 5.7-6.1) (Tables 1 and 2). With

Table 1 Prevalence of human bocavirus infection in respiratory infections and co-infections worldwide from 2005 to 2016

Country	Number of studies	Number of subjects	Prevalence estimates (%) (95%CI)	Number of studies	Number of HBoV+ subjects	Co-infections estimates (%) (95%CI)
Argentina	4	1536	13.5 (11.8-15.3)	4	208	55.8 (49.0-62.5)
Australia	10	2745	13.0 (11.7-14.2)	3	132	64.4 (56.2-72.6)
Belgium	1	445	11.5 (8.5-14.4)	1	51	49.0 (35.3-62.7)
Brazil	14	2718	10.8 (9.6-11.9)	10	293	90.1 (86.7-93.5)
Cambodia	4	3779	1.6 (1.2-2.0)	2	58	53.4 (40.6-66.3)
Canada	3	2825	4.1 (3.3-4.8)	2	97	59.8 (50.0-69.6)
China	62	106960	5.0 (4.9-5.2)	35	4088	50.3 (48.8-51.8)
Denmark	1	228	25.0 (19.4-30.6)	1	57	47.4 (34.4-60.3)
Egypt	1	95	56.8 (46.9-66.8)	NG	NG	-
Finland	11	4541	6.3 (5.6-7.0)	5	197	61.4 (54.6-68.2)
France	16	5826	6.1 (5.5-6.8)	13	282	31.2 (25.8-36.6)
Germany	17	4595	10.1 (9.2-10.9)	10	390	38.7 (33.9-43.6)
Greece	3	2039	5.8 (4.8-6.8)	3	118	48.3 (39.3-57.3)
Hong Kong	3	3709	7.6 (6.7-8.4)	1	95	18.9 (11.1-26.8)
Hungary	2	94	29.8 (20.5-39.0)	1	28	53.6 (35.1-72.0)
India	2	605	4.0 (2.4-5.5)	2	24	8.3 (0.0-19.4)
Iran	2	394	7.6 (5.0-10.2)	1	21	33.3 (13.2-53.5)
Israel	2	721	4.0 (2.6-5.5)	1	26	30.8 (13.0-48.5)
Italy	19	7354	7.4 (6.8-8.0)	14	513	62.8 (58.6-67.0)
Japan	8	5016	10.1 (9.3-10.9)	5	435	61.1 (56.6-65.7)
Jordan	1	312	18.3 (14.0-22.6)	1	57	89.5 (81.5-97.4)
Kenya	1	384	1.8 (0.5-3.2)	1	7	100.0 (-)
Kuwait	1	735	1.9 (0.9-2.9)	NG	NG	-
Malawi	1	95	6.3 (1.4-11.2)	NG	NG	-
Mexico	1	162	4.9 (1.6-8.3)	1	8	37.5 (4.0-71.0)
New Zealand	1	230	3.5 (1.1-5.8)	1	8	37.5 (4.0-71.0)
Nicaragua	1	192	33.3 (26.7-40.0)	NG	NG	-
Nigeria	1	246	2.4 (0.5-4.4)	1	6	83.3 (53.5-100)
Norway	3	1853	12.0 (10.5-13.5)	3	222	72.1 (66.2-78.0)
Peru	1	191	25.1 (19.0-31.3)	NG	NG	-
Portugal	3	428	6.5 (4.2-8.9)	NG	NG	-
Senegal	2	312	1.0 (0.0-2.0)	2	3	33.3 (0.0-86.7)
Shanghai	1	486	24.5 (20.7-28.3)	1	119	54.6 (45.7-63.6)
Singapore	1	500	8.0 (5.6-10.4)	1	40	30.0 (15.8-44.2)
Slovenia	1	891	18.4 (15.9-21.0)	1	164	59.8 (52.3-67.3)
South Africa	6	2880	10.3 (9.2-11.4)	5	278	56.8 (51.0-62.7)
South Arabia	5	833	5.3 (3.8-6.8)	4	39	53.8 (38.2-69.5)
South Korea	10	7594	5.8 (5.2-6.3)	10	437	43.0 (38.4-47.7)
Spain	16	13560	9.9 (9.4-10.4)	9	794	72.2 (69.0-75.3)
Sweden	3	956	12.1 (10.1-14.2)	3	116	38.8 (29.9-47.7)
Switzerland	3	279	4.3 (1.9-6.7)	NG	NG	-
Taiwan	5	1657	5.6 (4.4-6.7)	4	62	33.9 (22.1-45.7)
Thailand	8	2983	7.1 (6.2-8.0)	2	40	62.5 (47.5-77.5)
The Netherlands	5	2645	6.8 (5.9-7.8)	3	9	66.7 (35.9-97.5)
The Philippines	2	1284	1.0 (0.5-1.6)	1	7	71.4 (38.0-100)
Turkey	10	3949	2.3 (1.8-2.8)	4	39	30.8 (16.3-45.3)
United Kingdom	8	14923	1.5 (1.3-1.7)	4	102	32.4 (23.3-41.4)
Uruguay	1	1078	4.1 (2.9-5.3)	1	44	54.5 (39.8-69.3)
United States	21	13549	9.8 (9.3-10.3)	14	903	38.1 (34.9-41.3)
Vietnam	3	2349	5.5 (4.6-6.4)	2	128	45.3 (36.7-53.9)
Total	311	233761	6.3 (6.2-6.4)	193	10745	52.4 (51.5-53.4)

NG: Not given.

respect to the respiratory tract infections, prevalence averages < 2% have been reported for Cambodia (1.6%, CI: 1.2-2.0), Kenya (1.8%, CI: 0.5-3.2), Kuwait (1.9%, CI: 0.9-2.9), Senegal (1.0%, CI: 0.0-2.0) and the Philippines (1.0%, CI: 0.5-1.6). In contrast, the highest prevalence averages have been reported for Egypt (56.8%, CI: 46.9-66.8), Hungary (29.8%, CI: 20.5-39.0) and Nicaragua (33.3%, CI:

26.7-40.0) (Table 1).

Based on data available for gastrointestinal infections, countries such as Mexico and Russia have mostly low endemicity levels (1.3%, CI: 0.0-3.9 and 1.4%, CI: 1.1-1.6 respectively); conversely, high HBoV prevalence has been reported for Bangladesh (63.0%, CI: 55.0-77.1), Nigeria (29.2%, CI: 20.1-38.3) and Tunisia (58.3%, CI: 48.5-68.2) (Table 2).

Table 2 Prevalence of Human Bocavirus infection in gastrointestinal infections and co-infections worldwide from 2005 to 2016

Country	Number of studies	Number of subjects	Prevalence estimates (%) (95%CI)	Number of studies	Number of HBoV+ subjects	Co-infections estimates (%) (95%CI)
Albania	1	142	9.2 (4.4-13.9)	1	13	23.1 (0.2-46.0)
Australia	3	890	8.5 (6.7-10.4)	NG	NG	-
Bangladesh	1	138	63.0 (55.0-71.1)	NG	NG	-
Brazil	5	2469	4.8 (4.0-5.7)	5	119	15.1 (8.7-21.6)
Chile	1	462	19.3 (15.7-22.9)	NG	NG	-
China	16	8805	6.6 (6.1-7.1)	8	380	67.4 (62.7-72.1)
Finland	3	2493	10.3 (9.1-11.5)	2	251	32.7 (26.9-38.5)
Germany	2	338	8.3 (5.3-11.2)	2	28	28.6 (11.8-45.3)
Hong Kong	1	1600	6.4 (5.2-7.6)	NG	NG	-
Hungary	1	61	3.3 (0.0-7.7)	NG	NG	-
Iran	4	621	14.2 (11.4-16.9)	1	16	0.0 (0.0-0.0)
Ireland	1	155	7.7 (3.5-11.9)	1	12	100.0 (100.0-100.0)
Italy	2	1291	2.8 (1.9-3.7)	2	36	22.2 (8.6-35.8)
Japan	2	424	3.8 (2.0-5.6)	2	16	62.5 (38.8-86.2)
Mexico	1	76	1.3 (0.0-3.9)	1	1	100.0 (100.0-100.0)
Nepal	1	96	9.4 (3.5-15.2)	NG	NG	-
Nigeria	1	96	29.2 (20.1-38.3)	NG	NG	-
Pakistan	3	498	10.6 (7.9-13.4)	1	47	97.9 (93.7-100.0)
Paraguay	1	349	10.6 (7.4-13.8)	1	37	40.5 (24.7-56.4)
Russia	2	7031	1.4 (1.1-1.6)	2	95	49.5 (39.4-59.5)
South Korea	3	1640	3.8 (2.9-4.8)	2	55	14.5 (5.2-23.9)
Spain	1	520	9.2 (6.7-11.7)	1	48	52.1 (38.0-66.2)
Taiwan	1	110	3.6 (0.1-7.1)	NG	NG	-
Thailand	4	848	3.8 (2.5-5.1)	3	19	57.9 (35.7-80.1)
Tunisia	1	96	58.3 (48.5-68.2)	NG	NG	-
Turkey	1	150	8.7 (4.2-13.2)	NG	NG	-
United Kingdom	3	12459	5.4 (5.0-5.7)	1	324	46.0 (40.6-51.4)
United States	2	640	3.6 (2.2-5.0)	NG	NG	-
Total	68	44498	5.9 (5.7-6.1)	36	1497	46.7 (44.2-49.2)

NG: Not given.

The rate of co-infections in subjects with respiratory infections and HBoV-positivity ranges from 8.3% (CI: 0.0-19.4) to 100% (Table 1); meanwhile, the co-infection ratio is 46.7% (CI: 44.2-49.2) relative to the gastrointestinal manifestations (Table 2).

The seroprevalence of HBoV is age-related and ranges from about 40% in children between 18 and 23 mo of age up to virtually 100% in children older than 2 years, with an average of 76.6% in children and 96% in adults^[99,100]. A serological survey performed in Italy and involving 1206 participants was carried out with the aim of determining the presence of anti-HBoV IgG antibodies; the study showed a higher seropositive rate in children of 5-9 years of age (96.4%), with respect to those of 2-4 years of age (64.2%). Furthermore, the high seroprevalence observed in infants aged 0-5 mo (73.7%) and in children during the first months of life (51.4%) is assumed to be related to maternal antibodies^[101]. Kantola *et al*^[99] showed that the HBoVs infecting humans most frequently were, in descending order, HBoV1, HBoV2, HBoV3 and HBoV4. A recent seroprevalence study conducted in Beijing and Nanjing and involving 1391 samples showed the prevalence of HBoV1 and HBoV2 to be 73.4% (1021) and 70.5% (981) respectively^[102]; the results for HBoV1 were consistent with previous serological research conducted

in Japan (71.1%)^[100] and Jamaica (76.7%)^[103]. These findings indicate a high degree of antigenic cross-reactivity between HBoV1 and HBoV2^[102].

Conversely, the discrepancy observed in HBoV2 seroprevalence (70.5% vs 20.4%)^[104] is most likely due to differences in the methods used; in fact, while enzyme-linked immunosorbent assay (ELISA) indicate the exposure rate of accumulated infections, PCR results only reveal an on-going infection^[102].

DIAGNOSTICS

For many years, the diagnostic tools available for identification of the etiological agents associated with respiratory and gastroenteric diseases have been limited. At first, the main method to detect HBoV infections in respiratory and gastrointestinal samples was represented by a direct tool, namely conventional PCR^[5-7,13,19,61,62,72,80,105-114], which was followed by nested and real-time (RT)-PCR^[8,10,11,33,35,61,67,70,73,81,112,115-117].

PCR techniques enable isolation of viral genome fragments from NPA, broncho-alveolar, stool, serum and urine specimens through amplification of NP1, NS1 or/and the VP1/2 gene regions^[9], or *via* other nucleic acid-based detection HBoV diagnosis methods^[118-120]. NP1 and NS1 are more conserved than VP1/2, and

thus are commonly targeted for PCR-based detection of the virus^[13,111,121]. RT-PCR has an advantage over conventional PCR, being more specific and rapid, but its requirement for higher-cost oligoprobes is limiting^[11].

Application of RT-PCR to *NP1* and *VP1* genes for the detection of HBoV in swabs, faecal and whole blood samples allowed Tozer *et al.*^[11] to achieve clinical sensitivity of 100% and clinical specificity of 94% and 93% respectively for the NP1 and VP1 assays. Subsequently, multiplexing assays were developed to detect HBoV genotypes in respiratory infections; these included the commercially available Luminex RVP (Luminex Molecular Diagnostics, Toronto, Canada) and RespiFinder (Pathofinder, Maastricht, the Netherlands)^[69,122,123]. More recently, sequence-independent amplification techniques combined with next-generation sequencing platforms have been introduced, promising rapid and simultaneous detection of numerous pathogens. Despite their current drawbacks (*i.e.*, high cost, labour intensity, long turnaround time, specific training of personnel, etc.), as opposed to RT-PCR, these new approaches can provide more information regarding virus species/type; hence, they are of interest for virus diagnosis in clinical and public health settings^[124].

Serological methods have been developed for the estimation of HBoV-specific antibodies in serum samples; western blotting and immunofluorescence are among those^[104,125]. ELISA and enzyme immunoassay (EIA) are reliable qualitative and quantitative serologic assays used to detect IgG and IgM antibodies and IgG affinity^[99] using recombinant VP2 or VLP capsid proteins^[126]; the latter, for example, is produced by an insect cell line infected with a baculovirus vector and subsequently used to produce rabbit anti-HBoV antisera that is applied to develop an ELISA test^[127,128]. The IgG avidity test allows distinguishment between primary and secondary infections or immune-activations with high diagnostic specificity^[129]. Recent data have indicated that children with pre-existing HBoV2 immunity show cross-reactivity with HBoV1, which presents a paradigm of the hypothesized Original Antigenic Sin phenomenon^[130]. The few available serological studies to date have mainly addressed epidemiologic issues^[97,101,103,125,127,131]; however, considering that human bocaviruses are highly prevalent agents that can establish persistent infections, interpretation of the serological tests in the context of the clinical situation may be just as complicated as using the PCR results^[126].

Notably, Zaghloul^[132] determined the presence of HBoV in children *via* qualitative PCR detection of NPA and ELISA estimation of IgM antibodies in serum. Both assays were highly sensitive and specific; however, the ELISA had lower sensitivity than the PCR (81.8% vs 100%) but higher specificity (100% vs 78%).

CONCLUSION AND FUTURE CHALLENGE

Based on the current data, the pathogenic roles of the various HBoV genotypes in respiratory tract illness and gastrointestinal infections remain unresolved. It is possible that the virus may be both a passenger and a causative pathogen of acute respiratory tract and gastrointestinal diseases. The conflicting ideas on this pathogenic role mainly come from the fact that the Koch's revised postulates cannot be applied to HBoV, because neither an effective method for virus culture nor an animal model of infection is available in practice to date. Moreover, several studies have indicated that HBoV requires the presence of other agents to carry out the infection.

Recent studies have demonstrated that HBoV1 infection of HAE-ALI induces a DNA damage response that facilitates viral genome amplification^[68]. Nonetheless, further research to develop cell lines and animal models suitable for viral replication is needed in order to obtain more evidence to better understand the natural course of HBoV infection. In this respect, simpler culturing methods and infectious clones should be made available, since HBoV genomic analysis is difficult just for this reason^[38].

In spite of a relatively substantial amount of knowledge on the molecular basis of the HBoV life cycle, the function of several HBoV proteins still requires further investigation. For instance, only recently were three novel small NS proteins (NS2, NS3 and NS4) identified; among these, only one NS protein is critical to the replication of the virus in polarized human bronchial airway epithelium^[49]. The role of the other proteins remains rather uncertain.

Most of the studies to date have been performed on the HBoV1 genotype, whereas little information is available about the other agents. Notably, the presence of HBoV2, HBoV3 and HBoV4 in the respiratory tract should be further investigated, as well as their phylogenetic relationships. Our phylogenetic analysis suggests, as shown by other authors^[3,4], that HBoV3 may result from the recombination of HBoV1 and HBoV2; but, it may also be a hybrid of HBoV1, with a common ancestor of HBoV2 and HBoV4^[133]. In this respect, it would be appropriate for the future studies to test more, and simultaneously, (possibly all) genotypes and genes.

HBoV subtypes have been found worldwide, without any regional, geographic or border restrictions. HBoV1 is associated with paediatric respiratory illness but also with gastrointestinal symptoms^[6,134]. HBoV2, HBoV3 and HBoV4 are more frequently detected in stool samples and seem to be enteric^[4,107,135]. Moreover, the most typical age for HBoV infection is < 2-years-old; only rarely has it been found in adults and the elderly^[12]. In this respect, clinical studies would be useful to characterize disease pathogenesis and to understand

immunity in the various populations represented by infants, the elderly or immunocompromised individuals responding to HBoV infection.

There is also a need to optimize commercial diagnostic reagents and methods for HBoV identification. Overall, HBoV detection is mainly performed *via* molecular techniques (*i.e.*, PCR and RT-PCR)^[10]; only rarely is it done with serological methods (*i.e.*, ELISA, EIA, Western blotting and immunofluorescence), due to the lack of commercial kits^[99,103,125,126]. Furthermore, developing new sequence-independent amplification techniques combined with next generation sequencing platforms is worthy to achieve rapid and simultaneous detection of numerous pathogens^[124].

Finally, were the pathogenic role of HBoV to be confirmed, the development of an effective vaccine to control the spread of infection should be of primary importance. With the hope of achieving this goal, many studies have been performed on the HBoV viral capsid proteins. Previous research studies have confirmed that VLPs can be used as safe and effective vaccines. Recently, *in vitro* studies have demonstrated that HBoV VP2 VLPs have good immunogenicity and studies in mice have shown they can induce strong humoral and cellular immune responses, indicating their promise as candidate proteins for HBoV vaccine^[136]. Newer data suggest that the creation of non-replicating infectious HBoV1 mutants may represent a new approach for HBoV vaccine development^[49].

In conclusion, a better understanding of the natural course of HBoV infection, the implementation of experimental systems to analyse the replication cycle in more detail and the development of specific therapies are important and urgent needs.

REFERENCES

- 1 Allander T, Tammi MT, Eriksson M, Bjerkner A, Tiveljung-Lindell A, Andersson B. Cloning of a human parvovirus by molecular screening of respiratory tract samples. *Proc Natl Acad Sci USA* 2005; **102**: 12891-12896 [PMID: 16118271 DOI: 10.1073/pnas.0504666102]
- 2 Kapoor A, Slikas E, Simmonds P, Chiochansin T, Naeem A, Shaikat S, Alam MM, Sharif S, Angez M, Zaidi S, Delwart E. A newly identified bocavirus species in human stool. *J Infect Dis* 2009; **199**: 196-200 [PMID: 19072716 DOI: 10.1086/595831]
- 3 Arthur JL, Higgins GD, Davidson GP, Givney RC, Ratcliff RM. A novel bocavirus associated with acute gastroenteritis in Australian children. *PLoS Pathog* 2009; **5**: e1000391 [PMID: 19381259 DOI: 10.1371/journal.ppat.1000391]
- 4 Kapoor A, Simmonds P, Slikas E, Li L, Bodhidatta L, Sethabutr O, Triki H, Bahri O, Oderinde BS, Baba MM, Bukbuk DN, Besser J, Bartkus J, Delwart E. Human bocaviruses are highly diverse, dispersed, recombination prone, and prevalent in enteric infections. *J Infect Dis* 2010; **201**: 1633-1643 [PMID: 20415538 DOI: 10.1086/652416]
- 5 Bastien N, Brandt K, Dust K, Ward D, Li Y. Human Bocavirus infection, Canada. *Emerg Infect Dis* 2006; **12**: 848-850 [PMID: 16704852 DOI: 10.3201/eid1205.051424]
- 6 Lau SK, Yip CC, Que TL, Lee RA, Au-Yeung RK, Zhou B, So LY, Lau YL, Chan KH, Woo PC, Yuen KY. Clinical and molecular epidemiology of human bocavirus in respiratory and fecal samples from children in Hong Kong. *J Infect Dis* 2007; **196**: 986-993 [PMID: 17763318 DOI: 10.1086/521310]
- 7 Christensen A, Nordbø SA, Krokstad S, Rognlien AG, Døllner H. Human bocavirus commonly involved in multiple viral airway infections. *J Clin Virol* 2008; **41**: 34-37 [PMID: 18069054 DOI: 10.1016/j.jcv.2007.10.025]
- 8 Esposito S, Bosis S, Niesters HG, Tremolati E, Sabatini C, Porta A, Fossali E, Osterhaus AD, Principi N. Impact of human bocavirus on children and their families. *J Clin Microbiol* 2008; **46**: 1337-1342 [PMID: 18287315 DOI: 10.1128/JCM.02160-07]
- 9 Schildgen O, Müller A, Allander T, Mackay IM, Völz S, Kupfer B, Simon A. Human bocavirus: passenger or pathogen in acute respiratory tract infections? *Clin Microbiol Rev* 2008; **21**: 291-304, table of contents [PMID: 18400798 DOI: 10.1128/CMR.00030-07]
- 10 Chow BD, Esper FP. The human bocaviruses: a review and discussion of their role in infection. *Clin Lab Med* 2009; **29**: 695-713 [PMID: 19892229 DOI: 10.1016/j.cll.2009.07.010]
- 11 Tozer SJ, Lambert SB, Whiley DM, Bialasiewicz S, Lyon MJ, Nissen MD, Sloots TP. Detection of human bocavirus in respiratory, fecal, and blood samples by real-time PCR. *J Med Virol* 2009; **81**: 488-493 [PMID: 19152414 DOI: 10.1002/jmv.21409]
- 12 Martin ET, Taylor J, Kuypers J, Magaret A, Wald A, Zerr D, Englund JA. Detection of bocavirus in saliva of children with and without respiratory illness. *J Clin Microbiol* 2009; **47**: 4131-4132 [PMID: 19794045 DOI: 10.1128/JCM.01508-09]
- 13 Lee JI, Chung JY, Han TH, Song MO, Hwang ES. Detection of human bocavirus in children hospitalized because of acute gastroenteritis. *J Infect Dis* 2007; **196**: 994-997 [PMID: 17763319 DOI: 10.1086/521366]
- 14 Wang K, Wang W, Yan H, Ren P, Zhang J, Shen J, Deubel V. Correlation between bocavirus infection and humoral response, and co-infection with other respiratory viruses in children with acute respiratory infection. *J Clin Virol* 2010; **47**: 148-155 [PMID: 20022295 DOI: 10.1016/j.jcv.2009.11.015]
- 15 Hamza IA, Jurzik L, Wilhelm M, Uberla K. Detection and quantification of human bocavirus in river water. *J Gen Virol* 2009; **90**: 2634-2637 [PMID: 19656966 DOI: 10.1099/vir.0.013557-0]
- 16 Räsänen S, Lappalainen S, Kaikkonen S, Hämäläinen M, Salminen M, Vesikari T. Mixed viral infections causing acute gastroenteritis in children in a waterborne outbreak. *Epidemiol Infect* 2010; **138**: 1227-1234 [PMID: 20092670 DOI: 10.1017/S0950268809991671]
- 17 Li H, He M, Zeng P, Gao Z, Bian G, Yang C, Li W. The genomic and seroprevalence of human bocavirus in healthy Chinese plasma donors and plasma derivatives. *Transfusion* 2015; **55**: 154-163 [PMID: 25052026 DOI: 10.1111/trf.12785]
- 18 Jartti T, Hedman K, Jartti L, Ruuskanen O, Allander T, Söderlund-Venermo M. Human bocavirus-the first 5 years. *Rev Med Virol* 2012; **22**: 46-64 [PMID: 22038931 DOI: 10.1002/rmv.720]
- 19 Kesebir D, Vazquez M, Weibel C, Shapiro ED, Ferguson D, Landry ML, Kahn JS. Human bocavirus infection in young children in the United States: molecular epidemiological profile and clinical characteristics of a newly emerging respiratory virus. *J Infect Dis* 2006; **194**: 1276-1282 [PMID: 17041854 DOI: 10.1086/508213]
- 20 Fredricks DN, Relman DA. Sequence-based identification of microbial pathogens: a reconsideration of Koch's postulates. *Clin Microbiol Rev* 1996; **9**: 18-33 [PMID: 8665474]
- 21 Ong DS, Schuurman R, Heikens E. Human bocavirus in stool: A true pathogen or an innocent bystander? *J Clin Virol* 2016; **74**: 45-49 [PMID: 26655268 DOI: 10.1016/j.jcv.2015.11.027]
- 22 Dijkman R, Koekkoek SM, Molenkamp R, Schildgen O, van der Hoek L. Human bocavirus can be cultured in differentiated human airway epithelial cells. *J Virol* 2009; **83**: 7739-7748 [PMID: 19474096 DOI: 10.1128/JVI.00614-09]
- 23 Huang Q, Deng X, Yan Z, Cheng F, Luo Y, Shen W, Lei-Butters DC, Chen AY, Li Y, Tang L, Söderlund-Venermo M, Engelhardt JF, Qiu J. Establishment of a reverse genetics system for studying human bocavirus in human airway epithelia. *PLoS Pathog* 2012; **8**: e1002899 [PMID: 22956907 DOI: 10.1371/journal.ppat.1002899]
- 24 Cotmore SF, Agbandje-McKenna M, Chiorini JA, Mukha DV, Pintel DJ, Qiu J, Soderlund-Venermo M, Tattersall P, Tijssen

- P, Gatherer D, Davison AJ. The family Parvoviridae. *Arch Virol* 2014; **159**: 1239-1247 [PMID: 24212889 DOI: 10.1007/s00705-013-1914-1]
- 25 **Chen KC**, Shull BC, Moses EA, Lederman M, Stout ER, Bates RC. Complete nucleotide sequence and genome organization of bovine parvovirus. *J Virol* 1986; **60**: 1085-1097 [PMID: 3783814]
 - 26 **Fryer JF**, Kapoor A, Minor PD, Delwart E, Baylis SA. Novel parvovirus and related variant in human plasma. *Emerg Infect Dis* 2006; **12**: 151-154 [PMID: 16494735 DOI: 10.3201/eid1201.050916]
 - 27 **Lau SK**, Woo PC, Tse H, Fu CT, Au WK, Chen XC, Tsoi HW, Tsang TH, Chan JS, Tsang DN, Li KS, Tse CW, Ng TK, Tsang OT, Zheng BJ, Tam S, Chan KH, Zhou B, Yuen KY. Identification of novel porcine and bovine parvoviruses closely related to human parvovirus 4. *J Gen Virol* 2008; **89**: 1840-1848 [PMID: 18632954 DOI: 10.1099/vir.0.2008/000380-0]
 - 28 **Heegaard ED**, Brown KE. Human parvovirus B19. *Clin Microbiol Rev* 2002; **15**: 485-505 [PMID: 12097253 DOI: 10.1128/CMR.15.3.485-505.2002]
 - 29 **Riipinen A**, Väisänen E, Nuutila M, Sallmen M, Karikoski R, Lindbohm ML, Hedman K, Taskinen H, Söderlund-Venermo M. Parvovirus b19 infection in fetal deaths. *Clin Infect Dis* 2008; **47**: 1519-1525 [PMID: 18991512 DOI: 10.1086/593190]
 - 30 **Colmegna I**, Alberts-Grill N. Parvovirus B19: its role in chronic arthritis. *Rheum Dis Clin North Am* 2009; **35**: 95-110 [PMID: 19480999 DOI: 10.1016/j.rdc.2009.03.004]
 - 31 **Kueth F**, Lindner J, Matschke K, Wenzel JJ, Norja P, Ploetze K, Schaal S, Kamvissi V, Bornstein SR, Schwanebeck U, Modrow S. Prevalence of parvovirus B19 and human bocavirus DNA in the heart of patients with no evidence of dilated cardiomyopathy or myocarditis. *Clin Infect Dis* 2009; **49**: 1660-1666 [PMID: 19863443 DOI: 10.1086/648074]
 - 32 **Schildgen V**, Lüsebrink J, Tillmann RL, Wulfert M, Gattermann N, Schildgen O. Human bocavirus is not detectable in bone marrow from patients with myelodysplastic syndromes. *Influenza Other Respir Viruses* 2011; **5**: 221-222 [PMID: 21651730 DOI: 10.1111/j.1750-2659.2011.00200.x]
 - 33 **Lu X**, Gooding LR, Erdman DD. Human bocavirus in tonsillar lymphocytes. *Emerg Infect Dis* 2008; **14**: 1332-1334 [PMID: 18680679 DOI: 10.3201/eid1408.080300]
 - 34 **Falcone V**, Ridder GJ, Panning M, Bierbaum S, Neumann-Haefelin D, Huzly D. Human bocavirus DNA in paranasal sinus mucosa. *Emerg Infect Dis* 2011; **17**: 1564-1565 [PMID: 21801654 DOI: 10.3201/eid1708.101944]
 - 35 **Allander T**, Jartti T, Gupta S, Niesters HG, Lehtinen P, Osterback R, Vuorinen T, Waris M, Bjerkner A, Tiveljung-Lindell A, van den Hoogen BG, Hyypä T, Ruuskanen O. Human bocavirus and acute wheezing in children. *Clin Infect Dis* 2007; **44**: 904-910 [PMID: 17342639 DOI: 10.1086/512196]
 - 36 **Lüsebrink J**, Schildgen V, Tillmann RL, Wittleben F, Böhrer A, Müller A, Schildgen O. Detection of head-to-tail DNA sequences of human bocavirus in clinical samples. *PLoS One* 2011; **6**: e19457 [PMID: 21573237 DOI: 10.1371/journal.pone.0019457]
 - 37 **Kapoor A**, Hornig M, Asokan A, Williams B, Henriquez JA, Lipkin WI. Bocavirus episome in infected human tissue contains non-identical termini. *PLoS One* 2011; **6**: e21362 [PMID: 21738642 DOI: 10.1371/journal.pone.0021362]
 - 38 **Schildgen O**, Qiu J, Söderlund-Venermo M. Genomic features of the human bocaviruses. *Future Virol* 2012; **7**: 31-39 [PMID: 22389649 DOI: 10.2217/fvl.11.136]
 - 39 **Streiter M**, Malecki M, Prokop A, Schildgen V, Lüsebrink J, Guggemos A, Wisskirchen M, Weiss M, Cremer R, Brockmann M, Schildgen O. Does human bocavirus infection depend on helper viruses? A challenging case report. *Virol J* 2011; **8**: 417 [PMID: 21871135 DOI: 10.1186/1743-422X-8-417]
 - 40 **Babkin IV**, Tyumentsev AI, Tikunov AY, Zhirakovskaia EV, Netesov SV, Tikunova NV. A study of the human bocavirus replicative genome structures. *Virus Res* 2015; **195**: 196-202 [PMID: 25449911 DOI: 10.1016/j.virusres.2014.10.019]
 - 41 **Zhao H**, Zhao L, Sun Y, Qian Y, Liu L, Jia L, Zhang Y, Dong H. Detection of a bocavirus circular genome in fecal specimens from children with acute diarrhea in Beijing, China. *PLoS One* 2012; **7**: e48980 [PMID: 23133667 DOI: 10.1371/journal.pone.0048980]
 - 42 **Gurda BL**, Parent KN, Bladec H, Sinkovits RS, DiMattia MA, Rence C, Castro A, McKenna R, Olson N, Brown K, Baker TS, Agbandje-McKenna M. Human bocavirus capsid structure: insights into the structural repertoire of the parvoviridae. *J Virol* 2010; **84**: 5880-5889 [PMID: 20375175 DOI: 10.1128/JVI.02719-09]
 - 43 **Chen AY**, Cheng F, Lou S, Luo Y, Liu Z, Delwart E, Pintel D, Qiu J. Characterization of the gene expression profile of human bocavirus. *Virology* 2010; **403**: 145-154 [PMID: 20457462 DOI: 10.1016/j.virol.2010.04.014]
 - 44 **Tewary SK**, Zhao H, Shen W, Qiu J, Tang L. Structure of the NS1 protein N-terminal origin recognition/nickase domain from the emerging human bocavirus. *J Virol* 2013; **87**: 11487-11493 [PMID: 23966383 DOI: 10.1128/JVI.01770-13]
 - 45 **Smith RH**, Spano AJ, Kotin RM. The Rep78 gene product of adeno-associated virus (AAV) self-associates to form a hexameric complex in the presence of AAV ori sequences. *J Virol* 1997; **71**: 4461-4471 [PMID: 9151837]
 - 46 **Sun Y**, Chen AY, Cheng F, Guan W, Johnson FB, Qiu J. Molecular characterization of infectious clones of the minute virus of canines reveals unique features of bocaviruses. *J Virol* 2009; **83**: 3956-3967 [PMID: 19211770 DOI: 10.1128/JVI.02569-08]
 - 47 **Hsu TC**, Wu WJ, Chen MC, Tsay GJ. Human parvovirus B19 non-structural protein (NS1) induces apoptosis through mitochondria cell death pathway in COS-7 cells. *Scand J Infect Dis* 2004; **36**: 570-577 [PMID: 15370668 DOI: 10.1080/00365540410016230]
 - 48 **Hsu TC**, Tzang BS, Huang CN, Lee YJ, Liu GY, Chen MC, Tsay GJ. Increased expression and secretion of interleukin-6 in human parvovirus B19 non-structural protein (NS1) transfected COS-7 epithelial cells. *Clin Exp Immunol* 2006; **144**: 152-157 [PMID: 16542377 DOI: 10.1111/j.1365-2249.2006.03023.x]
 - 49 **Shen W**, Deng X, Zou W, Cheng F, Engelhardt JF, Yan Z, Qiu J. Identification and Functional Analysis of Novel Nonstructural Proteins of Human Bocavirus 1. *J Virol* 2015; **89**: 10097-10109 [PMID: 26223640 DOI: 10.1128/JVI.01374-15]
 - 50 **Sun B**, Cai Y, Li Y, Li J, Liu K, Li Y, Yang Y. The nonstructural protein NP1 of human bocavirus 1 induces cell cycle arrest and apoptosis in Hela cells. *Virology* 2013; **440**: 75-83 [PMID: 23507451 DOI: 10.1016/j.virol.2013.02.013]
 - 51 **Zou W**, Cheng F, Shen W, Engelhardt JF, Yan Z, Qiu J. Nonstructural Protein NP1 of Human Bocavirus 1 Plays a Critical Role in the Expression of Viral Capsid Proteins. *J Virol* 2016; **90**: 4658-4669 [PMID: 26912614 DOI: 10.1128/JVI.02964-15]
 - 52 **Shen W**, Deng X, Zou W, Engelhardt JF, Yan Z, Qiu J. Analysis of cis and trans Requirements for DNA Replication at the Right-End Hairpin of the Human Bocavirus 1 Genome. *J Virol* 2016; **90**: 7761-7777 [PMID: 27334591 DOI: 10.1128/JVI.00708-16]
 - 53 **Lindner J**, Modrow S. Human bocavirus—a novel parvovirus to infect humans. *Intervirology* 2008; **51**: 116-122 [PMID: 18536522 DOI: 10.1159/000137411]
 - 54 **Qiu J**, Cheng F, Johnson FB, Pintel D. The transcription profile of the bocavirus bovine parvovirus is unlike those of previously characterized parvoviruses. *J Virol* 2007; **81**: 12080-12085 [PMID: 17715221 DOI: 10.1128/JVI.00815-07]
 - 55 **Liu W**, Xie Y, Ma J, Luo X, Nie P, Zuo Z, Lahrmann U, Zhao Q, Zheng Y, Zhao Y, Xue Y, Ren J. IBS: an illustrator for the presentation and visualization of biological sequences. *Bioinformatics* 2015; **31**: 3359-3361 [PMID: 26069263 DOI: 10.1093/bioinformatics/btv362]
 - 56 **Lole KS**, Bollinger RC, Paranjape RS, Gadkari D, Kulkarni SS, Novak NG, Ingersoll R, Sheppard HW, Ray SC. Full-length human immunodeficiency virus type 1 genomes from subtype C-infected seroconverters in India, with evidence of intersubtype recombination. *J Virol* 1999; **73**: 152-160 [PMID: 9847317]
 - 57 **Sievers F**, Wilm A, Dineen D, Gibson TJ, Karplus K, Li W, Lopez R, McWilliam H, Remmert M, Söding J, Thompson JD, Higgins DG. Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. *Mol Syst Biol* 2011; **7**:

- 539 [PMID: 21988835 DOI: 10.1038/msb.2011.75]
- 58 **Tamura K**, Peterson D, Peterson N, Stecher G, Nei M, Kumar S. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol Biol Evol* 2011; **28**: 2731-2739 [PMID: 21546353 DOI: 10.1093/molbev/msr121]
 - 59 **Zehender G**, De Maddalena C, Canuti M, Zappa A, Amendola A, Lai A, Galli M, Tanzi E. Rapid molecular evolution of human bocavirus revealed by Bayesian coalescent inference. *Infect Genet Evol* 2010; **10**: 215-220 [PMID: 19932194 DOI: 10.1016/j.meegid.2009.11.011]
 - 60 **Lau SK**, Woo PC, Yip CC, Li KS, Fu CT, Huang Y, Chan KH, Yuen KY. Co-existence of multiple strains of two novel porcine bocaviruses in the same pig, a previously undescribed phenomenon in members of the family Parvoviridae, and evidence for inter- and intra-host genetic diversity and recombination. *J Gen Virol* 2011; **92**: 2047-2059 [PMID: 21632566 DOI: 10.1099/vir.0.033688-0]
 - 61 **Foulongne V**, Rodière M, Segondy M. Human Bocavirus in children. *Emerg Infect Dis* 2006; **12**: 862-863 [PMID: 16710957 DOI: 10.3201/eid1205.051523]
 - 62 **Ma X**, Endo R, Ishiguro N, Ebihara T, Ishiko H, Ariga T, Kikuta H. Detection of human bocavirus in Japanese children with lower respiratory tract infections. *J Clin Microbiol* 2006; **44**: 1132-1134 [PMID: 16517912 DOI: 10.1128/JCM.44.3.1132-1134.2006]
 - 63 **Ilyushina NA**, Ikizler MR, Kawaoka Y, Rudenko LG, Treanor JJ, Subbarao K, Wright PF. Comparative study of influenza virus replication in MDCK cells and in primary cells derived from adenoids and airway epithelium. *J Virol* 2012; **86**: 11725-11734 [PMID: 22915797 DOI: 10.1128/JVI.01477-12]
 - 64 **Pyrk K**, Sims AC, Dijkman R, Jebbink M, Long C, Deming D, Donaldson E, Vabret A, Baric R, van der Hoek L, Pickles R. Culturing the unculturable: human coronavirus HKU1 infects, replicates, and produces progeny virions in human ciliated airway epithelial cell cultures. *J Virol* 2010; **84**: 11255-11263 [PMID: 20719951 DOI: 10.1128/JVI.00947-10]
 - 65 **Zabner J**, Freimuth P, Puga A, Fabrega A, Welsh MJ. Lack of high affinity fiber receptor activity explains the resistance of ciliated airway epithelia to adenovirus infection. *J Clin Invest* 1997; **100**: 1144-1149 [PMID: 9276731 DOI: 10.1172/JCI83931]
 - 66 **Deng X**, Yan Z, Luo Y, Xu J, Cheng F, Li Y, Engelhardt JF, Qiu J. In vitro modeling of human bocavirus 1 infection of polarized primary human airway epithelia. *J Virol* 2013; **87**: 4097-4102 [PMID: 23345515 DOI: 10.1128/JVI.03132-12]
 - 67 **Deng X**, Li Y, Qiu J. Human bocavirus 1 infects commercially available primary human airway epithelium cultures productively. *J Virol Methods* 2014; **195**: 112-119 [PMID: 24134939 DOI: 10.1016/j.jviromet.2013.10.012]
 - 68 **Deng X**, Yan Z, Cheng F, Engelhardt JF, Qiu J. Replication of an Autonomously Human Parvovirus in Non-dividing Human Airway Epithelium Is Facilitated through the DNA Damage and Repair Pathways. *PLoS Pathog* 2016; **12**: e1005399 [PMID: 26765330 DOI: 10.1371/journal.ppat.1005399]
 - 69 **Schildgen O**. Human bocavirus: lessons learned to date. *Pathogens* 2013; **2**: 1-12 [PMID: 25436878 DOI: 10.3390/pathogens2010001]
 - 70 **Fry AM**, Lu X, Chittaganpitch M, Peret T, Fischer J, Dowell SF, Anderson LJ, Erdman D, Olsen SJ. Human bocavirus: a novel parvovirus epidemiologically associated with pneumonia requiring hospitalization in Thailand. *J Infect Dis* 2007; **195**: 1038-1045 [PMID: 17330795 DOI: 10.1086/512163]
 - 71 **Dina J**, Vabret A, Gouarin S, Petitjean J, Lecoq J, Brouard J, Arion A, Lafay-Delaire F, Freymuth F. Detection of human bocavirus in hospitalised children. *J Paediatr Child Health* 2009; **45**: 149-153 [PMID: 19210599 DOI: 10.1111/j.1440-1754.2008.01442.x]
 - 72 **Yu JM**, Li DD, Xu ZQ, Cheng WX, Zhang Q, Li HY, Cui SX, Miao-Jin SH, Fang ZY, Duan ZJ. Human bocavirus infection in children hospitalized with acute gastroenteritis in China. *J Clin Virol* 2008; **42**: 280-285 [PMID: 18499516 DOI: 10.1016/j.jcv.2008.03.032]
 - 73 **Campe H**, Hartberger C, Sing A. Role of Human Bocavirus infections in outbreaks of gastroenteritis. *J Clin Virol* 2008; **43**: 340-342 [PMID: 18835213 DOI: 10.1016/j.jcv.2008.07.014]
 - 74 **Proenca-Modena JL**, Martinez M, Amarilla AA, Espinola EE, Galeano ME, Fariña N, Russomando G, Aquino VH, Parra GI, Arruda E. Viral load of human bocavirus-1 in stools from children with viral diarrhoea in Paraguay. *Epidemiol Infect* 2013; **141**: 2576-2580 [PMID: 23425775 DOI: 10.1017/S095026881300023X]
 - 75 **Neske F**, Blessing K, Tollmann F, Schubert J, Rethwilm A, Kreth HW, Weissbrich B. Real-time PCR for diagnosis of human bocavirus infections and phylogenetic analysis. *J Clin Microbiol* 2007; **45**: 2116-2122 [PMID: 17475762 DOI: 10.1128/JCM.00027-07]
 - 76 **Alam MM**, Khurshid A, Shaikat S, Sharif S, Suleman RM, Angez M, Nisar N, Aamir UB, Naeem M, Zaidi SS. 'Human bocavirus in Pakistani children with gastroenteritis'. *J Med Virol* 2015; **87**: 656-663 [PMID: 25611467 DOI: 10.1002/jmv.24090]
 - 77 **Khamrin P**, Malasao R, Chaimongkol N, Ukarapol N, Kong-sricharoen T, Okitsu S, Hayakawa S, Ushijima H, Maneekarn N. Circulating of human bocavirus 1, 2, 3, and 4 in pediatric patients with acute gastroenteritis in Thailand. *Infect Genet Evol* 2012; **12**: 565-569 [PMID: 22333841 DOI: 10.1016/j.meegid.2012.01.025]
 - 78 **Santos N**, Peret TC, Humphrey CD, Albuquerque MC, Silva RC, Benati FJ, Lu X, Erdman DD. Human bocavirus species 2 and 3 in Brazil. *J Clin Virol* 2010; **48**: 127-130 [PMID: 20382557 DOI: 10.1016/j.jcv.2010.03.014]
 - 79 **Song JR**, Jin Y, Xie ZP, Gao HC, Xiao NG, Chen WX, Xu ZQ, Yan KL, Zhao Y, Hou YD, Duan ZJ. Novel human bocavirus in children with acute respiratory tract infection. *Emerg Infect Dis* 2010; **16**: 324-327 [PMID: 20113572 DOI: 10.3201/eid1602.090553]
 - 80 **Völz S**, Schildgen O, Klinkenberg D, Ditt V, Müller A, Tillmann RL, Kupfer B, Bode U, Lentze MJ, Simon A. Prospective study of Human Bocavirus (HBoV) infection in a pediatric university hospital in Germany 2005/2006. *J Clin Virol* 2007; **40**: 229-235 [PMID: 17851126 DOI: 10.1016/j.jcv.2007.07.017]
 - 81 **Regamey N**, Frey U, Deffernez C, Latzin P, Kaiser L. Isolation of human bocavirus from Swiss infants with respiratory infections. *Pediatr Infect Dis J* 2007; **26**: 177-179 [PMID: 17259883 DOI: 10.1097/01.inf.0000250623.43107.bc]
 - 82 **Chhabra P**, Payne DC, Szilagyi PG, Edwards KM, Staat MA, Shirley SH, Wikswo M, Nix WA, Lu X, Parashar UD, Vinjé J. Etiology of viral gastroenteritis in children < 5 years of age in the United States, 2008-2009. *J Infect Dis* 2013; **208**: 790-800 [PMID: 23757337 DOI: 10.1093/infdis/jit254]
 - 83 **Guido M**, Quattrocchi M, Campa A, Zizza A, Grima P, Romano A, De Donno A. Human metapneumovirus and human bocavirus associated with respiratory infection in Apulian population. *Virology* 2011; **417**: 64-70 [PMID: 21636105 DOI: 10.1016/j.virol.2011.04.016]
 - 84 **Jiang W**, Yin F, Zhou W, Yan Y, Ji W. Clinical significance of different virus load of human bocavirus in patients with lower respiratory tract infection. *Sci Rep* 2016; **6**: 20246 [PMID: 26832453 DOI: 10.1038/srep20246]
 - 85 **Ghietto LM**, Majul D, Ferreyra Soaje P, Baumeister E, Avaro M, Insfrán C, Mosca L, Cámara A, Moreno LB, Adamo MP. Comorbidity and high viral load linked to clinical presentation of respiratory human bocavirus infection. *Arch Virol* 2015; **160**: 117-127 [PMID: 25269520 DOI: 10.1007/s00705-014-2238-5]
 - 86 **Blessing K**, Neske F, Herre U, Kreth HW, Weissbrich B. Prolonged detection of human bocavirus DNA in nasopharyngeal aspirates of children with respiratory tract disease. *Pediatr Infect Dis J* 2009; **28**: 1018-1019 [PMID: 19730155 DOI: 10.1097/INF.0b013e3181a854ae]
 - 87 **Martin ET**, Fairchok MP, Kuypers J, Magaret A, Zerr DM, Wald A, Englund JA. Frequent and prolonged shedding of bocavirus in young children attending daycare. *J Infect Dis* 2010; **201**: 1625-1632 [PMID: 20415535 DOI: 10.1086/652405]
 - 88 **Peltola V**, Söderlund-Venermo M, Jartti T. Human bocavirus infections. *Pediatr Infect Dis J* 2013; **32**: 178-179 [PMID: 23328822 DOI: 10.1097/INF.0b013e31827fef67]
 - 89 **Manning A**, Willey SJ, Bell JE, Simmonds P. Comparison of tissue distribution, persistence, and molecular epidemiology of

- parvovirus B19 and novel human parvoviruses PARV4 and human bocavirus. *J Infect Dis* 2007; **195**: 1345-1352 [PMID: 17397006 DOI: 10.1086/513280]
- 90 **Gerna G**, Piralla A, Campanini G, Marchi A, Stronati M, Rovida F. The human bocavirus role in acute respiratory tract infections of pediatric patients as defined by viral load quantification. *New Microbiol* 2007; **30**: 383-392 [PMID: 18080673]
 - 91 **Arnott A**, Vong S, Rith S, Naughtin M, Ly S, Guillard B, Deubel V, Buchy P. Human bocavirus amongst an all-ages population hospitalised with acute lower respiratory infections in Cambodia. *Influenza Other Respir Viruses* 2013; **7**: 201-210 [PMID: 22531100 DOI: 10.1111/j.1750-2659.2012.00369.x]
 - 92 **Do AH**, van Doorn HR, Nghiem MN, Bryant JE, Hoang TH, Do QH, Van TL, Tran TT, Wills B, Nguyen VC, Vo MH, Vo CK, Nguyen MD, Farrar J, Tran TH, de Jong MD. Viral etiologies of acute respiratory infections among hospitalized Vietnamese children in Ho Chi Minh City, 2004-2008. *PLoS One* 2011; **6**: e18176 [PMID: 21455313 DOI: 10.1371/journal.pone.0018176]
 - 93 **Deng Y**, Gu X, Zhao X, Luo J, Luo Z, Wang L, Fu Z, Yang X, Liu E. High viral load of human bocavirus correlates with duration of wheezing in children with severe lower respiratory tract infection. *PLoS One* 2012; **7**: e34353 [PMID: 22479609 DOI: 10.1371/journal.pone.0034353]
 - 94 **Christensen A**, Nordbø SA, Krokstad S, Rognlien AG, Døllner H. Human bocavirus in children: mono-detection, high viral load and viraemia are associated with respiratory tract infection. *J Clin Virol* 2010; **49**: 158-162 [PMID: 20833582 DOI: 10.1016/j.jcv.2010.07.016]
 - 95 **Guidotti LG**, Chisari FV. Noncytolytic control of viral infections by the innate and adaptive immune response. *Annu Rev Immunol* 2001; **19**: 65-91 [PMID: 11244031 DOI: 10.1146/annurev.immunol.19.1.65]
 - 96 **Chung JY**, Han TH, Kim JS, Kim SW, Park CG, Hwang ES. Th1 and Th2 cytokine levels in nasopharyngeal aspirates from children with human bocavirus bronchiolitis. *J Clin Virol* 2008; **43**: 223-225 [PMID: 18650126 DOI: 10.1016/j.jcv.2008.06.008]
 - 97 **Lindner J**, Zehentmeier S, Franssila R, Barabas S, Schroeder J, Deml L, Modrow S. CD4+ T helper cell responses against human bocavirus viral protein 2 viruslike particles in healthy adults. *J Infect Dis* 2008; **198**: 1677-1684 [PMID: 18831690 DOI: 10.1086/592985]
 - 98 **Kumar A**, Filippone C, Lahtinen A, Hedman L, Söderlund-Venermo M, Hedman K, Franssila R. Comparison of Th-cell immunity against human bocavirus and parvovirus B19: proliferation and cytokine responses are similar in magnitude but more closely interrelated with human bocavirus. *Scand J Immunol* 2011; **73**: 135-140 [PMID: 21198754 DOI: 10.1111/j.1365-3083.2010.02483.x]
 - 99 **Kantola K**, Hedman L, Arthur J, Alibeto A, Delwart E, Jartti T, Ruuskanen O, Hedman K, Söderlund-Venermo M. Seroepidemiology of human bocaviruses 1-4. *J Infect Dis* 2011; **204**: 1403-1412 [PMID: 21921203 DOI: 10.1093/infdis/jir525]
 - 100 **Hustedt JW**, Christie C, Hustedt MM, Esposito D, Vazquez M. Seroepidemiology of human bocavirus infection in Jamaica. *PLoS One* 2012; **7**: e38206 [PMID: 22666484 DOI: 10.1371/journal.pone.0038206]
 - 101 **Guido M**, Zizza A, Bredl S, Lindner J, De Donno A, Quattrocchi M, Grima P, Modrow S. Seroepidemiology of human bocavirus in Apulia, Italy. *Clin Microbiol Infect* 2012; **18**: E74-E76 [PMID: 22309610 DOI: 10.1111/j.1469-0691.2011.03756.x]
 - 102 **Hao Y**, Gao J, Zhang X, Liu N, Li J, Zheng L, Duan Z. Seroepidemiology of human bocaviruses 1 and 2 in China. *PLoS One* 2015; **10**: e0122751 [PMID: 25923974 DOI: 10.1371/journal.pone.0122751]
 - 103 **Endo R**, Ishiguro N, Kikuta H, Teramoto S, Shirkoohi R, Ma X, Ebihara T, Ishiko H, Ariga T. Seroepidemiology of human bocavirus in Hokkaido prefecture, Japan. *J Clin Microbiol* 2007; **45**: 3218-3223 [PMID: 17699639 DOI: 10.1128/JCM.02140-06]
 - 104 **Jin Y**, Cheng WX, Xu ZQ, Liu N, Yu JM, Li HY, Jin M, Li DD, Zhang Q, Duan ZJ. High prevalence of human bocavirus 2 and its role in childhood acute gastroenteritis in China. *J Clin Virol* 2011; **52**: 251-253 [PMID: 21907613 DOI: 10.1016/j.jcv.2011.07.012]
 - 105 **Manning A**, Russell V, Eastick K, Leadbetter GH, Hallam N, Templeton K, Simmonds P. Epidemiological profile and clinical associations of human bocavirus and other human parvoviruses. *J Infect Dis* 2006; **194**: 1283-1290 [PMID: 17041855 DOI: 10.1086/508219]
 - 106 **Jacques J**, Moret H, Renois F, Lévêque N, Motte J, Andréoletti L. Human Bocavirus quantitative DNA detection in French children hospitalized for acute bronchiolitis. *J Clin Virol* 2008; **43**: 142-147 [PMID: 18644746 DOI: 10.1016/j.jcv.2008.05.010]
 - 107 **Arnold JC**, Singh KK, Spector SA, Sawyer MH. Human bocavirus: prevalence and clinical spectrum at a children's hospital. *Clin Infect Dis* 2006; **43**: 283-288 [PMID: 16804840 DOI: 10.1086/505399]
 - 108 **Maggi F**, Andreoli E, Pifferi M, Meschi S, Rocchi J, Bendinelli M. Human bocavirus in Italian patients with respiratory diseases. *J Clin Virol* 2007; **38**: 321-325 [PMID: 17336143 DOI: 10.1016/j.jcv.2007.01.008]
 - 109 **Arden KE**, McErlean P, Nissen MD, Sloots TP, Mackay IM. Frequent detection of human rhinoviruses, paramyxoviruses, coronaviruses, and bocavirus during acute respiratory tract infections. *J Med Virol* 2006; **78**: 1232-1240 [PMID: 16847968 DOI: 10.1002/jmv.20689]
 - 110 **Simon A**, Groneck P, Kupfer B, Kaiser R, Plum G, Tillmann RL, Müller A, Schildgen O. Detection of bocavirus DNA in nasopharyngeal aspirates of a child with bronchiolitis. *J Infect* 2007; **54**: e125-e127 [PMID: 16968654 DOI: 10.1016/j.jinf.2006.08.001]
 - 111 **Chieochansin T**, Chutinimitkul S, Payungporn S, Hiranras T, Samransamruajkit R, Theamboonlers A, Poovorawan Y. Complete coding sequences and phylogenetic analysis of Human Bocavirus (HBoV). *Virus Res* 2007; **129**: 54-57 [PMID: 17532505 DOI: 10.1016/j.virusres.2007.04.022]
 - 112 **Foulongne V**, Olejnik Y, Perez V, Elaerts S, Rodière M, Segondy M. Human bocavirus in French children. *Emerg Infect Dis* 2006; **12**: 1251-1253 [PMID: 16965707 DOI: 10.3201/eid1208.060213]
 - 113 **Terrosi C**, Fabbiani M, Cellesi C, Cusi MG. Human bocavirus detection in an atopic child affected by pneumonia associated with wheezing. *J Clin Virol* 2007; **40**: 43-45 [PMID: 17686654 DOI: 10.1016/j.jcv.2007.06.011]
 - 114 **Margaret IP**, Nelson EA, Cheuk ES, Leung E, Sung R, Chan PK. Pediatric hospitalization of acute respiratory tract infections with Human Bocavirus in Hong Kong. *J Clin Virol* 2008; **42**: 72-74 [PMID: 18296108 DOI: 10.1016/j.jcv.2007.12.016]
 - 115 **Hamano-Hasegawa K**, Morozumi M, Nakayama E, Chiba N, Murayama SY, Takayanagi R, Iwata S, Sunakawa K, Ubukata K. Comprehensive detection of causative pathogens using real-time PCR to diagnose pediatric community-acquired pneumonia. *J Infect Chemother* 2008; **14**: 424-432 [PMID: 19089556 DOI: 10.1007/s10156-008-0648-6]
 - 116 **Schenk T**, Huck B, Forster J, Berner R, Neumann-Haefelin D, Falcone V. Human bocavirus DNA detected by quantitative real-time PCR in two children hospitalized for lower respiratory tract infection. *Eur J Clin Microbiol Infect Dis* 2007; **26**: 147-149 [PMID: 17216422 DOI: 10.1007/s10096-006-0244-6]
 - 117 **Regamey N**, Kaiser L, Roiha HL, Deffèrnez C, Kuehni CE, Latzin P, Aebi C, Frey U. Viral etiology of acute respiratory infections with cough in infancy: a community-based birth cohort study. *Pediatr Infect Dis J* 2008; **27**: 100-105 [PMID: 18174876 DOI: 10.1097/INF.0b013e31815922c8]
 - 118 **Böhmer A**, Schildgen V, Lüsebrink J, Ziegler S, Tillmann RL, Kleines M, Schildgen O. Novel application for isothermal nucleic acid sequence-based amplification (NASBA). *J Virol Methods* 2009; **158**: 199-201 [PMID: 19428591 DOI: 10.1016/j.jviromet.2009.02.010]
 - 119 **Lassaunière R**, Kresfelder T, Venter M. A novel multiplex real-time RT-PCR assay with FRET hybridization probes for the detection and quantitation of 13 respiratory viruses. *J Virol Methods* 2010; **165**: 254-260 [PMID: 20153377 DOI: 10.1016/

- j.jviromet.2010.02.005]
- 120 **Loeffelholz MJ**, Pong DL, Pyles RB, Xiong Y, Miller AL, Bufton KK, Chonmaitree T. Comparison of the FilmArray Respiratory Panel and Prodesse real-time PCR assays for detection of respiratory pathogens. *J Clin Microbiol* 2011; **49**: 4083-4088 [PMID: 21998418 DOI: 10.1128/JCM.05010-11]
 - 121 **Gendrel D**, Guedj R, Pons-Catalano C, Emirian A, Raymond J, Rozenberg F, Lebon P. Human bocavirus in children with acute asthma. *Clin Infect Dis* 2007; **45**: 404-405 [PMID: 17599330 DOI: 10.1086/521125]
 - 122 **Babady NE**, Mead P, Stiles J, Brennan C, Li H, Shuptrav S, Stratton CW, Tang YW, Kamboj M. Comparison of the Luminex xTAG RVP Fast assay and the Idaho Technology FilmArray RP assay for detection of respiratory viruses in pediatric patients at a cancer hospital. *J Clin Microbiol* 2012; **50**: 2282-2288 [PMID: 22518855 DOI: 10.1128/JCM.06186-11]
 - 123 **Balada-Llasat JM**, LaRue H, Kamboj K, Rigali L, Smith D, Thomas K, Pancholi P. Detection of yeasts in blood cultures by the Luminex xTAG fungal assay. *J Clin Microbiol* 2012; **50**: 492-494 [PMID: 22170902 DOI: 10.1128/JCM.06375-11]
 - 124 **Prachayangprecha S**, Schapendonk CM, Koopmans MP, Osterhaus AD, Schürch AC, Pas SD, van der Eijk AA, Poovorawan Y, Haagmans BL, Smits SL. Exploring the potential of next-generation sequencing in detection of respiratory viruses. *J Clin Microbiol* 2014; **52**: 3722-3730 [PMID: 25100822 DOI: 10.1128/JCM.01641-14]
 - 125 **Kantola K**, Hedman L, Allander T, Jartti T, Lehtinen P, Ruuskanen O, Hedman K, Söderlund-Venermo M. Serodiagnosis of human bocavirus infection. *Clin Infect Dis* 2008; **46**: 540-546 [PMID: 18199037 DOI: 10.1086/526532]
 - 126 **Söderlund-Venermo M**, Lahtinen A, Jartti T, Hedman L, Kempainen K, Lehtinen P, Allander T, Ruuskanen O, Hedman K. Clinical assessment and improved diagnosis of bocavirus-induced wheezing in children, Finland. *Emerg Infect Dis* 2009; **15**: 1423-1430 [PMID: 19788810 DOI: 10.3201/eid1509.090204]
 - 127 **Kahn JS**, Kesebir D, Cotmore SF, D'Abramo A, Cosby C, Weibel C, Tattersall P. Seroepidemiology of human bocavirus defined using recombinant virus-like particles. *J Infect Dis* 2008; **198**: 41-50 [PMID: 18491974 DOI: 10.1086/588674]
 - 128 **Lüsebrink J**, Wittleben F, Schildgen V, Schildgen O. Human bocavirus - insights into a newly identified respiratory virus. *Viruses* 2009; **1**: 3-12 [PMID: 21994534 DOI: 10.3390/v1010003]
 - 129 **Hedman L**, Söderlund-Venermo M, Jartti T, Ruuskanen O, Hedman K. Dating of human bocavirus infection with protein-denaturing IgG-avidity assays-Secondary immune activations are ubiquitous in immunocompetent adults. *J Clin Virol* 2010; **48**: 44-48 [PMID: 20227338 DOI: 10.1016/j.jcv.2010.02.003]
 - 130 **Li X**, Kantola K, Hedman L, Arku B, Hedman K, Söderlund-Venermo M. Original antigenic sin with human bocaviruses 1-4. *J Gen Virol* 2015; **96**: 3099-3108 [PMID: 26224569 DOI: 10.1099/jgv.0.000253]
 - 131 **Lin F**, Guan W, Cheng F, Yang N, Pintel D, Qiu J. ELISAs using human bocavirus VP2 virus-like particles for detection of antibodies against HBoV. *J Virol Methods* 2008; **149**: 110-117 [PMID: 18289709 DOI: 10.1016/j.jviromet.2007.12.016]
 - 132 **Zaghloul MZ**. Human bocavirus (HBoV) in children with respiratory tract infection by enzyme linked immunosorbent assay (ELISA) and qualitative polymerase chain reaction (PCR). *Virol J* 2011; **8**: 239 [PMID: 21595869 DOI: 10.1186/1743-422X-8-239]
 - 133 **Cheng W**, Chen J, Xu Z, Yu J, Huang C, Jin M, Li H, Zhang M, Jin Y, Duan ZJ. Phylogenetic and recombination analysis of human bocavirus 2. *BMC Infect Dis* 2011; **11**: 50 [PMID: 21345238 DOI: 10.1186/1471-2334-11-50]
 - 134 **Jartti L**, Langen H, Söderlund-Venermo M, Vuorinen T, Ruuskanen O, Jartti T. New respiratory viruses and the elderly. *Open Respir Med J* 2011; **5**: 61-69 [PMID: 21760867 DOI: 10.2174/1874306401105010061]
 - 135 **Chow BD**, Ou Z, Esper FP. Newly recognized bocaviruses (HBoV, HBoV2) in children and adults with gastrointestinal illness in the United States. *J Clin Virol* 2010; **47**: 143-147 [PMID: 20036617 DOI: 10.1016/j.jcv.2009.11.030]
 - 136 **Deng ZH**, Hao YX, Yao LH, Xie ZP, Gao HC, Xie LY, Zhong LL, Zhang B, Cao YD, Duan ZJ. Immunogenicity of recombinant human bocavirus-1,2 VP2 gene virus-like particles in mice. *Immunology* 2014; **142**: 58-66 [PMID: 24843872 DOI: 10.1111/imm.12202]

P- Reviewer: Adamo MP, Garcia-Olmo D, Sun YN **S- Editor:** Yu J
L- Editor: A **E- Editor:** Wang CH



Improved glucose metabolism following bariatric surgery is associated with increased circulating bile acid concentrations and remodeling of the gut microbiome

Lukasz Kaska, Tomasz Sledzinski, Agnieszka Chomiczewska, Agnieszka Dettlaff-Pokora, Julian Swierczynski

Lukasz Kaska, General, Endocrine and Transplant Surgery, Medical University of Gdańsk, 80-214 Gdańsk, Poland

Tomasz Sledzinski, Agnieszka Chomiczewska, Department of Pharmaceutical Biochemistry, Medical University of Gdańsk, 80-211 Gdańsk, Poland

Agnieszka Dettlaff-Pokora, Julian Swierczynski, Department of Biochemistry, Medical University of Gdańsk, 80-211 Gdańsk, Poland

Julian Swierczynski, State School of Higher Vocational Education in Koszalin, 75-582 Koszalin, Poland

Author contributions: Kaska L, Sledzinski T and Chomiczewska A contributed to the background research, the formulation of the manuscript, and the revision of the manuscript; Dettlaff-Pokora A contributed to background research, formulation of the manuscript, and management of the images; Swierczynski J contributed to background research, formulation of the manuscript, revision of the manuscript, and final approval of the manuscript.

Supported by the Medical University of Gdańsk, No. ST-41 and No. ST-40; the Ministry of Science and Higher Education of the Republic of Poland under the Leading National Research Centre (KNOW) program, No. 2012-2017.

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

Open-Access: This article is an open-access article which was selected by an in-house editor and fully peer-reviewed by external reviewers. It is distributed in accordance with the Creative Commons Attribution Non Commercial (CC BY-NC 4.0) license, which permits others to distribute, remix, adapt, build upon this work non-commercially, and license their derivative works on different terms, provided the original work is properly cited and the use is non-commercial. See: <http://creativecommons.org/licenses/by-nc/4.0/>

Manuscript source: Invited manuscript

Correspondence to: Julian Swierczynski, Professor, Department of Biochemistry, Medical University of Gdańsk, ul. Dębinki 1, 80-211 Gdańsk, Poland. juls@gumed.edu.pl
Telephone: +48-58-34914 62
Fax: +48-58-34914 65

Received: July 12, 2016
Peer-review started: July 14, 2016
First decision: August 8, 2016
Revised: August 23, 2016
Accepted: September 14, 2016
Article in press: September 14, 2016
Published online: October 21, 2016

Abstract

Clinical studies have indicated that circulating bile acid (BA) concentrations increase following bariatric surgery, especially following malabsorptive procedures such as Roux-en-Y gastric bypasses (RYGB). Moreover, total circulating BA concentrations in patients following RYGB are positively correlated with serum glucagon-like peptide-1 concentrations and inversely correlated with postprandial glucose concentrations. Overall, these data suggest that the increased circulating BA concentrations following bariatric surgery - independently of calorie restriction and body-weight loss - could contribute, at least in part, to improvements in insulin sensitivity, incretin hormone secretion, and postprandial glycemia, leading to the remission of type-2 diabetes (T2DM). In humans, the primary and secondary BA pool size is dependent on the rate of biosynthesis and the enterohepatic circulation of BAs, as well as on the gut microbiota, which play a crucial role in BA biotransformation. Moreover, BAs and gut microbiota are closely integrated and affect each other. Thus, the alterations in bile flow that result from anatomical changes caused by bariatric surgery and changes in gut

microbiome may influence circulating BA concentrations and could subsequently contribute to T2DM remission following RYGB. Research data coming largely from animal and cell culture models suggest that BAs can contribute, *via* nuclear farnesoid X receptor (FXR) and membrane G-protein-receptor (TGR-5), to beneficial effects on glucose metabolism. It is therefore likely that FXR, TGR-5, and BAs play a similar role in glucose metabolism following bariatric surgery in humans. The objective of this review is to discuss in detail the results of published studies that show how bariatric surgery affects glucose metabolism and subsequently T2DM remission.

Key words: Bariatric surgery; Type-2 diabetes; Bile acids; RXR; TGR-5; Gut microbiota; Roux-en-Y gastric bypasses

© The Author(s) 2016. Published by Baishideng Publishing Group Inc. All rights reserved.

Core tip: Emerging evidence suggests that increased concentrations of circulating bile acids could, through their interaction with membrane (TGR-5) and nuclear (FXR) receptors, significantly contribute to improved glucose metabolism following bariatric surgery. This review presents information on the potential mechanism of bile acids on the remission of type-2 diabetes following bariatric surgery.

Kaska L, Sledzinski T, Chomiczewska A, Dettlaff-Pokora A, Swierczynski J. Improved glucose metabolism following bariatric surgery is associated with increased circulating bile acid concentrations and remodeling of the gut microbiome. *World J Gastroenterol* 2016; 22(39): 8698-8719 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v22/i39/8698.htm> DOI: <http://dx.doi.org/10.3748/wjg.v22.i39.8698>

INTRODUCTION

The number of overweight and obese individuals increased worldwide by approximately 28% between 1980 and 2013^[1]. Obesity is caused by many factors, such as lifestyle habits including physical activity, exposition to environmental factors, genetic and epigenetic factors, and hormones and gut microbiota, and is characterized by excessive fat accumulation and the development of several diseases, including type-2 diabetes (T2DM), which is a major public health problem at present^[2-6]. However, the primary inducer of obesity and its associated disorders is inappropriate food intake, especially of food containing high amounts of fat and carbohydrates^[7].

Bariatric surgery, which is currently the most effective treatment for obesity and its associated disorders, provides long-term control of T2DM in approximately 80% of patients, while the conventional therapy has never been as effective^[8]. The supremacy of the bariatric procedure in T2DM therapy has been confirmed by independent randomized trials

where it was compared to an intensive course of the conventional treatment^[9,10]. Very recently, the "Joint Statement by International Diabetes Organizations" regarding the role of bariatric (metabolic) surgery in the Treatment Algorithm for T2DM was published^[11]. The general conclusion of the statement is that bariatric (metabolic) surgery should be recommended for the treatment of patients with T2DM and obesity. Moreover, in news published recently in the *British Medical Journal*^[12], it is suggested that bariatric surgery "(...) is a highly cost effective therapy for patients with T2DM (...)".

Roux-en-Y gastric bypass (RYGB), a commonly used bariatric procedure, creates a small pouch in the stomach and connects it to the proximal jejunum to form the Roux limb, which is anastomosed to the duodenal limb, forming a Y configuration (Figure 1B). The anatomical changes resulting from RYGB (namely the exclusion of a long section of the small intestine from the passage of food) encourage bile to reach the distal intestine in relatively high concentrations (because the excreted bile has not mixed with ingested food). These events are frequently associated with: (1) body mass loss and improvement in associated disorders, including T2DM; (2) modification of secretion and action of some gut hormones and peptides, including incretin hormones^[13-15]; (3) an increase in circulating bile acid (BA) concentrations^[14-16]; and (4) alterations in gut microbiota^[14,17-20]. The alterations in gut microbiota, which play a crucial role in the conversion of primary BAs to secondary BAs, indirectly activate (or do not activate) nuclear and membrane receptors *via* modification of the structure of BAs, and may thus influence human glucose metabolism^[21].

It is generally believed that the improved glycemic control following RYGB, besides body weight loss and calorie restriction, can be the consequence of an increase in hepatic and peripheral insulin sensitivity, as well as of the increase in postprandial insulin secretion as a result of increased glucagon-like peptide-1 (GLP-1) secretion^[13]. Some data suggest that body mass loss is an important determinant of T2DM remission one year following RYGB^[22]. Other data indicate that changes in glycemic control following RYGB are mainly mediated by caloric restriction, but not by increased energy expenditure^[15]. Since the improvement in glucose metabolism consequent on bariatric surgery is observed earlier than body mass loss, it is likely that the beneficial effect of RYGB (or other types of bariatric surgery) is at least in part independent of calorie restriction and body mass loss. So far, several mechanisms independent of body-mass loss for the improvement of glucose metabolism following bariatric surgery have been discussed^[16]. However, the underlying mechanism of the action of bariatric surgery on glycemic control has not been fully elucidated.

Emerging evidence indicates that the increase in circulating bile acids and intestinal microbiota (*via* modification of the chemical structure and subsequently

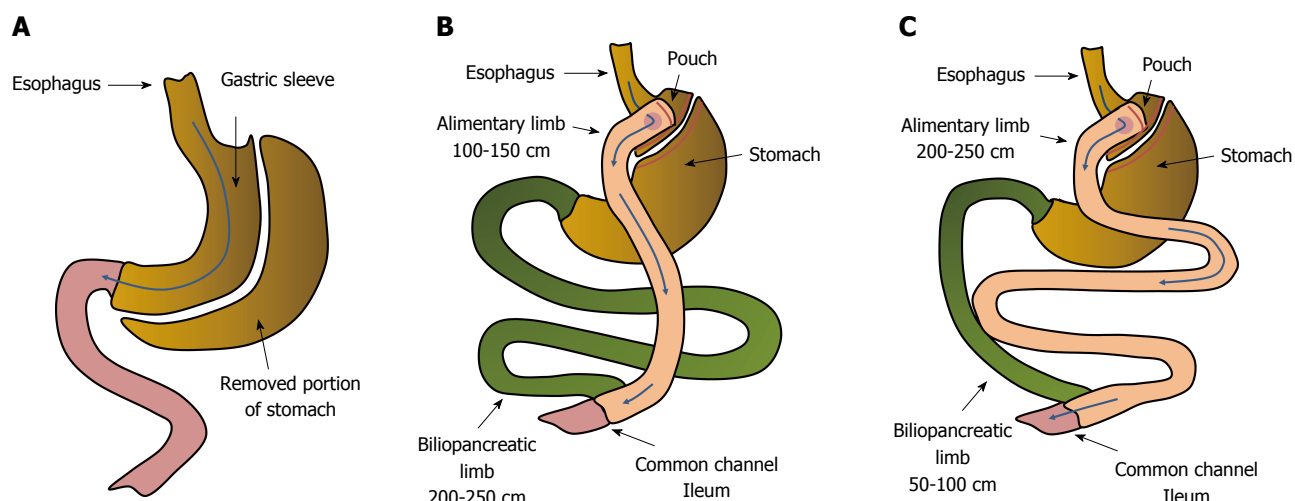


Figure 1 Anatomical changes in gastrointestinal tract resulting from: sleeve gastrectomy (A), Roux-en-Y gastric bypass - distal/scopinized (B), Roux en Y gastric bypass - long limb (C).

the biological activities of BAs) may play an important role in the improvement of glucose metabolism following bariatric surgery^[16,23-27]. It should be emphasized that restrictive bariatric procedures, such as sleeve gastrectomy and gastric banding, which have no effect or less effect on circulating BA concentrations, display less influence on T2DM remission. On the other hand, after malabsorptive procedures such as RYGB, significant increases in circulating BA concentrations and a simultaneous improvement of glucose metabolism have frequently been observed^[14,28-30]. Some authors have suggested that circulating BA concentrations increase following RYGB, independently of caloric restriction^[31].

In summary, there are several clinical indications that an increase in gut, and subsequently in circulating, BA concentrations can occur following bariatric surgery (especially after malabsorptive procedures). In turn, BAs may play an important role in the improvement of glucose metabolism, *via* their binding to the nuclear or membrane receptors present in many organs, including the intestine, liver, pancreas, adipose tissue, and skeletal muscle^[32-35]. The clinical observations were confirmed by animal studies indicating that: (1) RYGB contributes to increases in total circulating BAs in rats^[36]; and (2) farnesoid X receptors (FXR) - one of the types of nuclear receptor activated by BAs - may be involved in improving glycemic control following bariatric surgery. For instance, in FXR knockout mice, the ability of bariatric surgery to improve glucose tolerance was significantly reduced^[37]. Moreover, it has been shown that the administration of BAs to mice increases energy expenditure *via* the membrane G-protein - receptor (TGR-5) signaling pathway, preventing insulin resistance^[38]. Moreover, the antidiabetic effect of agonists of TGR-5 other than BAs (for instance, oleanolic acid) in mice was also observed^[39]. It is therefore likely that BAs and the nuclear and membrane receptors activated by

BAs could play an important role in the regulation of glucose metabolism, and subsequently in T2DM remission in humans following bariatric surgery. However, caution needs to be taken when translating animal results to humans^[36].

In recently published elegant review Penney *et al.*^[16] summarized the results regarding the role of BA and FXR and TGR-5 receptors in: (1) regulation of BA, lipid and energy metabolism; (2) glucose homeostasis; (3) incretin and other gut satiety hormones production; and (4) endoplasmic reticulum stress following bariatric surgery. The goal of this review is to focus on (1) BAs biosynthesis in liver; (2) BAs biotransformation in gut and enterohepatic circulation; and (3) the potential role of increased circulating BA concentrations and alterations of gut microbiota in improvement of glucose metabolism and subsequently in T2DM remission following bariatric surgery. In other words, this review offer a deeper understanding of the effect of bariatric surgery on T2DM remission.

EFFECT OF BARIATRIC SURGERY ON T2DM REMISSION: RESTRICTIVE PROCEDURES ARE LESS EFFECTIVE THAN MALABSORPTIVE PROCEDURES

As mentioned above, RYGB ameliorates most obesity related diseases, including T2DM^[40]. As far as the remission of T2DM is concerned, biliopancreatic diversion with duodenal switch (BPD-DS)-a procedure where the longer intestinal limb is excluded from the alimentary passage - and transiting concentrated bile provided better results than RYGB, a procedure with markedly shorter biliary limbs (Figure 1)^[41]. In turn, BPD-DS and RYGB are associated with a higher efficiency of T2DM remission than sleeve gastrectomy (SG), where no biliary exclusion is performed

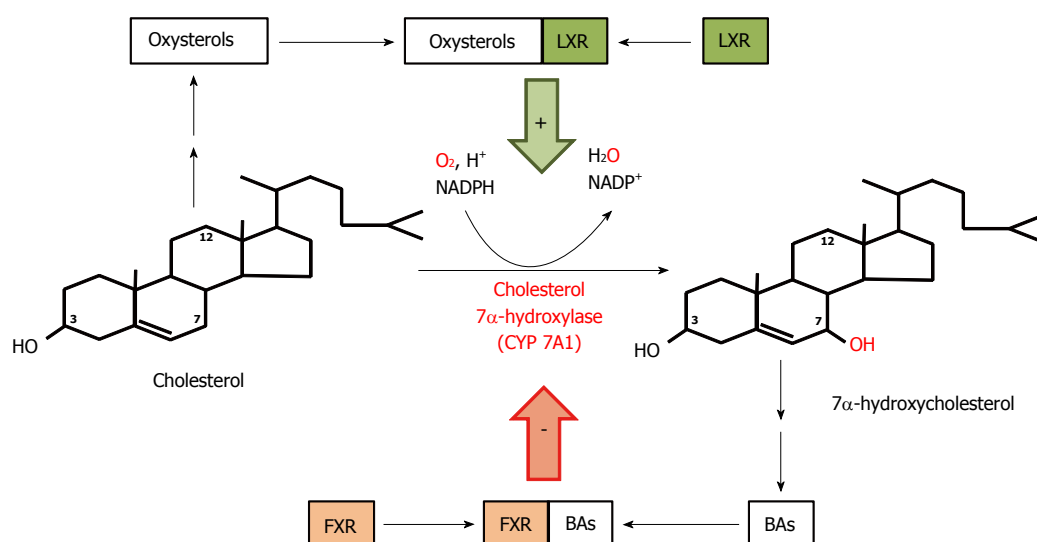


Figure 2 Course and regulation of reactions catalyzed by cholesterol 7 α -hydroxylase - the rate limiting enzyme in bile acid biosynthesis. Bile acids (BAs) formed from 7 α -hydroxycholesterol bind to farnesoid X receptor (FXR) and inhibit expression of the gene coding for 7 α -hydroxylase, subsequently diminishing the rate of BA biosynthesis in liver. Oxysterols formed from cholesterol bind to liver X receptor (LXR) and stimulate expression of the gene coding for 7 α -hydroxylase and the subsequent conversion of cholesterol to 7 α -hydroxycholesterol and to BAs.

(Figure 1A)^[42]. Several papers have indicated that improvements in insulin sensitivity were observed early after the surgery when the duodeno-jejunal exclusion is performed and cannot be only dependent on body mass loss^[43-46]. Some authors have reported that the homeostatic model assessment of insulin resistance (HOMA-IR) had significantly improved even as soon as a few days after the gastric bypass^[47-50]. Moreover, One Anastomosis Gastric Bypass (OAGB), where the concentrated bile transit is more than doubled in comparison to standard RYGB, provides greater improvement in HOMA-IR^[47]. The reduction of the HOMA-IR value after SG is not as spectacular as observed following BPD-DS and RYGB, and is associated rather with the extreme caloric restriction resulting from the surgery^[47].

The data suggest that the concentrated bile transit in the excluded jejunum, and the subsequently enhanced circulating concentrations of BAs, may play an important role in metabolic improvement following bariatric procedures. The mechanisms triggered by the altered digestive tract anatomy after the separation of the intestinal section from the alimentary passage are recognized as a key factor in foregut theory^[51]. An overview of the anatomical changes in the gastrointestinal tract caused by various types of bariatric surgery is presented in Figure 1.

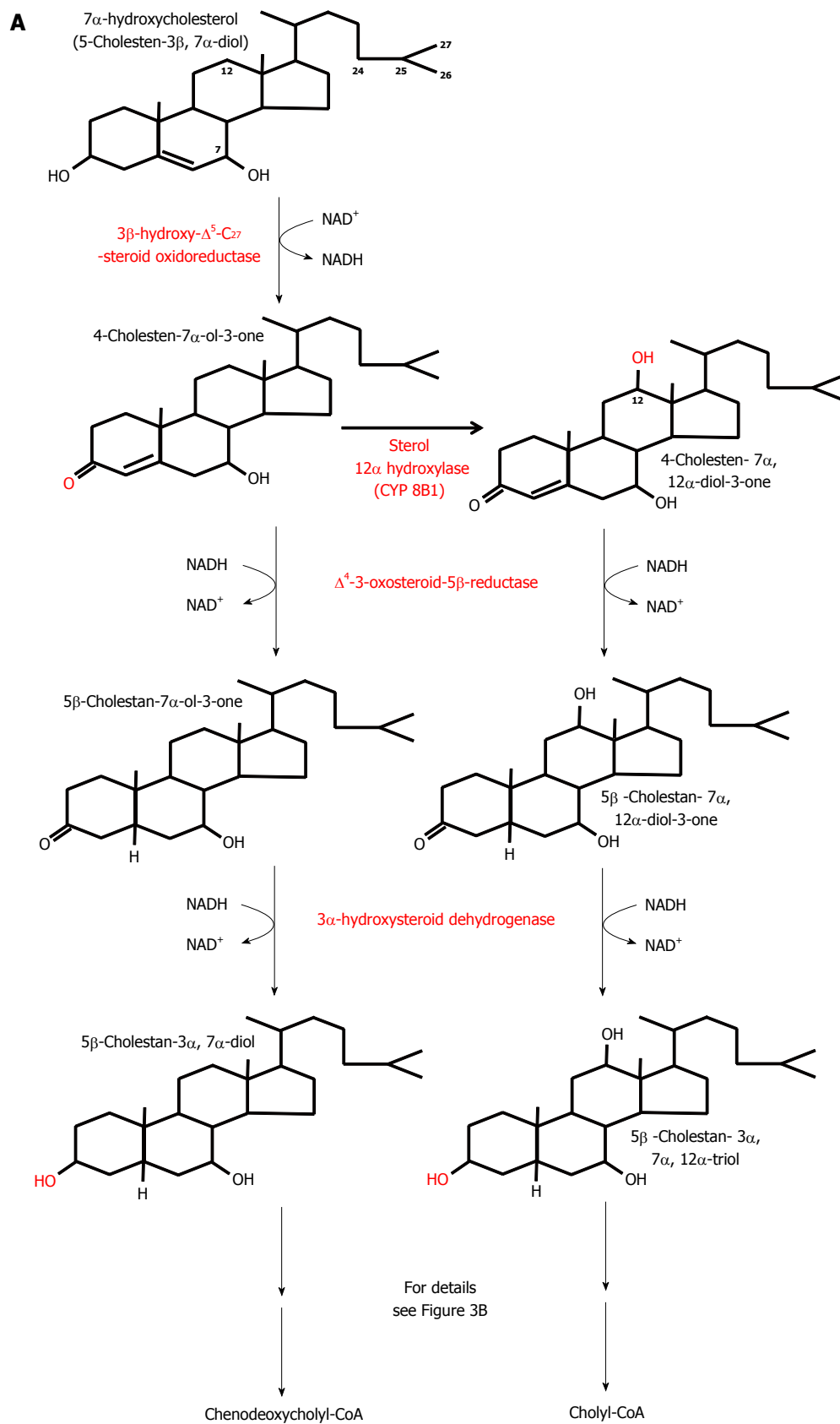
The effectiveness of gastric bypass in managing patients with type-1 diabetes mellitus or patients with low concentrations of C-peptide (below 1 ng/mL) has also been reported^[52,53]. Significant reductions in exogenous insulin administration have been observed shortly following the operation in the studied patients.

Overall, the clinical data reviewed above support the idea that bariatric surgery, especially malabsorptive

procedures, could be used in the treatment of both T1DM and T2DM.

ROLE OF THE LIVER IN PRIMARY BILE ACID BIOSYNTHESIS AND ENTEROHEPATIC BILE ACID CIRCULATION

In healthy subjects, cholic acid (CA) and chenodeoxycholic acid (CDCA) are the primary BAs synthesized from cholesterol mainly *via* the classic (or neutral) pathway in the liver endoplasmic reticulum. This pathway accounts for more than 90% of the total BA synthesis under physiological conditions^[54]. Cholesterol in the presence of NADPH and O₂ is converted to 7 α -hydroxycholesterol by cytochrome P₄₅₀ 7 α -hydroxylase (CYP7A1, encoded by *CYP7A1*), the rate limiting enzyme in both CA and CDCA biosynthesis (Figure 2). *CYP7A1* is under negative feedback regulation by BAs, *via* BAs binding to FXR^[55]. On the other hand, liver X receptor is involved in a feed-forward regulation of *CYP7A1* dependent on oxysterol, which is formed from cholesterol (Figure 2). The 7 α -hydroxycholesterol thus formed is then converted by 3 β -hydroxy- Δ^5 -C₂₇ steroid oxidoreductase (which isomerizes the Δ^5 bond to the Δ^4 position and oxidizes the 3 β -OH to a 3-oxo group) to 4-cholesten-7 α -ol-3-one, a precursor of both CA and CDCA (Figure 3A). In contrast to CDCA synthesis, CA requires the introduction of a hydroxyl group (OH group) in position 12 α on the steroid skeleton. Therefore, 4-cholesten-7 α -ol-3-one is hydroxylated to 4-cholesten-7 α , 12 α -diol-3-one by 12 α hydroxylase (CYP8B1, encoded by *CYP8B1*). Recently, it has been found that BA-



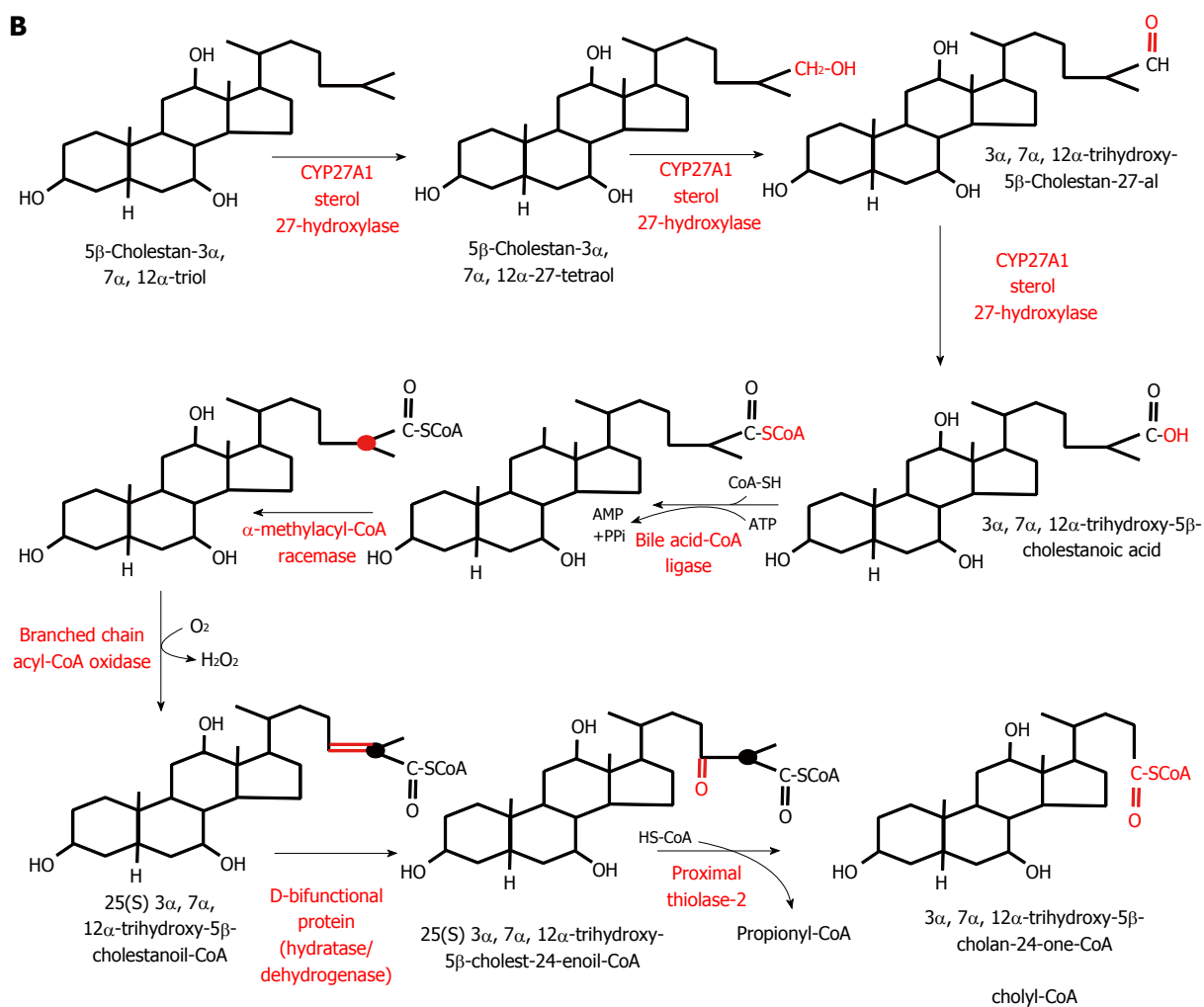


Figure 3 Classic pathway of bile acid biosynthesis in the liver. A: Conversion of 7α-hydroxycholesterol to 5β-cholestan-3α, 7α-diol - a precursor of chenodeoxycholy-CoA - and of 7α-hydroxycholesterol to 5β-cholestan-3α, 7α, 12α-triol - a precursor of cholelyl-CoA. B: Conversion of 5β-cholestan-3α, 7α, 12α-triol to cholelyl-CoA. Note that the conversion of 5β-cholestan-3α, 7α-diol to chenodeoxycholy-CoA biosynthesis takes place in the same manner and it is catalyzed by the same liver enzymes (not shown).

activated FXR increases the levels of MAFG (a product of the *Mafig* gene), which in turn inhibits CYP8B1 in mice^[56]. Moreover, it has been shown that MAFG has no effect on CYP7A1. This suggests that MAFG may regulate the CDCA:CA ratio (and subsequently BA composition), and thus the hydrophobicity of BAs (by inhibiting CA synthesis without affecting CDCA synthesis), though not the BA pool size^[56]. All the later steps in the formation of BAs, such as the conversion of 4-cholesten-7α-ol-3-one to chenodeoxycholy-CoA or 4-cholesten-7α, 12α-diol-3-one to cholelyl-CoA, are catalyzed by the same enzymes that are present in the hepatocytes (Figure 3B). Note that Figure 3B shows the pathway of cholelyl-CoA biosynthesis. Biosynthesis of chenodeoxycholy-CoA (which, unlike CA, does not contain a 12-OH group) takes place in the same manner and is catalyzed by the same liver enzymes (not shown). An alternative pathway (the acidic pathway) also begins in the hepatocytes with the mitochondrial 27-hydroxylation of cholesterol, catalyzed by CYP27A1 (encoded by *CYP27A1*) (not

shown). Moreover, the mitochondrial 27-hydroxylation of cholesterol may additionally occur in extrahepatic tissue^[57]. The conversion of cholesterol to BAs can also begin with 25-hydroxylation or 24-hydroxylation of cholesterol in extrahepatic tissue^[58], though these pathways are probably responsible for less than 1% of the total production of BAs. 27-OH-cholesterol, 25-OH-cholesterol, and 24-OH-cholesterol are then hydroxylated by specific hydroxylases (CYP7B1 in the case of 27-OH-cholesterol, 25-OH-cholesterol or CYP39A1 or CYP7A1 in the case of 24-OH-cholesterol)^[58,59]. Regardless of the initial hydroxylation of cholesterol, all the later steps in the biosynthesis of BAs occur in the liver, according to the scheme presented in detail in Figure 3B.

Most BAs, once formed, are immediately conjugated to glycine or taurine in liver peroxisomes, as illustrated in Figure 4. For example, Figure 4 shows the conversion of cholelyl-CoA to glycocholic acid or taurocholic acid (TCA). The conversion of chenodeoxycholy-CoA to glycochenodeoxycholic acid

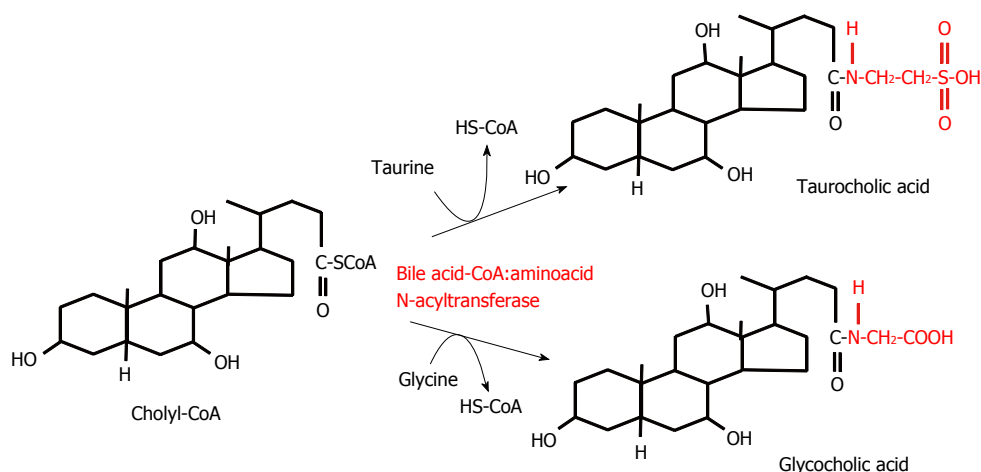


Figure 4 Conjugation reactions of cholesteryl-CoA with taurine or glycine.

(GCDCA) or taurochenodeoxycholic acid (TCDCA) proceeds in the same manner (not shown). It is worth noting that conjugation changes the ability of BA to activate nuclear and membrane receptors. Primary BAs preferentially activate FXR, whereas TGR-5 is principally activated by secondary BAs^[60].

BAs conjugated with taurine or glycine in the hepatocytes are excreted from the liver, chiefly *via* bile salts export proteins (BSEP) present at the canalicular membrane of the hepatocytes to canaliculi and then, *via* hepatic ducts, to the gallbladder, where the bile is concentrated during interdigestive periods before being released into the duodenum (Figure 5). Meals, especially those containing dietary fat, stimulate intestinal cells to secrete cholecystokinin, which in turn promotes the contraction of the gallbladder, facilitating the secretion of bile salts into the duodenum *via* the common bile duct (Figure 5). More than 95% of bile salts are reabsorbed by the intestinal cells (ileocytes) *via* apical sodium-dependent BA transporter (ASBT), also called ileal BA transporter (IBAT)^[61], or through passive diffusion, mostly in the upper small intestine and colon^[62,63] (Figure 5). In intestinal cells, BAs are transdiffused across the ileocytes or are bound to I-BABP (ileocyte BA-binding protein), also known as FABP6 (fatty-acid binding protein 6). The binding of BAs to I-BABP facilitates the transport of BAs across ileocytes. Interestingly, some BAs (such as TCA) increase I-BABP levels through activation of FXR and the subsequent stimulation of expression of the gene encoding I-BABP, suggesting that this protein plays an important role in regulation of enterohepatic BA circulation^[64]. BA efflux from intestinal cells is mediated *via* organic solute transporters α and β (OST α and OST β heterodimers) located in the basolateral membrane of intestinal cells^[54] (Figure 5). The reabsorbed BAs enter portal circulation and are transported back to the liver (hepatocytes). Na⁺-taurocholate cotransport peptide and the organic anion transporting proteins, located in basolateral membrane of the hepatocyte, are responsible

for sodium dependent uptake of conjugated and unconjugated BAs, respectively^[33,54]. Figure 5 presents an overview of enterohepatic BA circulation. Some data suggest that the altered enterohepatic circulation of BAs could contribute to improved insulin sensitivity and cholesterol metabolism following biliopancreatic diversion^[65].

After intestinal absorption and the return to the enterohepatic circulation, the conjugated and unconjugated BAs in the hepatocytes can undergo: (1) conversion to the corresponding CoA esters (CA to cholesteryl-CoA and CDCA to chenodeoxycholesteryl-CoA) by bile acid-CoA synthase (BACS; also called bile acid-CoA ligase, BACL) followed by re-conjugation with glycine or taurine catalyzed by bile acid-CoA:amino acid N-acyltransferase, as presented in Figure 4 (only unconjugated BAs); (2) re-epimerization (conversion of the 3 β -OH form to the 3 α -OH form); (3) reduction (conversion of the O = form to the OH form); and (4) 7 α -hydroxylation (in some species, though not in humans)^[66]. It should be emphasized that the enterohepatic circulation of BAs is regulated by BA-activated FXR, which influences the levels of BSEP, ASBT, and IBAP^[67].

ROLE OF INTESTINAL MICROBIOTA IN SECONDARY BILE ACID PRODUCTION-INTERACTIONS BETWEEN BILE ACIDS AND INTESTINAL MICROBIOTA

Approximately 5% of bile salts (bile acids conjugated with glycine or taurine) escape enterohepatic circulation and undergo biotransformation to secondary BAs by intestinal microbiota enzymes. CA is converted to deoxycholic acid (DCA) and CDCA to lithocholic (LCA)^[68]. In healthy adults, the daily fecal loss of BAs amounts to 0.4-0.8 g, which is replenished by *de novo* BA biosynthesis (that is, conversion of cholesterol to BAs) in the liver, as described above and presented in

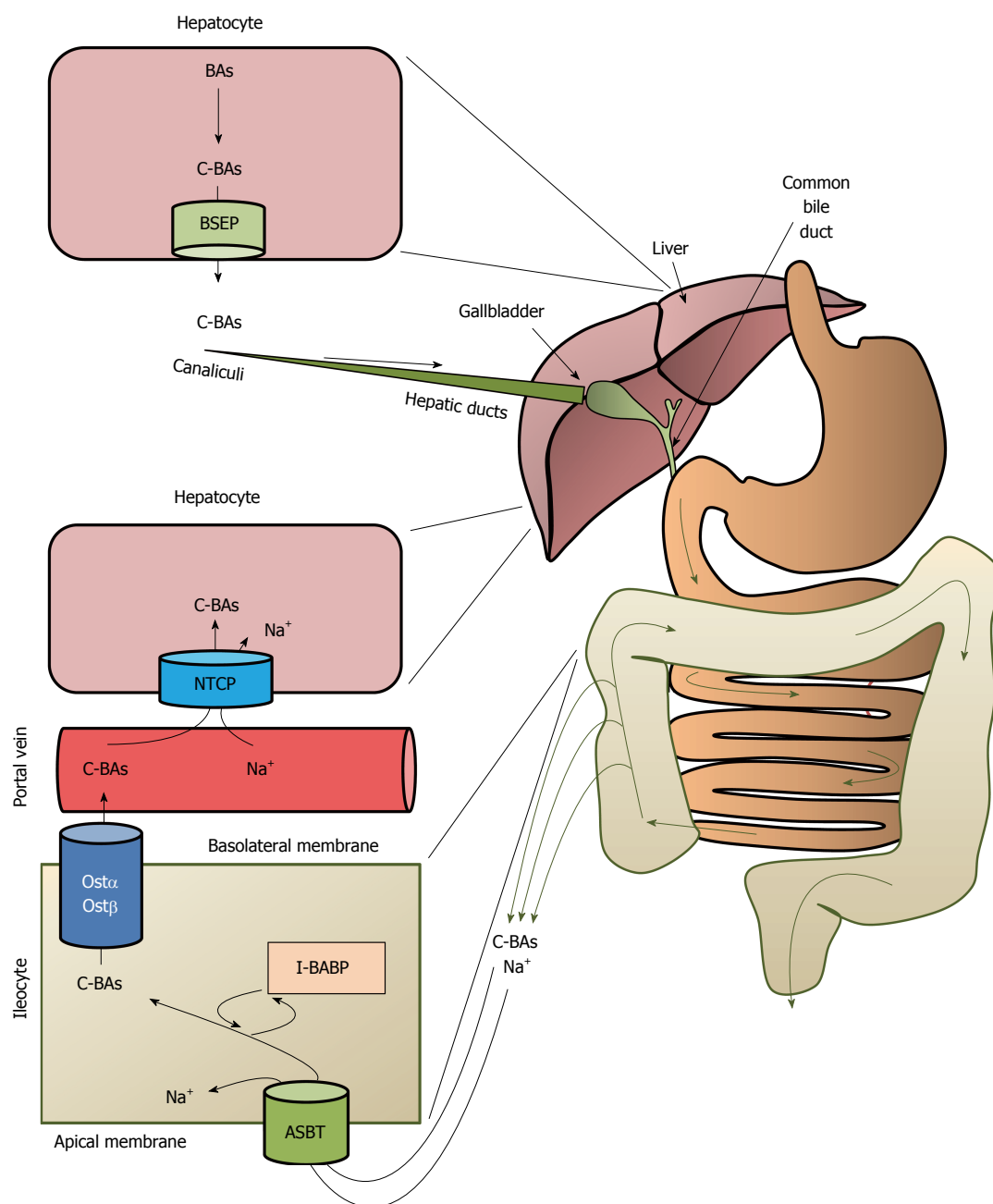


Figure 5 Overview of the enterohepatic circulation of bile acids. BAs: Bile acids; C-BAs: Conjugated bile acids; BSEP: Bile salt export proteins; ASBT: Bile acid transporter; I-BABP: Ileocyte bile-acid binding protein; OST α /OST β : Organic solute transporters α/β ; NTCP: Na⁺-taurocholate cotransport peptide.

Figures 2 and 3.

It is worth noting that the human intestine is colonized by about 100 trillion microbial cells^[69], whose total biomass is approximately 1 kg^[70]. The majority of gut microbiota are considered to be commensals, as they play important symbiotic roles, including: (1) the degradation of nondigested polysaccharides to short-chain fatty acids^[71]; (2) the production of some vitamins (B and K)^[72]; and (3) BA biotransformation. Six bacterial phyla-Firmicutes, Bacteroidetes, Proteobacteria, Actinobacteria, Fusobacteria, and Verrucomicrobia-dominate in the gut of adult healthy human. Firmicutes and Bacteroidetes together can constitute over 90% of the microbiota, whereas the

other phyla range between 2% and 10%^[73]. The composition and activity of the gut microbiota is affected by energetic substrate availability and the physicochemical conditions in the alimentary tract. Substrates required by gut bacteria come from food ingested by host. The host also provides mucins, desquamated cells, and digestive enzymes, which can be used by gut bacteria, as well as BAs, which influence bacterial growth and are metabolized (undergo biotransformation) by bacteria. Gut microbial changes in response to any intervention-for example anatomical changes caused by bariatric surgery^[19,36] or antibiotic treatment^[74]-may also affect BA biotransformation. In other words, it may be supposed that changes in gut

Table 1 Abundance of bacterial species in various sections of the human intestine

Intestine	Abundance of Bacteria	Bacteria
Small intestine		
Duodenum	About 10 ³ (bacteria/mL)	<i>Lactobacillus</i> ¹ <i>Streptococcus</i> ²
Jejunum	About 10 ⁴ (bacteria/mL)	<i>Lactobacillus</i> ¹ <i>Streptococcus</i> ² <i>Staphylococcus</i> <i>Veillonella</i> ²
Ileum	10 ⁶ -10 ⁸ (bacteria/mL)	<i>Enterobacter</i> ¹ <i>Enterococcus</i> ¹ <i>Bacteroides</i> ^{1,2} <i>Clostridium</i> ^{1,2} <i>Lactobacillus</i> ¹ <i>Veillonella</i> <i>Bacteroides</i> ^{1,2} <i>Eubacterium</i> ² <i>Bifidobacterium</i> ¹ <i>Ruminococcus</i> <i>Peptostreptococcus</i> <i>Propionibacterium</i> <i>Clostridium</i> ^{1,2} <i>Lactobacillus</i> ¹ <i>Escherichia</i> <i>Streptococcus</i> ² <i>Methanobrevibacter</i>
Large intestine	About 10 ¹¹ (bacteria/g)	

¹Bacteria displaying bile salt hydrolase (BSH) activity; ²Bacteria capable of catalyzing 7 α -dehydroxylation. Based on data presented in^[68].

microbiota composition can influence the composition and concentrations of circulating BAs, which in turn can affect obesity and related disorders. However, it remains to be clarified further how bariatric surgery (and other treatments) modulates the gut microbiota towards a beneficial or harmful composition.

BA biotransformation occurs mainly in the large intestine, which is rich in bacteria (Table 1). Intestinal biotransformation is a very complex process involving several reactions, including: (1) removal of the glycine or taurine side chain, a process commonly known as the deconjugation of BAs; (2) 7 α -dehydroxylation (removal of OH group at C-7 α); (3) oxidation and epimerization of the C-3, C-7, or C-12 OH groups of BAs; (4) reduction of steroid skeleton (insertion of H into steroid structure); and (5) hydroxylation of BAs (insertion of an OH group)^[68,75-78].

Bile salt deconjugation is catalyzed by bile salt hydrolase (BSH) (Figure 6). This enzyme is present in various gut bacteria including: *Clostridium*, *Bacteroides*, *Lactobacillus*, *Bifidobacterium*, and *Enterococcus* (Table 1)^[77]. In theory, deconjugation can begin in the small intestine, since bacteria displaying BSH activity are found there, though only in small numbers (Table 1). It is worth noting that deconjugated BAs are highly insoluble, toxic compounds and are excreted more rapidly than conjugated BAs^[79].

The 7 α -dehydroxylation contributes to the conversion of CA and CDCA to DCA and LCA, respectively. The formation of DCA and LCA - the secondary BAs that

Table 2 Composition of human biliary and fecal bile acids

Bile acids	Biliary bile acids composition (% of total)	Fecal bile acids composition (% of total)
Cholic acid (CA)	35%	2%
Chenodeoxycholic acid (CDCA)	35%	2%
Deoxycholic acid (DCA)	25%	34%
Ursodeoxycholic acid (UDCA)	2%	2%
Lithocholic acid (LCA)	1%	29%
12-oxo-Lithocholic acid (12-oxo-LCA)	-	3%
Other	2%	28%

Table shows percentage of both conjugated and unconjugated bile acids. Based on data presented in^[68].

predominate in human feces (Table 2)-is considered the most quantitatively important biotransformation process of BAs^[78]. This process is carried out by only a few strains of human intestinal bacteria, including *Bacteroides*, *Clostridium*, *Streptococcus fecalis*, and *Veillonella*^[78]. Theoretically, dehydroxylation can take place also in the small intestine, since bacteria capable of performing such activities (dehydroxylation of CA) are found there (Table 1).

The oxidation and epimerization of the C-3, C-7, or C-12 hydroxy groups of BAs are catalyzed by α -hydroxysteroid dehydrogenases (α -HSDH) or β -HSDH, which are also present in gut bacteria.

It is worth noting that bile (especially the BAs it contains) significantly affects the survival and, subsequently the colonization, of some intestinal bacteria, especially in the small intestine^[80]. Several mechanisms have been proposed for the direct antimicrobial action of BAs namely: (1) the interaction of BAs with bacterial membrane (where the BAs simply act as detergents); (2) acidification of bacterial cytoplasm; (3) bacterial DNA damage; and (4) alterations in bacterial proteins^[66]. Studies with mice lacking FXR suggest that, besides direct antimicrobial action, BAs have indirect effects through FXR-induced antimicrobial peptide synthesis in the intestine^[81]. Interestingly, Gram-positive bacteria are more sensitive to the deleterious effects of BAs than are Gram-negative bacteria^[77]. It is worth noting that the gut bacteria benefit from metabolizing BAs. For instance, in catalyzing the deconjugation of BAs, gut bacteria can use glycine or taurine for their own metabolism^[82]. Additionally, gut bacteria use BAs as sinks for the disposal of electrons liberated during bacterial fermentation^[82].

The results discussed above indicate that BA pool size is controlled through 3 processes: (1) regulation of BA biosynthesis in the human liver; (2) enterohepatic circulation; and (3) the involvement of intestinal microbiota in the biotransformation of BAs^[54]. As a result of their antimicrobial activity, BAs can affect the abundance and composition of gut microbiota^[54,83,84]. This means that BAs and gut microbiota are closely integrated and affect each other: bile affects the

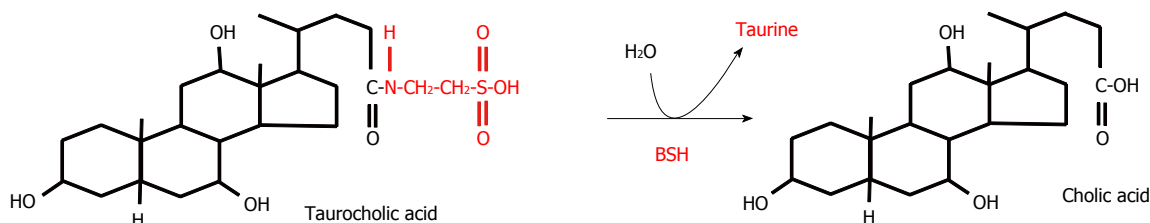


Figure 6 Deconjugation of taurocholic acid by bile salt hydrolase. BSH: Bile salt hydrolase.

growth and colonization of bacteria in the intestine, while bacteria contribute to the biotransformation of BAs. Theoretically, in pathological conditions, perturbations in the equilibrium between BA pool size and gut microbiota could occur. An intriguing example of such perturbation might be the state after bariatric surgery, when the gut contains more concentrated bile, which may affect the survival and subsequently the colonization of some gut bacteria, especially those involved in BA biotransformation and short-chain fatty acid production. As energetic substrates for colonocytes, short-chain fatty acids play an important role in maintaining intestinal barrier permeability^[85].

EFFECT OF BARIATRIC SURGERY ON INTESTINE MICROBIOTA ABUNDANCE AND COMPOSITION

Gut microbiota is now considered an important factor affecting human health^[86,87]. It is known that gut microbial composition is significantly altered in obese subjects, as compared to lean controls^[88]. Using 16S rRNA to quantitatively identify bacteria, it has been shown that obesity is associated with a decrease in the relative abundance of Bacteroidetes vs Firmicutes^[88-90]. Some data suggest that gut microbiota may play some role in the development of obesity^[91-93] and obesity-related diseases^[93,94], including T2DM^[95,96]. In mice, gut microbiota composition changes significantly in response to dietary changes^[97].

Several papers have pointed to significant changes in gut microbiota abundance and composition in humans and animal models as a possible beneficial effect of bariatric surgery^[17,20,98-102]. Moreover, following bariatric surgery, an association has been reported between increased circulating BA concentrations and changes in gut microbial composition^[19]. Altogether, the data discussed above suggest that changes in intestinal microbiota-possibly through the effects on BA pool size and primary:secondary BA ratio - may contribute to the beneficial effects of bariatric surgery on glucose metabolism^[103,104]. However further studies are necessary to confirm this suggestion. Changes in gut microbiota following bariatric surgery were also seen to be closely linked to improvements in diabetic parameters and body mass loss^[14,17-20].

It is worth noting that changes in gut microbiota

following bariatric surgery are related to short-chain fatty acid production (mainly of butyrate and propionate)^[19], which can influence glucose metabolism independently of BAs^[95,105]. Moreover, it has been reported that changes in short-chain fatty acid biosynthesis by gut bacteria following RYGB may promote GLP-1 secretion^[106]. It has recently been shown that the increased production of acetate by gut microbiota in rodents leads to activation of the parasympathetic nervous system, which in turn promotes insulin secretion^[107]. Thus, it is not impossible that changes in gut microbiota following bariatric surgery, together with the subsequent changes in short-chain fatty acid production in the gut, could affect glucose metabolism independently or in combination with BAs. However, further studies are needed to confirm this supposition.

BILE ACIDS: LIGANDS FOR MEMBRANE AND NUCLEAR RECEPTORS INVOLVED IN THE REGULATION OF GLUCOSE METABOLISM

For many years, BAs were considered to be involved in (1) the digestion and absorption of dietary lipids (including fat-soluble vitamins); (2) cholesterol solubilization in gallbladder bile; (3) excretion of cholesterol (following cholesterol conversion to BAs) from the human body; and (4) regulation of survival, and subsequently colonization, of some gut bacteria. BAs circulating in the blood are now considered to be important regulatory molecules which, *via* binding to membrane or nuclear receptors, contribute to the homeostasis of carbohydrates and lipids. BAs also have effects on the immune system^[108] and the apoptosis of colonic epithelial cells^[109]. The main BAs detected in human serum are presented in Table 3^[110].

It is worth noting that ursodeoxycholic acid (UDCA) is also used as a drug for cholesterol gallstone dissolution therapy^[111,112] and for treating patients with primary biliary cirrhosis^[113]. It has recently been reported that the treatment of patients with UDCA significantly decreases gallstone formation following sleeve gastrectomy^[114]. Moreover, the issue of using BA analogs as drugs in the treatment of NAFLD and NASH has also been discussed^[115].

The known nuclear receptors activated by BAs

Table 3 Bile acids present in human serum. Based on data presented in^[110]

Primary bile acids	Secondary bile acids
Cholic acid (CA)	Lithocholic acid (LCA)
Chenodeoxycholic acid (CDCA)	Deoxycholic acid (DCA)
Glycocholic acid (GCA)	Ursodeoxycholic acid (UDCA)
Glychenodeoxycholic acid (GCDCA)	Hyodeoxycholic acid (HDCA)
Taurocholic acid (TCA)	Glycolithocholic acid (GLCA)
Taurochenodeoxycholic acid (TCDCA)	Glycodeoxycholic acid (GDCA)
	Glycoursodeoxycholic acid (GUDCA)
	Taurolithocholic acid (TLCA)
	Taurodeoxycholic acid (TDCA)
	Tauroursodeoxycholic acid (TUDCA)
	Taurohyodeoxycholic acid (THDCA)

(generally called bile acid activated receptors or BARs) are FXR (also known as NR1H4), vitamin D receptor (VDR, also known as NR1H1); constitutive androstane receptor (CAR, also known as NR1H3); and pregnane X receptor (PXR, also known as NR1H2). Although the role of PXR, CAR and VDR in the regulation of metabolism, including glucose metabolism, cannot be excluded^[21,115-118], it seems that the activation of FXR by BAs (note that primary BA are preferential ligands of this receptor) plays an important role in the regulation of glucose metabolism^[119]. CDCA, which activates FXR at EC₅₀ in a concentration of approximately 10 µmol/L, appears to be the main physiological ligand for FXR. Other BAs could also activate FXR, but in significantly higher concentrations. The potency of BAs to activate FXR is as follow: CDCA > DCA > LCA > CA^[64]. Through activation of FXR, BAs play a key role in liver and intestinal biosynthesis, enterohepatic transport, and homeostasis of BAs^[64]. Accordingly, a deficiency in FXR may lead to cholestasis^[120]. In the intestine, BAs bind to FXR and induce synthesis of fibroblast growth factor 19 (FGF-19) in humans or FGF 15 in rodents and secretion, which in turn circulates to the liver where it regulates BA biosynthesis and glucose metabolism^[64]. In the liver, FGF 15/19 activates glycogen synthesis and inhibits gluconeogenesis *via* FGF receptor, which subsequently leads to a decrease in circulating glucose concentrations (Figure 7A)^[64]. Thus, FGF 15/19 is sometimes referred to as a downstream metabolic effector of BAs. When insulin is an early-acting hormone released after a meal, FGF 15/19 is a late-acting hormone released in the fed state (with a half-life of about 30 min)^[35]. By activating FXR, BAs also downregulate liver gluconeogenesis through inhibiting the gene expression of glucose 6-phosphatase, fructose 1,6-bisphosphatase, and phosphoenolpyruvate carboxykinase (PEPCK) (Figure 7B)^[121]. However, some data suggest that PEPCK is upregulated by BAs *via* FXR^[121]. In rodent hepatocytes, DCA-activated epidermal growth factor receptors ERB1/ERB2 and insulin receptor contribute to the activation of glycogen synthase through the PI3kinase/AKT/GSK3

signaling pathway^[122]. Accordingly, the agonists of FXR decrease blood glucose concentrations^[123]. Some authors suggest that BAs contribute to post-RYGB hypoglycemia, which sometimes occurs after bariatric surgery^[124].

In mice, BAs bind to FXR in pancreatic β-cells and thus stimulate insulin secretion (Figure 7B)^[125]. FXR knockout in mice leads to impaired glucose tolerance and insulin sensitivity^[126]. The activation of FXR in the liver and gallbladder is associated with the regulation of BA synthesis and excretion, respectively.

Apart from nuclear receptors, BAs (secondary BAs are preferential ligands) can bind to membrane, G-protein coupled receptors such as TGR-5 (Takeda-G-protein-receptor, also known as G-protein-coupled bile acid receptor 1, GPBAR1), muscarine receptor (M3R), and sphingosine 1-phosphate receptor-2 (S1PR2), as well as epidermal growth factor receptor (EGFR), which belongs to the ErbB family of tyrosine kinase receptors^[21,108,115,127,128]. The organ and tissue localizations of these nuclear and membrane receptors are presented in Figure 8.

As far as glucose metabolism is concerned, it seems that the activation of TGR-5 by BAs also plays an important role in the regulation of circulating glucose concentrations (Figure 7B)^[123]. Taurolithocholic acid and LCA activate TGR-5 in nanomolar concentrations, which suggests that they are physiological ligands of this receptor. *TGR5* is expressed in many organs including pancreatic β-cells, endocrine small intestine cells, the thyroid gland, the gallbladder, the liver, brown adipose tissue (BAT), cardiomyocytes, and macrophages^[108,129,130]. Recent studies have shown that tauroursodeoxycholic acid (TUDCA) increases glucose-induced insulin secretion *via* the cAMP/PKA pathway in pancreatic cells and that TGR-5 is likely involved in this process (Figure 7B)^[131]. Interestingly, this process is not associated with changes in glucose metabolism in pancreatic β-cells^[131]. It may thus be supposed that the activation of both TGR-5 and FXR (discussed above) in pancreatic β-cells by BAs contributes to hyperinsulinemia developing late after RYGB^[46]. Moreover, TGR-5 has been found in the brain, where it functions as a neurosteroid receptor^[132]. Considering that TGR-5 is present in the brain and that BAs cross the blood-brain barrier, it may be supposed that activation of brain TGR-5 by BAs can also contribute to the beneficial effects of bariatric surgery.

The activation of TGR-5 in skeletal muscle cells (and BAT) leads to the induction of the cAMP-dependent signaling pathway, which results in increased energy expenditure (Figure 7B)^[38,123,133]. The potency of BAs in activating TGR-5 is as follows: DCA>LCA>CDCA>CA^[21]. In the intestine, the activation of TGR-5 by endocrine L cells (and by BAs^[134]) leads to increased secretion of GLP-1 - an incretin hormone that increases the insulin secretion by pancreatic β-cells and inhibits the secretion of glucagon by pancreatic α-cells^[123,133,135], thus affecting blood glucose concentration (Figure 7C).

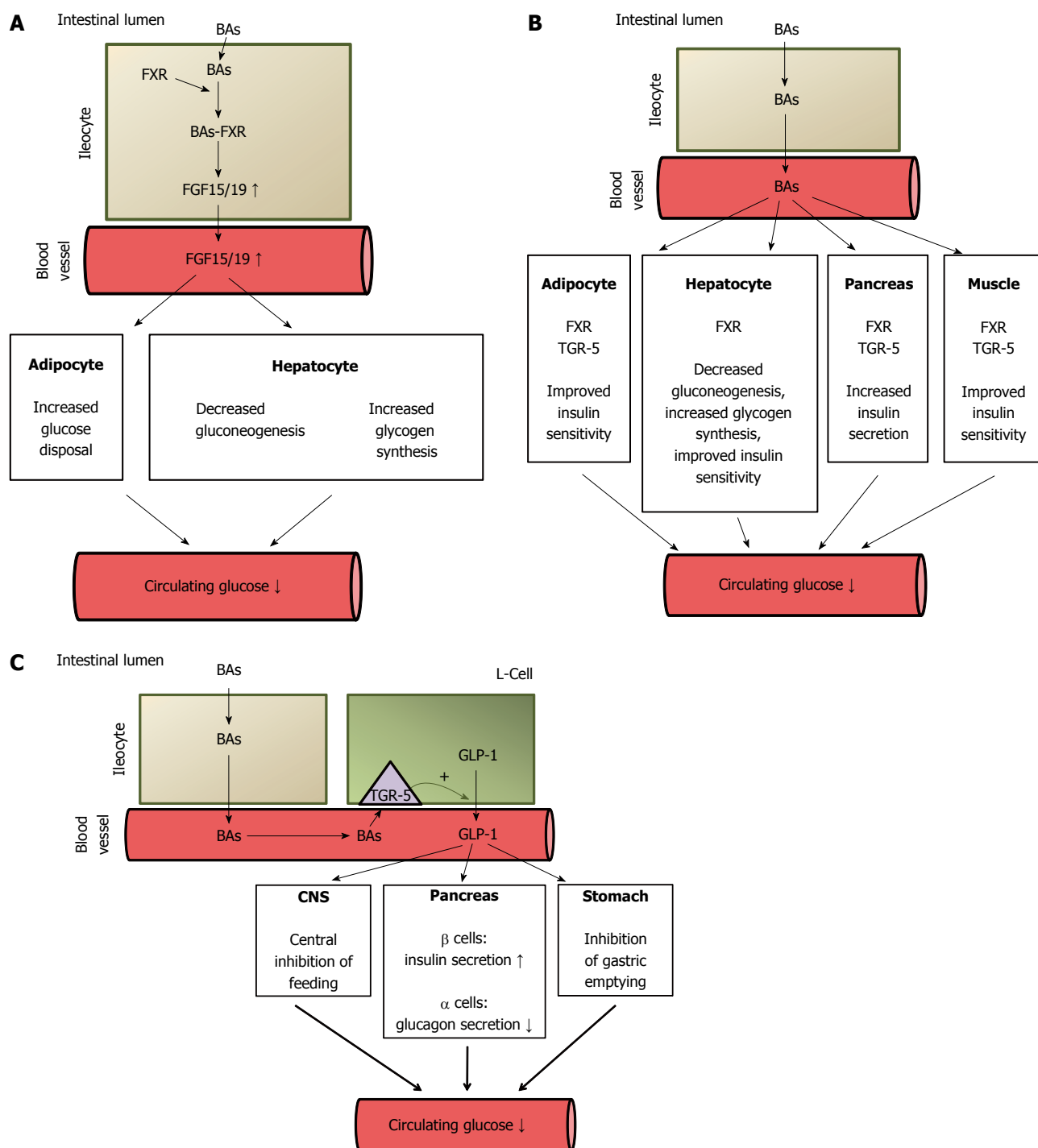


Figure 7 Potential mechanisms of bile acid mediated improvement of serum glucose concentration. A: Stimulatory effect of bile acids on FGF15/19 synthesis in intestinal cells (ileocytes). The activation of FXR in ileocytes by BAs leads to increased synthesis (via regulation of gene expression) and the release of fibroblast growth factor 15/19 (FGF 15/19) which, through the activation of the FGF-R present in hepatocyte and adipocyte membranes, regulates carbohydrate metabolism, leading to a decrease in circulating glucose concentrations. FGF 15/19 stimulates glycogen synthesis and inhibits gluconeogenesis in the liver and glucose disposal in adipose tissue. ↓: Decrease; ↑: Increase; B: Decreasing effect of BAs on circulating glucose concentration. BAs, by activating FXR, downregulate (via regulation of gene expression) liver gluconeogenesis and stimulate glycogen synthesis. BAs, by binding to FXR or to TGR-5 in pancreatic β-cells, stimulate insulin secretion. BAs, by binding to FXR or TGR-5 in adipose tissue and skeletal muscle, improve insulin sensitivity. ↓: Decrease; C: Potential mechanisms of BA-mediated decrease in circulating glucose concentrations after bariatric surgery caused by the increased release of GLP-1 by intestinal L-cells. ↓: Decrease; ↑: Increase. FXR: Farnesoid X receptor; FGR: Fibroblast growth factor; GLP: Glucagon-like peptide-1; BAs: Bile acids.

Moreover, BAs have been found to have a synergistic effect with glucose on the regulation of incretin hormone secretion^[134]. In addition, gastric emptying and satiety mediated by GLP-1 could also contribute to its effect on circulating glucose concentration

(Figure 7C)^[136,137]. However, whether LCA activates TGR-5 under physiological conditions is not clear^[55]; this may happen after bariatric surgery, when bile is concentrated and the subsequent circulating BA levels are elevated. An association has been found between

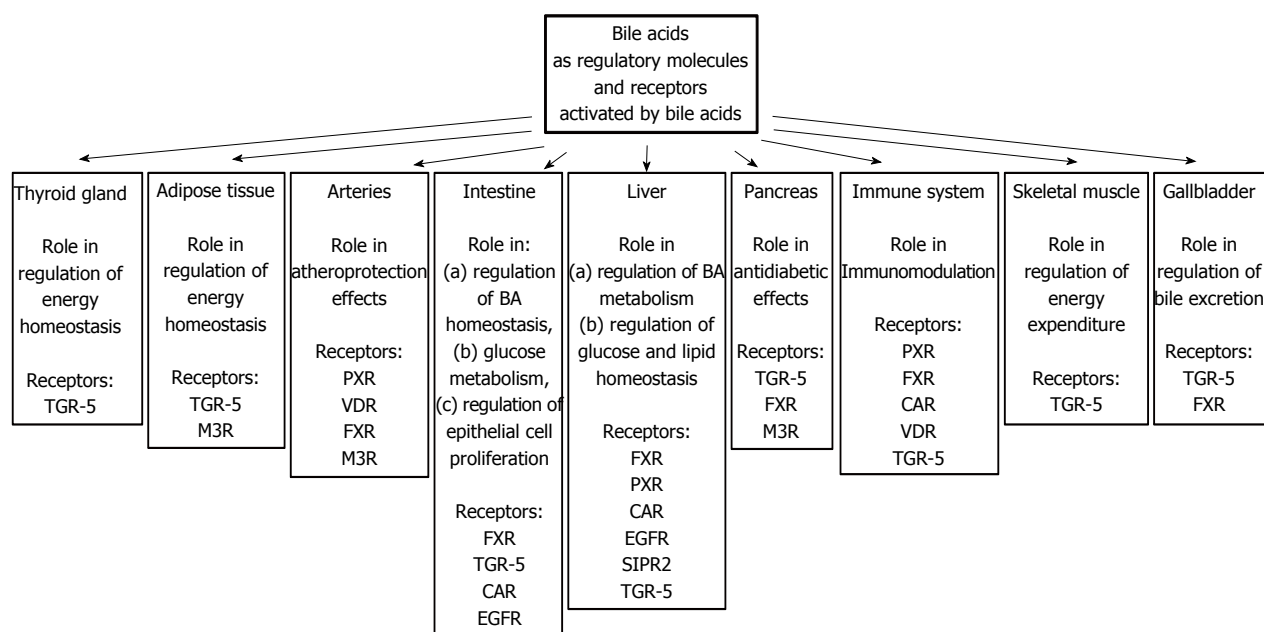


Figure 8 Bile acids as regulatory molecules and receptors activated by bile acids present in different organs.

circulating BAs and GLP-1 in patients who have undergone bariatric surgery^[28,103,138]. Moreover, rectal administration of taurocholate resulted in an increase in circulating GLP-1, PYY, and insulin, but decreased glucose concentrations in obese T2DM volunteers^[139]. It seems that TGR-5 also regulates BA pools, since these are significantly lower in TGR-5 (^{-/-}) mice than in wild-type animals^[115].

The activation of S1PR2 by BAs may also influence liver carbohydrate metabolism^[127]. Recent studies suggest that blocking S1PR2 signaling could function as a novel therapeutic strategy for T2DM^[140].

The activation of EGFR by BAs in the liver and intestine is associated with cell apoptosis^[141] and cell proliferation^[128], respectively. So far, the activation of muscarine receptors by BAs in context of the regulation of glucose homeostasis has not been reported.

Interestingly, BAs may exert many other biological functions *via* non-receptor-linked mechanisms involving the JNK1/2, ERK1/2, Akt1/2 signaling pathways, NO metabolism, and activation of cation channels^[108]. Moreover, it has been shown that some BAs, such as UDCA or CA, are able to reduce endoplasmic reticulum (ER) stress^[142,143]. Accordingly, significant reductions in ER stress have been observed following bariatric surgery on obese rats^[144]. Thus, it is not impossible that the increase in circulating BAs following bariatric surgery may ameliorate ER stress, and subsequently improve glucose homeostasis.

Overall, the data discussed above suggest that by binding to membrane or nuclear receptors, and also through non-receptor mechanisms, BAs may influence glucose metabolism and subsequently contribute to the remission of T2DM following bariatric surgery when

circulating BA concentrations are elevated.

EFFECT OF BARIATRIC SURGERY ON CIRCULATING BILE ACID CONCENTRATIONS AND DIABETES MARKERS: EVIDENCE THAT MALABSORPTIVE PROCEDURES ARE MORE EFFECTIVE THAN RESTRICTIVE PROCEDURES

Several papers have reported that circulating total BA concentrations significantly increase following bariatric surgery; however the results are inconsistent when BA composition is considered^[14,16]. Moreover, the effect of bariatric surgery on serum BA concentration depends on the type of surgical procedure. In patients who have undergone gastric banding, no changes or decreases in serum BA concentrations were reported (Table 4)^[28,138,145]. However, Nakatani *et al.*^[146] observed increased serum BAs one and three months after laparoscopic gastric banding (LAGB). Various effects of laparoscopic sleeve gastrectomy (LSG) on circulating BA concentrations have also been reported (Table 4). Steinert *et al.*^[147] found decreases in plasma BAs one week after LSG and increases three months and one year after the surgery. Belgaumkar *et al.*^[148] found decreases in primary BAs, and increases in secondary BAs six months after LSG. Increases in BA after LSG were documented by Nakatani *et al.*^[146] and Haluziková *et al.*^[29].

Most studies have shown increases in circulating BA concentrations following RYGB (Table 4)^[23,28,103,145,149-151].

Table 4 Effects of various bariatric procedures on diabetic parameters and serum bile acid concentrations

Patients	Type of bariatric procedure	Time interval from surgery to examination	Effect of surgery on diabetic parameters	Effect of surgery on serum bile acid concentrations	Ref.
Morbidly obese, <i>n</i> = 10	LAGB	Various (after losing 20% of body weight)	Not presented	Decreased fasting BAs; no change in postprandial BAs	[28]
Morbidly obese, <i>n</i> = 6, preoperative BMI = 44	Gastric banding	42 d	Not presented	No change	[138]
Morbidly obese, <i>n</i> = 28, BMI = 46.0	Gastric banding	6-28 mo	Decreased serum glucose Decreased serum insulin	Decreased primary BAs No change in deoxycholic BAs	[145]
Morbidly obese, <i>n</i> = 7, BMI = 43	LSG	1 wk, 3 mo, 1 yr	Decreased HOMA-IR	Decreased BAs after 1 wk Increased BAs after 3 mo and 1 yr	[147]
Morbidly obese, <i>n</i> = 18, BMI = 60	LSG	6 mo	Decreased: fasting glucose, fasting insulin, HOMA-IR and HbA1c	No change in total BAs Decreased primary BAs Increased secondary BAs	[148]
Obese females, <i>n</i> = 17, BMI = 43	LSG	24 mo	Decreased: HbA1c, insulin, HOMA-IR.	Increased total BAs	[29]
Morbidly obese, <i>n</i> = 15, BMI = 45	LSG and LAGB	1 and 3 mo	Decreased: HbA1c, insulin and HOMA-IR	Increased total, primary and secondary BAs	[146]
Morbidly obese, <i>n</i> = 8	RYGB	Various (after losing 20% of body weight)	Not presented	Increased fasting and postprandial total and conjugated BAs	[28]
Morbidly obese, <i>n</i> = 9, preoperative BMI = 50	RYGB	2-4 yr	Lower fasting glucose and insulin	Higher total BA concentration	[103]
Morbidly obese, <i>n</i> = 37, nondiabetic, preoperative BMI = 48	RYGB	About 200 d	No change in fasting serum glucose	No change in BAs after surgery	[150]
Morbidly obese, <i>n</i> = 75, diabetic, preoperative BMI = 48	RYGB	About 6 mo	Decreased fasting serum glucose and HbA1c	Increased total BAs	[150]
Severely obese women with T2DM, <i>n</i> = 13, preoperative BMI = 44	RYGB	1 mo and 2 yr	Decreased HOMA-IR	Reduced BAs after 1 mo Increased BAs after 2 yr	[32]
Obese patients, <i>n</i> = 63, BMI = 44	RYGB	15 mo	Decreased fasting glucose and HOMA-IR	Increased total BAs	[23]
Surgically obese, <i>n</i> = 5, BMI > 35	RYGB	1, 4, and 40 wk	Not presented	Increased conjugated BAs No changed unconjugated BAs	[152]
Obese females, <i>n</i> = 11, BMI = 44	RYGB	34 ± 16 mo	Increased postprandial insulin compared to controls	increased postprandial BAs comparing to controls	[149]
Morbidly obese, <i>n</i> = 30, BMI = 48	RYGB	8-13 mo	Decreased serum glucose and insulin concentration	Increased primary BAs, glycine BA, deoxycholic BA	[145]
Morbidly obese, <i>n</i> = 35, BMI = 48	RYGB	3 mo	Decreased HOMA-IR	Increased total BAs	[151]
Obese patients, <i>n</i> = 30, BMI = 46	RYGB	12 mo	Decreased fasting glucose and HOMA-IR	Increased total BAs, decreased taurine conjugated BAs	[153]
Morbidly obese, <i>n</i> = 7, BMI = 50	LRYGB	1 wk, 3 mo, 1 yr	Decreased HOMA-IR	Decreased BAs after 1 wk Increased BAs after 3 mo and 1 yr	[147]
Morbidly obese, <i>n</i> = 19, BMI = 43	LRYGB and LSG/DJB	1 and 3 mo	Decreased HbA1c, insulin, and HOMA-IR	Increased total, primary and secondary BA concentration	[146]
Morbidly obese, <i>n</i> = 12, preoperative BMI = 49	Gastric bypass	42 d	Not presented	Increased total BAs	[138]

LSG: Laparoscopic sleeve gastrectomy; LAGB: Laparoscopic adjustable gastric banding; LSG/DJB: LSG with duodeno-jejunal bypass; RYGB: Roux-en-Y gastric bypass; LRYGB: laparoscopic RYGB.

However, there are some reports with differing results. For instance, Gerhard *et al.*^[150] found there to be no change in serum BAs in nondiabetic subjects, but serum BA levels increased in diabetic subjects after RYGB. Ahmad *et al.*^[152] reported increased levels of conjugated BAs, but no changes in unconjugated BAs following RYGB. Dutia *et al.*^[32] even found decreased serum BAs 1 mo after RYGB, whereas 2 years after surgery their levels were higher than before surgery. Simonen *et al.*^[153] found an increase in total BAs after RYGB, but taurine conjugated BAs had decreased. Nonetheless, most published data suggest that

RYGB results in a postoperative increase of serum BAs in obese patients (Table 4). The increased levels of circulating BAs are probably due to the fact that BAs excreted into intestine have less time to mix with food prior to transit through the ileum, leaving more BAs free for reuptake and, probably more importantly, with the longer intestinal limb excluded from the alimentary passage and transited concentrated bile. Some studies have suggested that the BA concentrations were inversely correlated with 2 h post-meal serum glucose in patients after RYGB^[103]. Others found a positive correlation between the

increase in BA concentrations and fibroblast growth factor 19 (FGF-19), which regulates BA synthesis in the liver^[150]. The study of Pournaras *et al.*^[138] also confirmed increases in BAs and FGF-19 4 and 42 d following gastric bypass. Interestingly, circulating levels of FGF-19 and BAs increased following RYGB, but not after pharmacological management in patients with T2DM^[154]. Changes in serum BA levels were also positively correlated with FGF-19, incretin hormones, and peptide YY^[32]. The increase in serum BA concentrations following RYGB was also confirmed in an obese diabetic rat model^[155].

Overall, the majority of studies indicate that circulating BAs increase following RYGB (and other malabsorptive procedures) (Table 4). The increased circulating BA concentrations are usually associated with improvements in the glucose metabolism (Table 4), but are independent of caloric restriction^[31]. Data regarding circulating BA concentrations and the associations between circulating BA concentrations and glucose metabolism following sleeve gastrectomy are inconsistent (Table 4). Based on the data in Table 4, it can be concluded that there is an association between circulating BA concentration and T2DM remission following malabsorptive procedures. However, more research is needed to confirm the connection between circulating BA concentration and the improvement in glucose metabolism following different bariatric surgery procedures.

POTENTIAL MECHANISM OF BILE ACID ACTION IN THE IMPROVEMENT OF GLUCOSE METABOLISM AFTER BARIATRIC SURGERY

Bariatric procedures involving the transport of concentrated bile by a section of small intestine excluded from the passage of food lead to increased absorption of BAs by ileocytes, and subsequent increases in BA blood concentrations^[34]. In turn, BAs may interact with nuclear and membrane receptors—mainly FXR and TGR-5—in the intestines, liver, pancreas, adipose tissue, skeletal muscle cells, and other tissues, and improve glucose metabolism after bariatric surgery. Based on the data reported in the literature^[23,32,103,146,147,150,151,153], we calculated the correlation between the mean circulating concentration of BAs and HOMA-IR or insulin concentrations in groups of patients before and after RYGB. Circulating total BA concentrations were inversely correlated with (1) HOMA-IR ($R = -0.49$, $P < 0.05$); and (2) insulin concentrations ($R = -0.62$, $P < 0.01$). This confirms, at least in part, the notion that elevated circulating concentrations of BAs could contribute to improved glucose metabolism in patients who have undergone bariatric surgery.

Recent studies on FXR knockout mice have indicated

that FXR is necessary for improving glycemic control following bariatric surgery^[19]. This strongly supports the idea that BAs could contribute as FXR ligands to improved glucose metabolism following bariatric surgery. The activation by BAs of FXR in ileocytes leads to the increased release of FGF 15/19, which regulates carbohydrate metabolism^[32]. As mentioned above, FGF 15/19 stimulates glycogen synthesis and inhibits gluconeogenesis in the liver and glucose disposal in adipose tissue^[34]. Moreover, the plasma levels of FGF 19 have been found to correlate inversely with HbA_{1c} in diabetic patients^[156]. The potential role of FGF 15/19 in improving glucose metabolism following bariatric surgery is supported by the coordinated increase in BAs and FGF 19 and the improvement of glucose metabolism in patients following bariatric surgery^[32,138]. Another possible mechanism of the positive effect of elevated BAs following bariatric surgery is the direct activation of FXR in the liver, which leads to reduced gluconeogenesis^[123]. In turn, the activation of FXR in muscles, the liver, and adipose tissue leads to improvements in insulin sensitivity^[34,123]. The third potential mechanism of BA-mediated normalization of glycemia in bariatric patients is a through the stimulation of TGR-5 in L cells, which leads to the increased release of GLP-1^[34]. This assumption is supported by improved incretin effect after RYGB^[32] and the positive correlation seen between BAs and GLP-1 in patients who have undergone RYGB^[28]. The potential mechanisms of BA-mediated improvement of glucose metabolism after bariatric surgery are presented in Figure 7. However, it should be noted that FXR activation in L cells leads to the opposite effect - inhibition of GLP-1 production^[157]. This opposite effect of the BA induction of TGR-5 and FXR on GLP-1 release in L cells could possibly constitute some regulatory mechanism.

OTHER POTENTIAL MECHANISMS INVOLVED IN THE IMPROVEMENT IN GLUCOSE METABOLISM SUBSEQUENT UPON BARIATRIC SURGERY

Obesity is frequently associated with dyslipidemia, which includes hypertriglyceridemia, hypercholesterolemia, decreased HDL-cholesterol, and sometimes increased LDL-cholesterol concentrations in the blood^[158]. It is generally accepted that hyperlipidemia plays an important role in the loss of glucose-stimulated insulin secretion in T2DM^[159-161]. Bariatric surgery generally improves dyslipidemia^[162]. Thus, it may be supposed that improvements in glucose metabolism following bariatric surgery could be a consequence of the reduction of circulating lipid concentrations^[162]. A significant reduction in circulating triglyceride concentrations was observed from three months to four years after bariatric surgery^[162]. In

contrary, circulating non-esterified fatty acids (NEFA) concentrations, which also play an important role in the development of pancreatic β -cell dysfunctions and the subsequent progression of T2DM in obese subjects^[159], significantly increased one month after bariatric surgery, but their concentrations normalized after three months^[162]. Remission of T2DM has been shown to occur within a few days of bariatric surgery^[163]. Thus, it is likely that the beneficial effects of bariatric surgery on glucose metabolism are independent of the circulating NEFA normalization, especially shortly after surgery. However, it is not impossible that normalization of circulating NEFA (or other lipid) concentrations may play some role in improving glucose metabolism a few months after bariatric surgery.

The improvement in glucose metabolism following bariatric surgery, based on the enhanced incretin effect, is commonly accepted^[42,164]. However, the improvement in glucose metabolism following gastric bypass cannot be associated only with incretin hormones, because the incretin effect needs to be initiated through oral feeding, while patients cannot eat for the first few days after surgery, in order to avoid postoperative complications. Accordingly, the serum levels of GLP-1 (the main incretin hormone) is not markedly elevated in post-RYGB fasting patients, but is elevated after oral food intake^[43,164]. Moreover, the improvement in glucose metabolism following bariatric surgery has also been observed under conditions in which the incretin system was inactivated^[165].

Animal studies showed that bariatric surgery is associated with (1) increased production of GLP-1 in *A. muciniphila*^[20,43]; and (2) modification of gut microbiota composition and lower intestinal and systematic dipeptidyl peptidase-4 (DPP-4) activity^[166]. Thus, gut microbiota changes, leading to the elevated production of GLP-1 by intestinal endocrine cells (L cells) and decreased degradation of GLP-1 by DPP-4, may be another mechanism involved in the improvement of insulin resistance following bariatric surgery. However, further studies are needed to better understand the role of microbiota in incretin hormone modification after bariatric surgery.

Another potential mechanism for improving glucose metabolism following bariatric surgery may be associated with alterations in circulating adipokines. It has been shown that the concentrations of circulating proinflammatory adipokines (for instance, of leptin) increase, while those of anti-inflammatory adipokines (such as adiponectin) decrease in obesity and T2DM^[167-169]. The dysregulation of adipokine secretion (especially leptin and adiponectin), together with other factors, such as lipid disturbance, could thus be a causal factor mediating insulin resistance and subsequently T2DM in obese patients. Bariatric surgery may overcome these disturbances. Accordingly, recently published data indicates an association between circulating adiponectin concentrations and

the acute insulin response to intravenous glucose load in patients at one year and five years following bariatric surgery^[170]. Thus, in the long term following bariatric surgery, the increase in circulating adiponectin concentration might play some role in T2DM remission, though it is unlikely that this would occur in the short term.

Overall, the data discussed in this review suggest that several factors could be involved in the remission of T2DM following bariatric surgery. It seems that the increase in circulating BA concentrations and alterations in gut microbiota play important role in this phenomenon.

CONCLUSION

Recent research indicates that the improvements in insulin sensitivity observed in patients who have undergone bariatric surgery, especially after gastric bypass procedures, are associated with elevated circulating BA concentration and changes in gut microbiota. Through the activation of FXR in enterocytes, the BAs increase the release of FGF 15/19, which in turn bind to FGFR, leading to (1) decreased gluconeogenesis and increased glycogen synthesis and glucose catabolism in the liver; and (2) improved insulin sensitivity and glucose disposal in adipose tissue. Moreover, BAs activate TGR-5 or FXR and thus lead to increases in (1) insulin secretion by pancreatic cells; (2) GLP-1 production in intestinal endocrine L-cells; (3) glucose catabolism in muscle and adipose tissue; and (4) insulin sensitivity with decreases of gluconeogenesis in the liver. Moreover, changes in gut microbiota following bariatric surgery also could be beneficial, as far as T2DM remission is concerned. Thus, increases in circulating BA concentrations and the interaction between BAs and gut microbiota (especially the role of microbiota in the biotransformation of BAs and the antimicrobial effects of BAs) following bariatric surgery would seem to be important factors leading to T2DM remission, besides body mass loss, calorie restriction, and changes in the concentrations of serum lipids and adipokines.

REFERENCES

- 1 Ng M, Fleming T, Robinson M, Thomson B, Graetz N, Margono C, Mullany EC, Biryukov S, Abbafati C, Abera SF, Abraham JP, Abu-Rmeileh NM, Achoki T, AlBuhairan FS, Alemu ZA, Alfonso R, Ali MK, Ali R, Guzman NA, Ammar W, Anwar P, Banerjee A, Barquera S, Basu S, Bennett DA, Bhutta Z, Blore J, Cabral N, Nonato IC, Chang JC, Chowdhury R, Courville KJ, Criqui MH, Cundiff DK, Dabhadkar KC, Dandona L, Davis A, Dayama A, Dharmaratne SD, Ding EL, Durrani AM, Esteghamati A, Farzadfar F, Fay DF, Feigin VL, Flaxman A, Forouzanfar MH, Goto A, Green MA, Gupta R, Hafezi-Nejad N, Hankey GJ, Harewood HC, Havmoeller R, Hay S, Hernandez L, Husseini A, Idrisov BT, Ikeda N, Islami F, Jahangir E, Jassal SK, Jee SH, Jeffreys M, Jonas JB, Kabagambe EK, Khalifa SE, Kengne AP, Khader YS, Khang YH, Kim D, Kimokoti RW, Kinge JM, Kokubo Y, Kosen S, Kwan G, Lai T, Leinsalu M, Li Y, Liang X, Liu S, Logroscino

- G, Lotufo PA, Lu Y, Ma J, Mainoo NK, Mensah GA, Merriman TR, Mokdad AH, Moschandreas J, Naghavi M, Naheed A, Nand D, Narayan KM, Nelson EL, Neuhauser ML, Nisar MI, Ohkubo T, Oti SO, Pedroza A, Prabhakaran D, Roy N, Sampson U, Seo H, Sepanlou SG, Shibuya K, Shiri R, Shieue I, Singh GM, Singh JA, Skirbekk V, Stapelberg NJ, Sturua L, Sykes BL, Tobias M, Tran BX, Trasande L, Toyoshima H, van d, V, Vasankari TJ, Veerman JL, Velasquez-Melendez G, Vlassov VV, Vollset SE, Vos T, Wang C, Wang X, Weiderpass E, Werdecker A, Wright JL, Yang YC, Yatsuya H, Yoon J, Yoon SJ, Zhao Y, Zhou M, Zhu S, Lopez AD, Murray CJ, Gakidou E. Global, regional, and national prevalence of overweight and obesity in children and adults during 1980-2013: a systematic analysis for the Global Burden of Disease Study 2013. *Lancet* 2014; **384**: 766-781 [PMID: 24880830 DOI: 10.1016/S0140-6736(14)60460-8]
- 2 **Kaska L**, Mika A, Stepnowski P, Proczko M, Ratnicki-Sklucki K, Sledzinski T, Goyke E, Swierczynski J. The relationship between specific Fatty acids of serum lipids and serum high sensitivity C- reactive protein levels in morbidly obese women. *Cell Physiol Biochem* 2014; **34**: 1101-1108 [PMID: 25228402 DOI: 10.1159/000366324]
- 3 **Sledzinski T**, Sledzinski M, Smolenski RT, Swierczynski J. Increased serum nitric oxide concentration after bariatric surgery-a potential mechanism for cardiovascular benefit. *Obes Surg* 2010; **20**: 204-210 [PMID: 19997784 DOI: 10.1007/s11695-009-0041-2]
- 4 **Swierczynski J**, Sledzinski T, Slominska E, Smolenski R, Sledzinski Z. Serum phenylalanine concentration as a marker of liver function in obese patients before and after bariatric surgery. *Obes Surg* 2009; **19**: 883-889 [PMID: 18431611 DOI: 10.1007/s11695-008-9521-z]
- 5 **Swierczynski J**, Sledzinski T. The Role of Adipokines and Gastrointestinal Tract Hormones in Obesity. In: Karcz K, Thomusch O, editors. Principles of metabolic surgery: Springer, 2012
- 6 **Ley RE**, Turnbaugh PJ, Klein S, Gordon JI. Microbial ecology: human gut microbes associated with obesity. *Nature* 2006; **444**: 1022-1023 [PMID: 17183309 DOI: 10.1038/4441022a]
- 7 **Rial SA**, Karelis AD, Bergeron KF, Mounier C. Gut Microbiota and Metabolic Health: The Potential Beneficial Effects of a Medium Chain Triglyceride Diet in Obese Individuals. *Nutrients* 2016; **8**: pii: E281 [PMID: 27187452 DOI: 10.3390/nu8050281]
- 8 **Mutch DM**, Clément K. Unraveling the genetics of human obesity. *PLoS Genet* 2006; **2**: e188 [PMID: 17196040 DOI: 10.1371/journal.pgen.0020188]
- 9 **Mingrone G**, Panunzi S, De Gaetano A, Guidone C, Iaconelli A, Leccesi L, Nanni G, Pomp A, Castagneto M, Ghirlanda G, Rubino F. Bariatric surgery versus conventional medical therapy for type 2 diabetes. *N Engl J Med* 2012; **366**: 1577-1585 [PMID: 22449317 DOI: 10.1056/NEJMoa1200111]
- 10 **Schauer PR**, Kashyap SR, Wolski K, Brethauer SA, Kirwan JP, Pothier CE, Thomas S, Abood B, Nissen SE, Bhatt DL. Bariatric surgery versus intensive medical therapy in obese patients with diabetes. *N Engl J Med* 2012; **366**: 1567-1576 [PMID: 22449319 DOI: 10.1056/NEJMoa1200225]
- 11 **Rubino F**, Nathan DM, Eckel RH, Schauer PR, Alberti KG, Zimmet PZ, Del Prato S, Ji L, Sadikot SM, Herman WH, Amiel SA, Kaplan LM, Taroncher-Oldenburg G, Cummings DE. Metabolic Surgery in the Treatment Algorithm for Type 2 Diabetes: A Joint Statement by International Diabetes Organizations. *Diabetes Care* 2016; **39**: 861-877 [PMID: 27222544 DOI: 10.2337/dc16-0236]
- 12 **Kmietowicz Z**. Surgery for obese people with diabetes could save the NHS £100,000 a patient, finds study. *BMJ* 2016; **353**: i3150 [PMID: 27268789]
- 13 **Quercia I**, Dutia R, Kotler DP, Belsley S, Laferrère B. Gastrointestinal changes after bariatric surgery. *Diabetes Metab* 2014; **40**: 87-94 [PMID: 24359701 DOI: 10.1016/j.diabet.2013.11.003]
- 14 **Sweeney TE**, Morton JM. Metabolic surgery: action via hormonal milieu changes, changes in bile acids or gut microbiota? A summary of the literature. *Best Pract Res Clin Gastroenterol* 2014; **28**: 727-740 [PMID: 25194186 DOI: 10.1016/j.bpg.2014.07.016]
- 15 **Schmidt JB**, Pedersen SD, Gregersen NT, Vestergaard L, Nielsen MS, Ritz C, Madsbad S, Worm D, Hansen DL, Clausen TR, Rehfeld JF, Astrup A, Holst JJ, Sjödin A. Effects of RYGB on energy expenditure, appetite and glycaemic control: a randomized controlled clinical trial. *Int J Obes (Lond)* 2016; **40**: 281-290 [PMID: 26303352 DOI: 10.1038/ijo.2015.162]
- 16 **Penney NC**, Kinross J, Newton RC, Purkayastha S. The role of bile acids in reducing the metabolic complications of obesity after bariatric surgery: a systematic review. *Int J Obes (Lond)* 2015; **39**: 1565-1574 [PMID: 26081915 DOI: 10.1038/ijo.2015.115]
- 17 **Furet JP**, Kong LC, Tap J, Poitou C, Basdevant A, Bouillot JL, Mariat D, Corthier G, Doré J, Henegar C, Rizkalla S, Clément K. Differential adaptation of human gut microbiota to bariatric surgery-induced weight loss: links with metabolic and low-grade inflammation markers. *Diabetes* 2010; **59**: 3049-3057 [PMID: 20876719 DOI: 10.2337/db10-0253]
- 18 **Li JV**, Ashrafian H, Bueter M, Kinross J, Sands C, le Roux CW, Bloom SR, Darzi A, Athanasiou T, Marchesi JR, Nicholson JK, Holmes E. Metabolic surgery profoundly influences gut microbial-host metabolic cross-talk. *Gut* 2011; **60**: 1214-1223 [PMID: 21572120 DOI: 10.1136/gut.2010.234708]
- 19 **Ryan KK**, Tremaroli V, Clemmensen C, Kovatcheva-Datchary P, Myronovych A, Karns R, Wilson-Pérez HE, Sandoval DA, Kohli R, Bäckhed F, Seeley RJ. FXR is a molecular target for the effects of vertical sleeve gastrectomy. *Nature* 2014; **509**: 183-188 [PMID: 24670636 DOI: 10.1038/nature13135]
- 20 **Zhang H**, DiBaise JK, Zuccolo A, Kudrna D, Braidotti M, Yu Y, Parameswaran P, Crowell MD, Wing R, Rittmann BE, Krajmalnik-Brown R. Human gut microbiota in obesity and after gastric bypass. *Proc Natl Acad Sci USA* 2009; **106**: 2365-2370 [PMID: 19164560 DOI: 10.1073/pnas.0812600106]
- 21 **Fiorucci S**, Distrutti E. Bile Acid-Activated Receptors, Intestinal Microbiota, and the Treatment of Metabolic Disorders. *Trends Mol Med* 2015; **21**: 702-714 [PMID: 26481828 DOI: 10.1016/j.molmed.2015.09.001]
- 22 **Nosso G**, Griffo E, Cotugno M, Saldalamacchia G, Lupoli R, Pacini G, Riccardi G, Angrisani L, Capaldo B. Comparative Effects of Roux-en-Y Gastric Bypass and Sleeve Gastrectomy on Glucose Homeostasis and Incretin Hormones in Obese Type 2 Diabetic Patients: A One-Year Prospective Study. *Horm Metab Res* 2016; **48**: 312-317 [PMID: 26788926 DOI: 10.1055/s-0041-111505]
- 23 **Werling M**, Vincent RP, Cross GF, Marschall HU, Fändriks L, Lönroth H, Taylor DR, Alagband-Zadeh J, Olbers T, Le Roux CW. Enhanced fasting and post-prandial plasma bile acid responses after Roux-en-Y gastric bypass surgery. *Scand J Gastroenterol* 2013; **48**: 1257-1264 [PMID: 24044585 DOI: 10.3109/00365521.2013.833647]
- 24 **Luo P**, Yu H, Zhao X, Bao Y, Hong CS, Zhang P, Tu Y, Yin P, Gao P, Wei L, Zhuang Z, Jia W, Xu G. Metabolomics Study of Roux-en-Y Gastric Bypass Surgery (RYGB) to Treat Type 2 Diabetes Patients Based on Ultraperformance Liquid Chromatography-Mass Spectrometry. *J Proteome Res* 2016; **15**: 1288-1299 [PMID: 26889720 DOI: 10.1021/acs.jproteome.6b00022]
- 25 **Yu H**, Ni Y, Bao Y, Zhang P, Zhao A, Chen T, Xie G, Tu Y, Zhang L, Su M, Wei L, Jia W, Jia W. Chenodeoxycholic Acid as a Potential Prognostic Marker for Roux-en-Y Gastric Bypass in Chinese Obese Patients. *J Clin Endocrinol Metab* 2015; **100**: 4222-4230 [PMID: 26425885 DOI: 10.1210/jc.2015-2884]
- 26 **Cole AJ**, Teigen LM, Jahansouz C, Earthman CP, Sibley SD. The Influence of Bariatric Surgery on Serum Bile Acids in Humans and Potential Metabolic and Hormonal Implications: a Systematic Review. *Curr Obes Rep* 2015; **4**: 441-450 [PMID: 26335653 DOI: 10.1007/s13679-015-0171-x]
- 27 **Argyropoulos G**. Bariatric surgery: prevalence, predictors, and mechanisms of diabetes remission. *Curr Diab Rep* 2015; **15**: 15 [PMID: 25702097 DOI: 10.1007/s11892-015-0590-9]
- 28 **Kohli R**, Bradley D, Setchell KD, Eagon JC, Abumrad N, Klein S. Weight loss induced by Roux-en-Y gastric bypass but not laparoscopic adjustable gastric banding increases circulating bile

- acids. *J Clin Endocrinol Metab* 2013; **98**: E708-E712 [PMID: 23457410 DOI: 10.1210/jc.2012-3736]
- 29 **Haluzíková D**, Lacinová Z, Kaválková P, Drápalová J, Křížová J, Bártlová M, Mráz M, Petr T, Vitek L, Kasalický M, Haluzík M. Laparoscopic sleeve gastrectomy differentially affects serum concentrations of FGF-19 and FGF-21 in morbidly obese subjects. *Obesity* (Silver Spring) 2013; **21**: 1335-1342 [PMID: 23670968 DOI: 10.1002/oby.20208]
 - 30 **Dixon JB**, le Roux CW, Rubino F, Zimmet P. Bariatric surgery for type 2 diabetes. *Lancet* 2012; **379**: 2300-2311 [PMID: 22683132 DOI: 10.1016/S0140-6736(12)60401-2]
 - 31 **Jahansouz C**, Xu H, Hertzog AV, Serrot FJ, Kvalheim N, Cole A, Abraham A, Luthra G, Ewing K, Leslie DB, Bernlohr DA, Ikramuddin S. Bile Acids Increase Independently From Hypocaloric Restriction After Bariatric Surgery. *Ann Surg* 2015; Epub ahead of print [PMID: 26655924 DOI: 10.1097/SLA.0000000000001552]
 - 32 **Dutia R**, Embrey M, O'Brien CS, Haeusler RA, Agénor KK, Homel P, McGinty J, Vincent RP, Alaghband-Zadeh J, Staels B, le Roux CW, Yu J, Laferrère B. Temporal changes in bile acid levels and 12 α -hydroxylation after Roux-en-Y gastric bypass surgery in type 2 diabetes. *Int J Obes* (Lond) 2015; **39**: 806-813 [PMID: 25599611 DOI: 10.1038/ijo.2015.1]
 - 33 **Fiorucci S**, Cipriani S, Baldelli F, Mencarelli A. Bile acid-activated receptors in the treatment of dyslipidemia and related disorders. *Prog Lipid Res* 2010; **49**: 171-185 [PMID: 19932133 DOI: 10.1016/j.plipres.2009.11.001]
 - 34 **Kuipers F**, Groen AK. FXR: the key to benefits in bariatric surgery? *Nat Med* 2014; **20**: 337-338 [PMID: 24710375 DOI: 10.1038/nm.3525]
 - 35 **Zhang F**, Yu L, Lin X, Cheng P, He L, Li X, Lu X, Tan Y, Yang H, Cai L, Zhang C. Minireview: Roles of Fibroblast Growth Factors 19 and 21 in Metabolic Regulation and Chronic Diseases. *Mol Endocrinol* 2015; **29**: 1400-1413 [PMID: 26308386 DOI: 10.1210/me.2015-1155]
 - 36 **Spinelli V**, Lalloyer F, Baud G, Osto E, Kouach M, Daoudi M, Vallez E, Raverdy V, Goossens JF, Descat A, Doytcheva P, Hubert T, Lutz TA, Lestavel S, Staels B, Pattou F, Tailleux A. Influence of Roux-en-Y gastric bypass on plasma bile acid profiles: a comparative study between rats, pigs and humans. *Int J Obes* (Lond) 2016; **40**: 1260-1267 [PMID: 27089995 DOI: 10.1038/ijo.2016.46]
 - 37 **Raghow R**. Ménage-à-trois of bariatric surgery, bile acids and the gut microbiome. *World J Diabetes* 2015; **6**: 367-370 [PMID: 25897347 DOI: 10.4239/wjd.v6.i3.367]
 - 38 **Watanabe M**, Houten SM, Matakaki C, Christoffolete MA, Kim BW, Sato H, Messaddeq N, Harney JW, Ezaki O, Kodama T, Schoonjans K, Bianco AC, Auwerx J. Bile acids induce energy expenditure by promoting intracellular thyroid hormone activation. *Nature* 2006; **439**: 484-489 [PMID: 16400329 DOI: 10.1038/nature04330]
 - 39 **Sato H**, Genet C, Strehle A, Thomas C, Lobstein A, Wagner A, Mioskowski C, Auwerx J, Saladin R. Anti-hyperglycemic activity of a TGR5 agonist isolated from *Olea europaea*. *Biochem Biophys Res Commun* 2007; **362**: 793-798 [PMID: 17825251 DOI: 10.1016/j.bbrc.2007.06.130]
 - 40 **Christou NV**. Impact of obesity and bariatric surgery on survival. *World J Surg* 2009; **33**: 2022-2027 [PMID: 19440652 DOI: 10.1007/s00268-009-0050-2]
 - 41 Clinical Guidelines - Recommendations of International Diabetes Federation, Chapter 06 Glucose Control Levels. Available from: URL: https://www.idf.org/sites/default/files/IDF_T2DM_Guideline.pdf
 - 42 **Cho YM**. A gut feeling to cure diabetes: potential mechanisms of diabetes remission after bariatric surgery. *Diabetes Metab J* 2014; **38**: 406-415 [PMID: 25541603 DOI: 10.4093/dmj.2014.38.6.406]
 - 43 **Rhee NA**, Vilsbøll T, Knop FK. Current evidence for a role of GLP-1 in Roux-en-Y gastric bypass-induced remission of type 2 diabetes. *Diabetes Obes Metab* 2012; **14**: 291-298 [PMID: 21951387 DOI: 10.1111/j.1463-1326.2011.01505.x]
 - 44 **Hansen M**, Sonne DP, Knop FK. Bile acid sequestrants: glucose-lowering mechanisms and efficacy in type 2 diabetes. *Curr Diab Rep* 2014; **14**: 482 [PMID: 24623198 DOI: 10.1007/s11892-014-0482-4]
 - 45 **Docherty NG**, le Roux CW. Improvements in the metabolic milieu following Roux-en-Y gastric bypass and the arrest of diabetic kidney disease. *Exp Physiol* 2014; **99**: 1146-1153 [PMID: 25085842 DOI: 10.1113/expphysiol.2014.078790]
 - 46 **Cummings DE**. Endocrine mechanisms mediating remission of diabetes after gastric bypass surgery. *Int J Obes* (Lond) 2009; **33** Suppl 1: S33-S40 [PMID: 19363506 DOI: 10.1038/ijo.2009.15]
 - 47 **Kaska L**, Proczko M, Wiśniewski P, Stankiewicz M, Gill D, Śledziński Z. A prospective evaluation of the influence of three bariatric procedures on insulin resistance improvement. Should the extent of undiluted bile transit be considered a key postoperative factor altering glucose metabolism? *Wideochir Inne Tech Maloinwazyjne* 2015; **10**: 213-228 [PMID: 26240621 DOI: 10.5114/wiitm.2015.52062]
 - 48 **Faria G**, Preto J, da Costa EL, Guimarães JT, Calhau C, Taveira-Gomes A. Acute improvement in insulin resistance after laparoscopic Roux-en-Y gastric bypass: is 3 days enough to correct insulin metabolism? *Obes Surg* 2013; **23**: 103-110 [PMID: 23114971 DOI: 10.1007/s11695-012-0803-0]
 - 49 **Gómez-Abril S**, Morillas-Ariño C, Ponce-Marco JL, Torres-Sánchez T, Delgado-Gomis F, Hernández-Mijares A, Rocha M. Short- and Long-Term Effects of Weight Loss on the Complement Component C3 After Laparoscopic Gastric Bypass in Obese Patients. *Obes Surg* 2016; Epub ahead of print [PMID: 27143095 DOI: 10.1007/s11695-016-2195-z]
 - 50 **Severino A**, Castagneto-Gissey L, Raffaelli M, Gastaldelli A, Capristo E, Iaconelli A, Guidone C, Callari C, Bellantone R, Mingrone G. Early effect of Roux-en-Y gastric bypass on insulin sensitivity and signaling. *Surg Obes Relat Dis* 2016; **12**: 42-47 [PMID: 26483070 DOI: 10.1016/j.soard.2015.06.005]
 - 51 **Mingrone G**, Castagneto-Gissey L. Mechanisms of early improvement/resolution of type 2 diabetes after bariatric surgery. *Diabetes Metab* 2009; **35**: 518-523 [PMID: 20152737 DOI: 10.1016/S1262-3636(09)73459-7]
 - 52 **Robert M**, Belanger P, Hould FS, Marceau S, Tcherno A, Biertho L. Should metabolic surgery be offered in morbidly obese patients with type I diabetes? *Surg Obes Relat Dis* 2015; **11**: 798-805 [PMID: 25868828 DOI: 10.1016/j.soard.2014.12.016]
 - 53 **Garciaaballero M**, Martínez-Moreno JM, Toval JA, Miralles F, Mínguez A, Osorio D, Mata JM, Reyes-Ortiz A. Improvement of C peptide zero BMI 24-34 diabetic patients after tailored one anastomosis gastric bypass (BAGUA). *Nutr Hosp* 2013; **28** Suppl 2: 35-46 [PMID: 23834045 DOI: 10.3305/nh.2013.28.sup2.6712]
 - 54 **Li T**, Chiang JY. Bile acid signaling in metabolic disease and drug therapy. *Pharmacol Rev* 2014; **66**: 948-983 [PMID: 25073467 DOI: 10.1124/pr.113.008201]
 - 55 **Li T**, Chiang JY. Bile acids as metabolic regulators. *Curr Opin Gastroenterol* 2015; **31**: 159-165 [PMID: 25584736 DOI: 10.1097/MOG.0000000000000156]
 - 56 **de Aguiar Vallim TQ**, Tarling EJ, Ahn H, Hagey LR, Romanoski CE, Lee RG, Graham MJ, Motohashi H, Yamamoto M, Edwards PA. MAFG is a transcriptional repressor of bile acid synthesis and metabolism. *Cell Metab* 2015; **21**: 298-310 [PMID: 25651182 DOI: 10.1016/j.cmet.2015.01.007]
 - 57 **Norlin M**, Wikvall K. Enzymes in the conversion of cholesterol into bile acids. *Curr Mol Med* 2007; **7**: 199-218 [PMID: 17346171]
 - 58 **Russell DW**. Fifty years of advances in bile acid synthesis and metabolism. *J Lipid Res* 2009; **50** Suppl: S120-S125 [PMID: 18815433 DOI: 10.1194/jlr.R800026-JLR200]
 - 59 **Norlin M**, Toll A, Björkhem I, Wikvall K. 24-hydroxycholesterol is a substrate for hepatic cholesterol 7 α -hydroxylase (CYP7A). *J Lipid Res* 2000; **41**: 1629-1639 [PMID: 11013305]
 - 60 **Sagar NM**, Cree IA, Covington JA, Arasaradnam RP. The interplay of the gut microbiome, bile acids, and volatile organic compounds. *Gastroenterol Res Pract* 2015; **2015**: 398585 [PMID: 25821460 DOI: 10.1155/2015/398585]
 - 61 **Camilleri M**, Gores GJ. Therapeutic targeting of bile acids. *Am J*

- Physiol Gastrointest Liver Physiol* 2015; **309**: G209-G215 [PMID: 26138466 DOI: 10.1152/ajpgi.00121.2015]
- 62 **Makishima M**, Okamoto AY, Repa JJ, Tu H, Learned RM, Luk A, Hull MV, Lustig KD, Mangelsdorf DJ, Shan B. Identification of a nuclear receptor for bile acids. *Science* 1999; **284**: 1362-1365 [PMID: 10334992]
 - 63 **Grober J**, Zaghini I, Fujii H, Jones SA, Kliewer SA, Willson TM, Ono T, Besnard P. Identification of a bile acid-responsive element in the human ileal bile acid-binding protein gene. Involvement of the farnesoid X receptor/9-cis-retinoic acid receptor heterodimer. *J Biol Chem* 1999; **274**: 29749-29754 [PMID: 10514450]
 - 64 **Matsubara T**, Li F, Gonzalez FJ. FXR signaling in the enterohepatic system. *Mol Cell Endocrinol* 2013; **368**: 17-29 [PMID: 22609541 DOI: 10.1016/j.mce.2012.05.004]
 - 65 **Ferrannini E**, Camastra S, Astiarraga B, Nannipieri M, Castro-Perez J, Xie D, Wang L, Chakravarthy M, Haeusler RA. Increased Bile Acid Synthesis and Deconjugation After Biliopancreatic Diversion. *Diabetes* 2015; **64**: 3377-3385 [PMID: 26015549 DOI: 10.2337/db15-0214]
 - 66 **Dawson PA**, Karpen SJ. Intestinal transport and metabolism of bile acids. *J Lipid Res* 2015; **56**: 1085-1099 [PMID: 25210150 DOI: 10.1194/jlr.R054114]
 - 67 **Chiang JY**. Bile acids: regulation of synthesis. *J Lipid Res* 2009; **50**: 1955-1966 [PMID: 19346330 DOI: 10.1194/jlr.R900010-JLR200]
 - 68 **Ridlon JM**, Kang DJ, Hylemon PB. Bile salt biotransformations by human intestinal bacteria. *J Lipid Res* 2006; **47**: 241-259 [PMID: 16299351 DOI: 10.1194/jlr.R500013-JLR200]
 - 69 **Guinane CM**, Cotter PD. Role of the gut microbiota in health and chronic gastrointestinal disease: understanding a hidden metabolic organ. *Therap Adv Gastroenterol* 2013; **6**: 295-308 [PMID: 23814609 DOI: 10.1177/1756283X13482996]
 - 70 **Chow J**, Lee SM, Shen Y, Khosravi A, Mazmanian SK. Host-bacterial symbiosis in health and disease. *Adv Immunol* 2010; **107**: 243-274 [PMID: 21034976 DOI: 10.1016/B978-0-12-381300-8.0008-3]
 - 71 **Kelly CJ**, Zheng L, Campbell EL, Saeedi B, Scholz CC, Bayless AJ, Wilson KE, Glover LE, Kominsky DJ, Magnuson A, Weir TL, Ehrentauf SF, Pickel C, Kuhn KA, Lanis JM, Nguyen V, Taylor CT, Colgan SP. Crosstalk between Microbiota-Derived Short-Chain Fatty Acids and Intestinal Epithelial HIF Augments Tissue Barrier Function. *Cell Host Microbe* 2015; **17**: 662-671 [PMID: 25865369 DOI: 10.1016/j.chom.2015.03.005]
 - 72 **LeBlanc JG**, Milani C, de Giori GS, Sesma F, van Sinderen D, Ventura M. Bacteria as vitamin suppliers to their host: a gut microbiota perspective. *Curr Opin Biotechnol* 2013; **24**: 160-168 [PMID: 22940212 DOI: 10.1016/j.copbio.2012.08.005]
 - 73 **Turnbaugh PJ**, Hamady M, Yatsunenko T, Cantarel BL, Duncan A, Ley RE, Sogin ML, Jones WJ, Roe BA, Affourtit JP, Egholm M, Henrissat B, Heath AC, Knight R, Gordon JI. A core gut microbiome in obese and lean twins. *Nature* 2009; **457**: 480-484 [PMID: 19043404 DOI: 10.1038/nature07540]
 - 74 **Allegretti JR**, Kearney S, Li N, Bogart E, Bullock K, Gerber GK, Bry L, Clish CB, Alm E, Korzenik JR. Recurrent *Clostridium difficile* infection associates with distinct bile acid and microbiome profiles. *Aliment Pharmacol Ther* 2016; **43**: 1142-1153 [PMID: 27086647 DOI: 10.1111/apt.13616]
 - 75 **Ridlon JM**, Kang DJ, Hylemon PB, Bajaj JS. Bile acids and the gut microbiome. *Curr Opin Gastroenterol* 2014; **30**: 332-338 [PMID: 24625896 DOI: 10.1097/MOG.0000000000000057]
 - 76 **Hagey LR**, Krasowski MD. Microbial biotransformations of bile acids as detected by electrospray mass spectrometry. *Adv Nutr* 2013; **4**: 29-35 [PMID: 23319120 DOI: 10.3945/an.112.003061]
 - 77 **Begley M**, Gahan CG, Hill C. The interaction between bacteria and bile. *FEMS Microbiol Rev* 2005; **29**: 625-651 [PMID: 16102595 DOI: 10.1016/j.femsre.2004.09.003]
 - 78 **Bortolini O**, Medici A, Poli S. Biotransformations on steroid nucleus of bile acids. *Steroids* 1997; **62**: 564-577 [PMID: 9292932]
 - 79 **Chikai T**, Nakao H, Uchida K. Deconjugation of bile acids by human intestinal bacteria implanted in germ-free rats. *Lipids* 1987; **22**: 669-671 [PMID: 3312906]
 - 80 **Lorenzo-Zúñiga V**, Bartolí R, Planas R, Hofmann AF, Viñado B, Hagey LR, Hernández JM, Mañé J, Alvarez MA, Ausina V, Gassull MA. Oral bile acids reduce bacterial overgrowth, bacterial translocation, and endotoxemia in cirrhotic rats. *Hepatology* 2003; **37**: 551-557 [PMID: 12601352 DOI: 10.1053/jhep.2003.50116]
 - 81 **Inagaki T**, Moschetta A, Lee YK, Peng L, Zhao G, Downes M, Yu RT, Shelton JM, Richardson JA, Repa JJ, Mangelsdorf DJ, Kliewer SA. Regulation of antibacterial defense in the small intestine by the nuclear bile acid receptor. *Proc Natl Acad Sci USA* 2006; **103**: 3920-3925 [PMID: 16473946 DOI: 10.1073/pnas.0509592103]
 - 82 **Philipp B**. Bacterial degradation of bile salts. *Appl Microbiol Biotechnol* 2011; **89**: 903-915 [PMID: 21088832 DOI: 10.1007/s00253-010-2998-0]
 - 83 **Merritt ME**, Donaldson JR. Effect of bile salts on the DNA and membrane integrity of enteric bacteria. *J Med Microbiol* 2009; **58**: 1533-1541 [PMID: 19762477 DOI: 10.1099/jmm.0.014092-0]
 - 84 **Islam KB**, Fukiya S, Hagio M, Fujii N, Ishizuka S, Ooka T, Ogura Y, Hayashi T, Yokota A. Bile acid is a host factor that regulates the composition of the cecal microbiota in rats. *Gastroenterology* 2011; **141**: 1773-1781 [PMID: 21839040 DOI: 10.1053/j.gastro.2011.07.046]
 - 85 **den Besten G**, van Eunen K, Groen AK, Venema K, Reijngoud DJ, Bakker BM. The role of short-chain fatty acids in the interplay between diet, gut microbiota, and host energy metabolism. *J Lipid Res* 2013; **54**: 2325-2340 [PMID: 23821742 DOI: 10.1194/jlr.R036012]
 - 86 **Hur KY**, Lee MS. Gut Microbiota and Metabolic Disorders. *Diabetes Metab J* 2015; **39**: 198-203 [PMID: 26124989 DOI: 10.4093/dmj.2015.39.3.198]
 - 87 **Arslan N**. Obesity, fatty liver disease and intestinal microbiota. *World J Gastroenterol* 2014; **20**: 16452-16463 [PMID: 25469013 DOI: 10.3748/wjg.v20.i44.16452]
 - 88 **Ley RE**, Bäckhed F, Turnbaugh P, Lozupone CA, Knight RD, Gordon JI. Obesity alters gut microbial ecology. *Proc Natl Acad Sci USA* 2005; **102**: 11070-11075 [PMID: 16033867 DOI: 10.1073/pnas.0504978102]
 - 89 **Delzenne NM**, Cani PD. Interaction between obesity and the gut microbiota: relevance in nutrition. *Annu Rev Nutr* 2011; **31**: 15-31 [PMID: 21568707 DOI: 10.1146/annurev-nutr-072610-145146]
 - 90 **Turnbaugh PJ**, Ley RE, Mahowald MA, Magrini V, Mardis ER, Gordon JI. An obesity-associated gut microbiome with increased capacity for energy harvest. *Nature* 2006; **444**: 1027-1031 [PMID: 17183312 DOI: 10.1038/nature05414]
 - 91 **Bäckhed F**, Ding H, Wang T, Hooper LV, Koh GY, Nagy A, Semenkovich CF, Gordon JI. The gut microbiota as an environmental factor that regulates fat storage. *Proc Natl Acad Sci USA* 2004; **101**: 15718-15723 [PMID: 15505215 DOI: 10.1073/pnas.0407076101]
 - 92 **Ridaura VK**, Faith JJ, Rey FE, Cheng J, Duncan AE, Kau AL, Griffin NW, Lombard V, Henrissat B, Bain JR, Muehlbauer MJ, Ilkayeva O, Semenkovich CF, Funai K, Hayashi DK, Lyle BJ, Martini MC, Ursell LK, Clemente JC, Van Treuren W, Walters WA, Knight R, Newgard CB, Heath AC, Gordon JI. Gut microbiota from twins discordant for obesity modulate metabolism in mice. *Science* 2013; **341**: 1241214 [PMID: 24009397 DOI: 10.1126/science.1241214]
 - 93 **Caricilli AM**, Saad MJ. Gut microbiota composition and its effects on obesity and insulin resistance. *Curr Opin Clin Nutr Metab Care* 2014; **17**: 312-318 [PMID: 24848531 DOI: 10.1097/MCO.0000000000000067]
 - 94 **Aron-Wisniewsky J**, Gaborit B, Dutour A, Clement K. Gut microbiota and non-alcoholic fatty liver disease: new insights. *Clin Microbiol Infect* 2013; **19**: 338-348 [PMID: 23452163 DOI: 10.1111/1469-0691.12140]
 - 95 **Karlsson FH**, Tremaroli V, Nookaew I, Bergström G, Behre CJ, Fagerberg B, Nielsen J, Bäckhed F. Gut metagenome in European women with normal, impaired and diabetic glucose control. *Nature* 2013; **498**: 99-103 [PMID: 23719380 DOI: 10.1038/nature12198]
 - 96 **Han JL**, Lin HL. Intestinal microbiota and type 2 diabetes:

- from mechanism insights to therapeutic perspective. *World J Gastroenterol* 2014; **20**: 17737-17745 [PMID: 25548472 DOI: 10.3748/wjg.v20.i47.17737]
- 97 **Parks BW**, Nam E, Org E, Kostem E, Norheim F, Hui ST, Pan C, Civelek M, Rau CD, Bennett BJ, Mehrabian M, Ursell LK, He A, Castellani LW, Zinker B, Kirby M, Drake TA, Drevon CA, Knight R, Gargalovic P, Kirchgeßner T, Eskin E, Lusis AJ. Genetic control of obesity and gut microbiota composition in response to high-fat, high-sucrose diet in mice. *Cell Metab* 2013; **17**: 141-152 [PMID: 23312289 DOI: 10.1016/j.cmet.2012.12.007]
 - 98 **Graessler J**, Qin Y, Zhong H, Zhang J, Licinio J, Wong ML, Xu A, Chavakis T, Bornstein AB, Ehrhart-Bornstein M, Lamounier-Zepter V, Lohmann T, Wolf T, Bornstein SR. Metagenomic sequencing of the human gut microbiome before and after bariatric surgery in obese patients with type 2 diabetes: correlation with inflammatory and metabolic parameters. *Pharmacogenomics J* 2013; **13**: 514-522 [PMID: 23032991 DOI: 10.1038/tpj.2012.43]
 - 99 **Liou AP**, Paziuk M, Luevano JM, Machineni S, Turnbaugh PJ, Kaplan LM. Conserved shifts in the gut microbiota due to gastric bypass reduce host weight and adiposity. *Sci Transl Med* 2013; **5**: 178ra41 [PMID: 23536013 DOI: 10.1126/scitranslmed.3005687]
 - 100 **Kong LC**, Tap J, Aron-Wisnewsky J, Pelloux V, Basdevant A, Bouillot JL, Zucker JD, Doré J, Clément K. Gut microbiota after gastric bypass in human obesity: increased richness and associations of bacterial genera with adipose tissue genes. *Am J Clin Nutr* 2013; **98**: 16-24 [PMID: 23719559 DOI: 10.3945/ajcn.113.058743]
 - 101 **Aron-Wisnewsky J**, Doré J, Clement K. The importance of the gut microbiota after bariatric surgery. *Nat Rev Gastroenterol Hepatol* 2012; **9**: 590-598 [PMID: 22926153 DOI: 10.1038/nrgastro.2012.161]
 - 102 **Aron-Wisnewsky J**, Clement K. The effects of gastrointestinal surgery on gut microbiota: potential contribution to improved insulin sensitivity. *Curr Atheroscler Rep* 2014; **16**: 454 [PMID: 25214424 DOI: 10.1007/s11883-014-0454-9]
 - 103 **Patti ME**, Houten SM, Bianco AC, Bernier R, Larsen PR, Holst JJ, Badman MK, Maratos-Flier E, Mun EC, Pihlajamäki J, Auwerx J, Goldfine AB. Serum bile acids are higher in humans with prior gastric bypass: potential contribution to improved glucose and lipid metabolism. *Obesity* (Silver Spring) 2009; **17**: 1671-1677 [PMID: 19360006 DOI: 10.1038/oby.2009.102]
 - 104 **Myronovych A**, Kirby M, Ryan KK, Zhang W, Jha P, Setchell KD, Dexheimer PJ, Aronow B, Seeley RJ, Kohli R. Vertical sleeve gastrectomy reduces hepatic steatosis while increasing serum bile acids in a weight-loss-independent manner. *Obesity* (Silver Spring) 2014; **22**: 390-400 [PMID: 23804416 DOI: 10.1002/oby.20548]
 - 105 **Qin J**, Li Y, Cai Z, Li S, Zhu J, Zhang F, Liang S, Zhang W, Guan Y, Shen D, Peng Y, Zhang D, Jie Z, Wu W, Qin Y, Xue W, Li J, Han L, Lu D, Wu P, Dai Y, Sun X, Li Z, Tang A, Zhong S, Li X, Chen W, Xu R, Wang M, Feng Q, Gong M, Yu J, Zhang Y, Zhang M, Hansen T, Sanchez G, Raes J, Falony G, Okuda S, Almeida M, LeChatelier E, Renault P, Pons N, Batto JM, Zhang Z, Chen H, Yang R, Zheng W, Li S, Yang H, Wang J, Ehrlich SD, Nielsen R, Pedersen O, Kristiansen K, Wang J. A metagenome-wide association study of gut microbiota in type 2 diabetes. *Nature* 2012; **490**: 55-60 [PMID: 23023125 DOI: 10.1038/nature11450]
 - 106 **Kaji I**, Karaki S, Kuwahara A. Short-chain fatty acid receptor and its contribution to glucagon-like peptide-1 release. *Digestion* 2014; **89**: 31-36 [PMID: 24458110 DOI: 10.1159/000356211]
 - 107 **Perry RJ**, Peng L, Barry NA, Cline GW, Zhang D, Cardone RL, Petersen KF, Kibbey RG, Goodman AL, Shulman GI. Acetate mediates a microbiome-brain- β -cell axis to promote metabolic syndrome. *Nature* 2016; **534**: 213-217 [PMID: 27279214 DOI: 10.1038/nature18309]
 - 108 **Vitek L**, Haluzik M. The role of bile acids in metabolic regulation. *J Endocrinol* 2016; **228**: R85-R96 [PMID: 26733603 DOI: 10.1530/JOE-15-0469]
 - 109 **Barrasa JI**, Olmo N, Lizarbe MA, Turnay J. Bile acids in the colon, from healthy to cytotoxic molecules. *Toxicol In Vitro* 2013; **27**: 964-977 [PMID: 23274766 DOI: 10.1016/j.tiv.2012.12.020]
 - 110 **Scherer M**, Gnewuch C, Schmitz G, Liebisch G. Rapid quantification of bile acids and their conjugates in serum by liquid chromatography-tandem mass spectrometry. *J Chromatogr B Analyt Technol Biomed Life Sci* 2009; **877**: 3920-3925 [PMID: 19819765 DOI: 10.1016/j.jchromb.2009.09.038]
 - 111 **Yamamoto R**, Tazuma S, Kanno K, Igarashi Y, Inui K, Ohara H, Tsuyuguchi T, Ryoza S. Ursodeoxycholic acid after bile duct stone removal and risk factors for recurrence: a randomized trial. *J Hepatobiliary Pancreat Sci* 2016; **23**: 132-136 [PMID: 26705893 DOI: 10.1002/jhbp.316]
 - 112 **Stokes CS**, Gluud LL, Casper M, Lammert F. Ursodeoxycholic acid and diets higher in fat prevent gallbladder stones during weight loss: a meta-analysis of randomized controlled trials. *Clin Gastroenterol Hepatol* 2014; **12**: 1090-1100.e2; quiz e61 [PMID: 24321208 DOI: 10.1016/j.cgh.2013.11.031]
 - 113 **Jackson H**, Solaymani-Dodaran M, Card TR, Aithal GP, Logan R, West J. Influence of ursodeoxycholic acid on the mortality and malignancy associated with primary biliary cirrhosis: a population-based cohort study. *Hepatology* 2007; **46**: 1131-1137 [PMID: 17685473 DOI: 10.1002/hep.21795]
 - 114 **Adams LB**, Chang C, Pope J, Kim Y, Liu P, Yates A. Randomized, Prospective Comparison of Ursodeoxycholic Acid for the Prevention of Gallstones after Sleeve Gastrectomy. *Obes Surg* 2016; **26**: 990-994 [PMID: 26342481 DOI: 10.1007/s11695-015-1858-5]
 - 115 **Yuan L**, Bambha K. Bile acid receptors and nonalcoholic fatty liver disease. *World J Hepatol* 2015; **7**: 2811-2818 [PMID: 26668692 DOI: 10.4254/wjh.v7.i28.2811]
 - 116 **Makishima M**, Lu TT, Xie W, Whitfield GK, Domoto H, Evans RM, Haussler MR, Mangelsdorf DJ. Vitamin D receptor as an intestinal bile acid sensor. *Science* 2002; **296**: 1313-1316 [PMID: 12016314 DOI: 10.1126/science.1070477]
 - 117 **Gao J**, Xie W. Targeting xenobiotic receptors PXR and CAR for metabolic diseases. *Trends Pharmacol Sci* 2012; **33**: 552-558 [PMID: 22889594 DOI: 10.1016/j.tips.2012.07.003]
 - 118 **Gao J**, He J, Zhai Y, Wada T, Xie W. The constitutive androstane receptor is an anti-obesity nuclear receptor that improves insulin sensitivity. *J Biol Chem* 2009; **284**: 25984-25992 [PMID: 19617349 DOI: 10.1074/jbc.M109.016808]
 - 119 **Hylemon PB**, Zhou H, Pandak WM, Ren S, Gil G, Dent P. Bile acids as regulatory molecules. *J Lipid Res* 2009; **50**: 1509-1520 [PMID: 19346331 DOI: 10.1194/jlr.R900007-JLR200]
 - 120 **Jansen PL**, Sturm E. Genetic cholestasis, causes and consequences for hepatobiliary transport. *Liver Int* 2003; **23**: 315-322 [PMID: 14708891]
 - 121 **Keitel V**, Kubitz R, Häussinger D. Endocrine and paracrine role of bile acids. *World J Gastroenterol* 2008; **14**: 5620-5629 [PMID: 18837077 DOI: 10.3748/wjg.14.5620]
 - 122 **Han SI**, Studer E, Gupta S, Fang Y, Qiao L, Li W, Grant S, Hylemon PB, Dent P. Bile acids enhance the activity of the insulin receptor and glycogen synthase in primary rodent hepatocytes. *Hepatology* 2004; **39**: 456-463 [PMID: 14767998 DOI: 10.1002/hep.20043]
 - 123 **Fiorucci S**, Mencarelli A, Palladino G, Cipriani S. Bile-acid-activated receptors: targeting TGR5 and farnesoid-X-receptor in lipid and glucose disorders. *Trends Pharmacol Sci* 2009; **30**: 570-580 [PMID: 19758712 DOI: 10.1016/j.tips.2009.08.001]
 - 124 **Romero D**, Korytkowski M. Perioperative Glycemic Management of Patients Undergoing Bariatric Surgery. *Curr Diab Rep* 2016; **16**: 23 [PMID: 26879306 DOI: 10.1007/s11892-016-0718-6]
 - 125 **Schittenhelm B**, Wagner R, Kähny V, Peter A, Krippeit-Drews P, Düfer M, Drews G. Role of FXR in β -cells of lean and obese mice. *Endocrinology* 2015; **156**: 1263-1271 [PMID: 25599407 DOI: 10.1210/en.2014-1751]
 - 126 **Ding L**, Pang S, Sun Y, Tian Y, Yu L, Dang N. Coordinated Actions of FXR and LXR in Metabolism: From Pathogenesis to Pharmacological Targets for Type 2 Diabetes. *Int J Endocrinol* 2014; **2014**: 751859 [PMID: 24872814 DOI: 10.1155/2014/751859]
 - 127 **Zhou H**, Hylemon PB. Bile acids are nutrient signaling hormones. *Steroids* 2014; **86**: 62-68 [PMID: 24819989 DOI: 10.1016/

- j.steroids.2014.04.016]
- 128 **Dossa AY**, Escobar O, Golden J, Frey MR, Ford HR, Gayer CP. Bile acids regulate intestinal cell proliferation by modulating EGFR and FXR signaling. *Am J Physiol Gastrointest Liver Physiol* 2016; **310**: G81-G92 [PMID: 26608185 DOI: 10.1152/ajpgi.00065.2015]
 - 129 **Maruyama T**, Miyamoto Y, Nakamura T, Tamai Y, Okada H, Sugiyama E, Nakamura T, Itadani H, Tanaka K. Identification of membrane-type receptor for bile acids (M-BAR). *Biochem Biophys Res Commun* 2002; **298**: 714-719 [PMID: 12419312]
 - 130 **Vassileva G**, Golovko A, Markowitz L, Abbondanzo SJ, Zeng M, Yang S, Hoos L, Tetzloff G, Levitan D, Murgolo NJ, Keane K, Davis HR, Hedrick J, Gustafson EL. Targeted deletion of Gpbar1 protects mice from cholesterol gallstone formation. *Biochem J* 2006; **398**: 423-430 [PMID: 16724960 DOI: 10.1042/BJ20060537]
 - 131 **Vettorazzi JF**, Ribeiro RA, Borck PC, Branco RC, Soriano S, Merino B, Boschero AC, Nadal A, Quesada I, Carneiro EM. The bile acid TUDCA increases glucose-induced insulin secretion via the cAMP/PKA pathway in pancreatic beta cells. *Metabolism* 2016; **65**: 54-63 [PMID: 26892516 DOI: 10.1016/j.metabol.2015.10.021]
 - 132 **Keitel V**, Görg B, Bidmon HJ, Zemtsova I, Spomer L, Zilles K, Häussinger D. The bile acid receptor TGR5 (Gpbar-1) acts as a neurosteroid receptor in brain. *Glia* 2010; **58**: 1794-1805 [PMID: 20665558 DOI: 10.1002/glia.21049]
 - 133 **Stepanov V**, Stankov K, Mikov M. The bile acid membrane receptor TGR5: a novel pharmacological target in metabolic, inflammatory and neoplastic disorders. *J Recept Signal Transduct Res* 2013; **33**: 213-223 [PMID: 23782454 DOI: 10.3109/10799893.2013.802805]
 - 134 **Parker HE**, Wallis K, le Roux CW, Wong KY, Reimann F, Gribble FM. Molecular mechanisms underlying bile acid-stimulated glucagon-like peptide-1 secretion. *Br J Pharmacol* 2012; **165**: 414-423 [PMID: 21718300 DOI: 10.1111/j.1476-5381.2011.01561.x]
 - 135 **Holst JJ**. The physiology of glucagon-like peptide 1. *Physiol Rev* 2007; **87**: 1409-1439 [PMID: 17928588 DOI: 10.1152/physrev.00034.2006]
 - 136 **Nauck MA**, Niedereichholz U, Ettler R, Holst JJ, Orskov C, Ritzel R, Schmiegell WH. Glucagon-like peptide 1 inhibition of gastric emptying outweighs its insulinotropic effects in healthy humans. *Am J Physiol* 1997; **273**: E981-E988 [PMID: 9374685]
 - 137 **Tang-Christensen M**, Vrang N, Larsen PJ. Glucagon-like peptide 1(7-36) amide's central inhibition of feeding and peripheral inhibition of drinking are abolished by neonatal monosodium glutamate treatment. *Diabetes* 1998; **47**: 530-537 [PMID: 9568683]
 - 138 **Pournaras DJ**, Glicksman C, Vincent RP, Kuganopava S, Alaghband-Zadeh J, Mahon D, Bekker JH, Ghatei MA, Bloom SR, Walters JR, Welbourn R, le Roux CW. The role of bile after Roux-en-Y gastric bypass in promoting weight loss and improving glycaemic control. *Endocrinology* 2012; **153**: 3613-3619 [PMID: 22673227 DOI: 10.1210/en.2011-2145]
 - 139 **Adrian TE**, Gariballa S, Parekh KA, Thomas SA, Saadi H, Al Kaabi J, Nagelkerke N, Gedulin B, Young AA. Rectal taurocholate increases L cell and insulin secretion, and decreases blood glucose and food intake in obese type 2 diabetic volunteers. *Diabetologia* 2012; **55**: 2343-2347 [PMID: 22696033 DOI: 10.1007/s00125-012-2593-2]
 - 140 **Kitada Y**, Kajita K, Taguchi K, Mori I, Yamauchi M, Ikeda T, Kawashima M, Asano M, Kajita T, Ishizuka T, Banno Y, Kojima I, Chun J, Kamata S, Ishii I, Morita H. Blockade of Sphingosine 1-Phosphate Receptor 2 Signaling Attenuates High-Fat Diet-Induced Adipocyte Hypertrophy and Systemic Glucose Intolerance in Mice. *Endocrinology* 2016; **157**: 1839-1851 [PMID: 26943364 DOI: 10.1210/en.2015-1768]
 - 141 **Qiao L**, Studer E, Leach K, McKinsty R, Gupta S, Decker R, Kukreja R, Valerie K, Nagarkatti P, El Deiry W, Molkentin J, Schmidt-Ullrich R, Fisher PB, Grant S, Hylemon PB, Dent P. Deoxycholic acid (DCA) causes ligand-independent activation of epidermal growth factor receptor (EGFR) and FAS receptor in primary hepatocytes: inhibition of EGFR/mitogen-activated protein kinase-signaling module enhances DCA-induced apoptosis. *Mol Biol Cell* 2001; **12**: 2629-2645 [PMID: 11553704]
 - 142 **Cummings BP**, Bettaieb A, Graham JL, Kim J, Ma F, Shibata N, Stanhope KL, Giulivi C, Hansen F, Jelsing J, Vrang N, Kowala M, Chouinard ML, Haj FG, Havel PJ. Bile-acid-mediated decrease in endoplasmic reticulum stress: a potential contributor to the metabolic benefits of ileal interposition surgery in UCD-T2DM rats. *Dis Model Mech* 2013; **6**: 443-456 [PMID: 23264565 DOI: 10.1242/dmm.010421]
 - 143 **Cao AL**, Wang L, Chen X, Wang YM, Guo HJ, Chu S, Liu C, Zhang XM, Peng W. Ursodeoxycholic acid and 4-phenylbutyrate prevent endoplasmic reticulum stress-induced podocyte apoptosis in diabetic nephropathy. *Lab Invest* 2016; **96**: 610-622 [PMID: 26999661 DOI: 10.1038/labinvest.2016.44]
 - 144 **Kohli R**, Setchell KD, Kirby M, Myronovych A, Ryan KK, Ibrahim SH, Berger J, Smith K, Toure M, Woods SC, Seeley RJ. A surgical model in male obese rats uncovers protective effects of bile acids post-bariatric surgery. *Endocrinology* 2013; **154**: 2341-2351 [PMID: 23592746 DOI: 10.1210/en.2012-2069]
 - 145 **Scholtz S**, Miras AD, Chhina N, Precht CG, Sleeth ML, Daud NM, Ismail NA, Durighel G, Ahmed AR, Olbers T, Vincent RP, Alaghband-Zadeh J, Ghatei MA, Waldman AD, Frost GS, Bell JD, le Roux CW, Goldstone AP. Obese patients after gastric bypass surgery have lower brain-hedonic responses to food than after gastric banding. *Gut* 2014; **63**: 891-902 [PMID: 23964100 DOI: 10.1136/gutjnl-2013-305008]
 - 146 **Nakatani H**, Kasama K, Oshiro T, Watanabe M, Hirose H, Itoh H. Serum bile acid along with plasma incretins and serum high-molecular weight adiponectin levels are increased after bariatric surgery. *Metabolism* 2009; **58**: 1400-1407 [PMID: 19570554 DOI: 10.1016/j.metabol.2009.05.006]
 - 147 **Steinert RE**, Peterli R, Keller S, Meyer-Gerspach AC, Drewe J, Peters T, Beglinger C. Bile acids and gut peptide secretion after bariatric surgery: a 1-year prospective randomized pilot trial. *Obesity* (Silver Spring) 2013; **21**: E660-E668 [PMID: 23804517 DOI: 10.1002/oby.20522]
 - 148 **Belgaumkar AP**, Vincent RP, Carswell KA, Hughes RD, Alaghband-Zadeh J, Mitry RR, le Roux CW, Patel AG. Changes in Bile Acid Profile After Laparoscopic Sleeve Gastrectomy are Associated with Improvements in Metabolic Profile and Fatty Liver Disease. *Obes Surg* 2016; **26**: 1195-1202 [PMID: 26337697 DOI: 10.1007/s11695-015-1878-1]
 - 149 **De Giorgi S**, Campos V, Egli L, Toepel U, Carrel G, Cariou B, Rainteau D, Schneiter P, Tappy L, Giusti V. Long-term effects of Roux-en-Y gastric bypass on postprandial plasma lipid and bile acids kinetics in female non diabetic subjects: A cross-sectional pilot study. *Clin Nutr* 2015; **34**: 911-917 [PMID: 25306425 DOI: 10.1016/j.clnu.2014.09.018]
 - 150 **Gerhard GS**, Styer AM, Wood GC, Roesch SL, Petrick AT, Gabrielsen J, Strodel WE, Still CD, Argyropoulos G. A role for fibroblast growth factor 19 and bile acids in diabetes remission after Roux-en-Y gastric bypass. *Diabetes Care* 2013; **36**: 1859-1864 [PMID: 23801799 DOI: 10.2337/dc12-2255]
 - 151 **Jansen PL**, van Werven J, Aarts E, Berends F, Janssen I, Stoker J, Schaap FG. Alterations of hormonally active fibroblast growth factors after Roux-en-Y gastric bypass surgery. *Dig Dis* 2011; **29**: 48-51 [PMID: 21691104 DOI: 10.1159/000324128]
 - 152 **Ahmad NN**, Pfaller A, Kaplan LM. Roux-en-Y gastric bypass normalizes the blunted postprandial bile acid excursion associated with obesity. *Int J Obes (Lond)* 2013; **37**: 1553-1559 [PMID: 23567924 DOI: 10.1038/ijo.2013.38]
 - 153 **Simonen M**, Dali-Youcef N, Kaminska D, Venesmaa S, Käkälä P, Pääkkönen M, Hallikainen M, Kolehmainen M, Uusitupa M, Moilanen L, Laakso M, Gylling H, Patti ME, Auwerx J, Pihlajamäki J. Conjugated bile acids associate with altered rates of glucose and lipid oxidation after Roux-en-Y gastric bypass. *Obes Surg* 2012; **22**: 1473-1480 [PMID: 22638681 DOI: 10.1007/s11695-012-0673-5]
 - 154 **Sachdev S**, Wang Q, Billington C, Connert J, Ahmed L, Inabnet W, Chua S, Ikramuddin S, Korner J. FGF 19 and Bile Acids Increase Following Roux-en-Y Gastric Bypass but Not After Medical

- Management in Patients with Type 2 Diabetes. *Obes Surg* 2016; **26**: 957-965 [PMID: 26259981 DOI: 10.1007/s11695-015-1834-0]
- 155 **Bhutta HY**, Rajpal N, White W, Freudenberg JM, Liu Y, Way J, Rajpal D, Cooper DC, Young A, Tavakkoli A, Chen L. Effect of Roux-en-Y gastric bypass surgery on bile acid metabolism in normal and obese diabetic rats. *PLoS One* 2015; **10**: e0122273 [PMID: 25798945 DOI: 10.1371/journal.pone.0122273]
 - 156 **Barutcuoglu B**, Basol G, Cakir Y, Cetinkalp S, Parildar Z, Kabaroglu C, Ozmen D, Mutaf I, Bayindir O. Fibroblast growth factor-19 levels in type 2 diabetic patients with metabolic syndrome. *Ann Clin Lab Sci* 2011; **41**: 390-396 [PMID: 22166511]
 - 157 **Trabelsi MS**, Daoudi M, Prawitt J, Ducastel S, Touche V, Sayin SI, Perino A, Brighton CA, Sebt Y, Kluza J, Briand O, Dehondt H, Vallez E, Dorchie E, Baud G, Spinelli V, Hennuyer N, Caron S, Bantubungi K, Caiazza R, Reimann F, Marchetti P, Lefebvre P, Bäckhed F, Gribble FM, Schoonjans K, Pattou F, Tailleux A, Staels B, Lestavel S. Farnesoid X receptor inhibits glucagon-like peptide-1 production by enteroendocrine L cells. *Nat Commun* 2015; **6**: 7629 [PMID: 26134028 DOI: 10.1038/ncomms8629]
 - 158 **Howard BV**, Ruotolo G, Robbins DC. Obesity and dyslipidemia. *Endocrinol Metab Clin North Am* 2003; **32**: 855-867 [PMID: 14711065]
 - 159 **Janikiewicz J**, Hanzelka K, Kozinski K, Kolczynska K, Dobrzyn A. Islet β -cell failure in type 2 diabetes--Within the network of toxic lipids. *Biochem Biophys Res Commun* 2015; **460**: 491-496 [PMID: 25843796 DOI: 10.1016/j.bbrc.2015.03.153]
 - 160 **Poitout V**, Robertson RP. Minireview: Secondary beta-cell failure in type 2 diabetes--a convergence of glucotoxicity and lipotoxicity. *Endocrinology* 2002; **143**: 339-342 [PMID: 11796484 DOI: 10.1210/endo.143.2.8623]
 - 161 **Boucher A**, Lu D, Burgess SC, Telemaque-Potts S, Jensen MV, Mulder H, Wang MY, Unger RH, Sherry AD, Newgard CB. Biochemical mechanism of lipid-induced impairment of glucose-stimulated insulin secretion and reversal with a malate analogue. *J Biol Chem* 2004; **279**: 27263-27271 [PMID: 15073188 DOI: 10.1074/jbc.M401167200]
 - 162 **Carswell KA**, Belgaumkar AP, Amiel SA, Patel AG. A Systematic Review and Meta-analysis of the Effect of Gastric Bypass Surgery on Plasma Lipid Levels. *Obes Surg* 2016; **26**: 843-855 [PMID: 26210195 DOI: 10.1007/s11695-015-1829-x]
 - 163 **Wickremesekera K**, Miller G, Naotunne TD, Knowles G, Stubbs RS. Loss of insulin resistance after Roux-en-Y gastric bypass surgery: a time course study. *Obes Surg* 2005; **15**: 474-481 [PMID: 15946424 DOI: 10.1381/0960892053723402]
 - 164 **Laferrère B**. Do we really know why diabetes remits after gastric bypass surgery? *Endocrine* 2011; **40**: 162-167 [PMID: 21853297 DOI: 10.1007/s12020-011-9514-x]
 - 165 **Gale EA**. GLP-1-based therapies and the exocrine pancreas: more light, or just more heat? *Diabetes* 2012; **61**: 986-988 [PMID: 22517653 DOI: 10.2337/db11-1838]
 - 166 **Osto M**, Abegg K, Bueter M, le Roux CW, Cani PD, Lutz TA. Roux-en-Y gastric bypass surgery in rats alters gut microbiota profile along the intestine. *Physiol Behav* 2013; **119**: 92-96 [PMID: 23770330 DOI: 10.1016/j.physbeh.2013.06.008]
 - 167 **Sledzinski T**, Korczynska J, Hallmann A, Kaska L, Proczko-Markuszevska M, Stefaniak T, Sledzinski M, Swierczynski J. The increase of serum chemerin concentration is mainly associated with the increase of body mass index in obese, non-diabetic subjects. *J Endocrinol Invest* 2013; **36**: 428-434 [PMID: 23211604 DOI: 10.3275/8770]
 - 168 **Zabrocka L**, Raczyńska S, Goyke E, Sledzinski Z, Swierczynski J. BMI is the main determinant of the circulating leptin in women after vertical banded gastroplasty. *Obes Res* 2004; **12**: 505-512 [PMID: 15044668 DOI: 10.1038/oby.2004.57]
 - 169 **Matsuzawa Y**. The role of fat topology in the risk of disease. *Int J Obes (Lond)* 2008; **32** Suppl 7: S83-S92 [PMID: 19136997 DOI: 10.1038/ijo.2008.243]
 - 170 **Adami GF**, Gradaschi R, Andraghetti G, Scopinaro N, Cordera R. Serum Leptin and Adiponectin Concentration in Type 2 Diabetes Patients in the Short and Long Term Following Biliopancreatic Diversion. *Obes Surg* 2016; **26**: 2442-2448 [PMID: 26989058 DOI: 10.1007/s11695-016-2126-z]

P- Reviewer: Bishu S, Hu S, Konishi T S- Editor: Qi Y

L- Editor: A E- Editor: Wang CH



Update on occult hepatitis B virus infection

Manoochehr Makvandi

Manoochehr Makvandi, Health Research Institute, Infectious and Tropical Disease Research Center, Ahvaz Jundishapur University of Medical Sciences, Ahvaz 15794-61357, Iran

Manoochehr Makvandi, Department of Medical Virology, School of Medicine, Ahvaz Jundishapur University of Medical Sciences, Ahvaz 15794-61357, Iran

Author contributions: Makvandi M solely wrote this paper.

Conflict-of-interest statement: The author declares that there are no conflicts of interest in the content of this review.

Open-Access: This article is an open-access article which was selected by an in-house editor and fully peer-reviewed by external reviewers. It is distributed in accordance with the Creative Commons Attribution Non Commercial (CC BY-NC 4.0) license, which permits others to distribute, remix, adapt, build upon this work non-commercially, and license their derivative works on different terms, provided the original work is properly cited and the use is non-commercial. See: <http://creativecommons.org/licenses/by-nc/4.0/>

Manuscript source: Invited manuscript

Correspondence to: Dr. Manoochehr Makvandi, Health Research Institute, Infectious and Tropical Disease Research Center, Ahvaz Jundishapur University of Medical Sciences, Boulevard Golestan, Ahvaz 15794-61357, Iran. manoochehrmakvandi29@yahoo.com
Telephone: +98-9166181683
Fax: +98-6113738313

Received: March 28, 2016

Peer-review started: March 29, 2016

First decision: May 30, 2016

Revised: June 13, 2016

Accepted: July 20, 2016

Article in press: July 21, 2016

Published online: October 21, 2016

Abstract

The event of mutations in the surface antigen gene of hepatitis B virus (HBV) results in undetectable hepatitis B surface antigen with positive/negative anti-hepatitis

B core (anti-HBc) antibody status in serum and this phenomenon is named occult hepatitis B infection (OBI). The presence of anti-HBc antibody in serum is an important key for OBI tracking, although about 20% of OBI cases are negative for anti-HBc antibody. The diagnosis of OBI is mainly based on polymerase chain reaction (PCR) and real-time PCR assays. However, real-time PCR is a more reliable method than PCR. OBI is a great issue for the public health problem and a challenge for the clinical entity worldwide. The persistence of OBI may lead to the development of cirrhosis and hepatocellular carcinoma. With regard to OBI complications, the screening of HBV DNA by the highly sensitive molecular means should be implemented for: (1) patients with a previous history of chronic or acute HBV infection; (2) patients co-infected with hepatitis C virus/human immunodeficiency virus; (3) patients undergoing chemotherapy or anti-CD20 therapy; (4) recipients of organ transplant; (5) blood donors; (6) organ transplant donors; (7) thalassemia and hemophilia patients; (8) health care workers; (9) patients with liver related disease (cryptogenic); (10) hemodialysis patients; (11) patients undergoing lamivudine or interferon therapy; and (12) children in time of HBV vaccination especially in highly endemic areas of HBV. Active HBV vaccination should be implemented for the close relatives of patients who are negative for OBI markers. Thus, the goal of this review is to evaluate the rate of OBI with a focus on status of high risk groups in different regions of the world.

Key words: Nested polymerase chain reaction; Occult hepatitis B infection; Cryptogenic; Real-time polymerase chain reaction

© The Author(s) 2016. Published by Baishideng Publishing Group Inc. All rights reserved.

Core tip: Occult hepatitis B infection (OBI) is defined as negative hepatitis B surface antigen and positive/negative anti-hepatitis B core immunoglobulin G status but hepatitis B virus (HBV) DNA is detectable in serum and liver tissue. Genotypes A, C, G, E and D have been

found among patients with OBI in different regions of the world. Genotype D is the only dominant genotype among Iranian OBI patients. OBI has been reported among many high risk groups, including blood donors, liver transplant recipients, patients co-infected with hepatitis C virus/human immunodeficiency virus, patients undergoing immunosuppressive therapy or hemodialysis, patients with liver cirrhosis, cryptogenic liver disease, or abnormal alanine transaminase, healthcare workers, patients with lymphoma or rheumatoid arthritis. It is recommended that to manage and reduce OBI and HBV carriage, the screening of HBV DNA be implemented among high risk groups by means of highly sensitive molecular assays periodically. In addition, comprehensive investigations are needed to understand the epidemiology of OBI worldwide.

Makvandi M. Update on occult hepatitis B virus infection. *World J Gastroenterol* 2016; 22(39): 8720-8734 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v22/i39/8720.htm> DOI: <http://dx.doi.org/10.3748/wjg.v22.i39.8720>

INTRODUCTION

Hepatitis B virus (HBV) infection is a considerable global health problem and approximately two billion of the world population have been infected, of which 250 million live with HBV infection^[1]. HBV infection is linked with a wide range of clinical manifestations, including acute or fulminant hepatitis to various forms of chronic infection, including asymptomatic carriers, chronic hepatitis, cirrhosis, and hepatocellular carcinoma (HCC). Although the implementation of screening tests for hepatitis B surface antigen (HBsAg) has significantly reduced the spread of HBV infection among blood donors, it fails to detect occult HBV infection (OBI) cases. In the 1970s, a new form of clinical HBV infection was reported in a patient with acute hepatitis, who was positive for anti-hepatitis B core (anti-HBc) immunoglobulin G (IgG), but negative for HBsAg^[2]. Subsequently, by developing highly sensitive molecular means, the clinical entity of OBI was characterized, which resulted in the concept of "occult" or "silent" HBV infection^[3,4]. The presence of mutations was demonstrated in the preS1, preS2 and S regions of the *HBsAg* gene, which results in undetectable HBsAg by enzyme-linked immunosorbent assay^[5-8]. In the absence of serum HBsAg, low quantity of HBV DNA even < 200 IU/mL was detected in the serum and liver tissue biopsy by real-time polymerase chain reaction (PCR), and this new form of clinical entity of HBV infection was called OBI^[9,10]. OBI is a clinical class of HBV infection and can appear in two forms: seropositive OBI and seronegative OBI. In seropositive OBI, serum HBV DNA is detectable and both anti-HBc/anti-hepatitis B surface (HBs) IgGs are positive or only anti-HBc IgG is positive, while

in seronegative OBI, only HBV DNA is detectable in serum/or liver tissue, but anti-HBc IgG/anti-HBs IgGs are negative in serum^[4]. The clinical feature of OBI remains unknown and more studies are required to understand the characteristics of OBI among the high risk group worldwide. With the present data on the OBI, several groups are believed to be at risk of OBI. The reactivation of OBI may take place in individuals with a previous history of HBV infection along with immunosuppression or chemotherapy status. Lastly, to prevent the spread of OBI, the screening of HBV DNA should be implemented in blood donors, immunosuppressive patients, organ transplant donors, organ transplant recipients, and individuals with acute rheumatoid arthritis before and after treatment with anti-tumor necrosis factor (TNF)- α ^[11]. In this paper, a search of MEDLINE database was performed to retrieve suitable articles to explain the epidemiology, diagnosis and prevention of OBI.

DEFINITION OF OBI

Most of OBI cases are asymptomatic and clinically not well defined. OBI has been investigated only in high risk groups with different serological and molecular descriptions. Several definitions of OBI have been described. In the international workshop (2008) in Italy, OBI was defined as the detection of HBV DNA in the liver (with or without HBV DNA in serum) without HBsAg^[12]. OBI can be defined by the presence of HBV DNA in serum or liver tissue with either seropositive or seronegative status. Seropositive OBI is characterized by the detection of anti-HBc antibody with or without anti-HBs antibody, while seronegative OBI is described by undetectable both anti-HBc and anti-HBs antibodies. Seropositive OBI accounts for the enormous majority of OBI cases which can be attributed to the larger proportion of resolved HBV infections. It has been reported that more than 20% of OBI cases are seronegative for all the HBV markers^[13]. In chronic occult infections, viral covalently closed circular DNA (cccDNA) persists as an episome in the nucleus of infected cells. Although the clinical features between OBI-seropositive and OBI-seronegative cases remain entirely cryptic, OBI may be exhibited in one of three clinical forms: (1) in a window period of acute HBV infection; (2) detectable HBV DNA and undetectable HBsAg in patient serum without a previous history of overt HBV infection; and (3) in patients with a history of chronic HBV infection. At present there is no standard assay for diagnosis of OBI in liver tissue or in serum, and the only reliable method is the detection of HBV DNA by nested PCR or real-time PCR. It has been illustrated that the application of real-time PCR possesses better outcomes provided that the specific primers are capable to cover all HBV genotypes^[14]. The viral load lower than 200 IU/mL has been defined for OBI diagnosis, interestingly, in more than 90%

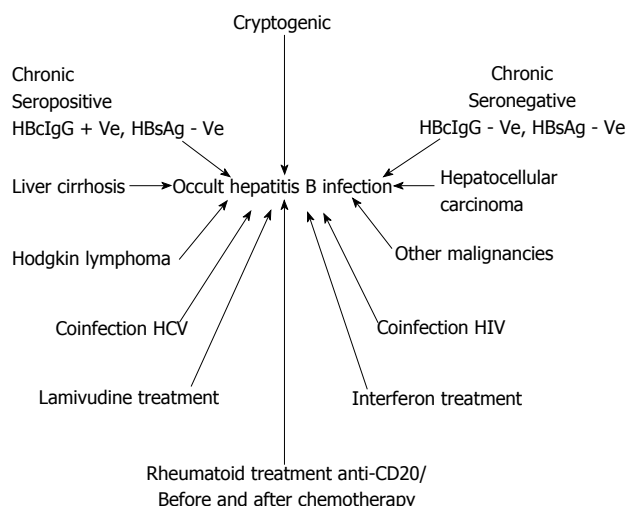


Figure 1 Schematic representation of clinical entity of occult hepatitis B infection. HCV: Hepatitis C virus; HIV: Human immunodeficiency virus; HBsAg: Hepatitis B surface antigen.

of OBI patients, the viral load in serum was reported to be around 20 IU/mL^[15]. Several mechanisms and factors may affect or suppress the HBV replication, which result in mutations in the *HBsAg* gene, although host immune response and epigenetic factors also play crucial roles in OBI (Figure 1).

PREVALENCE OF OBI

The prevalence of OBI varies from region to region worldwide. This variability relies upon the sensitivity of HBV DNA detection assays, the sample size, and the detection of HBV DNA in liver tissue and serum by nested PCR or real-time PCR. The prevalence of OBI varies from 1% to 87% in different regions of the world^[16,17]. OBI has been reported even in some geographical regions with low HBV endemicity^[16]. The prevalence of OBI among the general population has been reported to be 45.5% with genotypes B and C^[18] in China, and 1.7%-6.6% with genotype C2^[19,20] in South Korea. In Taiwan, the prevalence was 10.9% in HBV vaccinated children^[21] and 0.11% in blood donors^[22]. In Egypt, it varied from a low 4.1% to high 26.8% in hemodialysis patients^[23,24]. In Iran the prevalence of OBI has been reported to be 2 in 50000 in blood donors^[25] and 14% in cryptogenic patients^[26], while the prevalence of seropositive OBI was 2.27%^[27] and 0% among blood donors^[28].

MOLECULAR MECHANISMS OF OBI

Mutations in the “a” determinant of HBsAg

A mutation in the “a” determinant of the surface antigen is one of the known mechanisms which may result in OBI. Mutations in the *HBsAg* gene bring about the structural arrangement of the protein, which may lead to undetectable HBsAg by commercially HBsAg test kits^[29]. The occurrence of sG145R mutation in

the “a” determinant of the HBsAg gene also results in OBI^[29]. It has been shown that the sG145R mutation in the HBsAg gene leads to a low binding affinity to monoclonal antibody against HBsAg^[30]. In addition, within the “a” determinant several other mutations have been shown to cause a low affinity to monoclonal antibody against HBsAg^[30-32].

Mutations in the pre S1 and preS2 regions

Mutations in the S region have been associated with reduced expression of HBV surface proteins. Subsequently, mutations in preS1/preS2 promoters are frequently observed in OBI patients, which make HBsAg become undetectable^[33,34].

RNA splicing

Splicing steps have a critical effect on gene expression in HBV. In patients with OBI, it has been found that the substitution of nt G-to-A at position 458 of the surface gene interferes with the splicing of S gene mRNA and was associated with a lack of HBsAg expression and low replication of HBV DNA^[35].

POSSIBLE OBI OUTCOMES OF

LAMIVUDINE OR INTERFERON THERAPY

Treatment of chronic patients with lamivudine may result in amino acid changes in YMDD motif, in HBV polymerase Q563S and in sS207R surface genes, and thus contributes to OBI^[36]. The nucleotide deletions in the pre-S1 and pre-S2 regions following the interferon therapy have resulted in the low replication of HBV DNA with low detection of HBsAg in cell culture systems^[37].

OBI and chronic hepatitis C virus infection

The mutations in the *HBsAg* gene have been observed among patients coinfecting with hepatitis C virus (HCV)^[38-40]. Several studies have reported that low HBV DNA replication occurs in patients coinfecting with HCV infection. It has been described that about one-third of patients with chronic HCV infection had detectable serum HBV DNA but undetectable HBsAg^[41,42]. The presence of OBI in chronic HCV infected patients increases the risk of HCC^[43,44]. When the coexistence of both HBV and HCV genomes occurs in the same hepatocyte, the replication of HBV is inhibited due to the interference of HCV molecules, which therefore results in the creation of OBI with low replication of HBV DNA^[45]. Moreover, the HBX protein is a transactivator and activates HBV promoters and enhances HBV gene transcription^[46,47]. The HCV core protein can interact with HBV X gene and prevent HBV gene transcription^[48]. In addition, HCV “core”, NS2 and NS5A proteins could strongly inhibit HBV replication^[34,43,49-51]. Table 1 shows the distribution of OBI among patients with HCV infection^[52-66].

HCC

While the prevalence of OBI among patients with

Table 1 Profile of various studies on occult hepatitis B infection in patients with hepatitis C virus infection

Ref.	Years	Study population	OBI
Fukuda <i>et al</i> ^[52]	1999	65 patients with HCV-related liver disease	34/65 (52.3%)
Kao <i>et al</i> ^[53]	2002	210 patients with HCV-related liver disease	31/210 (14.8%)
Besisik <i>et al</i> ^[54]	2003	33 HCV positive patients on hemodialysis	12/33 (36.4%)
Georgiadou <i>et al</i> ^[55]	2004	187 patients with HCV-related liver disease	49/187
Khattab <i>et al</i> ^[56]	2005	53 patients with chronic HCV infection	4/53 (7.5%)
Goral <i>et al</i> ^[57]	2006	50 HCV positive patients on hemodialysis	0/50
Branco <i>et al</i> ^[43]	2007	46 patients with HCV-related liver disease	9/46 (19.5%)
Toyoda <i>et al</i> ^[58]	2007	95 HCV positive patients with HCC	2/95 (2.1%)
Shetty <i>et al</i> ^[59]	2008	44 HCV positive patients with liver cirrhosis	22/44 (50%)
Tamori <i>et al</i> ^[60]	2009	50 HCV positive patients with HCC	21/50 (42%)
Chen <i>et al</i> ^[61]	2010	126 patients with chronic HCV infection	6/126 (5%)
Jang <i>et al</i> ^[62]	2011	32 patients with chronic HCV infection	9/32 (28.1%)
Joukar <i>et al</i> ^[63]	2012	59 HCV positive patients on hemodialysis	0/59
Vakili Ghartavol <i>et al</i> ^[64]	2013	50 patients with chronic HCV infection	18/50 (36%)
Kishk <i>et al</i> ^[65]	2014	162 patients with chronic HCV infection	3/162 (1.85%)
Mandour <i>et al</i> ^[66]	2015	210 patients with chronic HCV infection	53/210 (25.2%)

HCC: Hepatocellular carcinoma; HCV: Hepatitis C virus.

Table 2 Rates of occult hepatitis B infection among HIV positive patients in some countries

Ref.	Country	Prevalence %	Year
Vargas <i>et al</i> ^[73]	Chile	0/192 (0)	2016
Alvarez-Muñoz <i>et al</i> ^[74]	Mexico	24/49 (49.0)	2014
Chadwick <i>et al</i> ^[75]	England	15/335 (4.5)	2014
Coffin <i>et al</i> ^[76]	Canada	19/45 (42.0)	2014
Dapena <i>et al</i> ^[77]	Spain	6/254 (2.4)	2013
Khamduang <i>et al</i> ^[78]	Thailand	47/200 (23.5)	2013
Bell <i>et al</i> ^[79]	Africa	45/298 (15.1)	2012
Panigrahi <i>et al</i> ^[80]	India	12/112 (10.7)	2012
Bagaglio <i>et al</i> ^[81]	Italy	9/29 (31.0)	2011
Gupta <i>et al</i> ^[82]	India	24/53 (45.3)	2010
Hakeem <i>et al</i> ^[83]	Scotland	2/70 (2.8)	2010
Morsica <i>et al</i> ^[84]	Italy	27/175 (15)	2009
Azadmanesh <i>et al</i> ^[85]	Iran	3/22 (13.6)	2008
Tsui <i>et al</i> ^[86]	United States	8/400 (2.0)	2007

HCV related liver disease is controversial, some data have approved this issue. For improving treatment and consequence of OBI, it is recommended that the screening of anti-HBc and HBV DNA be implemented for pretreatment of HCV-infected patients.

Coinfection of OBI with human immunodeficiency virus infection

Both HBV and human immunodeficiency virus (HIV) share the same rout of transmission. Mostly the coinfection of OBI and HIV occurs among intravenous drug users. It was found that coinfection of HIV and HBV may lead to faster progression of liver fibrosis, development of cirrhosis and HCC, even without coinfection with HIV^[67]. The persistence of coinfection of OBI and HIV may result in severe and sometimes fulminant hepatitis^[68]. The low HBV replication and undetectable surface antigen may be due to host cell epigenetic genome and polymorphisms in host cytokine and chemokine receptors^[68-70]. Mutations in

the X, precore/core, and Pol regions of HBV genome may result in mutations within the preS/S open reading frame and bring about OBI^[29-51]. However, the effect of HIV components in HBV genome to lead to OBI remains unknown. While the occurrence of OBI may be related to host immune response and co-infections with HCV^[50,51] or HIV^[67,68], it has been postulated that HIV components are not major factors for the occurrence of OBI^[71,72]. Table 2 shows the distribution of co-existence of OBI with HIV infection in different countries^[73-86].

Thus, with regard to the aforementioned data for improving treatment and outcomes of OBI, it is recommended that the screening of anti-HBc and HBV DNA be carried out for pretreatment of HIV patients.

BLOOD TRANSFUSION AND OBI PROBLEM

Blood transfusion is a main risk factor for transmission of OBI provided that the screening of blood donors is done with less security^[87,88]. In the most developed countries, to boost blood safety, the nucleic acid amplification testing (NAT) has been established for screening of blood donors for detection of HCV, HIV and HBV or OBI. It is well documented that the application of NAT for HBV DNA, HCV RNA and HIV RNA detection is more sensitive than serological HBsAg, HCVAb, and HIV Ab tests^[89,90]. Thus, the implementation of HBV-DNA detection by NAT is more sensitive than HBsAg assay as a preventive measure for HBV or OBI transmission *via* blood transfusion^[89,90]. The prevalence of OBI among blood donors varies from country to country and has been reported in South Korea (HBV DNA, 0.016%)^[91], India (anti-HBc, 10.22%; HBV DNA, 0.15%)^[92], Turkey (anti-HBc, 20%; HBV DNA, 0%)^[93], Egypt (anti-HBc, 22.7%; HBV DNA, 22.7%)^[94] and Iran (0% OBI)^[95], (HBVDNA, 2/5000)^[25], (anti-HBc, 20%; HBV DNA, 0%)^[93], (HBV DNA, 12.2%)^[96]. In

the most developing countries the screening of HBV among blood donors relies only on serological detection of HBsAg. While the screening of HBV by the NAT is expensive, it is effective in reducing the transmission of OBI *via* blood transfusion and blood products^[89,90]. The detection of anti-HBc is a good test for OBI tracking, but it accounts for about 80% of OBI cases^[97,98]. Thus, with regard to what was stated previously the implementation of anti-HBc test for blood donors can be considered a second safeguard policy for reducing the transmission of HBV *via* blood transfusion^[99,100], although NAT is more sensitive and effective than serological HBsAg test as a preventive measure for HBV or OBI transmission *via* blood transfusion.

OBI AND HEAMODIALYSIS

Heamodialysis (HD) patients are at high risk of viral bloodborne infections (HBV, HIV, and HCV)^[101-103] and tracking of the diagnosed liver disease based on aminotransferase levels in HD patients is difficult. Mostly aminotransferase is suppressed by reduced immune competence which results in weak inflammatory reactions and consequently reduces hepatocyte destruction^[104]. It has been hypothesized that status of chronic uremia in HD patients may suppress the inflammatory reactions in the liver and consequently, no hepatocyte destruction will occur^[105,106]. Therefore, the evaluation of quantitative HBV DNA was found to be the most efficient method to evaluate OBI in HD patients^[101]. The prevalence of OBI has been studied in many countries, but varies from region to region worldwide. In Tehran, OBI was studied among HD patients by Ramezani *et al*^[107], and they isolated HBV DNA in 1% of 100 HBsAg negative HD patients. In Tehran, Aghakhani *et al*^[108] detected HBV DNA in 9/289 (3.1%) HD patients, who were negative for HBsAg but positive for HBcIgG. In Ahvaz, Neisi *et al*^[109] detected HBV DNA in 10/250 (4%) of HD patients, who were negative for HBsAg but positive for anti-HBc. Also in Ahvaz, Rastegarvand *et al*^[110] isolated HBV DNA in 6/216 (2.9%) HD patients, who were negative for HBsAg but positive for anti-HBc. The prevalence of OBI in HD patients was also reported in Spain (58%)^[111], Egypt (26.9%)^[24], United Kingdom (2.2%)^[101], Greece (20.4%)^[112] and Italy (0%)^[113].

It is recommended that all the patients on HD be routinely screened for viral bloodborne infections (HBV, HIV and HCV), including OBI, using highly sensitive molecular techniques to prevent nosocomial transmission.

OBI AND CRYPTOGENIC LIVER DISEASE

The rate of cryptogenic liver diseases varies greatly in different regions of the world. Patients with long-term persistent ALT abnormality or with the lack of overt viral detection and autoimmune markers, have been

shown to be positive for HBV DNA (OBI)^[114]. While the etiology of cryptogenic liver disease remains unknown, the association of occult hepatitis C has been reported in patients with abnormal alanin aminotransaminase^[115]. OBI has been regarded as an additional risk factor for progression of liver cirrhosis and HCC^[26,116]. The prevalence of OBI in cryptogenic chronic liver disease varies from 3.88% to 55.6%^[117,118]: in Brazil, 4.4%^[119]; in China, 28.3%^[120]; and in Iran, 1.9%^[121], 10%^[114], and India 9.5%^[122]. With regard to the mentioned data, it is recommended that for improving treatment and management, the sera and PBMCs or liver biopsy of patients with cryptogenic hepatitis be screened for HBV DNA by highly sensitive molecular means before developing signs of cirrhosis or HCC.

OBI IN CRYPTOGENIC CIRRHOSIS AND HCC

Liver cirrhosis is an endangering public health problem worldwide. Most of liver cirrhosis patients may progress to upper gastrointestinal bleeding, hepatic encephalopathy, and HCC. HBV infection or OBI, HCV infection or occult HCV, and alcohol consumption are major etiologies for development of liver cirrhosis^[123-125].

During the last phase of the natural course of chronic HBV infection, the inactive carrier phase is represented by HBeAg negativity, anti-HBe positivity, low HBV DNA levels (< 2000 IU/mL) with minimal or no fibrosis. The rates of spontaneous seroclearance of HBsAg (OBI) among inactive carriers range from 0.5% to 40% per year^[126,127]. There have been reports on progression of inactive carriers to cirrhosis^[128,129].

The prevalence of OBI among cirrhotic patients varies from region to region worldwide. The prevalence rates of OBI in cirrhotic patients have been reported: in Iran, 14% and 38%^[26,130]; India, 38%^[131]; Italy, 23.4% and 27%^[132,133]; Egypt, 2.7%^[134]; Japan, 18.1%^[135]; France, 60%^[136]; United States, 19.4%^[137]; Brazil, 20%^[138]; China, 32%^[139]; and China, 3.88%^[117].

The mechanism of liver damage due to OBI is still not well elucidated, but there are some data that described the persistence and transcription of HBV cccDNA in hepatocytes and subsequently, production of cytokines, such as TNF- α and interferon- γ may result in damage to hepatocytes^[140,141]. The occurrence of mutations in the X region of HBV may bring about a reduction in the ability of the transactivation of X protein, which is essential for viral replication, and also result in low HBV DNA replication and undetectable HBsAg in serum^[142].

Liver cancer is considered a major global health problem. Viral hepatitis B and C are main risk factors for the development of liver cancer^[135,143]. The prolonged persistence of cccDNA in the hepatocyte nucleus has been detected in patients with HCC^[144]. In addition, HBV DNA has been found to be integrated within the host chromosomes of individuals with HCC^[145]. Most

findings described that OBI is an important risk factor for hastening the progression of liver disease and the development of cirrhosis and HCC^[146]. Several studies have documented that in patients with HCC who were negative for all HBV serum markers, including HBsAg, HBV DNA was detected in hepatocytes^[147-160].

Several mechanisms may be involved in OBI-induced hepatocarcinogenesis. When HBV DNA is integrated into the host genome, the integrated HBX and truncated pre-S2/S genomic sequences may alter the cellular gene expression and result in the development of HCC^[161-163]. OBI DNA, either in the form of free episomes or in integrated forms, is able to replicate, transcribe, and synthesize proteins, at very low levels^[144-165].

The advances in molecular approaches have made it possible to disclose several virological features of OBI, and describe different clinical settings. Thus the persistence of OBI is an important risk factor for development of cirrhosis and HCC. But more investigations are needed to understand the relationship between OBI and cryptogenic liver disease. It is recommended that for improving and management of patients in the initial stage of cryptogenic liver diseases, the sera and PBMCs of the patients be screened for HBV DNA by highly sensitive molecular means as a preventive measure before the development of cirrhosis and HCC.

OBI AND TRANSPLANT

Liver transplantation is the only option for patients with end-stage chronic liver disease. But in liver transplant recipients with OBI, the reactivation of HBV is enhanced by the induced immunosuppression factors and rapidly leads to graft failure and death^[166-168].

The occult HBV transmission from HBsAg-negative and anti-HBc-positive liver organ donors is possible, especially when the organ liver recipient is negative for all HBV serum markers^[169]. Dickson *et al*^[170] reported the *de novo* HBV infection was developed in 18/23 (78%) liver organ transplant recipients from donors who were positive for anti-HBc compared with 3/651 (0.5%) recipients of organ transplant liver from donors who were negative for anti-HBc ($P < 0.0001$). Although the prevalence of OBI among kidney or bone marrow transplant recipients is controversial, limited data are available on this subject. Franz *et al*^[171] detected HBV DNA in 1% of 207 kidney transplant recipients negative for HBsAg. Cinzia Lo Giudice *et al*^[5] detected HBV DNA in a bone marrow transplant recipient who was negative for HBsAg and required constant blood transfusion.

For the management and prevention of the consequences of OBI in organ transplant recipients, it is suggested that the screening of HBV DNA be carried out in both donors and organ transplant recipients by highly sensitive molecular means.

EPIGENETIC CHANGES

Methylation

Methylation of cytosines in CpG dinucleotides within CpG islands affects the HBV DNA promoter, which may lead to gene silencing^[172]. Methylation was found in both HBV DNA integrated in the host hepatocyte genome as well in the free episomal form of HBV cccDNA^[159,168]. Methylation of HBV DNA symbolizes a novel epigenetic mechanism, and it can alter HBV proteins, HBV replication, and HBV virion production, which may lead to OBI^[173]. Hypermethylated HBV DNA sequences are frequently detected in HCC patients with OBI^[174]. The integrated HBX and carboxy-terminally truncated preS or S polypeptide genes in the host genome may modify the host gene expression and cellular phenotypes and result in the acceleration of growth factor-independent proliferation, metastasis and the development of HCC^[175,176].

Acetylation

Both experimental *in vivo* and *in vitro* data have shown that HBV replication is regulated by the acetylation of H3/H4 histones bound to viral cccDNA^[177]. Besides, the histone deacetylase onto the cccDNA is associated with low HBV replication *in vitro* and low viremia *in vivo*^[178].

OBI AND HODGKIN AND NON-HODGKIN LYMPHOMAS

The etiology of lymphoma remains unknown, although genetic, environment, and some infectious agents have been implicated in the development of Hodgkin and non-Hodgkin lymphomas. The association between viruses and lymphomas has been investigated, although the precise mechanisms behind this association are still unknown. The hepatotropism and lymphotropism of HBV have been well documented^[179,180].

The association between HBV and non-Hodgkin lymphoma has been well investigated^[181,182]. In a study conducted by Elbedewy *et al*^[182] in Egypt, HBV DNA was detected in 5/72 (6.94%) of patients with diffuse large B-cell lymphoma who were positive for anti HBc (191). In a study conducted by Kamyar *et al*^[183] in Ahvaz, Iran, HBV DNA was isolated in 3/12 (25%) of patients with Hodgkin lymphoma and in 7/ 29 (24.13%) of patients with non-Hodgkin lymphoma. In this study, the results of sequencing exhibited a substitution of the amino acid proline with leucine in position 88 of the HBs gene in six patients with Hodgkin or non-Hodgkin lymphoma. Cheung *et al*^[184] in Hong Kong detected HBV DNA in 10/47 (21%) patients with lymphoma who were negative for HBsAg but positive for anti-HBc.

With regard to the aforementioned data, it is recommended that for improving the treatment, patients with Hodgkin and non-Hodgkin lymphomas be screened for HBV DNA by highly sensitive molecular means prior to chemotherapy treatment.

Table 3 Prevalence of occult hepatitis B infection among healthcare workers

Ref.	Country	No. of samples	No. of OBI cases	No. of cases positive for anti-HBc	Year
Borzooy <i>et al</i> ^[188]	Iran	120	4 (3.3)	0 (0)	2015
Chiarakul <i>et al</i> ^[189]	Thailand	36	4 (11)	4 (100)	2011
Slusarczyk <i>et al</i> ^[185]	Poland	961	6 (4)	4 (100%)	2012
Shim <i>et al</i> ^[190]	Korea	334	0	0	2011
Sukriti <i>et al</i> ^[191]	India	120	6 (5)	6 (100)	2008
Yen <i>et al</i> ^[192]	Taiwan	250	16 (6.4)	13 (81)	2005

OBI: Occult hepatitis B infection. HBc: Hepatitis B core.

OBI AND HEALTH CARE WORKERS

Health care workers are more often at high risk of HBV infection/OBI than the general population^[185,186]. They may contract HBV transmission *via* exposure to potentially infected material as well as mucosal-cutaneous and percutaneous exposure to HBV from HBV carriers^[187]. Most of individuals with OBI are clinically asymptomatic and remain undiagnosed unless a sudden development of cirrhosis or HCC occurred^[188]. The prevalence of OBI among health care workers varies from region to region worldwide. The occurrence of OBI was mostly reported in regions of high endemicity of HBV^[117]. Table 3 shows the prevalence of OBI among healthcare workers^[188-192].

Based on the mentioned data, it is recommended that the screening of HBV DNA be implemented for health care workers. Besides, regardless to OBI, effective HBV vaccination should be carried out for health care workers. Also a booster dose of HBV vaccine should be put into practice for individuals with a low titer of anti-HBs (< 100 IU/mL).

OBI REACTIVATION

In OBI patients, HBV DNA may persist in two forms: episomal free cccDNA or integration into the DNA of hepatocytes. OBI may be generated by subsequently resolved acute HBV infection, occurrence of mutation in "a" determinant of the HBsAg gene, coinfection with HCV or HIV, and cellular epigenetic changes. In OBI, HBsAg is undetectable in serum with positive/negative anti-HBc status^[172,175,193]. OBI reactivation may take place with increasing HBV DNA replication in patients during immunosuppression therapy^[185,186,189-199]. OBI reactivation was described with enhancing HBV DNA replication in HIV patients during anti-retroviral therapy^[200]. OBI reactivation resulted in the development of fulminant hepatitis in patients with cancer who underwent chemotherapy^[200,201]. The risk of HBV reactivation is considered as high as 21% to 67% when immunosuppression is distinct, particularly in onco-hematological patients, in those receiving hematopoietic stem cell transplantation and in those treated with the anti-CD20 monoclonal

antibody rituximab or with the monoclonal anti-CD52 antibody alemtuzumab, which account for long-lasting immunosuppression^[193,194,202-208]. Under these situations, HBV reactivation causes a mortality rate about 20%, due to hepatic failure^[209,210]. Seto *et al*^[211] reported reactivation of hepatitis B in lymphoma patients with a past history of HBV infection, who were treated with rituximab-containing chemotherapy. Hsu *et al*^[212] studied the chemotherapy-induced hepatitis B reactivation in lymphoma patients with resolved HBV infection. Dominguez *et al*^[213] (2015) observed reactivation of OBI in a patient with chronic lymphocytic leukemia after treatment with a rituximab and fludarabine-based regimen. A recent study conducted by Liu *et al*^[214] revealed that reactivation of HBV was observed in patients with breast cancer receiving chemotherapy.

The risk of HBV reactivation in cancer patients receiving chemotherapy is impressed by inducing factors related to the virus, the host and specific immunosuppressive treatment, although the complete dimension of risk remains unknown.

With regard to the aforementioned data, the screening of HBV DNA by highly sensitive molecular means be implemented in all patients before and after immunosuppression status.

CONCLUSION

OBI is a life-threatening public health problem worldwide. The detection of OBI is costly, especially for developing countries, therefore many patients with OBI may remain undiagnosed. OBI is an important risk factor for developing cirrhosis and HCC.

OBI can be controlled in high risk groups, provided that the implementation of highly sensitive molecular means used for detection HBV DNA as a preventive measure.

With regard to the consequence of OBI, for improving the treatment and management, the screening of HBV DNA by real-time PCR should be implemented in the following groups: (1) patients with a previous history of HBV infection; (2) HBV patients coinfecting with HCV/HIV; (3) patients undergoing chemotherapy anti-CD20 therapy; (4) recipients of organ transplant; (5) blood donors; (6) organ transplant donors; (7) thalassemia or hemophilia patients; h) health care workers; (8) patients with cryptogenic hepatitis or cryptogenic liver related disease (cirrhosis and HCC); (9) HD patients; (10) patients treated with lamivudine or interferon; and (11) children in time of HBV vaccination, especially in highly endemic areas of HBV. Besides, recent data revealed that reactivation of HBV was observed in patients with breast cancer receiving chemotherapy. Therefore, the screening of OBI should be implemented in patients with breast cancer.

In addition, proper disinfection should be performed for dialysis, endoscopy, colonoscopy and endoscopy

units.

The effective HBV vaccination program should be carried out for the close relatives of patients who are negative for OBI. The third generation HBV vaccines containing preS1 and preS2 antigens have been developed with excellent immunogenicity in humans, and rapid antibody responses may be able to control the further incidence of OBI^[215,216].

REFERENCES

- 1 **World Health Organization.** Hepatitis B Fact Sheet N204: Hepatitis B. World Health Organization, 2013. Accessed January 23, 2013. Available from: URL: <http://www.who.int/mediacentre/factsheets/fs204/en/>
- 2 **Tabor E,** Hoofnagle JH, Smallwood LA, Drucker JA, Pineda-Tamondong GC, Ni LY, Greenwalt TJ, Barker LF, Gerety RJ. Studies of donors who transmit posttransfusion hepatitis. *Transfusion* 1979; **19**: 725-731 [PMID: 230620 DOI: 10.1046/j.1537-2995.1979.19680104098.x]
- 3 **Grob P,** Jilg W, Bornhak H, Gerken G, Gerlich W, Günther S, Hess G, Hüdig H, Kitchen A, Margolis H, Michel G, Trepo C, Will H, Zanetti A, Mushahwar I. Serological pattern “anti-HBc alone”: report on a workshop. *J Med Virol* 2000; **62**: 450-455 [PMID: 11074473 DOI: 10.1002/1096-9071(200012)62:4<450::AID-JMV9>3.0.CO;2-Y]
- 4 **Hu KQ.** Occult hepatitis B virus infection and its clinical implications. *J Viral Hepat* 2002; **9**: 243-257 [PMID: 12081601 DOI: 10.1046/j.1365-2893.2002.00344.x]
- 5 **Giudice CL,** Martinengo M, Pietrasanta P, Bocciardo L, Malavasi C, Rastelli S, Faraci M, Tripodi G. Occult hepatitis B virus infection: a case of reactivation in a patient receiving immunosuppressive treatment for allogeneic bone marrow transplantation. *Blood Transfus* 2008; **6**: 46-50 [PMID: 18661923]
- 6 **Raimondo G,** Pollicino T, Romanò L, Zanetti AR. A 2010 update on occult hepatitis B infection. *Pathol Biol* (Paris) 2010; **58**: 254-257 [PMID: 20303674 DOI: 10.1016/j.patbio.2010.02.003]
- 7 **Kim H,** Lee SA, Kim DW, Lee SH, Kim BJ. Naturally occurring mutations in large surface genes related to occult infection of hepatitis B virus genotype C. *PLoS One* 2013; **8**: e54486 [PMID: 23349904 DOI: 10.1371/journal.pone.0054486]
- 8 **Raimondo G,** Pollicino T, Cacciola I, Squadrito G. Occult hepatitis B virus infection. *J Hepatol* 2007; **46**: 160-170 [PMID: 17112622 DOI: 10.1016/j.jhep.2006.10.007]
- 9 **Kim H,** Lee SA, Won YS, Lee H, Kim BJ. Occult infection related hepatitis B surface antigen variants showing lowered secretion capacity. *World J Gastroenterol* 2015; **21**: 1794-1803 [PMID: 25684944 DOI: 10.3748/wjg.v21.i6.1794]
- 10 **Zhu HL,** Li X, Li J, Zhang ZH. Genetic variation of occult hepatitis B virus infection. *World J Gastroenterol* 2016; **22**: 3531-3546 [PMID: 27053845 DOI: 10.3748/wjg.v22.i13.3531]
- 11 **Sarin SK,** Kumar M, Lau GK, Abbas Z, Chan HL, Chen CJ, Chen DS, Chen HL, Chen PJ, Chien RN, Dokmeci AK, Gane E, Hou JL, Jafri W, Jia J, Kim JH, Lai CL, Lee HC, Lim SG, Liu CJ, Locarnini S, Al Mahtab M, Mohamed R, Omata M, Park J, Piratvisuth T, Sharma BC, Sollano J, Wang FS, Wei L, Yuen MF, Zheng SS, Kao JH. Asian-Pacific clinical practice guidelines on the management of hepatitis B: a 2015 update. *Hepatol Int* 2016; **10**: 1-98 [PMID: 26563120]
- 12 **Raimondo G,** Allain JP, Brunetto MR, Buendia MA, Chen DS, Colombo M, Craxi A, Donato F, Ferrari C, Gaeta GB, Gerlich WH, Levrero M, Locarnini S, Michalak T, Mondelli MU, Pawlotsky JM, Pollicino T, Prati D, Puoti M, Samuel D, Shouval D, Smedile A, Squadrito G, Trépo C, Villa E, Will H, Zanetti AR, Zoulim F. Statements from the Taormina expert meeting on occult hepatitis B virus infection. *J Hepatol* 2008; **49**: 652-657 [PMID: 18715666 DOI: 10.1016/j.jhep.2008.07.014]
- 13 **Torbenson M,** Thomas DL. Occult hepatitis B. *Lancet Infect Dis* 2002; **2**: 479-486 [PMID: 12150847]
- 14 **Morales-Romero J,** Vargas G, García-Román R. Occult HBV infection: a faceless enemy in liver cancer development. *Viruses* 2014; **6**: 1590-1611 [PMID: 24717680 DOI: 10.3390/v6041590]
- 15 **Yuen MF,** Lee CK, Wong DK, Fung J, Hung I, Hsu A, But DY, Cheung TK, Chan P, Yuen JC, Fung FK, Seto WK, Lin CK, Lai CL. Prevalence of occult hepatitis B infection in a highly endemic area for chronic hepatitis B: a study of a large blood donor population. *Gut* 2010; **59**: 1389-1393 [PMID: 20675695 DOI: 10.1136/gut.2010.209148]
- 16 **Minuk GY,** Sun DF, Uhanova J, Zhang M, Caouette S, Nicolle LE, Gutkin A, Doucette K, Martin B, Giulivi A. Occult hepatitis B virus infection in a North American community-based population. *J Hepatol* 2005; **42**: 480-485 [PMID: 15763333 DOI: 10.1016/j.jhep.2004.11.037]
- 17 **Escobedo-Melendez G,** Panduro A, Fierro NA, Roman S. High prevalence of occult hepatitis B virus genotype H infection among children with clinical hepatitis in west Mexico. *Mem Inst Oswaldo Cruz* 2014; **109**: 728-737 [PMID: 25099333]
- 18 **Fang ZL,** Sabin CA, Dong BQ, Wei SC, Chen QY, Fang KX, Yang JY, Huang J, Wang XY, Harrison TJ. Hepatitis B virus pre-S deletion mutations are a risk factor for hepatocellular carcinoma: a matched nested case-control study. *J Gen Virol* 2008; **89**: 2882-2890 [PMID: 18931087 DOI: 10.1099/vir.0.2008/002824-0]
- 19 **Kang SY,** Kim MH, Lee WI. The prevalence of “anti-HBc alone” and HBV DNA detection among anti-HBc alone in Korea. *J Med Virol* 2010; **82**: 1508-1514 [PMID: 20648604 DOI: 10.1002/jmv.21862]
- 20 **Kim H,** Kim BJ. Association of preS/S Mutations with Occult Hepatitis B Virus (HBV) Infection in South Korea: Transmission Potential of Distinct Occult HBV Variants. *Int J Mol Sci* 2015; **16**: 13595-13609 [PMID: 26084041 DOI: 10.3390/ijms160613595]
- 21 **Mu SC,** Lin YM, Jow GM, Chen BF. Occult hepatitis B virus infection in hepatitis B vaccinated children in Taiwan. *J Hepatol* 2009; **50**: 264-272 [PMID: 19070923]
- 22 **Su TH,** Chen PJ, Chen TC, Cheng HR, Li L, Lin KS, Kao JH, Chen DS, Liu CJ. The clinical significance of occult hepatitis B transfusion in Taiwan—a look-back study. *Transfus Med* 2011; **21**: 33-41 [PMID: 20726954 DOI: 10.1111/j.1365-3148.2010.01036.x]
- 23 **Abu El Makarem MA,** Abdel Hamid M, Abdel Aleem A, Ali A, Shatat M, Sayed D, Deaf A, Hamdy L, Tony EA. Prevalence of occult hepatitis B virus infection in hemodialysis patients from Egypt with or without hepatitis C virus infection. *Hepat Mon* 2012; **12**: 253-258 [PMID: 22690232 DOI: 10.5812/hepatmon.5805]
- 24 **Elgohry I,** Elbanna A, Hashad D. Occult hepatitis B virus infection in a cohort of Egyptian chronic hemodialysis patients. *Clin Lab* 2012; **58**: 1057-1061 [PMID: 23163124]
- 25 **Alizadeh Z,** Milani S, Sharifi Z. Occult hepatitis B virus infection among Iranian blood donors: a preliminary study. *Arch Iran Med* 2014; **17**: 106-107 [PMID: 24527970]
- 26 **Hashemi JS,** Hajiani E, Masjedizadeh A, Makvandi M, Shayesteh AA, Alavinejad SP, Kadkhodaei A, Shahbazian H, Jasefi F, Karimi M. Occult Hepatitis B Infection in Patients With Cryptogenic Liver Cirrhosis in Southwest of Iran. *Jundishapur J Microbiol* 2015; **8**: e16873 [PMID: 25861432 DOI: 10.5812/jjm.16873]
- 27 **Sharifi-Mood B,** Sanei-Moghaddam E, Khosravi S. Occult hepatitis B Virus infection among anti-HBc only positive individuals in the southeast of Iran in high prevalence of HBV infection region. *IRCMJ* 2009; **11**: 90-92
- 28 **Vaezjalali M,** Rashidpour S, Rezaee H, Hajibeigi B, Zeidi M, Gachkar L, Aghamohamad S, Najafi R, Goudarzi H. Hepatitis B viral DNA among HBs antigen negative healthy blood donors. *Hepat Mon* 2013; **13**: e6590 [PMID: 23675384 DOI: 10.5812/hepatmon.6590]
- 29 **Carman WF,** Zanetti AR, Karayiannis P, Waters J, Manziello G, Tanzi E, Zuckerman AJ, Thomas HC. Vaccine-induced escape mutant of hepatitis B virus. *Lancet* 1990; **336**: 325-329 [PMID: 1697396]
- 30 **Elbahrawy A,** Alaboudy A, El Moghazy W, Elwassief A, Alashker A, Abdallah AM. Occult hepatitis B virus infection in Egypt. *World J Hepatol* 2015; **7**: 1671-1678 [PMID: 26140086 DOI: 10.4254/

- wjh.v7.i12.1671]
- 31 **Sagnelli E**, Pisaturo M, Martini S, Filippini P, Sagnelli C, Coppola N. Clinical impact of occult hepatitis B virus infection in immunosuppressed patients. *World J Hepatol* 2014; **6**: 384-393 [PMID: 25018849 DOI: 10.4254/wjh.v6.i6.384]
 - 32 **Schilling R**, Ijaz S, Davidoff M, Lee JY, Locarnini S, Williams R, Naoumov NV. Endocytosis of hepatitis B immune globulin into hepatocytes inhibits the secretion of hepatitis B virus surface antigen and virions. *J Virol* 2003; **77**: 8882-8892 [PMID: 12885906 DOI: 10.1128/JVI.77.16.8882-8892.2003]
 - 33 **Chaudhuri V**, Tayal R, Nayak B, Acharya SK, Panda SK. Occult hepatitis B virus infection in chronic liver disease: full-length genome and analysis of mutant surface promoter. *Gastroenterology* 2004; **127**: 1356-1371 [PMID: 15521005 DOI: 10.1053/j.gastro.2004.08.003]
 - 34 **Vivekanandan P**, Kannangai R, Ray SC, Thomas DL, Torbenson M. Comprehensive genetic and epigenetic analysis of occult hepatitis B from liver tissue samples. *Clin Infect Dis* 2008; **46**: 1227-1236 [PMID: 18444860 DOI: 10.1086/529437]
 - 35 **Hass M**, Hannoun C, Kalinina T, Sommer G, Manegold C, Günther S. Functional analysis of hepatitis B virus reactivating in hepatitis B surface antigen-negative individuals. *Hepatology* 2005; **42**: 93-103 [PMID: 15962285 DOI: 10.1002/hep.20748]
 - 36 **Wakil SM**, Kazim SN, Khan LA, Raisuddin S, Parvez MK, Gupta RC, Thakur V, Hasnain SE, Sarin SK. Prevalence and profile of mutations associated with lamivudine therapy in Indian patients with chronic hepatitis B in the surface and polymerase genes of hepatitis B virus. *J Med Virol* 2002; **68**: 311-318 [PMID: 12226816 DOI: 10.1002/jmv.10205]
 - 37 **Melegari M**, Bruno S, Wands JR. Properties of hepatitis B virus pre-S1 deletion mutants. *Virology* 1994; **199**: 292-300 [PMID: 8122362 DOI: 10.1006/viro.1994.1127]
 - 38 **Obika M**, Shinji T, Fujioka S, Terada R, Ryuko H, Lwin AA, Shiraha H, Koide N. Hepatitis B virus DNA in liver tissue and risk for hepatocarcinogenesis in patients with hepatitis C virus-related chronic liver disease. A prospective study. *Intervirology* 2008; **51**: 59-68 [PMID: 18349544 DOI: 10.1159/000121363]
 - 39 **Jansen K**, Thamm M, Bock CT, Scheufele R, Kücherer C, Muenstermann D, Hagedorn HJ, Jessen H, Dupke S, Hamouda O, Günsenheimer-Bartmeyer B, Meixenberger K. High Prevalence and High Incidence of Coinfection with Hepatitis B, Hepatitis C, and Syphilis and Low Rate of Effective Vaccination against Hepatitis B in HIV-Positive Men Who Have Sex with Men with Known Date of HIV Seroconversion in Germany. *PLoS One* 2015; **10**: e0142515 [PMID: 26555244 DOI: 10.1371/journal.pone.0142515]
 - 40 **Lok AS**, Everhart JE, Di Bisceglie AM, Kim HY, Hussain M, Morgan TR. Occult and previous hepatitis B virus infection are not associated with hepatocellular carcinoma in United States patients with chronic hepatitis C. *Hepatology* 2011; **54**: 434-442 [PMID: 21374690 DOI: 10.1002/hep.24257]
 - 41 **Marusawa H**, Osaki Y, Kimura T, Ito K, Yamashita Y, Eguchi T, Kudo M, Yamamoto Y, Kojima H, Seno H, Moriyasu F, Chiba T. High prevalence of anti-hepatitis B virus serological markers in patients with hepatitis C virus related chronic liver disease in Japan. *Gut* 1999; **45**: 284-288 [PMID: 10403743]
 - 42 **Raouf HE**, Yassin AS, Megahed SA, Ashour MS, Mansour TM. Seroprevalence of occult hepatitis B among Egyptian paediatric hepatitis C cancer patients. *J Viral Hepat* 2015; **22**: 103-111 [PMID: 24754376 DOI: 10.1111/jvh.12260]
 - 43 **Branco F**, Mattos AA, Coral GP, Vanderborcht B, Santos DE, França P, Alexandre C. Occult hepatitis B virus infection in patients with chronic liver disease due to hepatitis C virus and hepatocellular carcinoma in Brazil. *Arq Gastroenterol* 2007; **44**: 58-63 [PMID: 17639185]
 - 44 **Miura Y**, Shibuya A, Adachi S, Takeuchi A, Tsuchihashi T, Nakazawa T, Saigenji K. Occult hepatitis B virus infection as a risk factor for hepatocellular carcinoma in patients with chronic hepatitis C in whom viral eradication fails. *Hepatol Res* 2008; **38**: 546-556 [PMID: 18179561 DOI: 10.1111/j.1872-034X]
 - 45 **Rodríguez-Iñigo E**, Bartolomé J, Ortiz-Movilla N, Platero C, López-Alcorocho JM, Pardo M, Castillo I, Carreño V. Hepatitis C virus (HCV) and hepatitis B virus (HBV) can coinfect the same hepatocyte in the liver of patients with chronic HCV and occult HBV infection. *J Virol* 2005; **79**: 15578-15581 [PMID: 16306629 DOI: 10.1128/JVI.79.24.15578-15581.2005]
 - 46 **Choi BH**, Park GT, Rho HM. Interaction of hepatitis B viral X protein and CCAAT/ enhancer-binding protein alpha synergistically activates the hepatitis B viral enhancer II/pregenomic promoter. *J Biol Chem* 1999; **274**: 2858-2865 [PMID: 9915821 DOI: 10.1074/jbc.274.5.2858]
 - 47 **Nakatake H**, Chisaka O, Yamamoto S, Matsubara K, Koshy R. Effect of X protein on transactivation of hepatitis B virus promoters and on viral replication. *Virology* 1993; **195**: 305-314 [PMID: 8337816 DOI: 10.1006/viro.1993.1381]
 - 48 **Chen SY**, Kao CF, Chen CM, Shih CM, Hsu MJ, Chao CH, Wang SH, You LR, Lee YH. Mechanisms for inhibition of hepatitis B virus gene expression and replication by hepatitis C virus core protein. *J Biol Chem* 2003; **278**: 591-607 [PMID: 12401801]
 - 49 **Schüttler CG**, Fiedler N, Schmidt K, Repp R, Gerlich WH, Schaefer S. Suppression of hepatitis B virus enhancer 1 and 2 by hepatitis C virus core protein. *J Hepatol* 2002; **37**: 855-862 [PMID: 12445429 DOI: 10.1016/S0168-8278(02)00296-9]
 - 50 **Dumoulin FL**, von dem Bussche A, Li J, Khamzina L, Wands JR, Sauerbruch T, Spengler U. Hepatitis C virus NS2 protein inhibits gene expression from different cellular and viral promoters in hepatic and nonhepatic cell lines. *Virology* 2003; **305**: 260-266 [PMID: 12573571 DOI: 10.1006/viro.2002.1701]
 - 51 **Pan Y**, Wei W, Kang L, Wang Z, Fang J, Zhu Y, Wu J. NS5A protein of HCV enhances HBV replication and resistance to interferon response. *Biochem Biophys Res Commun* 2007; **359**: 70-75 [PMID: 17532300 DOI: 10.1016/j.bbrc.2007.05.052]
 - 52 **Fukuda R**, Ishimura N, Niigaki M, Hamamoto S, Satoh S, Tanaka S, Kushiya Y, Uchida Y, Iihara S, Akagi S, Watanabe M, Kinoshita Y. Serologically silent hepatitis B virus coinfection in patients with hepatitis C virus-associated chronic liver disease: clinical and virological significance. *J Med Virol* 1999; **58**: 201-207 [PMID: 10447413]
 - 53 **Kao JH**, Chen PJ, Lai MY, Chen DS. Occult hepatitis B virus infection and clinical outcomes of patients with chronic hepatitis C. *J Clin Microbiol* 2002; **40**: 4068-4071 [PMID: 12409376 DOI: 10.1128/JCM.40.11.4068-4071.2002]
 - 54 **Besisk F**, Karaca C, Akyüz F, Horosanli S, Onel D, Badur S, Sever MS, Danalioglu A, Demir K, Kaymakoglu S, Cakaloglu Y, Okten A. Occult HBV infection and YMDD variants in hemodialysis patients with chronic HCV infection. *J Hepatol* 2003; **38**: 506-510 [PMID: 12663244]
 - 55 **Georgiadou SP**, Zachou K, Rigopoulou E, Liaskos C, Mina P, Gerovalis F, Makri E, Dalekos GN. Occult hepatitis B virus infection in Greek patients with chronic hepatitis C and in patients with diverse nonviral hepatic diseases. *J Viral Hepat* 2004; **11**: 358-365 [PMID: 15230859 DOI: 10.1111/j.1365-2893.2004]
 - 56 **Khattab E**, Chemin I, Vuillermoz I, Vieux C, Mrani S, Guillaud O, Trepo C, Zoulim F. Analysis of HCV co-infection with occult hepatitis B virus in patients undergoing IFN therapy. *J Clin Virol* 2005; **33**: 150-157 [PMID: 15911431]
 - 57 **Goral V**, Ozkul H, Tekes S, Sit D, Kadiroglu AK. Prevalence of occult HBV infection in haemodialysis patients with chronic HCV. *World J Gastroenterol* 2006; **12**: 3420-3424 [PMID: 16733862 DOI: 10.3748/wjg.v12.i21.3420]
 - 58 **Toyoda H**, Kumada T, Kiriya S, Sone Y, Tanikawa M, Hisanaga Y, Kanamori A. Prevalence of low-level hepatitis B viremia in patients with HBV surface antigen-negative hepatocellular carcinoma with and without hepatitis C virus infection in Japan: analysis by COBAS TaqMan real-time PCR. *Intervirology* 2007; **50**: 241-244 [PMID: 17446712]
 - 59 **Shetty K**, Hussain M, Nei L, Reddy KR, Lok AS. Prevalence and significance of occult hepatitis B in a liver transplant population with chronic hepatitis C. *Liver Transpl* 2008; **14**: 534-540 [PMID: 18324677 DOI: 10.1002/lt.21284]
 - 60 **Tamori A**, Hayashi T, Shinzaki M, Kobayashi S, Iwai S, Enomoto

- M, Morikawa H, Sakaguchi H, Shiomi S, Takemura S, Kubo S, Kawada N. Frequent detection of hepatitis B virus DNA in hepatocellular carcinoma of patients with sustained virologic response for hepatitis C virus. *J Med Virol* 2009; **81**: 1009-1014 [PMID: 19382258 DOI: 10.1002/jmv.21488]
- 61 **Chen LW**, Chien RN, Yen CL, Chang JJ, Liu CJ, Lin CL. Therapeutic effects of pegylated interferon plus ribavirin in chronic hepatitis C patients with occult hepatitis B virus dual infection. *J Gastroenterol Hepatol* 2010; **25**: 259-263 [PMID: 19817959 DOI: 10.1111/j.1440-1746.2009.06006.x]
 - 62 **Jang JY**, Jeong SW, Cheon SR, Lee SH, Kim SG, Cheon YK, Kim YS, Cho YD, Kim HS, Jin SY, Kim YS, Kim BS. Clinical significance of occult hepatitis B virus infection in chronic hepatitis C patients. *Korean J Hepatol* 2011; **17**: 206-212 [PMID: 22102387 DOI: 10.3350/kjhep.2011.17.3.206]
 - 63 **Joukar F**, Mansour-Ghanaei F, Besharati S, Khosh-Sorur M. Occult hepatitis B infection in a hemodialysis population in Guilan province, northern Iran. *Hemodial Int* 2012; **16**: 294-297 [PMID: 22118428 DOI: 10.1111/j.1542-4758.2011.00645.x]
 - 64 **Vakili Ghartavol Z**, Alavian SM, Amini S, Vahabpour R, Bahramali G, Mostafavi E, Aghasadeghi MR. Prevalence of occult hepatitis B virus in plasma and peripheral blood mononuclear cell compartments of patients with chronic hepatitis C infection in tehran-iran. *Hepat Mon* 2013; **13**: e10134 [PMID: 23967017 DOI: 10.5812/hepatmon.10134]
 - 65 **Kishk R**, Atta HA, Ragheb M, Kamel M, Metwally L, Nemr N. Genotype characterization of occult hepatitis B virus strains among Egyptian chronic hepatitis C patients. *East Mediterr Health J* 2014; **20**: 130-138 [PMID: 24945562]
 - 66 **Mandour M**, Nemr N, Shehata A, Kishk R, Badran D, Hawass N. Occult HBV infection status among chronic hepatitis C and hemodialysis patients in Northeastern Egypt: regional and national overview. *Rev Soc Bras Med Trop* 2015; **48**: 258-264 [PMID: 26108002 DOI: 10.1590/0037-8682-0037-2015]
 - 67 **Mallet V**, Vallet-Pichard A, Pol S. The impact of human immunodeficiency virus on viral hepatitis. *Liver Int* 2011; **31** Suppl 1: 135-139 [PMID: 21205151 DOI: 10.1111/j.1478-3231.2010.02394.x]
 - 68 **Mphahlele MJ**, Lukhwareni A, Burnett RJ, Moropeng LM, Ngobeni JM. High risk of occult hepatitis B virus infection in HIV-positive patients from South Africa. *J Clin Virol* 2006; **35**: 14-20 [PMID: 15916918]
 - 69 **Arababadi MK**, Pourfathollah AA, Jafarzadeh A, Hassanshahi G, Rezvani ME. Association of exon 9 but not intron 8 VDR polymorphisms with occult HBV infection in south-eastern Iranian patients. *J Gastroenterol Hepatol* 2010; **25**: 90-93 [PMID: 19793172 DOI: 10.1111/j.1440-1746.2009.05950.x]
 - 70 **Ahmadabadi BN**, Hassanshahi G, Arababadi MK, Leanza C, Kennedy D. The IL-10 promoter polymorphism at position -592 is correlated with susceptibility to occult HBV infection. *Inflammation* 2012; **35**: 818-821 [PMID: 21901441 DOI: 10.1007/s10753-011-9381-x]
 - 71 **Pollicino T**, Raffa G, Costantino L, Lisa A, Campello C, Squadrito G, Levvero M, Raimondo G. Molecular and functional analysis of occult hepatitis B virus isolates from patients with hepatocellular carcinoma. *Hepatology* 2007; **45**: 277-285 [PMID: 17256766 DOI: 10.1002/hep.21529]
 - 72 **Raimondo G**, Caccamo G, Filomia R, Pollicino T. Occult HBV infection. *Semin Immunopathol* 2013; **35**: 39-52 [PMID: 22829332 DOI: 10.1007/s00281-012-0327-7]
 - 73 **Vargas JI**, Jensen D, Sarmiento V, Peirano F, Acuña P, Fuster F, Soto S, Ahumada R, Huilcaman M, Bruna M, Jensen W, Fuster F. Presence of anti-HBc is associated to high rates of HBV resolved infection and low threshold for Occult HBV Infection in HIV patients with negative HBsAg in Chile. *J Med Virol* 2016; **88**: 639-646 [PMID: 26381185 DOI: 10.1002/jmv.24384]
 - 74 **Alvarez-Muñoz MT**, Maldonado-Rodríguez A, Rojas-Montes O, Torres-Ibarra R, Gutierrez-Escobedo F, Vazquez-Rosales G, Gomez A, Muñoz O, Torres J, Lira R. Occult hepatitis B virus infection among Mexican human immunodeficiency virus-1-infected patients. *World J Gastroenterol* 2014; **20**: 13530-13537 [PMID: 25309083 DOI: 10.3748/wjg.v20.i37.13530]
 - 75 **Chadwick D**, Doyle T, Ellis S, Price D, Abbas I, Valappil M, Geretti AM. Occult hepatitis B virus coinfection in HIV-positive African migrants to the UK: a point prevalence study. *HIV Med* 2014; **15**: 189-192 [PMID: 24118868 DOI: 10.1111/hiv.12093]
 - 76 **Coffin CS**, Mulrooney-Cousins PM, Osiowy C, van der Meer F, Nishikawa S, Michalak TI, van Marle G, Gill MJ. Virological characteristics of occult hepatitis B virus in a North American cohort of human immunodeficiency virus type 1-positive patients on dual active anti-HBV/HIV therapy. *J Clin Virol* 2014; **60**: 347-353 [PMID: 24881491 DOI: 10.1016/j.jcv.2014.04.021]
 - 77 **Dapena M**, Figueras C, Noguera-Julian A, Fortuny C, de José MI, Mellado MJ, Gavilán C, Falcón-Neyra MD, Navarro ML, de Ory SJ, López C, Mayol L, Méndez M, Ciria LM, Coll MT, García L, Nuñez E, Espiau M, Soler-Palacín P. Implementation of occult hepatitis screening in the Spanish cohort of HIV-infected pediatric patients. *Pediatr Infect Dis J* 2013; **32**: e377-e379 [PMID: 23446444 DOI: 10.1097/INF.0b013e31828e9b99]
 - 78 **Khamduang W**, Ngo-Giang-Huong N, Gaudy-Graffin C, Jourdain G, Suwankornsakul W, Jarupanich T, Chalermprapra V, Nanta S, Puarattana-Aroonkorn N, Tonmat S, Lallemand M, Goudeau A, Sirirungsri W. Prevalence, risk factors, and impact of isolated antibody to hepatitis B core antigen and occult hepatitis B virus infection in HIV-1-infected pregnant women. *Clin Infect Dis* 2013; **56**: 1704-1712 [PMID: 23487379 DOI: 10.1093/cid/cit166]
 - 79 **Bell TG**, Makondo E, Martinson NA, Kramvis A. Hepatitis B virus infection in human immunodeficiency virus infected southern African adults: occult or overt--that is the question. *PLoS One* 2012; **7**: e45750 [PMID: 23049685 DOI: 10.1371/journal.pone.0045750]
 - 80 **Panigrahi R**, Majumder S, Goopu M, Biswas A, Datta S, Chandra PK, Banerjee A, Chakrabarti S, Bandopadhyay D, De BK, Chakravarty R. Occult HBV infection among anti-HBc positive HIV-infected patients in apex referral centre, Eastern India. *Ann Hepatol* 2012; **11**: 870-875 [PMID: 23109450]
 - 81 **Bagaglio S**, Bianchi G, Danise A, Porrino L, Uberti-Foppa C, Lazzarin A, Castagna A, Morsica G. Longitudinal evaluation of occult hepatitis B infection in HIV-1 infected individuals during highly active antiretroviral treatment interruption and after HAART resumption. *Infection* 2011; **39**: 121-126 [PMID: 21424854 DOI: 10.1007/s15010-011-0093-9]
 - 82 **Gupta S**, Singh S. Occult hepatitis B virus infection in ART-naïve HIV-infected patients seen at a tertiary care centre in north India. *BMC Infect Dis* 2010; **10**: 53 [PMID: 20205948 DOI: 10.1186/1471-2334-10-53]
 - 83 **Hakeem L**, Thomson G, McCleary E, Bhattacharyya D, Banerjee I. Prevalence and Immunization Status of Hepatitis B Virus in the HIV Cohort in Fife, Scotland. *J Clin Med Res* 2010; **2**: 34-38 [PMID: 22457699 DOI: 10.4021/jocmr.12.1282]
 - 84 **Morsica G**, Ancarani F, Bagaglio S, Maracci M, Cicconi P, Cozzi Lepri A, Antonucci G, Bruno R, Santantonio T, Tacconi L, Baldelli F, Piscopo R, Santoro D, Lazzarin A, D'Arminio Monforte A. Occult hepatitis B virus infection in a cohort of HIV-positive patients: correlation with hepatitis C virus coinfection, virological and immunological features. *Infection* 2009; **37**: 445-449 [PMID: 19669092 DOI: 10.1007/s15010-008-8194-9]
 - 85 **Azadmanesh K**, Mohraz M, Aghakhani A, Edalat R, Jam S, Eslamifar A, Banifazl M, Moradmamand-Badie B, Ramezani A. Occult hepatitis B virus infection in HIV-infected patients with isolated hepatitis B core antibody. *Intervirology* 2008; **51**: 270-274 [PMID: 18841029 DOI: 10.1159/000160217]
 - 86 **Tsui JI**, French AL, Seaberg EC, Augenbraun M, Nowicki M, Peters M, Tien PC. Prevalence and long-term effects of occult hepatitis B virus infection in HIV-infected women. *Clin Infect Dis* 2007; **45**: 736-740 [PMID: 17712758 DOI: 10.1086/520989]
 - 87 **Candotti D**, Allain JP. Transfusion-transmitted hepatitis B virus infection. *J Hepatol* 2009; **51**: 798-809 [PMID: 19615780 DOI: 10.1016/j.jhep.2009.05.020]
 - 88 **Allain JP**, Candotti D. Diagnostic algorithm for HBV safe transfusion. *Blood Transfus* 2009; **7**: 174-182 [PMID: 19657480]

DOI: 10.2450/2008.0062-08]

- 89 **Yoshikawa A**, Gotanda Y, Minegishi K, Taira R, Hino S, Tadokoro K, Ohnuma H, Miyakawa K, Tachibana K, Mizoguchi H. Lengths of hepatitis B viremia and antigenemia in blood donors: preliminary evidence of occult (hepatitis B surface antigen-negative) infection in the acute stage. *Transfusion* 2007; **47**: 1162-1171 [PMID: 17581150 DOI: 10.1111/j.1537-2995.2007]
- 90 **González R**, Torres P, Castro E, Barbolla L, Candotti D, Koppelman M, Zaaijer HL, Lelie N, Allain JP, Echevarría JM. Efficacy of hepatitis B virus (HBV) DNA screening and characterization of acute and occult HBV infections among blood donors from Madrid, Spain. *Transfusion* 2010; **50**: 221-230 [PMID: 19682332 DOI: 10.1111/j.1537-2995.2009]
- 91 **Seo DH**, Whang DH, Song EY, Kim HS, Park Q. Prevalence of antibodies to hepatitis B core antigen and occult hepatitis B virus infections in Korean blood donors. *Transfusion* 2011; **51**: 1840-1846 [PMID: 21332731 DOI: 10.1111/j.1537-2995.2010.03056.x]
- 92 **Makroo RN**, Chowdhry M, Bhatia A, Arora B, Rosamma NL. Hepatitis B core antibody testing in Indian blood donors: A double-edged sword! *Asian J Transfus Sci* 2012; **6**: 10-13 [PMID: 22623835 DOI: 10.4103/0973-6247.95043]
- 93 **Findik D**, Arslan U, Baykan M. Determination of hepatitis B virus DNA incidence, viral load, and mutations in blood donors with HBsAg and anti-HBs-negative serology and antibodies to hepatitis B core antigen. *Eur J Intern Med* 2007; **18**: 571-575 [PMID: 18054706 DOI: 10.1016/j.ejim.2007.07.001]
- 94 **Kishk R**, Nemr N, Elkady A, Mandour M, Aboelmagd M, Ramsis N, Hassan M, Soliman N, Iijima S, Murakami S, Tanaka Y, Ragheb M. Hepatitis B surface gene variants isolated from blood donors with overt and occult HBV infection in north eastern Egypt. *Virol J* 2015; **12**: 153 [PMID: 26420301]
- 95 **Sofian M**, Aghakhani A, Izadi N, Banifazl M, Kalantar E, Eslamifard A, Ramezani A. Lack of occult hepatitis B virus infection among blood donors with isolated hepatitis B core antibody living in an HBV low prevalence region of Iran. *Int J Infect Dis* 2010; **14**: e308-e310 [PMID: 19656713]
- 96 **Behzad-Behbahani A**, Mafi-Nejad A, Tabei SZ, Lankarani KB, Torab A, Moaddab A. Anti-HBc & amp; HBV-DNA detection in blood donors negative for hepatitis B virus surface antigen in reducing risk of transfusion associated HBV infection. *Indian J Med Res* 2006; **123**: 37-42 [PMID: 16567866]
- 97 **Hollinger FB**, Sood G. Occult hepatitis B virus infection: a covert operation. *J Viral Hepat* 2010; **17**: 1-15 [PMID: 20002296 DOI: 10.1111/j.1365-2893]
- 98 **Meidani M**, Rostami M, Hemmati S, Ashrafi F, Gholamnezhad M, Emadi M, Ghasemian R, Ahmadian M. Screening and evaluation of chronic and occult Hepatitis B in chemo - radiotherapy patients with cancer. *Adv Biomed Res* 2016; **5**: 85 [PMID: 27274500 DOI: 10.4103/2277-9175.182216]
- 99 **Said ZN**, El-Sayed MH, El-Bishbishi IA, El-Fouhil DF, Abdel-Rheem SE, El-Abedin MZ, Salama II. High prevalence of occult hepatitis B in hepatitis C-infected Egyptian children with haematological disorders and malignancies. *Liver Int* 2009; **29**: 518-524 [PMID: 19192168 DOI: 10.1111/j.1478-3231.2009.01975.x]
- 100 **El-Sherif AM**, Abou-Shady MA, Al-Hiatmy MA, Al-Bahrawy AM, Motawea EA. Screening for hepatitis B virus infection in Egyptian blood donors negative for hepatitis B surface antigen. *Hepatol Int* 2007; **1**: 469-470 [PMID: 19669344 DOI: 10.1007/s12072-007-9017-2]
- 101 **Sowole L**, Labbett W, Patel M, O'Riordan A, Cross J, Davenport A, Haque T. The prevalence of occult hepatitis B virus (HBV) infection in a large multi-ethnic haemodialysis cohort. *BMC Nephrol* 2015; **16**: 12 [PMID: 25884422 DOI: 10.1186/s12882-015-0010-z]
- 102 **Marinaki S**, Boletis JN, Sakellariou S, Delladetsima IK. Hepatitis C in hemodialysis patients. *World J Hepatol* 2015; **7**: 548-558 [PMID: 25848478 DOI: 10.4254/wjh.v7.i3.548]
- 103 **Lucas GM**, Ross MJ, Stock PG, Shlipak MG, Wyatt CM, Gupta SK, Atta MG, Wools-Kaloustian KK, Pham PA, Bruggeman LA, Lennox JL, Ray PE, Kalayjian RC. Clinical practice guideline for the management of chronic kidney disease in patients infected with HIV: 2014 update by the HIV Medicine Association of the Infectious Diseases Society of America. *Clin Infect Dis* 2014; **59**: e96-138 [PMID: 25234519 DOI: 10.1093/cid/ciu617]
- 104 **Yakaryilmaz F**, Gurbuz OA, Guliter S, Mert A, Songur Y, Karakan T, Keles H. Prevalence of occult hepatitis B and hepatitis C virus infections in Turkish hemodialysis patients. *Ren Fail* 2006; **28**: 729-735 [PMID: 17162434 DOI: 10.1080/08860220600925602]
- 105 **Fabrizi F**, Lunghi G, Martin P. Hepatitis B virus infection in hemodialysis: recent discoveries. *J Nephrol* 2002; **15**: 463-468 [PMID: 12455711]
- 106 **Fabrizi F**, Poordad FF, Martin P. Hepatitis C infection and the patient with end-stage renal disease. *Hepatology* 2002; **36**: 3-10 [PMID: 12085342 DOI: 10.1053/jhep.2002.34613]
- 107 **Ramezani A**, Aghasadeghi MR, Ahmadi F, Razeghi E, Eslamifard A, Banifazl M, Sofian M, Bahramali G, Hekmat S, Aghakhani A. Isolated anti-hbc and occult HBV infection in dialysis patients. *Nephrourol Mon* 2015; **7**: e22674 [PMID: 25738121 DOI: 10.5812/numonthly.22674]
- 108 **Aghakhani A**, Banifazl M, Kalantar E, Eslamifard A, Ahmadi F, Razeghi E, Atabak S, Amini M, Khadem-Sadegh A, Ramezani A. Occult hepatitis B virus infection in hemodialysis patients with isolated hepatitis B core antibody: a multicenter study. *Ther Apher Dial* 2010; **14**: 349-353 [PMID: 20609190 DOI: 10.1111/j.1744-9987]
- 109 **Neisi N**, Makvandi M, Samarbarf-Zadeh AR. A study on genotypes of hepatitis B virus among hemodialysis patients in Khuzestan province. *Jundishapur J Microbiol* 2011; **4**: 65-70
- 110 **Rastegarvand N**, Makvandi M, Samarbarfzadeh A, Rasti M, Neisi N, Pouremamali A, Teimoori A, Shabani A. Molecular Characterization of Pre-Core/Core and S Region of Hepatitis B Virus in Hemodialysis Patients With Occult Hepatitis B Infection. *Jundishapur J Microbiol* 2015; **8**: e23686 [PMID: 26587212 DOI: 10.5812/jjm.23686]
- 111 **Cabrero M**, Bartolomé J, De Sequera P, Caramelo C, Carreño V. Hepatitis B virus DNA in serum and blood cells of hepatitis B surface antigen-negative hemodialysis patients and staff. *J Am Soc Nephrol* 1997; **8**: 1443-1447 [PMID: 9294837]
- 112 **Siagris D**, Christofidou M, Triga K, Pagoni N, Theocharis GJ, Goumenos D, Lekkou A, Thomopoulos K, Tsamandas AC, Vlachojannis J, Labropoulou-Karatza C. Occult hepatitis B virus infection in hemodialysis patients with chronic HCV infection. *J Nephrol* 2006; **19**: 327-333 [PMID: 16874693]
- 113 **Fabrizi F**, Messa PG, Lunghi G, Aucella F, Bisegna S, Mangano S, Villa M, Barbisoni F, Rusconi E, Martin P. Occult hepatitis B virus infection in dialysis patients: a multicentre survey. *Aliment Pharmacol Ther* 2005; **21**: 1341-1347 [PMID: 15932364 DOI: 10.1111/j.1365-2036]
- 114 **Makvandi M**, Neisi N, Khalafkhany D, Makvandi K, Hajiani E, Shayesteh AA, Masjedi Zadeh A, Sina AH, Hamidifard M, Rasti M, Aryan E, Ahmadi K, Yad Yad MJ. Occult hepatitis B virus among the patients with abnormal alanine transaminase. *Jundishapur J Microbiol* 2014; **7**: e11648 [PMID: 25485052 DOI: 10.5812/jjm.11648]
- 115 **Makvandi M**, Khalafkhany D, Rasti M, Neisi N, Omidvarinia A, Mirghaed AT, Masjedizadeh A, Shyesteh AA. Detection of Hepatitis C virus RNA in peripheral blood mononuclear cells of patients with abnormal alanine transaminase in Ahvaz. *Indian J Med Microbiol* 2014; **32**: 251-255 [PMID: 25008816 DOI: 10.4103/0255-0857.136553]
- 116 **Saitta C**, Tripodi G, Barbera A, Bertuccio A, Smedile A, Ciancio A, Raffa G, Sangiovanni A, Navarra G, Raimondo G, Pollicino T. Hepatitis B virus (HBV) DNA integration in patients with occult HBV infection and hepatocellular carcinoma. *Liver Int* 2015; **35**: 2311-2317 [PMID: 25677098 DOI: 10.1111/liv.12807]
- 117 **Hou J**, Wang Z, Cheng J, Lin Y, Lau GK, Sun J, Zhou F, Waters J, Karayiannis P, Luo K. Prevalence of naturally occurring surface gene variants of hepatitis B virus in nonimmunized surface antigen-negative Chinese carriers. *Hepatology* 2001; **34**: 1027-1034 [PMID:

- 11679975 DOI: 10.1053/jhep.2001.28708]
- 118 **Youssef A**, Yano Y, Utsumi T, abd El-alah EM, abd El-Hameed Ael-E, Serwah Ael-H, Hayashi Y. Molecular epidemiological study of hepatitis viruses in Ismailia, Egypt. *Intervirology* 2009; **52**: 123-131 [PMID: 19468235 DOI: 10.1159/000219385]
 - 119 **Ferrari TC**, Xavier MA, Vidigal PV, Amaral NS, Diniz PA, Resende AP, Miranda DM, Faria AC, Lima AS, Faria LC. Occult hepatitis B virus infection in liver transplant patients in a Brazilian referral center. *Braz J Med Biol Res* 2014; **47**: 990-994 [PMID: 25296362 DOI: 10.1590/1414-431X20143782]
 - 120 **Fang Y**, Shang QL, Liu JY, Li D, Xu WZ, Teng X, Zhao HW, Fu LJ, Zhang FM, Gu HX. Prevalence of occult hepatitis B virus infection among hepatopathy patients and healthy people in China. *J Infect* 2009; **58**: 383-388 [PMID: 19329189 DOI: 10.1016/j.jinf.2009.02.013]
 - 121 **Kaviani MJ**, Behbahani B, Mosallai MJ, Sari-Aslani F, Taghavi SA. Occult hepatitis B virus infection and cryptogenic chronic hepatitis in an area with intermediate prevalence of HBV infection. *World J Gastroenterol* 2006; **12**: 5048-5050 [PMID: 16937504 DOI: 10.3748/wjg.v12.i31.5048]
 - 122 **Srivastava A**, Mathias A, Yachha SK, Aggarwal R. Occult hepatitis B infection in children with chronic liver disease. *Eur J Gastroenterol Hepatol* 2015; **27**: 375-377 [PMID: 25874508 DOI: 10.1097/MEG.0000000000000294]
 - 123 **Ansari N**, Makvandi M, Samarbaft-Zadeh AR. Hepatitis B virus genotyping among patients with cirrhosis. *Jundishapur J Microbiol* 2015; **8**: e14571 [PMID: 25964845 DOI: 10.5812/jjm.14571]
 - 124 **Carreño V**. Seronegative occult hepatitis C virus infection: clinical implications. *J Clin Virol* 2014; **61**: 315-320 [PMID: 25304062 DOI: 10.1016/j.jcv.2014.09.007]
 - 125 **Rasineni K**, Penrice DD, Natarajan SK, McNiven MA, McVicker BL, Kharbada KK, Casey CA, Harris EN. Alcoholic vs non-alcoholic fatty liver in rats: distinct differences in endocytosis and vesicle trafficking despite similar pathology. *BMC Gastroenterol* 2016; **16**: 27 [PMID: 26924554 DOI: 10.1186/s12876-016-0433-4]
 - 126 **Chu CM**, Liaw YF. HBsAg seroclearance in asymptomatic carriers of high endemic areas: appreciably high rates during a long-term follow-up. *Hepatology* 2007; **45**: 1187-1192 [PMID: 17465003 DOI: 10.1002/hep.21612]
 - 127 **McMahon BJ**, Holck P, Bulkow L, Snowball M. Serologic and clinical outcomes of 1536 Alaska Natives chronically infected with hepatitis B virus. *Ann Intern Med* 2001; **135**: 759-768 [PMID: 11694101 DOI: 10.7326/0003-4819-135-9-200111060-00006]
 - 128 **Liaw YF**, Tai DI, Chu CM, Chen TJ. The development of cirrhosis in patients with chronic type B hepatitis: a prospective study. *Hepatology* 1988; **8**: 493-496 [PMID: 3371868 DOI: 10.1002/hep.1840080310]
 - 129 **Weissberg JI**, Andres LL, Smith CI, Weick S, Nichols JE, Garcia G, Robinson WS, Merigan TC, Gregory PB. Survival in chronic hepatitis B. An analysis of 379 patients. *Ann Intern Med* 1984; **101**: 613-616 [PMID: 6486592 DOI: 10.7326/0003-4819-101-5-613]
 - 130 **Anvari FA**, Alavian SM, Norouzi M, Mahabadi M, Jazayeri SM. Prevalence and molecular analysis of occult hepatitis B virus infection isolated in a sample of cryptogenic cirrhosis patients in Iran. *Oman Med J* 2014; **29**: 92-96 [PMID: 24715933]
 - 131 **Agarwal N**, Naik S, Aggarwal R, Singh H, Somani SK, Kini D, Pandey R, Choudhuri G, Saraswat VA, Naik SR. Occult hepatitis B virus infection as a cause of cirrhosis of liver in a region with intermediate endemicity. *Indian J Gastroenterol* 2003; **22**: 127-131 [PMID: 12962434]
 - 132 **Squadrito G**, Cacciola I, Alibrandi A, Pollicino T, Raimondo G. Impact of occult hepatitis B virus infection on the outcome of chronic hepatitis C. *J Hepatol* 2013; **59**: 696-700 [PMID: 23751755 DOI: 10.1016/j.jhep.2013.05.043]
 - 133 **Sagnelli E**, Imperato M, Coppola N, Pisapia R, Sagnelli C, Messina V, Piai G, Stanzione M, Bruno M, Moggio G, Caprio N, Pasquale G, Del Vecchio Blanco C. Diagnosis and clinical impact of occult hepatitis B infection in patients with biopsy proven chronic hepatitis C: a multicenter study. *J Med Virol* 2008; **80**: 1547-1553 [PMID: 18649338 DOI: 10.1002/jmv.21239]
 - 134 **Emara MH**, El-Gammal NE, Mohamed LA, Bahgat MM. Occult hepatitis B infection in Egyptian chronic hepatitis C patients: prevalence, impact on pegylated interferon/ribavirin therapy. *Virol J* 2010; **7**: 324 [PMID: 21083926 DOI: 10.1186/1743-422X-7-324]
 - 135 **Matsuoka S**, Nirei K, Tamura A, Nakamura H, Matsumura H, Oshiro S, Arakawa Y, Yamagami H, Tanaka N, Moriyama M. Influence of occult hepatitis B virus coinfection on the incidence of fibrosis and hepatocellular carcinoma in chronic hepatitis C. *Intervirology* 2008; **51**: 352-361 [PMID: 19127078 DOI: 10.1159/000187720]
 - 136 **Mrani S**, Chemin I, Menouar K, Guillaud O, Pradat P, Borghi G, Traubad MA, Chevallier P, Chevallier M, Zoulim F, Trépo C. Occult HBV infection may represent a major risk factor of non-response to antiviral therapy of chronic hepatitis C. *J Med Virol* 2007; **79**: 1075-1081 [PMID: 17596829]
 - 137 **Hui CK**, Lau E, Wu H, Monto A, Kim M, Luk JM, Lau GK, Wright TL. Fibrosis progression in chronic hepatitis C patients with occult hepatitis B co-infection. *J Clin Virol* 2006; **35**: 185-192 [PMID: 16103008]
 - 138 **Silva CD**, Gonçalves NS, Pereira JS, Escanhoela CA, Pavan MH, Gonçalves FL. The influence of occult infection with hepatitis B virus on liver histology and response to interferon treatment in chronic hepatitis C patients. *Braz J Infect Dis* 2004; **8**: 431-439 [PMID: 15880234]
 - 139 **Chan HL**, Tsang SW, Leung NW, Tse CH, Hui Y, Tam JS, Chan FK, Sung JJ. Occult HBV infection in cryptogenic liver cirrhosis in an area with high prevalence of HBV infection. *Am J Gastroenterol* 2002; **97**: 1211-1215 [PMID: 12014730 DOI: 10.1111/j.1572-0241.2002.05706.x]
 - 140 **Germanidis G**, Htiroglou P, Zakalka M, Settas L. Reactivation of occult hepatitis B virus infection, following treatment of refractory rheumatoid arthritis with abatacept. *J Hepatol* 2012; **56**: 1420-1421 [PMID: 22127282 DOI: 10.1016/j.jhep.2011.10.011]
 - 141 **Martin CM**, Welge JA, Shire NJ, Shata MT, Sherman KE, Blackard JT. Cytokine expression during chronic versus occult hepatitis B virus infection in HIV co-infected individuals. *Cytokine* 2009; **47**: 194-198 [PMID: 19625194 DOI: 10.1016/j.cyto.2009.06.005]
 - 142 **Uchida T**, Saitoh T, Shinzawa H. Mutations of the X region of hepatitis B virus and their clinical implications. *Pathol Int* 1997; **47**: 183-193 [PMID: 9103208]
 - 143 **Tan YJ**. Hepatitis B virus infection and the risk of hepatocellular carcinoma. *World J Gastroenterol* 2011; **17**: 4853-4857 [PMID: 22171125 DOI: 10.3748/wjg.v17.i44.4853]
 - 144 **Wong DK**, Yuen MF, Poon RT, Yuen JC, Fung J, Lai CL. Quantification of hepatitis B virus covalently closed circular DNA in patients with hepatocellular carcinoma. *J Hepatol* 2006; **45**: 553-559 [PMID: 16904225 DOI: 10.1016/j.jhep.2006.05.014]
 - 145 **Raimondo G**, Burk RD, Lieberman HM, Muschel J, Hadziyannis SJ, Will H, Kew MC, Dusheiko GM, Shafritz DA. Interrupted replication of hepatitis B virus in liver tissue of HBsAg carriers with hepatocellular carcinoma. *Virology* 1988; **166**: 103-112 [PMID: 2842938]
 - 146 **Shi Y**, Wu YH, Wu W, Zhang WJ, Yang J, Chen Z. Association between occult hepatitis B infection and the risk of hepatocellular carcinoma: a meta-analysis. *Liver Int* 2012; **32**: 231-240 [PMID: 21745272 DOI: 10.1111/j.1478-3231.2011.02481.x]
 - 147 **Shafritz DA**, Shouval D, Sherman HI, Hadziyannis SJ, Kew MC. Integration of hepatitis B virus DNA into the genome of liver cells in chronic liver disease and hepatocellular carcinoma. Studies in percutaneous liver biopsies and post-mortem tissue specimens. *N Engl J Med* 1981; **305**: 1067-1073 [PMID: 6268980 DOI: 10.1056/NEJM198110293051807]
 - 148 **Paterlini P**, Driss F, Nalpas B, Pisi E, Franco D, Berthelot P, Bréchet C. Persistence of hepatitis B and hepatitis C viral genomes in primary liver cancers from HBsAg-negative patients: a study of a low-endemic area. *Hepatology* 1993; **17**: 20-29 [PMID: 8380790 DOI: 10.1002/hep.1840170106]
 - 149 **Paterlini P**, Poussin K, Kew M, Franco D, Brechet C. Selective accumulation of the X transcript of hepatitis B virus in patients negative for hepatitis B surface antigen with hepatocellular

- carcinoma. *Hepatology* 1995; **21**: 313-321 [PMID: 7843699]
- 150 **Poussin K**, Dienes H, Sirma H, Urban S, Beaugrand M, Franco D, Schirmacher P, Bréchot C, Paterlini Bréchot P. Expression of mutated hepatitis B virus X genes in human hepatocellular carcinomas. *Int J Cancer* 1999; **80**: 497-505 [PMID: 9935147 DOI: 10.1002/(SICI)1097-0215(19990209)80]
 - 151 **Bläckberg J**, Kidd-Ljunggren K. Occult hepatitis B virus after acute self-limited infection persisting for 30 years without sequence variation. *J Hepatol* 2000; **33**: 992-997 [PMID: 11131464 DOI: 10.1016/S0168-8278(00)80134-8]
 - 152 **Mulrooney-Cousins PM**, Chauhan R, Churchill ND, Michalak TI. Primary seronegative but molecularly evident hepadnaviral infection engages liver and induces hepatocarcinoma in the woodchuck model of hepatitis B. *PLoS Pathog* 2014; **10**: e1004332 [PMID: 25165821 DOI: 10.1371/journal.ppat.1004332]
 - 153 **Pollicino T**, Vegetti A, Saitta C, Ferrara F, Corradini E, Raffa G, Pietrangelo A, Raimondo G. Hepatitis B virus DNA integration in tumour tissue of a non-cirrhotic HFE-haemochromatosis patient with hepatocellular carcinoma. *J Hepatol* 2013; **58**: 190-193 [PMID: 22989571 DOI: 10.1016/j.jhep.2012.09.005]
 - 154 **Tamori A**, Nishiguchi S, Kubo S, Koh N, Moriyama Y, Fujimoto S, Takeda T, Shiomi S, Hirohashi K, Kinoshita H, Otani S, Kuroki T. Possible contribution to hepatocarcinogenesis of X transcript of hepatitis B virus in Japanese patients with hepatitis C virus. *Hepatology* 1999; **29**: 1429-1434 [PMID: 10216126 DOI: 10.1002/hep.510290520]
 - 155 **Squadrito G**, Pollicino T, Cacciola I, Caccamo G, Villari D, La Masa T, Restuccia T, Cucinotta E, Scisca C, Magazzu D, Raimondo G. Occult hepatitis B virus infection is associated with the development of hepatocellular carcinoma in chronic hepatitis C patients. *Cancer* 2006; **106**: 1326-1330 [PMID: 16453330 DOI: 10.1002/cncr.21702]
 - 156 **Ikeda K**, Marusawa H, Osaki Y, Nakamura T, Kitajima N, Yamashita Y, Kudo M, Sato T, Chiba T. Antibody to hepatitis B core antigen and risk for hepatitis C-related hepatocellular carcinoma: a prospective study. *Ann Intern Med* 2007; **146**: 649-656 [PMID: 17470833 DOI: 10.7326/0003-4819-146-9-2007]
 - 157 **Kitab B**, Ezzikouri S, Alaoui R, Nadir S, Badre W, Trepo C, Chemin I, Benjelloun S. Occult HBV infection in Morocco: from chronic hepatitis to hepatocellular carcinoma. *Liver Int* 2014; **34**: e144-e150 [PMID: 24502524 DOI: 10.1111/liv.12482]
 - 158 **Chen CH**, Changchien CS, Lee CM, Tung WC, Hung CH, Hu TH, Wang JH, Wang JC, Lu SN. A study on sequence variations in pre-S/surface, X and enhancer II/core promoter/precore regions of occult hepatitis B virus in non-B, non-C hepatocellular carcinoma patients in Taiwan. *Int J Cancer* 2009; **125**: 621-629 [PMID: 19431214 DOI: 10.1002/ijc.24416]
 - 159 **Lee SB**, Kim KM, An J, Lee D, Shim JH, Lim YS, Lee HC, Chung YH, Lee YS. Clinical characteristics and potential aetiologies of non-B non-C hepatocellular carcinoma in hepatitis B virus endemic area. *Liver Int* 2016; **36**: 1351-1361 [PMID: 26913702 DOI: 10.1111/liv.13099]
 - 160 **Kondo R**, Nakashima O, Sata M, Imazeki F, Yokosuka O, Tanikawa K, Kage M, Yano H. Pathological characteristics of patients who develop hepatocellular carcinoma with negative results of both serous hepatitis B surface antigen and hepatitis C virus antibody. *Hepatol Res* 2014; **44**: 1039-1046 [PMID: 23937266 DOI: 10.1111/hepr.12219]
 - 161 **Pollicino T**, Saitta C, Raimondo G. Hepatocellular carcinoma: the point of view of the hepatitis B virus. *Carcinogenesis* 2011; **32**: 1122-1132 [PMID: 21665892 DOI: 10.1093/carcin/bgr108]
 - 162 **Pan J**, Clayton M, Feitelson MA. Hepatitis B virus X antigen promotes transforming growth factor-beta1 (TGF-beta1) activity by up-regulation of TGF-beta1 and down-regulation of alpha2-macroglobulin. *J Gen Virol* 2004; **85**: 275-282 [PMID: 14769885 DOI: 10.1099/vir.0.19650-0]
 - 163 **Fallot G**, Neuveut C, Bendia MA. Diverse roles of hepatitis B virus in liver cancer. *Curr Opin Virol* 2012; **2**: 467-473 [PMID: 22722078 DOI: 10.1016/j.coviro.2012.05.008]
 - 164 **Wong DK**, Huang FY, Lai CL, Poon RT, Seto WK, Fung J, Hung IF, Yuen MF. Occult hepatitis B infection and HBV replicative activity in patients with cryptogenic cause of hepatocellular carcinoma. *Hepatology* 2011; **54**: 829-836 [PMID: 21809355 DOI: 10.1002/hep.24551]
 - 165 **Gozuacik D**, Murakami Y, Saigo K, Chami M, Mugnier C, Lagorce D, Okanoue T, Urashima T, Bréchot C, Paterlini-Bréchot P. Identification of human cancer-related genes by naturally occurring Hepatitis B Virus DNA tagging. *Oncogene* 2001; **20**: 6233-6240 [PMID: 11593432 DOI: 10.1038/sj.onc.1204835]
 - 166 **García-Fulgueiras A**, García-Pina R, Morant C, García-Ortuzar V, Génova R, Alvarez E. Hepatitis C and hepatitis B-related mortality in Spain. *Eur J Gastroenterol Hepatol* 2009; **21**: 895-901 [PMID: 19357523 DOI: 10.1097/MEG.0b013e328313139d]
 - 167 **Marcellin P**, Peignot F, Delarocque-Astagneau E, Zarski JP, Ganne N, Hillon P, Antona D, Bovet M, Mechain M, Asselah T, Desenclos JC, Jougla E. Mortality related to chronic hepatitis B and chronic hepatitis C in France: evidence for the role of HIV coinfection and alcohol consumption. *J Hepatol* 2008; **48**: 200-207 [PMID: 18086507 DOI: 10.1016/j.jhep.2007.09.010]
 - 168 **Xie M**, Rao W, Yang T, Deng Y, Zheng H, Pan C, Liu Y, Shen Z, Jia J. Occult hepatitis B virus infection predicts de novo hepatitis B infection in patients with alcoholic cirrhosis after liver transplantation. *Liver Int* 2015; **35**: 897-904 [PMID: 24750566 DOI: 10.1111/liv.12567]
 - 169 **Cholongitas E**, Papatheodoridis GV, Burroughs AK. Liver grafts from anti-hepatitis B core positive donors: a systematic review. *J Hepatol* 2010; **52**: 272-279 [PMID: 20034693 DOI: 10.1016/j.jhep.2009.11.009]
 - 170 **Dickson RC**, Everhart JE, Lake JR, Wei Y, Seaberg EC, Wiesner RH, Zetterman RK, Pruett TL, Ishitani MB, Hoofnagle JH. Transmission of hepatitis B by transplantation of livers from donors positive for antibody to hepatitis B core antigen. The National Institute of Diabetes and Digestive and Kidney Diseases Liver Transplantation Database. *Gastroenterology* 1997; **113**: 1668-1674 [PMID: 9352871]
 - 171 **Franz C**, Perez Rde M, Zalis MG, Zalona AC, Rocha PT, Gonçalves RT, Nabuco LC, Villela-Nogueira CA. Prevalence of occult hepatitis B virus infection in kidney transplant recipients. *Mem Inst Oswaldo Cruz* 2013; **108**: 657-660 [PMID: 23903984 DOI: 10.1590/0074-0276108052013019]
 - 172 **Zhang YJ**, Li H, Wu HC, Shen J, Wang L, Yu MW, Lee PH, Bernard Weinstein I, Santella RM. Silencing of Hint1, a novel tumor suppressor gene, by promoter hypermethylation in hepatocellular carcinoma. *Cancer Lett* 2009; **275**: 277-284 [PMID: 19081673 DOI: 10.1016/j.canlet.2008.10.042]
 - 173 **Samal J**, Kandpal M, Vivekanandan P. Molecular mechanisms underlying occult hepatitis B virus infection. *Clin Microbiol Rev* 2012; **25**: 142-163 [PMID: 22232374 DOI: 10.1128/CMR.00018-11]
 - 174 **Fang S**, Huang SF, Cao J, Wen YA, Zhang LP, Ren GS. Silencing of PCDH10 in hepatocellular carcinoma via de novo DNA methylation independent of HBV infection or HBX expression. *Clin Exp Med* 2013; **13**: 127-134 [PMID: 22543497 DOI: 10.1007/s10238-012-0182-9]
 - 175 **Vivekanandan P**, Thomas D, Torbenson M. Methylation regulates hepatitis B viral protein expression. *J Infect Dis* 2009; **199**: 1286-1291 [PMID: 19301974 DOI: 10.1086/597614]
 - 176 **Han YF**, Zhao J, Ma LY, Yin JH, Chang WJ, Zhang HW, Cao GW. Factors predicting occurrence and prognosis of hepatitis-B-virus-related hepatocellular carcinoma. *World J Gastroenterol* 2011; **17**: 4258-4270 [PMID: 22090781 DOI: 10.3748/wjg.v17.i38.4258]
 - 177 **Johnstone RW**, Licht JD. Histone deacetylase inhibitors in cancer therapy: is transcription the primary target? *Cancer Cell* 2003; **4**: 13-18 [PMID: 12892709 DOI: 10.1016/S1535-6108(03)00165-X]
 - 178 **Pontisso P**, Vidalino L, Quarta S, Gatta A. Biological and clinical implications of HBV infection in peripheral blood mononuclear cells. *Autoimmun Rev* 2008; **8**: 13-17 [PMID: 18706529 DOI: 10.1016/j.autrev.2008.07.016]
 - 179 **Wang F**, Yuan S, Teng KY, García-Prieto C, Luo HY, Zeng MS, Rao HL, Xia Y, Jiang WQ, Huang HQ, Xia ZJ, Sun XF, Xu RH. High hepatitis B virus infection in B-cell lymphoma tissue and its

- potential clinical relevance. *Eur J Cancer Prev* 2012; **21**: 261-267 [PMID: 22433629 DOI: 10.1097/CEJ.0b013e3283498e87]
- 180 **Engels EA**, Cho ER, Jee SH. Hepatitis B virus infection and risk of non-Hodgkin lymphoma in South Korea: a cohort study. *Lancet Oncol* 2010; **11**: 827-834 [PMID: 20688564 DOI: 10.1016/S1470-2045(10)70167-4]
 - 181 **Ulcickas Yood M**, Quesenberry CP, Guo D, Caldwell C, Wells K, Shan J, Sanders L, Skovron ML, Iloeje U, Manos MM. Incidence of non-Hodgkin's lymphoma among individuals with chronic hepatitis B virus infection. *Hepatology* 2007; **46**: 107-112 [PMID: 17526021 DOI: 10.1002/hep.21642]
 - 182 **Elbedewy TA**, Elashtokhy HE, Rabee ES, Kheder GE. Prevalence and chemotherapy-induced reactivation of occult hepatitis B virus among hepatitis B surface antigen negative patients with diffuse large B-cell lymphoma: significance of hepatitis B core antibodies screening. *J Egypt Natl Canc Inst* 2015; **27**: 11-18 [PMID: 25716703 DOI: 10.1016/j.jnci.2015.01.004]
 - 183 **Makvandi K**, Ranjbari N, Makvandi M, Ashraf Teimori A, Neisi N, Rasti M, Alipour V, Albokord M, Kanani M, Ahadi R, Habibi A. Study of the Association of Mutant HBsAg Gene and Hodgkin and Non-Hodgkin Lymphoma. *Jundishapur J Microbiol* 2015; **8**: e25726 [PMID: 26862382 DOI: 10.5812/jjm.25726]
 - 184 **Cheung WI**, Lin SY, Leung VK, Fung KS, Lam YK, Lo FH, Chau TN. Prospective evaluation of seropositive occult hepatitis B viral infection in lymphoma patients receiving chemotherapy. *Hong Kong Med J* 2011; **17**: 376-380 [PMID: 21979474]
 - 185 **Slusarczyk J**, Małkowski P, Bobilewicz D, Juszczak G. Cross-sectional, anonymous screening for asymptomatic HCV infection, immunity to HBV, and occult HBV infection among health care workers in Warsaw, Poland. *Przegl Epidemiol* 2012; **66**: 445-451 [PMID: 23230715]
 - 186 **US Public Health Service**. Updated U.S. Public Health Service Guidelines for the Management of Occupational Exposures to HBV, HCV, and HIV and Recommendations for Postexposure Prophylaxis. *MMWR Recomm Rep* 2001; **50**: 1-52 [PMID: 11442229]
 - 187 **Squadrito G**, Spinella R, Raimondo G. The clinical significance of occult HBV infection. *Ann Gastroenterol* 2014; **27**: 15-19 [PMID: 24714731]
 - 188 **Borzooy Z**, Jazayeri SM, Mirshafiey A, Khamseh A, Mahmoudie MK, Azimzadeh P, Geravand B, Boroumand MA, Afshar M, Poortahmasebi V, Hosseini M, Streinu-Cercel A. Identification of occult hepatitis B virus (HBV) infection and viral antigens in healthcare workers who presented low to moderate levels of anti-HBs after HBV vaccination. *Germs* 2015; **5**: 134-140 [PMID: 26716102 DOI: 10.1159/germs.2015.1081]
 - 189 **Chiarakul S**, Eunumjitkul K, Vorapimol AR, Kaewkungwal J, Chimparlee N, Poovorawan Y. Response of health care workers with isolated antibody to hepatitis B core antigen to hepatitis B vaccine. *Southeast Asian J Trop Med Public Health* 2011; **42**: 831-838 [PMID: 22299465]
 - 190 **Shim J**, Kim KY, Kim BH, Chun H, Lee MS, Hwangbo Y, Jang JY, Dong SH, Kim HJ, Chang YW, Chang R. Anti-hepatitis B core antibody is not required for prevaccination screening in healthcare workers. *Vaccine* 2011; **29**: 1721-1726 [PMID: 21147128 DOI: 10.1016/j.vaccine.2010.11.044]
 - 191 **Sukriti NT**, Sethi A, Agrawal K, Agrawal K, Kumar GT, Kumar M, Kaanan AT, Sarin SK. Low levels of awareness, vaccine coverage, and the need for boosters among health care workers in tertiary care hospitals in India. *J Gastroenterol Hepatol* 2008; **23**: 1710-1715 [PMID: 18761556 DOI: 10.1111/j.1440-1746.2008.05483]
 - 192 **Yen YH**, Chen CH, Wang JH, Lee CM, Changchien CS, Lu SN. Study of hepatitis B (HB) vaccine non-responsiveness among health care workers from an endemic area (Taiwan). *Liver Int* 2005; **25**: 1162-1168 [PMID: 16343067 DOI: 10.1111/j.1478-3231.2005]
 - 193 **Yeo W**, Chan TC, Leung NW, Lam WY, Mo FK, Chu MT, Chan HL, Hui EP, Lei KI, Mok TS, Chan PK. Hepatitis B virus reactivation in lymphoma patients with prior resolved hepatitis B undergoing anticancer therapy with or without rituximab. *J Clin Oncol* 2009; **27**: 605-611 [PMID: 19075267 DOI: 10.1200/JCO.2008.18.0182]
 - 194 **Coppola N**, Tonziello G, Pisaturo M, Messina V, Guastafierro S, Fiore M, Iodice V, Sagnelli C, Stanzone M, Capoluongo N, Pasquale G, Sagnelli E. Reactivation of overt and occult hepatitis B infection in various immunosuppressive settings. *J Med Virol* 2011; **83**: 1909-1916 [PMID: 21915865 DOI: 10.1002/jmv.22199]
 - 195 **Pei SN**, Chen CH, Lee CM, Wang MC, Ma MC, Hu TH, Kuo CY. Reactivation of hepatitis B virus following rituximab-based regimens: a serious complication in both HBsAg-positive and HBsAg-negative patients. *Ann Hematol* 2010; **89**: 255-262 [PMID: 19697028 DOI: 10.1007/s00277-009-0806-7]
 - 196 **Palmore TN**, Shah NL, Loomba R, Borg BB, Lopatin U, Feld JJ, Khokhar F, Lutchman G, Kleiner DE, Young NS, Childs R, Barrett AJ, Liang TJ, Hoofnagle JH, Heller T. Reactivation of hepatitis B with reappearance of hepatitis B surface antigen after chemotherapy and immunosuppression. *Clin Gastroenterol Hepatol* 2009; **7**: 1130-1137 [PMID: 19577007 DOI: 10.1016/j.cgh.2009.06.027]
 - 197 **Talotta R**, Atzeni F, Sarzi Puttini P. Reactivation of occult hepatitis B virus infection under treatment with abatacept: a case report. *BMC Pharmacol Toxicol* 2016; **17**: 17 [PMID: 27098382]
 - 198 **Chaulet P**. Tuberculosis: a six-month cure. *World Health Forum* 1989; **10**: 116-122 [PMID: 2665766 DOI: 10.1016/j.critrevonc.2015.10.017]
 - 199 **Filippini P**, Coppola N, Pisapia R, Scolastico C, Marrocco C, Zaccariello A, Nacca C, Sagnelli C, De Stefano G, Ferraro T, De Stefano C, Sagnelli E. Impact of occult hepatitis B virus infection in HIV patients naive for antiretroviral therapy. *AIDS* 2006; **20**: 1253-1260 [PMID: 16816553 DOI: 10.1097/01.aids.0000232232.41877.2a]
 - 200 **Kubo S**, Tamori A, Ohba K, Shuto T, Yamamoto T, Tanaka H, Nishiguchi S, Wakasa K, Hirohashi K, Kinoshita H. Previous or occult hepatitis B virus infection in hepatitis C virus-associated hepatocellular carcinoma without hepatic fibrosis. *Dig Dis Sci* 2001; **46**: 2408-2414 [PMID: 11713944 DOI: 10.1023/A:1012359400193]
 - 201 **Lalazar G**, Rund D, Shouval D. Screening, prevention and treatment of viral hepatitis B reactivation in patients with haematological malignancies. *Br J Haematol* 2007; **136**: 699-712 [PMID: 17338776 DOI: 10.1111/j.1365-2141.2006]
 - 202 **Kusumoto S**, Tanaka Y, Mizokami M, Ueda R. Reactivation of hepatitis B virus following systemic chemotherapy for malignant lymphoma. *Int J Hematol* 2009; **90**: 13-23 [PMID: 19544079 DOI: 10.1007/s12185-009-0359-5]
 - 203 **Liang R**. How I treat and monitor viral hepatitis B infection in patients receiving intensive immunosuppressive therapies or undergoing hematopoietic stem cell transplantation. *Blood* 2009; **113**: 3147-3153 [PMID: 19144986 DOI: 10.1182/blood-2008-10-163493]
 - 204 **Lau GK**, Leung YH, Fong DY, Au WY, Kwong YL, Lie A, Hou JL, Wen YM, Nanj A, Liang R. High hepatitis B virus (HBV) DNA viral load as the most important risk factor for HBV reactivation in patients positive for HBV surface antigen undergoing autologous hematopoietic cell transplantation. *Blood* 2002; **99**: 2324-2330 [PMID: 11895763 DOI: 10.1182/blood.V99.7.2324]
 - 205 **Ma SY**, Lau GK, Cheng VC, Liang R. Hepatitis B reactivation in patients positive for hepatitis B surface antigen undergoing autologous hematopoietic cell transplantation. *Leuk Lymphoma* 2003; **44**: 1281-1285 [PMID: 12952220 DOI: 10.1080/1042819031000083343]
 - 206 **Knöll A**, Boehm S, Hahn J, Holler E, Jilg W. Reactivation of resolved hepatitis B virus infection after allogeneic hematopoietic stem cell transplantation. *Bone Marrow Transplant* 2004; **33**: 925-929 [PMID: 15004543]
 - 207 **Hui CK**, Sun J, Au WY, Lie AK, Yueng YH, Zhang HY, Lee NP, Hou JL, Liang R, Lau GK. Occult hepatitis B virus infection in hematopoietic stem cell donors in a hepatitis B virus endemic area. *J Hepatol* 2005; **42**: 813-819 [PMID: 15885351 DOI: 10.1016/j.jhep.2005.01.018]
 - 208 **Onozawa M**, Hashino S, Izumiyama K, Kahata K, Chuma M, Mori A, Kondo T, Toyoshima N, Ota S, Kobayashi S, Hige

- S, Toubai T, Tanaka J, Imamura M, Asaka M. Progressive disappearance of anti-hepatitis B surface antigen antibody and reverse seroconversion after allogeneic hematopoietic stem cell transplantation in patients with previous hepatitis B virus infection. *Transplantation* 2005; **79**: 616-619 [PMID: 15753855 DOI: 10.1097/01.TP.0000151661.52601.FB]
- 209 **Aksoy S**, Harputluoglu H, Kilickap S, Dede DS, Dizdar O, Altundag K, Barista I. Rituximab-related viral infections in lymphoma patients. *Leuk Lymphoma* 2007; **48**: 1307-1312 [PMID: 17613758 DOI: 10.1080/10428190701411441]
- 210 **Francisci D**, Falcinelli F, Schiaroli E, Capponi M, Belfiori B, Flenghi L, Baldelli F. Management of hepatitis B virus reactivation in patients with hematological malignancies treated with chemotherapy. *Infection* 2010; **38**: 58-61 [PMID: 19904491 DOI: 10.1007/s15010-009-9019-1]
- 211 **Seto WK**, Chan TS, Hwang YY, Wong DK, Fung J, Liu KS, Gill H, Lam YF, Lie AK, Lai CL, Kwong YL, Yuen MF. Hepatitis B reactivation in patients with previous hepatitis B virus exposure undergoing rituximab-containing chemotherapy for lymphoma: a prospective study. *J Clin Oncol* 2014; **32**: 3736-3743 [PMID: 25287829 DOI: 10.1200/JCO.2014.56.7081]
- 212 **Hsu C**, Tsou HH, Lin SJ, Wang MC, Yao M, Hwang WL, Kao WY, Chiu CF, Lin SF, Lin J, Chang CS, Tien HF, Liu TW, Chen PJ, Cheng AL. Chemotherapy-induced hepatitis B reactivation in lymphoma patients with resolved HBV infection: a prospective study. *Hepatology* 2014; **59**: 2092-2100 [PMID: 24002804]
- 213 **Dominguez N**, Manzano ML, Muñoz R, Martín A, Fernández I, Castellano G. Late reactivation of occult hepatitis B virus infection in a patient with chronic lymphocytic leukemia after rituximab and fludarabine-based regimen. *Leuk Lymphoma* 2015; **56**: 1160-1163 [PMID: 25115508 DOI: 10.3109/10428194.2014.947978]
- 214 **Liu JY**, Sheng YJ, Ding XC, Tang H, Tong SW, Zhang DZ, Zhou Z, Hu P, Liao Y, Ren H, Hu HD. The efficacy of lamivudine prophylaxis against hepatitis B reactivation in breast cancer patients undergoing chemotherapy: a meta-analysis. *J Formos Med Assoc* 2015; **114**: 164-173 [PMID: 25678179 DOI: 10.1016/j.jfma.2012.10.007]
- 215 **Madaliński K**, Sylvan SP, Hellstrom U, Mikołajewicz J, Dzierzanowska-Fangrat K. Presence of anti-preS1, anti-preS2, and anti-HBs antibodies in newborns immunized with Bio-Hep-B vaccine. *Med Sci Monit* 2004; **10**: PI10-PI17 [PMID: 14704645]
- 216 **Ge G**, Wang S, Han Y, Zhang C, Lu S, Huang Z. Removing N-terminal sequences in pre-S1 domain enhanced antibody and B-cell responses by an HBV large surface antigen DNA vaccine. *PLoS One* 2012; **7**: e41573 [PMID: 22844502 DOI: 10.1371/journal.pone.0041573]

P- Reviewer: Franz C, Hoshina T, Yeung CY **S- Editor:** Yu J

L- Editor: Wang TQ **E- Editor:** Zhang FF



Metastasis-associated long noncoding RNAs in gastrointestinal cancer: Implications for novel biomarkers and therapeutic targets

Fei-Fei Zhang, Yu-Hao Luo, Hui Wang, Liang Zhao

Fei-Fei Zhang, Yu-Hao Luo, Liang Zhao, Department of Pathology, Nanfang Hospital, Southern Medical University, Guangzhou 510000, Guangdong Province, China

Hui Wang, Department of Medical Oncology, Affiliated Tumor Hospital of Guangzhou Medical University, Guangzhou 510000, Guangdong Province, China

Liang Zhao, Department of Pathology, School of Basic Medical Sciences, Southern Medical University, Guangzhou 510000, Guangdong Province, China

Author contributions: Zhang FF and Luo YH contributed equally to this work; Zhang FF, Luo YH, Wang H and Zhao L prepared the manuscript.

Supported by the National Natural Science Foundation of China, No. 81272762 and No.81201635; and the Guangdong Natural Science Funds for Distinguished Young Scholar, No. S20120011334.

Conflict-of-interest statement: Authors declare no conflict of interests for this article.

Open-Access: This article is an open-access article which was selected by an in-house editor and fully peer-reviewed by external reviewers. It is distributed in accordance with the Creative Commons Attribution Non Commercial (CC BY-NC 4.0) license, which permits others to distribute, remix, adapt, build upon this work non-commercially, and license their derivative works on different terms, provided the original work is properly cited and the use is non-commercial. See: <http://creativecommons.org/licenses/by-nc/4.0/>

Manuscript source: Invited manuscript

Correspondence to: Liang Zhao, MD, Department of Pathology, Nanfang Hospital, Southern Medical University, No. 1838 Guangzhoudao South Road, Guangzhou 510000, Guangdong Province, China. liangsmu@foxmail.com
Telephone: +86-20-62789365
Fax: +86-20-62789365

Received: March 17, 2016

Peer-review started: March 20, 2016

First decision: June 20, 2016

Revised: July 5, 2016

Accepted: August 23, 2016

Article in press: August 23, 2016

Published online: October 21, 2016

Abstract

Long non-coding RNAs (lncRNAs), a newly discovered class of ncRNA molecules, have been widely accepted as crucial regulators of various diseases including cancer. Increasing numbers of studies have demonstrated that lncRNAs are involved in diverse physiological and pathophysiological processes, such as cell cycle progression, chromatin remodeling, gene transcription, and posttranscriptional processing. Aberrant expression of lncRNAs frequently occurs in gastrointestinal cancer and plays emerging roles in cancer metastasis. In this review, we focus on and outline the regulatory functions of recently identified metastasis-associated lncRNAs, and evaluate the potential roles of lncRNAs as novel diagnostic biomarkers and therapeutic targets in gastrointestinal cancer.

Key words: Gastrointestinal cancer; Tumor metastasis; Long noncoding RNAs; Epithelial-to-mesenchymal transition; MicroRNAs

© **The Author(s) 2016.** Published by Baishideng Publishing Group Inc. All rights reserved.

Core tip: Long non-coding RNAs (lncRNAs), a newly discovered class of non-coding RNA molecules, have been widely identified as crucial regulators of various

diseases including cancer. Aberrant expression of lncRNAs frequently occurs in gastrointestinal cancer and plays emerging roles in cancer metastasis. We focus on and outline the regulatory functions of recently identified metastasis-associated lncRNAs and evaluate the potential roles of lncRNAs as novel diagnostic biomarkers and therapeutic targets in gastrointestinal cancer.

Zhang FF, Luo YH, Wang H, Zhao L. Metastasis-associated long noncoding RNAs in gastrointestinal cancer: Implications for novel biomarkers and therapeutic targets. *World J Gastroenterol* 2016; 22(39): 8735-8749 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v22/i39/8735.htm> DOI: <http://dx.doi.org/10.3748/wjg.v22.i39.8735>

INTRODUCTION

Gastrointestinal cancers (GCs) are among the most lethal cancers worldwide. In 2012, there were estimated more than 2.8 million new cases and 1 817 200 deaths worldwide^[1]. Although multimodal therapeutic regimens have been applied, survival from gastrointestinal cancers remains in a narrow range of 25% to 30% in most countries^[2]. One reason for this poor survival rate is distant metastasis before diagnosis. Metastasis is one of the most significant features of cancer and frequently occurs in multiple sequential steps. Thus, a detailed understanding of the precise molecular mechanisms underlying GC metastasis is urgently required.

Recent studies have found that protein-coding genes and non-protein-coding genes are abundantly transcribed in the human genome, especially ncRNAs, which account for more than 70% of the human genome^[3-5]. A large number of transcripts are ncRNAs, including miRNAs (miRNAs) and lncRNAs, as well as several other types of RNAs. MiRNAs are mostly known ncRNAs, with molecules of around 22 nucleotides in length that regulate target gene expression at the post-transcriptional level^[6]. lncRNAs are roughly defined as molecules greater than 200 nucleotides in length that are devoid of evident open reading frames^[5]. With the development of high-throughput sequencing, increasing numbers of lncRNAs are being revealed and extensively investigated. It is now recognized that lncRNA alterations are highly tumor- and lineage-specific and regulate various stages of cancer, including chromatin remodeling, gene transcription, and post-transcriptional modifications^[7-10]. Over the past decades, multiple studies have demonstrated that lncRNAs are aberrantly expressed in GCs and are associated with tumor metastasis. In this article, we summarize the molecular mechanisms of lncRNAs identified in GCs, especially those that are significantly associated with tumor metastasis, and discuss the potential role of lncRNAs as novel diagnostic

biomarkers and therapeutic targets.

OVERVIEW OF lncRNAs

lncRNAs can be generated at any region of the genome by RNA polymerase II/III, and most of them are polyadenylated and located within nuclear or cytosolic fractions^[3]. Interestingly, lncRNAs can undergo alternative splicing similarly to protein coding RNAs, and develop secondary and even tertiary structures to form specific functional domains. These domains provide greater potential for interaction with DNA, RNA or proteins^[11,12]. The detailed classification of lncRNAs can be complex. According to the genome position with their neighboring protein-coding genes, lncRNAs can be divided into sense, antisense, intronic, intergenic, and divergent lncRNAs (Figure 1). Sense lncRNAs are transcribed from the same strand as protein-coding genes, while antisense lncRNAs are generated from antisense strands. Both can overlap with exonic or intronic regions, or cover entire protein-coding regions. Intronic lncRNAs are entirely generated from introns of protein-coding genes, while intergenic lncRNAs are transcribed from the regions between two protein-coding genes. Divergent lncRNAs are located on the opposite strands of the protein-coding genes, the transcription of which starts within 1000 base pairs. Compared with protein-coding genes, lncRNAs are lower in abundance and poorer in interspecies sequence conservation, and frequently occur in the nucleus^[13]. lncRNAs can be located in the nucleus or cytoplasm, and different biological functions are implicated depending on their subcellular localization (Figure 2).

NUCLEAR lncRNAs

Multiple lines of evidence have indicated that nuclear lncRNAs function by guiding chromatin modifiers to their specific genomic loci^[10]. Genomic DNA is surrounded by histone proteins and packaged into an advanced structure termed chromatin. Histones can be modified in various ways, and as a result can influence the potential DNA functional state. Recent studies have demonstrated that most nuclear lncRNAs function through interacting with and modulating the activity of chromatin regulatory complexes, thereby guiding them to their specific genomic loci^[8,10]. For example, they can recruit protein complexes from the Trithorax group (TrxG) or Polycomb group (PcG), and guide these protein complexes to specific sites for action^[14,15]. Generally, TrxG proteins are essential for active gene expression, while PcG proteins function by repressing expression^[16,17]. Transcriptional activation may be promoted by lncRNAs *via* recruitment of chromatin regulatory complexes such as histone H3K4 methyltransferase, which is frequently located at the promoter regions of the transcribed genes. Furthermore, the common

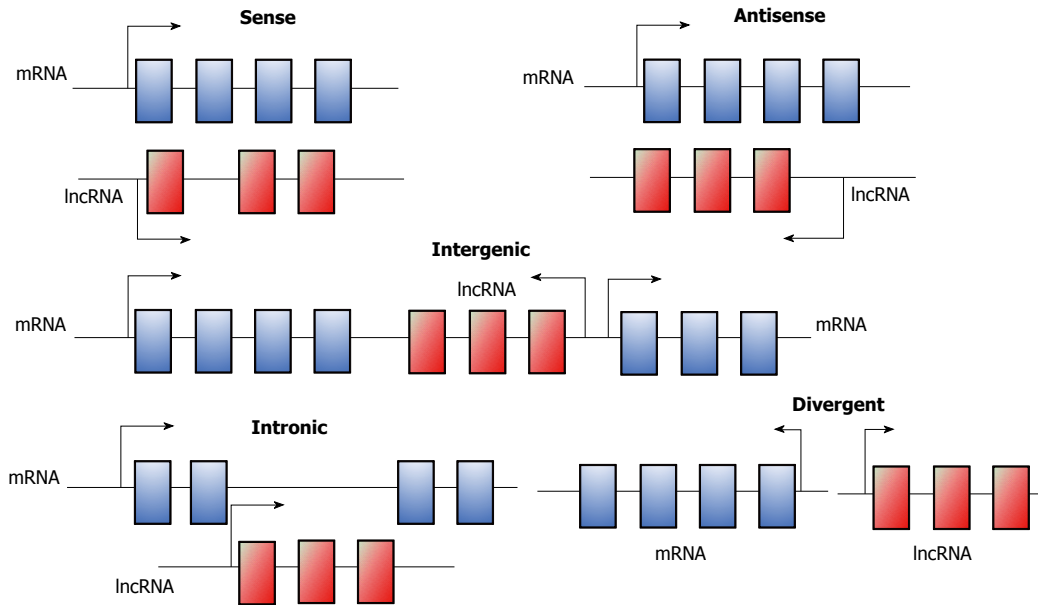


Figure 1 Five brief categories of long noncoding RNAs based on the genome position. lncRNAs: Long noncoding RNAs.

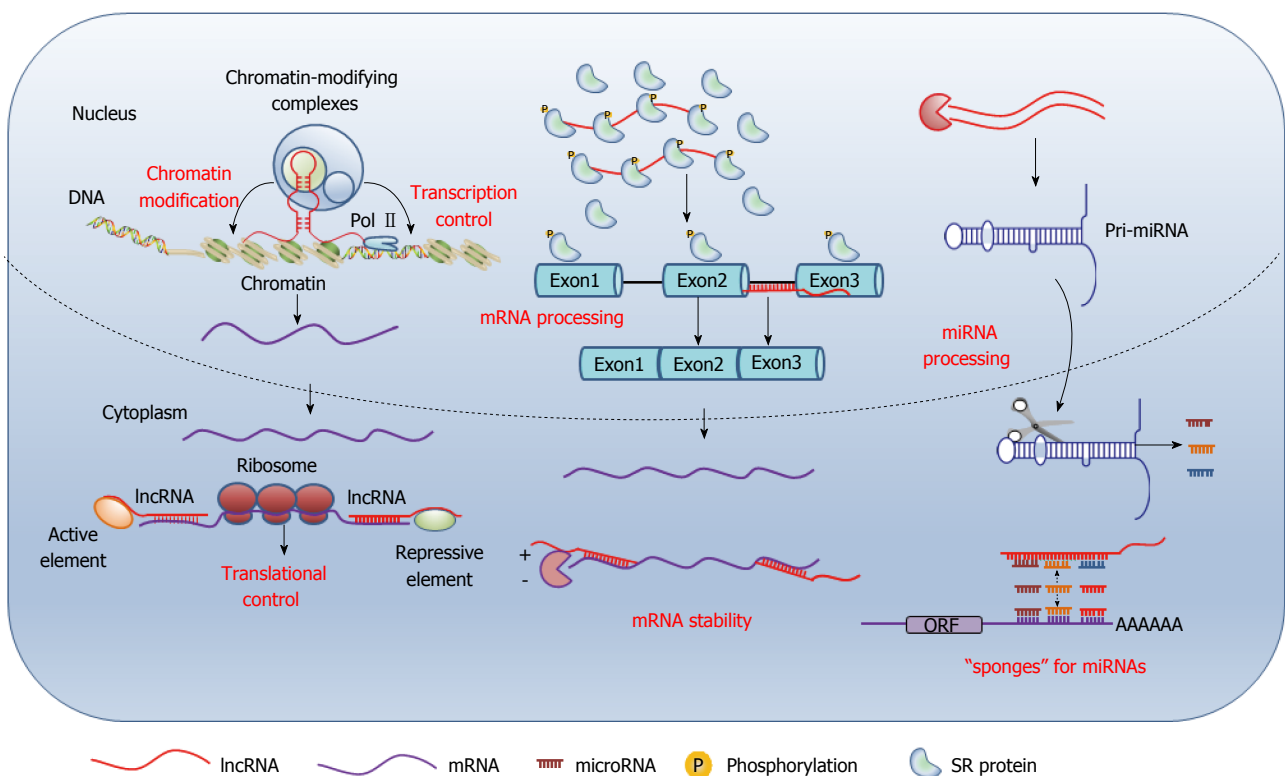


Figure 2 Mechanisms of long noncoding RNAs according to the subcellular localization. lncRNAs in nucleus frequently interact with protein complexes (such as PRC2) to modify chromatin structures. lncRNAs can also recruit transcription factors to promote or repress gene transcription, through regulating SR proteins, or form RNA-RNA duplexes with pre-mRNAs. Some lncRNAs (such as H19) are spliced into pri-miRNAs, which contributed to miRNA processing. In cytoplasm, lncRNAs may form specific structures with mRNA and interact with some active elements or repressive elements to modulate mRNA translation. By forming RNA-RNA complementary sites with mRNA or microRNA, lncRNAs can promote or inhibit mRNA stability and serve as "sponges" for miRNAs. lncRNAs: Long noncoding RNAs; PRC2: Polycomb repressive complex2.

marks of silenced heterochromatin contain di- and trimethylated H3K9, trimethylated H3K27 and Polycomb Repressive Complex 2 (PRC2). PRC2 is composed of four core components including Enhancer of zeste, Suppressor of zeste 12 homolog, Embryonic ectoderm

development and RbAp46/48^[18,19]. As lncRNAs are frequently located in the nucleus, their molecular functions can be divided into three main categories: modification of chromatin structures, transcriptional control, and mRNA/miRNA processing. For example,

the X-inactive specific transcript (*Xist*) gene was the first lncRNA identified to be directly involved in the formation of repressive chromatin domains, which is necessary for reducing X-chromosome gene expression in female genomes^[20]. *Xist* is expressed in the X chromosome targeted for inactivation (Xi), and produces multiple transcripts to “coat” Xi^[21]. As lncRNAs can develop secondary or even tertiary structures, they are proposed to be molecular scaffolds through combinations of regulatory proteins. During X inactivation, one of the steps in initiating the process is the recruitment of PRC2 *in cis* by RepA RNA, which originates from the 5′ end of *Xist*^[22]. Furthermore, the transcriptional repressor Yin Yang 1 (YY1) may be involved in the interaction between the *Xist* and chromatin, because its deletion leads to a loss of *Xist* loading on Xi^[23]. Recent studies have revealed that YY1 can facilitate the loading of PRC2 to DNA and initiate DNA methylation and chromatin silencing, these findings suggest that YY1 is the docking factor for the *cis*-acting nature of *Xist*^[22,23]. Due to the development of RNA technology, increasing numbers of lncRNAs are being discovered, and are verified to be associated with modification of chromatin. HOX transcript antisense intergenic RNA (HOTAIR) is another classical case. HOTAIR is a 2.2 kb lncRNA expressed in the HOXC locus, which cooperates with PRC2 to repress transcription of *HOX* genes^[24]. Tsai *et al.*^[25] demonstrated that besides the 5′ domain of HOTAIR binding to PRC2, the 3′ domain binds to LSD1, resulting in H3K27me3 and H3K4me2 methylation, which in turn leads to HOXD silencing^[25,26].

Nuclear lncRNAs also play important roles in transcriptional control. Some lncRNAs can form RNA-protein complexes with transcription factors and modulate gene transcription. For example, the lncRNA heat shock RNA 1 interacts with heat-shock transcription factor 1 to form a protein complex that induces expression of heat-shock proteins during cellular heat-shock stress^[27]. As well as roles in positive regulation, lncRNAs can function as transcription co-repressors. Those transcribed from the Cyclin D1 (CCND1) promoter region are reported to change the RNA-binding protein translocated in liposarcoma (TLS) from an inactive to an active conformation. This causes TLS to bind to and inhibit the enzymatic activities of CBP and p300, which in turn leads to the silencing of CCND^[28].

Interestingly, lncRNAs are not only involved in gene transcription but also in mRNA/miRNA processing. For example, metastasis-associated lung adenocarcinoma transcript 1 (MALAT1) is a cancer-related lncRNA, whose expression is deregulated in many cancers including GCs^[29,30]. MALAT1 regulates the phosphorylation of the serine/arginine-rich (SR) family of nuclear phosphoproteins (SR proteins) and influences the distribution of splicing factors in nuclear speckle domains^[31]. In addition, lncRNA FGFR2

impairs binding of a repressive chromatin-splicing adaptor complex *via* recruitment of PRC2 and KDM2a, leading to the epithelial-specific alternative splicing of FGFR2^[32]. In some cases, lncRNAs are located antisense to their known protein-coding genes. These lncRNAs are also called natural antisense transcripts (NATs). Recent studies indicated that NATs are associated with mRNA processing. For instance, they influence the splicing patterns of mRNAs at v-myc avian myelocytomatosis viral oncogene homolog (MYC)^[33], tyrosine kinase containing immunoglobulin and epidermal growth factor homology domain-1 (Tie-1)^[34], and zinc-finger E-box binding homeobox 2 (ZEB2)^[35]. In these cases, lncRNAs form RNA-RNA duplexes with pre-mRNAs and inhibit splicing. Processing of miRNAs can also be affected by lncRNAs. For example, lncRNA H19 can be spliced into pri-miRNA of miR-675, and miR-675 is derived from nucleotides 1014-1036 of human H19 RNA^[36].

CYTOPLASMIC lncRNAs

Many lncRNAs are located in the cytoplasm, and these lncRNAs often regulate gene expression through sequence complementarity with target genes by base pairing. In the cytoplasm, lncRNAs are responsible for modulating translational control, promoting or inhibiting mRNA stability and serving as molecular “sponges” for miRNAs. For example, ubiquitin carboxy-terminal hydrolase L1 antisense RNA 1 (Uchl1-AS1) lncRNA is produced from the Uchl1 (ubiquitin carboxy-terminal hydrolase L1) locus. The mature lncRNA contains a 73-nucleotide complementary site with the 5′ end of the Uchl1 mRNA, which serves to upregulate translation of Uchl1 mRNA^[37]. By contrast, tumor protein p53 pathway corepressor 1 (Trp53cor1, also known as lincRNA-p21) lncRNA negatively regulates gene translation in human cervical carcinoma^[38]. Similarly, lncRNAs can modulate mRNA stability positively or negatively. The lncRNA-activated by TGF-β (lncRNA-ATB) is upregulated in hepatocellular carcinoma (HCC). It binds IL-11 mRNA and triggers STAT3 signaling, promoting the colonization of organs by disseminated tumor cells^[39]. By contrast, Alu repeat-containing lncRNAs are associated with targeting mRNA transcripts for Staufen-mediated decay, and destabilizing the target mRNA^[40]. Aside from binding to mRNA, lncRNAs can act as decoys to attenuate miRNA regulation. For example, HOTAIR functions as a competing endogenous RNA by “sponging” miR-331-3p, thereby modulating the derepression of human epidermal growth factor receptor 2 (HER2) and promoting GC progression^[41]. Furthermore, another study demonstrated that HOTAIR is involved in the upregulation of human leukocyte antigen (HLA-G) through inhibition of miR-152 in GC^[42]. As increasing numbers of lncRNAs are being identified, we believe that new perspectives on the mechanisms of lncRNA

function will come to light in the near future.

ABERRANT EXPRESSION OF lncRNAs IN GASTROINTESTINAL CANCER

The development of GC is a complex process, although several serum tumor markers such as carcinoembryonic antigen (CEA) and CA19.9 are recommended for clinical applications, their low sensitivity and specificity remain a severe challenge for clinicians. Recent observations demonstrate that lncRNAs are aberrantly expressed in GCs, and have roles in tumorigenesis. Among the most prominent deregulated lncRNAs in gastrointestinal cancer are HOTAIR, MALAT1, and H19, which are upregulated in various cancers and are involved in migration, invasion, metastasis, dissemination and are associated with a more advanced tumor, node, metastasis (TNM) stage. For example, high HOTAIR expression in cancerous tissues closely correlates with poor prognosis in GC^[43-45]. Surprisingly, for colorectal cancer (CRC) patients, plasma levels of HOTAIR were significantly higher than those in healthy controls, and high levels of HOTAIR were associated with an unfavorable prognosis. This indicated that the HOTAIR plasma level could act as a new biomarker for CRC patients^[46]. In GC, circulating H19 in plasma was also higher than in healthy controls, and after surgery, circulating H19 was reduced^[47]. In GC tissues, lncRNA AA174084 expression was found to be downregulated compared to expression in the paired normal tissues, although the AA174084 plasma level was positively associated with invasion and metastasis. Plasma levels of AA174084 dropped after surgical treatment, and A174084 levels in gastric juice were significantly higher in GC patients. These observations provide evidence that A174084 could be a potential biomarker for early diagnosis in GC^[48]. Resveratrol, a monomer extracted from a Chinese herbal medicine *Polygonum cuspidatum*, is known to inhibit metastasis of CRC. Studies have shown that Resveratrol exerts anti-tumor effects *via* MALAT1-mediated Wnt/ β -Catenin signaling; co-repression of MALAT1 led to the enhancement of Resveratrol-induced anti-cancer effects^[49]. These emerging studies elegantly indicate that lncRNAs could be promising future diagnostic markers or therapeutic targets, and that combining lncRNA-based strategies with existing methods would improve the accuracy of diagnosis and efficacy of treatments in a clinical setting. Increasing evidence demonstrates that lncRNA expression levels correlate with GC. A summary of aberrantly expressed lncRNAs, their gene locus, product size, expression status, and biological function are shown in Table 1^[41-46,48-90].

lncRNAs ARE ASSOCIATED WITH CANCER-RELATED PATHWAYS

Cancer-related pathways play essential roles in

cancer metastasis. For example, miR-29b can inactivate the MAPK/ERK and PI3K/AKT pathways *via* dephosphorylation of ERK1/2 and AKT in CRC, which leads to repression of metastatic capacity in cancer cells^[91]. Pathway regulation is mediated not only by miRNAs, but also by lncRNAs. PVT-1 is located at 8q24, whose copy number is amplified in CRC. As observed using gene expression microarray analysis, the TGF- β signaling pathway is upregulated by PVT-1 knockdown^[65]. MALAT1 was first identified in lung cancer, and is an indicator of probability of survival and metastasis in early-stage non-small cell lung cancer^[92]. However, it also plays oncogenic roles in other cancers. MALAT1 was demonstrated to be an independent prognostic parameter in gallbladder carcinoma (GBC); its downregulation reduced phosphorylated MEK1/2, ERK 1/2, MAPK, and JNK 1/2/3, and inactivation of the ERK/MAPK pathway led to the partial impairment of proliferation and metastasis^[76].

Wnt/ β -catenin signaling is a classical pathway in cancer, and frequently correlates with cancer metastasis. During Wnt/ β -catenin signaling activation, β -catenin often translocates to the nucleus from the cytoplasm, and cooperates with transcription factors such as TCF/LEF to induce downstream target genes such as *c-Myc*, *MMP-7*, and *CD44*. In CRC, MALAT1 was found to increase the nuclear localization of β -catenin, activating Wnt/ β -catenin signaling to enhance expression of *c-Myc* and *MMP-7*, which are involved in CRC cell metastasis^[49]. HOTAIR cooperates with the PRC2 complex, leading to H3K27me3 and H3K4me2 methylation and HOXD gene silencing^[25,26]. In esophageal squamous cell carcinoma (ESCC), HOTAIR is significantly upregulated, and has been shown to promote migration and invasion of ESCC cells *in vitro*^[43]. Gene microarray analysis indicated that the expression of WNT/ β -catenin associated gene WIF-1 changed most significantly in HOTAIR overexpressing cells. A further study showed that HOTAIR promoted H3K27 methylation in the promoter region of WIF-1 and inhibited WIF-1 expression, and that the low expression of WIF-1 triggered Wnt/ β -catenin signaling, thereby promoting ESCC invasion and migration.

lncRNAs PARTICIPATE IN EPITHELIAL-TO-MESENCHYMAL TRANSITION

The epithelial-mesenchymal transition (EMT) is a biological process in which polarized epithelial cells acquire a mesenchymal cell phenotype. It is a process known to enhance cancer cell metastasis, invasiveness, and chemoresistance. Recent studies have verified that noncoding RNAs are frequently involved in the EMT process, especially miRNAs. For example, miR-187, as a downstream target of TGF- β , inhibits EMT by suppressing the expression of multiple targets in CRC^[93]. The maturation of high throughput genomic tools, such as high-resolution microarray and

Table 1 Aberrant expression of lncRNAs and their gene locus, product size, expression status and biological function

lncRNAs	Cancer type	Gene locus	Size	Expression	Correlation with metastasis	Biological function
91H	CRC	11p15.5 (H19/IGF2 locus)	119392nt	Up	Positive	Migration and invasiveness ^[50]
AK123657	CRC	NA	2126 nt	Down	Negative	Inhibit cell invasion, and serve as promising biomarkers for prognosis ^[51]
BX649059			3967 nt	Down	Negative	
BX648207			5032 nt	Down	Negative	
CCAT1	CRC	8q24.21	2628 nt	Up	Positive	
CCAT2	CRC	8q24.21	1752 nt	Up	Positive	Up-regulated across the colonadenoma-carcinoma sequence and activated by C-myc to promote metastatic process ^[52,53]
FER1L4	CRC	20q11.22	6717 nt	Down	Negative	Interacts with TCF7L2 which leads to genomic instability, cell invasion and forms a feedback loop with WNT signaling ^[54]
HOTAIR	CRC	12q13.13	2370 nt	Up	Positive	Suppresses oncogenesis <i>via</i> inhibiting miR-106a-5p ^[55]
LOC285194	CRC	3q13.31	2105 nt	Down	NA	Interacts with PRC2 complex and participates in EMT ^[45,56] ; the blood levels of HOTAIR also serves as a negative prognostic factor ^[46]
MALAT1	CRC	11q13.1	8758 nt	Up	Positive	Novel biomarker of cancer metastasis ^[57]
MEG3	CRC	14q32	1595 nt	Down	NA	3' end of MALAT-1 (6918 nt-8441 nt) promotes migration and invasion and AKAP-5 is a downstream target protein ^[58,59] ; Mediates Wnt/ β -catenin signalling ^[49] ; Upregulated by tumor-associated dendritic cells expressed CCL5 and enhances Snail to promote migration, invasion and EMT ^[60]
ncRAN	CRC	17q25.1	2538 nt	Up	Positive	Potential marker for prognosis ^[61]
ncRuPAR	CRC	5q13.3	486 nt	Down	NA	Promotes migration and invasion ^[62]
NEAT1	CRC	11q13.1	3756 nt	Up	NA	Potential biomarker ^[63]
PVT-1	CRC	8q24	1957 nt	Up	NA	Indicator of tumor differentiation, invasion and metastasis ^[64]
TUG1	CRC	22q12.2	7598 nt	Up	Positive	Antiaoptotic activity ^[65]
91H	ESCC	11p15.5	119,392kb	Down	Negative	Activates EMT and promotes migration, invasion ^[66]
AFAP1-AS1	ESCC	(H19/IGF2 locus)	6810 nt	Down	Positive	Inhibits IGF2 expression and regulates tumor development ^[67]
CCAT2	ESCC	8q24.21	1752 nt	Up	Negative	Hypomethylated and overexpressed in ESCC, promotes migration and invasion in a AFAP1 independent manner ^[68]
H19	ESCC	11p15.5	2362 nt	Up	Positive	Potential prognostic biomarker and therapeutic target ^[69]
HNF1A-AS1	ESCC	(IGF2 locus)	2455 nt	Up	Positive	H19 CBS6 hypermethylation leads to IGF2 overexpression and cancer progression ^[70]
HOTAIR	ESCC	(HNF1A locus)	2370 nt	Up	Positive	Upregulated in primary ESCC , affects assembly of chromatin and the nucleosome process by H19 induction ^[71]
MALAT1	ESCC	11q13.1	> 8 kb	Up	Positive	Medicates migratory capacity ^[44,72,73] ; promotes histone H3K27 methylation and activates Wnt/ β -catenin signaling and decreases WIF-1, lead to metastasis ^[43]
MALAT1	GBC	11q13.1	8758 nt	Up	Positive	Target of miR-101 and miR-217 and promotes migration, invasion and metastasis ^[74]
AA174084	GC	13q33.1	601 nt	Up	NA	Binds to SFPQ, leading to PTBP2 release from SFPQ/PTBP2 complex ^[75] ; Activates the ERK/MAPK pathway and promotes metastasis ^[76]
AC130710	GC	2p24.3	984 nt	Up	NA	The expression level in gastric juice is a potential marker for the early diagnosis of GC ^[48]
AK058003	GC	10q22	1197 nt	Up	NA	As a target of miR-129-5p and may be a potential biomarker for GC prognosis ^[77]
BM742401	GC	18q11.2	1788 nt	Down	Negative	Induced by hypoxia and regulates SNCG to promote metastasis ^[78]
FENDRR	GC	3q13.31	3099 nt	Down	Negative	Decreases expression of MMP9 and inhibits metastasis-related phenotypes ^[79]
GAPLINC	GC	18p11.31	924 nt	Up	Positive	Downregulates FN1 and MMP2/MMP9 to suppress invasion and migration ^[80]
H19	GC	11p15.5	2362 nt	Up	Positive	Competes to bind with miR-211-3p, which leads to the increased translation of CD44 and promotes proliferation, migration, and angiogenesis ^[81]
HOTAIR	GC	(IGF2 locus)	2370 nt	Up	Positive	Target of c-Myc and promotes migration, invasion and metastasis by the direct upregulation of ISM1 and indirect suppression of CALN1 <i>via</i> miR-675 ^[82,83]
HULC	GC	6p24.3	About 1.6 kb	Up	Positive	Novel biomarker for prognosis and promotes metastasis <i>via</i> PCBP1 inhibition ^[61,84-87] ; as a ceRNA of miR-331-3p and modulates the derepression of HER2 ^[41] ; Upregulates HLA-G <i>via</i> inhibiting miR-152 ^[42] ; binds to PRC2 complex and epigenetically represses miR34a, which inhibits HGF/Met/ Snail pathway and Snail, leads to EMT and metastasis ^[55]

LEIGC	GC	2q14.1	2659 nt	Down	Negative	Inhibits EMT ^[89]
OR3A4	GC	17p13.3	1202 nt	Up	Positive	Promotes migration, invasion, tubule formation, vasculogenic mimicry, angiogenesis by regulating PDLIM2, MACC1, NTN4, and GNB2L1 ^[90]

CRC: Colorectal cancer; GBC: Gallbladder carcinoma; GC: Gastric cancer; ESCC: Esophageal squamous cell carcinoma; NA: Data not available.

massively parallel sequencing technology, has led to the discovery of an extensive range of lncRNAs. There is a growing understanding that lncRNAs and their associated signaling networks may participate in the induction and regulation of EMT to modulate cancer metastasis^[94]. Esophagus epithelial intergenic specific transcript (Epist) acts as a tumor suppressor by inhibiting EMT^[95], and TGF- β stimulation significantly suppresses the expression of Epist. These findings reveal a new plausible mechanism by which TGF- β -induced EMT could take place.

Taurine upregulated gene 1 (TUG1) was originally reported as a transcript that is upregulated by taurine, and it was also observed to be up-regulated in CRC^[66]. TUG1 overexpression was shown to enhance the migration and invasion capacity of CRC cells significantly. A decrease in the epithelial hallmark E-cadherin combined with an increase in the mesenchymal markers N-cadherin, vimentin, and fibronectin, indicated that TUG1 promoted CRC metastasis by mediating EMT. Another two studies suggested that HOTAIR^[56] and highly upregulated in liver cancer (HULC)^[88] promoted metastasis of CRC by participating in EMT, although the underlying mechanisms require further investigation. In GC, microarray and quantitative real-time PCR identified a new, specific, differentially expressed lncRNA termed LEIGC^[89]. LEIGC is a 2659 nt transcript located at 2q14.1, and contains two exons. Intriguingly, LEIGC knockdown in MGC803 cells induced them to assume a spindle-shaped morphology, whereas LEIGC-overexpression induced a cobblestone-like phenotype, suggesting that LEIGC may be involved in EMT. Further investigation revealed that LEIGC knockdown reduced E-cadherin expression while increasing expression of vimentin, Snail, Slug, Zeb, and Twist. These data indicated that LEIGC may be a potential EMT inhibitor in GC.

Mounting evidence suggests that tumor-associated dendritic cells and the tumor microenvironment are critical factors for cancer metastasis. Many inflammatory factors such as chemokines and cytokines have been implicated as being strongly associated with EMT and metastasis. Increasing numbers of studies support the hypothesis that tumor-associated dendritic cells (TADCs) influence the inflammatory cancer microenvironment and contribute to an aggressive cancer phenotype. Kan *et al.*^[60] found that TADCs secreted high levels of Chemokine (C-C motif) ligand 5 (CCL5), and that CCL5 stimulation upregulated MALAT1 expression in CRC cells. This in turn led to an increase in Snail expression, resulting in enhanced EMT and cancer progression.

HOTAIR is known to play important roles in epigenetic regulation^[24-26]. In GC tissue, HOTAIR expression was shown to correlate negatively with E-cadherin expression^[96]. Western blot assays confirmed that HOTAIR could promote EMT. Bioinformatics analysis showed that miR-34a, which is known to be involved in the C-Met (HGF/C-Met/Snail pathway) and Snail pathway and to repress EMT, was a downstream microRNA of HOTAIR. RNA immunoprecipitation (RIP) assays indicated that HOTAIR could recruit and bind to the PRC2 complex and repress miR-34a epigenetically in GC. This research provided a new insight into the RNA regulatory network, and supported the hypothesis that chromatin modification not only affects protein levels, but also controls miRNA expression.

MALAT1

MALAT1 is a highly conserved lncRNA that was originally identified and is highly expressed in metastatic non-small-cell lung cancer. Recent studies have demonstrated that MALAT1 is overexpressed in other human malignancies, including CRC, GC, and ESCC, and is associated with tumor metastasis. The underlying mechanism of MALAT1 in promoting metastasis is complex. One fragment (6918 nt-8441 nt) of MALAT1 located at the 3' end of the *MALAT1* gene has biological functions in CRC, providing an excellent example for MALAT1 as an accurate therapeutic target in CRC^[58]. Using genome-wide expression profiling analysis, a novel downstream target, PRKA kinase anchor protein 9 (AKAP-9) was identified, providing a novel insight into the mechanism of MALAT1 involvement in CRC development^[59]. MALAT1 can activate several cancer-related pathways such as the ERK/MAPK pathway^[76] and Wnt/ β -catenin pathway^[49]. MALAT1 can also interact with other proteins, such as splicing factor proline/glutamine rich (SFPQ), leading to the release of polypyrimidine tract binding protein 2 (PTBP2) from the SFPQ/PTBP2 complex^[75]. In addition to proteins, MALAT1 also interacts with RNAs, such as the tumor suppressor miRNAs miR-101 and miR-217, and affects their post-transcriptional regulation^[74]. One study also showed that MALAT1 is regulated by tumor-associated dendritic cells (TADCs)^[60], providing a novel insight into the interaction between TADCs and cancer metastasis, and indicating that targeting MALAT1 is a potential strategy to improve immunotherapeutic efficacy. Taken together, the results of these studies support an oncogenic role for MALAT1 in GC (Figure 3), presenting it as a promising biomarker and therapeutic target for metastatic cancers.

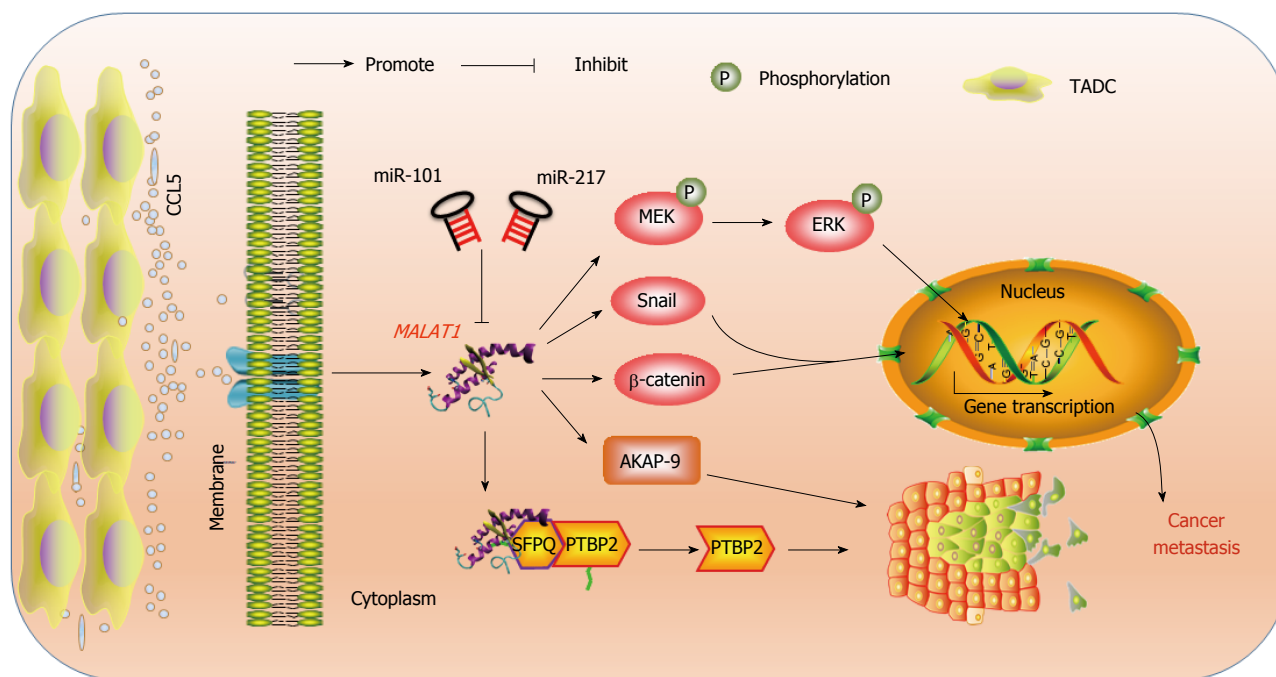


Figure 3 Regulating function of metastasis-associated lung adenocarcinoma transcript 1 in gastrointestinal cancer. CCL5 secreted by tumor-associated dendritic cells (TADCs) can promote MALAT1 expression. MALAT1 activates the ERK/MAPK pathway and Wnt/β-catenin pathway. Snail and AKAP-9 are downstream targets of MALAT1. miR-101 and miR-217 can regulate MALAT1 in 3'UTR region. MALAT1 can also bind to SFPQ/PTBP2 complex and promote the release of PTBP2. MALAT1: Metastasis-associated lung adenocarcinoma transcript 1.

HOTAIR

HOTAIR is a 2.2 kb lncRNA expressed in HOXC locus, which is known to participate in epigenetic regulation in cooperation with the PRC2 complex, leading to H3K27me3 and H3K4me2 methylation and gene silencing^[24]. Overexpression of HOTAIR has been observed in GC^[84,85], ESCC^[72], gastrointestinal stromal tumor^[97] and CRC^[56]. Notably, the level of HOTAIR expression in the blood as well as within the primary tumors was also a reliable biomarker for metastasis in CRC and GC^[46,47]. Although HOTAIR is known to cooperate with the PRC2 complex, it is still not known whether this regulation exists in CRC. A cDNA microarray and gene set enrichment analysis provided evidence for a correlation between HOTAIR and PRC2 complex expression in CRC^[45]. HOTAIR can also modulate HER2 and HLA-G expression by competitively binding to their miRNAs, such as miR-331-3p and miR-152^[41,42]. Therefore, HOTAIR could be a promising prognostic biomarker and a therapeutic target for GC (Figure 4).

H19

H19 is localized at 11p15.5, where a highly conserved cluster of imprinted genes is encoded, such as paternally expressed insulin-like growth factor 2 (IGF2) and maternally expressed H19. The oncogenic role of H19 has been found in a number of cancers. A case-control study in the Chinese Han

population revealed that the presence of H19 single nucleotide polymorphisms (SNPs) rs217727C>T and rs2839698C>T significantly correlated with susceptibility to GC^[98]. These findings indicated an important role for H19 variants in gastric carcinogenesis. In ESCC, Gao *et al.*^[70] found that hypermethylation of CBS6 on H19 was related to loss of imprinting (LOI) of IGF2, which indicated overexpression of the gene. Enhanced expression of IGF2 was closely correlated with cancer metastasis, suggesting that H19 CBS6 methylation might be a novel, clinically relevant epigenetic marker for poor prognosis and ESCC metastasis. In GC, H19 was a novel target of c-Myc, in SGC-7901 and in BGC-823 cells. Exogenous c-Myc significantly induced H19 expression to increase by approximately 3-folds compared to controls. However, the underlying molecular mechanisms remain to be fully elucidated^[82]. Investigations showed that H19 is a precursor of miR-675, and that H19 is involved in biological functions that are partly dependent on the activity of miR-675. Li *et al.*^[83] developed a H19/miR-675 knockdown model in the GC cell line MKN45, and found that calneuron 1 (CALN1) was a target of miR-675. H19 suppressed CALN1 by promoting the generation of miR-675. Conversely, H19 also upregulated expression of its binding protein isthmin 1 (ISM1) directly, suggesting that this function of H19 could be independent of miR-675 activity. In addition, recent reports confirmed that eukaryotic translation initiation factor 4A3 (eIF4A3) also binds to H19 in CRC^[99], and that H19 recruits eIF4A3, resulting in

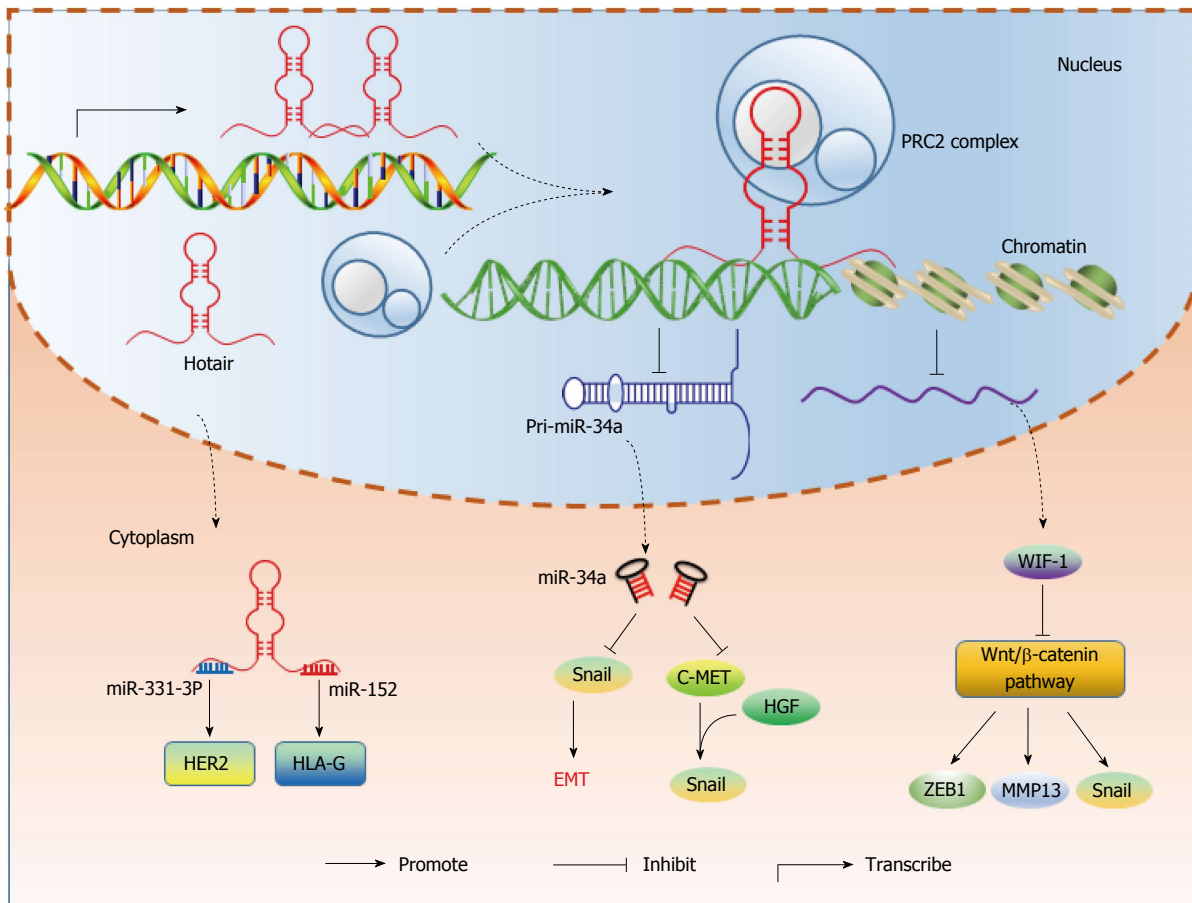


Figure 4 Regulating function of HOTAIR in gastrointestinal cancer. In nucleus, HOTAIR recruits PRC2 complex and represses pri-miR-34a transcription epigenetically. By inducing histone H3K27 methylation in WIF-1's promoter region, HOTAIR decreases WIF-1 expression. In cytoplasm, HOTAIR serves as "sponges" for miR-331-3p and miR-152, leading to the increase of target genes HER2 and HLA-G. PRC2: Polycomb repressive complex2.

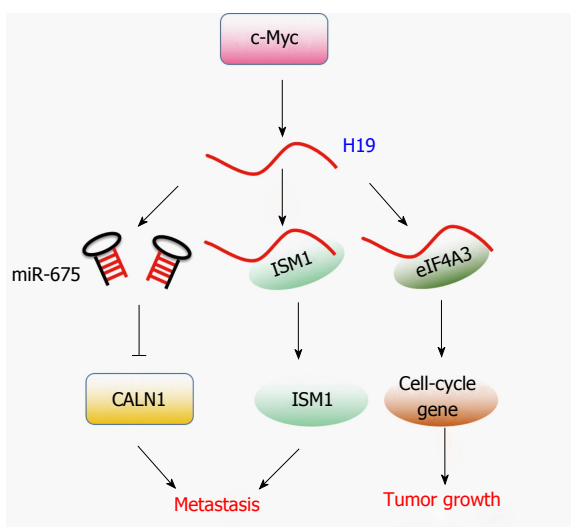


Figure 5 Regulating function of H19 in gastrointestinal cancer. H19 is transcribed by transcription factor c-Myc. H19 can be spliced into pre-miRNA of miR-675, and suppresses miR-675 target gene CALN1. H19 also binds with its binding protein ISM1 or Eif4A3 and promotes metastasis or growth.

obstructing the interaction between eIF4A3 and cell-cycle gene mRNA (Figure 5).

OTHER POTENTIAL METASTASIS-ASSOCIATED lncRNAs

lncRNA 91H is located in the H19/IGF2 locus, and low expression of 91H is associated with imprinting control region methylation of H19^[67], which is known to cause IGF2 overexpression *via* loss of genomic imprinting^[70]. Through the indirect inhibition of IGF-2 in ESCC, 91H inhibits the occurrence, progression, and prognosis of ESCC. Conversely, another study suggested that 91H was overexpressed in CRC tissues as well as cell lines^[50], and might be associated with copy number variation (CNV) in the 11p15.5 region. These findings showed that lncRNAs are highly tumor and lineage specific, and that different regulatory mechanisms might lead to different biological functions in cancer.

A novel specific lncRNA expressed in GC is termed gastric adenocarcinoma predictive long intergenic noncoding RNA (GAPLINC). High expression of GAPLINC indicates severe lymph node invasion and poor prognosis. Ectopic expression of GAPLINC in MGC803 and SGC901 cell lines significantly enhances

their invasive capacity. GAPLINC regulates CD44 mRNA at post-transcriptional level, and miR-221-3p targets both GAPLINC and CD44. GAPLINC increases CD44 expression by competing for miR-221-3p, which contributes to a reduced rate of degradation of CD44 mRNA in cytoplasm^[81].

Olfactory receptor, family 3, subfamily A, member 4 (OR3A4) is a novel lncRNA overexpressed in GC. High OR3A4 expression levels are significantly associated with metastasis. Functional experiments performed in SGC7901 and NCI-N87 indicated that OR3A4 promotes GC cell proliferation, migration, invasion, tubule formation, vasculogenic mimicry, angiogenesis, and tumorigenicity. Four target genes of OR3A4 have been identified: PDLIM2, MACC1, NTN4, and GNB2L1. These results suggest that OR3A4 might play an important role in GC progression^[90].

In order to search lncRNA signatures to predict prognosis of CRC, Hu *et al.*^[51] analyzed lncRNA expression in large CRC cohorts obtained from Gene Expression Omnibus databases. Six of the identified lncRNAs included two genes with enhanced expression in CRC, AK024680, AK026784, and four lower expressed genes with reduced expression, AK123657, CR622106, BX649059, and BX648207. These six genes were significantly correlated (positively or negatively) with disease free survival. Among them, functional experiments demonstrated that three downregulated lncRNAs, AK123657, BX648207 and BX649059, were required for suppressing invasion and proliferation in CRC cell lines. The lncRNAs identified in this study could be potential biomarkers and therapeutic targets for CRC in the future^[80].

MOLECULAR MECHANISMS OF lncRNAs IN GASTROINTESTINAL CANCER

Previously, we have categorized the molecular mechanisms of lncRNAs into modification of chromatin structures, transcriptional control, mRNA/miRNA processing, translational control, mRNA stability control, and roles as molecular “sponges” for miRNAs. However, these mechanisms are not absolutely independent of each other, and one lncRNA may use several processing controls. In this section, we will focus on the molecular mechanisms of metastasis-associated-lncRNAs in GC, and provide insights into interpreting results of previous investigation of lncRNAs.

ROLE OF lncRNAs IN CHROMATIN MODIFICATION

Many lncRNAs are located in the nucleus and form secondary or tertiary structures. They can interact with specific chromatin regulatory complexes and

participate in chromatin modification, leading to DNA methylation and gene control, including expression of miRNAs. HOTAIR is known to cooperate with the PRC2 complex and repress transcription of HOX genes^[24-26]. In ESCC, WIF-1 expression is decreased by HOTAIR^[43]. Mechanistically, HOTAIR promotes histone H3K27 methylation in the promoter region of the WIF-1 gene, and directly decreases WIF-1 expression. In GC, HOTAIR recruits and binds to the PRC2 complex and epigenetically represses miR34a, leading to the enhancement of C-Met (HGF/C-Met/Snail pathway) and Snail activation, and thus contributes to EMT and metastasis^[55]. This work provided a new insight into the RNA regulation network, and demonstrated that chromatin modification not only affects protein levels, but also controls miRNA expression. Another study showed that HNF1A antisense RNA 1 (HNF1A-AS1), a 2455 nt lncRNA located at chromosomal band 12q24.31, is markedly upregulated in esophageal adenocarcinoma (EAC) and that HNF1A-AS1 knockdown significantly inhibits cell proliferation, migration, and invasion. Microarray analysis and GO enrichment analysis revealed that HNF1A-AS1 knockdown affected chromatin/nucleosome assembly by inhibiting lncRNA H19, which contributed to cancer metastasis^[71].

ROLE OF lncRNAs IN TRANSLATIONAL CONTROL

Colon cancer associated transcript 2 (CCAT2), a novel lncRNA encompassing the rs6983267 SNP, is highly overexpressed in microsatellite-stable (MSS) CRC, and promotes cancer growth and metastasis. Genomics analysis showed that the oncogene MYC is located in the same region as CCAT2. CCAT2 regulated MYC expression and affected MYC downstream genes such as miR-17-5p and miR-20a. Transcription factor 7 like 2 (TCF7L2) was confirmed as the promoter of MYC, and physical interaction between CCAT2 and TCF7L2 enhanced TCF7L2 transcriptional activity. This resulted in an increase in MYC and a change in downstream gene expression and pathway activity^[54]. Notably, CCAT2 itself was a downstream target of TCF7L2-mediated WNT signaling, indicating existence of a feedback loop during carcinogenesis.

ROLE OF lncRNAs IN mRNA/miRNA PROCESSING

H19 was reported to be the precursor of miR-675, a regulator of CALN1. H19 promoted metastasis of GC via regulation of the miR-675/CALN1 axis. However, H19 banded to ISM1 and upregulated ISM1 expression directly, which suggested that H19 could also work in an miR-675 independent manner^[83].

ROLE OF lncRNAs AS “SPONGES” FOR miRNAs

lncRNAs and mRNAs may share some of the same response elements of miRNAs. In these cases, lncRNAs may serve as competing endogenous RNAs (ceRNAs) to sponge miRNAs, regulating target gene expression in post-transcriptional regulation. The function of ceRNAs is one of the most commonly investigated mechanisms of lncRNA activity, and completes the post-transcriptional regulatory network.

miR-331-3p and miR-124 directly target HOTAIR, which is reported to play a critical role in GC metastasis. Moreover, miR-331-3p also targets HER2 at the 3'-UTR region, providing compelling evidence that targeting HOTAIR would be a useful therapeutic strategy in GC^[41]. HOTAIR competitively binds to miR-152 and attenuates its expression in GC, leading to the release of HLA-G, which has been identified as a target of miR-152 and is associated with cancer metastasis^[42].

lncRNA AC130710 is overexpressed in GC, and its expression is significantly associated with tumor size, TNM stage, and distal metastasis. Bioinformatics analysis uncovered that AC130710 had nine complementary binding sites to miR-129-5p. A miR-129-5p mimic significantly decreased AC130710 expression, suggesting that miR-129-5p could regulate AC130710 expression through binding with complementary sites^[77].

A newly described lncRNA, termed gastric adenocarcinoma associated, positive CD44 regulator, long intergenic non-coding RNA (GAPLINC) is associated with CNV and expression of oncogenic transcription factors in GC^[81]. In GC cells, GAPLINC and CD44 were identified as common targets of miR-211-3p^[81]. GAPLINC regulated CD44 as a decoy for miR-211-3p.

MALAT1, an acknowledged metastasis-associated lncRNA, was predicted as a target of miR-101, miR-217, miR-383, and miR-503 using bioinformatics analysis. Among them, miR-101 and miR-217 mimics decreased MALAT1 expression and were identified as tumor suppressor genes in GC. This was proposed as the mechanism involved in MALAT1 suppression at a post-transcriptional level^[74].

Through combining lncRNA microarray, bioinformatics algorithm databases such as miRcode, and the miRNA target database TarBase, Xia *et al.*^[100] constructed an lncRNA-miRNA-mRNA network in GC. Furthermore, *in vitro* experiments showed that lncRNA-FER1L4 promoted the target gene RB transcriptional corepressor 1 (RB1) expression by binding to miR-106a-5p. Based on the previous report, FER1L4 suppresses oncogenesis by inhibiting miR-106a-5p expression^[55].

CONCLUSION

GCs are still one of the greatest challenges to human

health, and metastasis is the leading cause of death from these cancers worldwide. Recently, lncRNAs have attracted remarkable attention from researchers, and increasing evidence suggests that lncRNAs are frequently aberrantly expressed in cancers, and play vital roles in various biological processes including cancer metastasis. In this review, we focus on the metastasis-associated lncRNAs in GCs. Characterization of their deregulated expression, especially in blood or other fluids, could provide novel biomarkers for cancer diagnosis and prognosis, including early diagnosis and observation of cancer progression^[46-48]. Many lncRNAs have been demonstrated to participate in cancer metastasis by influencing the process of EMT and affecting pathways required for cancer metastasis. The molecular mechanisms of lncRNA involvement in cancer include chromatin modification, transcriptional control, mRNA/miRNA processing, translational control, mRNA stability control and acting as “sponges” for miRNAs. However, the roles and mechanisms of metastasis-associated lncRNAs in GC still require further investigation and evaluation. Through promoting the understanding of lncRNAs in cancer, lncRNAs may be implicated as new targets for biological therapeutics.

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 **Allemani C**, Weir HK, Carreira H, Harewood R, Spika D, Wang XS, Bannon F, Ahn JV, Johnson CJ, Bonaventure A, Marcos-Gragera R, Stiller C, Azevedo e Silva G, Chen WQ, Ogunbiyi OJ, Rachet B, Soeberg MJ, You H, Matsuda T, Bielska-Lasota M, Storm H, Tucker TC, Coleman MP. Global surveillance of cancer survival 1995-2009: analysis of individual data for 25,676,887 patients from 279 population-based registries in 67 countries (CONCORD-2). *Lancet* 2015; **385**: 977-1010 [PMID: 25467588 DOI: 10.1016/S0140-6736(14)62038-9]
- 3 **Derrien T**, Johnson R, Bussotti G, Tanzer A, Djebali S, Tilgner H, Guernec G, Martin D, Merkel A, Knowles DG, Lagarde J, Veeravalli L, Ruan X, Ruan Y, Lassmann T, Carninci P, Brown JB, Lipovich L, Gonzalez JM, Thomas M, Davis CA, Shiekhata R, Gingeras TR, Hubbard TJ, Notredame C, Harrow J, Guigó R. The GENCODE v7 catalog of human long noncoding RNAs: analysis of their gene structure, evolution, and expression. *Genome Res* 2012; **22**: 1775-1789 [PMID: 22955988 DOI: 10.1101/gr.132159.111]
- 4 **Ezkurdia I**, Juan D, Rodriguez JM, Frankish A, Diekhans M, Harrow J, Vazquez J, Valencia A, Tress ML. Multiple evidence strands suggest that there may be as few as 19,000 human protein-coding genes. *Hum Mol Genet* 2014; **23**: 5866-5878 [PMID: 24939910 DOI: 10.1093/hmg/ddu309]
- 5 **Mattick JS**, Rinn JL. Discovery and annotation of long noncoding RNAs. *Nat Struct Mol Biol* 2015; **22**: 5-7 [PMID: 25565026 DOI: 10.1038/nsmb.2942]
- 6 **He L**, Hannon GJ. MicroRNAs: small RNAs with a big role in gene regulation. *Nat Rev Genet* 2004; **5**: 522-531 [PMID: 15211354 DOI: 10.1038/nrg1379]
- 7 **Prensner JR**, Chinnaiyan AM. The emergence of lncRNAs in cancer biology. *Cancer Discov* 2011; **1**: 391-407 [PMID: 22096659 DOI: 10.1158/2159-8290.CD-11-0209]
- 8 **Khorkova O**, Hsiao J, Wahlestedt C. Basic biology and therapeutic

- implications of lncRNA. *Adv Drug Deliv Rev* 2015; **87**: 15-24 [PMID: 26024979 DOI: 10.1016/j.addr.2015.05.012]
- 9 **Yan X**, Hu Z, Feng Y, Hu X, Yuan J, Zhao SD, Zhang Y, Yang L, Shan W, He Q, Fan L, Kandalaft LE, Tanyi JL, Li C, Yuan CX, Zhang D, Yuan H, Hua K, Lu Y, Katsaros D, Huang Q, Montone K, Fan Y, Coukos G, Boyd J, Sood AK, Rebbeck T, Mills GB, Dang CV, Zhang L. Comprehensive Genomic Characterization of Long Non-coding RNAs across Human Cancers. *Cancer Cell* 2015; **28**: 529-540 [PMID: 26461095 DOI: 10.1016/j.ccell.2015.09.006]
 - 10 **Guttman M**, Rinn JL. Modular regulatory principles of large non-coding RNAs. *Nature* 2012; **482**: 339-346 [PMID: 22337053 DOI: 10.1038/nature10887]
 - 11 **Cabili MN**, Trapnell C, Goff L, Koziol M, Tazon-Vega B, Regev A, Rinn JL. Integrative annotation of human large intergenic noncoding RNAs reveals global properties and specific subclasses. *Genes Dev* 2011; **25**: 1915-1927 [PMID: 21890647 DOI: 10.1101/gad.17446611]
 - 12 **Novikova IV**, Hennelly SP, Sanbonmatsu KY. Sizing up long non-coding RNAs: do lncRNAs have secondary and tertiary structure? *Bioarchitecture* 2012; **2**: 189-199 [PMID: 23267412 DOI: 10.4161/bioa.22592]
 - 13 **Nakagawa S**, Kageyama Y. Nuclear lncRNAs as epigenetic regulators-beyond skepticism. *Biochim Biophys Acta* 2014; **1839**: 215-222 [PMID: 24200874 DOI: 10.1016/j.bbagr.2013.10.009]
 - 14 **Mattick JS**, Amaral PP, Dinger ME, Mercer TR, Mehler MF. RNA regulation of epigenetic processes. *Bioessays* 2009; **31**: 51-59 [PMID: 19154003 DOI: 10.1002/bies.080099]
 - 15 **Wang KC**, Chang HY. Molecular mechanisms of long noncoding RNAs. *Mol Cell* 2011; **43**: 904-914 [PMID: 21925379 DOI: 10.1016/j.molcel.2011.08.018]
 - 16 **Bracken AP**, Helin K. Polycomb group proteins: navigators of lineage pathways led astray in cancer. *Nat Rev Cancer* 2009; **9**: 773-784 [PMID: 19851313 DOI: 10.1038/nrc2736]
 - 17 **Schuettengruber B**, Martinez AM, Iovino N, Cavalli G. Trithorax group proteins: switching genes on and keeping them active. *Nat Rev Mol Cell Biol* 2011; **12**: 799-814 [PMID: 22108599 DOI: 10.1038/nrm3230]
 - 18 **Cao R**, Wang L, Wang H, Xia L, Erdjument-Bromage H, Tempst P, Jones RS, Zhang Y. Role of histone H3 lysine 27 methylation in Polycomb-group silencing. *Science* 2002; **298**: 1039-1043 [PMID: 12351676 DOI: 10.1126/science.1076997]
 - 19 **Kuzmichev A**, Nishioka K, Erdjument-Bromage H, Tempst P, Reinberg D. Histone methyltransferase activity associated with a human multiprotein complex containing the Enhancer of Zeste protein. *Genes Dev* 2002; **16**: 2893-2905 [PMID: 12435631 DOI: 10.1101/gad.1035902]
 - 20 **Penny GD**, Kay GF, Sheardown SA, Rastan S, Brockdorff N. Requirement for Xist in X chromosome inactivation. *Nature* 1996; **379**: 131-137 [PMID: 8538762 DOI: 10.1038/379131a0]
 - 21 **Lee JT**. Lessons from X-chromosome inactivation: long ncRNA as guides and tethers to the epigenome. *Genes Dev* 2009; **23**: 1831-1842 [PMID: 19684108 DOI: 10.1101/gad.1811209]
 - 22 **Zhao J**, Sun BK, Erwin JA, Song JJ, Lee JT. Polycomb proteins targeted by a short repeat RNA to the mouse X chromosome. *Science* 2008; **322**: 750-756 [PMID: 18974356 DOI: 10.1126/science.1163045]
 - 23 **Jeon Y**, Lee JT. YY1 tethers Xist RNA to the inactive X nucleation center. *Cell* 2011; **146**: 119-133 [PMID: 21729784 DOI: 10.1016/j.cell.2011.06.026]
 - 24 **Rinn JL**, Kertesz M, Wang JK, Squazzo SL, Xu X, Bruggmann SA, Goodnough LH, Helms JA, Farnham PJ, Segal E, Chang HY. Functional demarcation of active and silent chromatin domains in human HOX loci by noncoding RNAs. *Cell* 2007; **129**: 1311-1323 [PMID: 17604720 DOI: 10.1016/j.cell.2007.05.022]
 - 25 **Tsai MC**, Manor O, Wan Y, Mosammamapara N, Wang JK, Lan F, Shi Y, Segal E, Chang HY. Long noncoding RNA as modular scaffold of histone modification complexes. *Science* 2010; **329**: 689-693 [PMID: 20616235 DOI: 10.1126/science.1192002]
 - 26 **Wu SC**, Kallin EM, Zhang Y. Role of H3K27 methylation in the regulation of lncRNA expression. *Cell Res* 2010; **20**: 1109-1116 [PMID: 20680032 DOI: 10.1038/cr.2010.114]
 - 27 **Shamovsky I**, Ivannikov M, Kandel ES, Gershon D, Nudler E. RNA-mediated response to heat shock in mammalian cells. *Nature* 2006; **440**: 556-560 [PMID: 16554823 DOI: 10.1038/nature04518]
 - 28 **Wang X**, Arai S, Song X, Reichart D, Du K, Pascual G, Tempst P, Rosenfeld MG, Glass CK, Kurokawa R. Induced ncRNAs allosterically modify RNA-binding proteins in cis to inhibit transcription. *Nature* 2008; **454**: 126-130 [PMID: 18509338 DOI: 10.1038/nature06992]
 - 29 **Fang XY**, Pan HF, Leng RX, Ye DQ. Long noncoding RNAs: novel insights into gastric cancer. *Cancer Lett* 2015; **356**: 357-366 [PMID: 25444905 DOI: 10.1016/j.canlet.2014.11.005]
 - 30 **Xie X**, Tang B, Xiao YF, Xie R, Li BS, Dong H, Zhou JY, Yang SM. Long non-coding RNAs in colorectal cancer. *Oncotarget* 2016; **7**: 5226-5239 [PMID: 26637808 DOI: 10.18632/oncotarget.6446]
 - 31 **Tripathi V**, Ellis JD, Shen Z, Song DY, Pan Q, Watt AT, Freier SM, Bennett CF, Sharma A, Bubulya PA, Blencowe BJ, Prasanth SG, Prasanth KV. The nuclear-retained noncoding RNA MALAT1 regulates alternative splicing by modulating SR splicing factor phosphorylation. *Mol Cell* 2010; **39**: 925-938 [PMID: 20797886 DOI: 10.1016/j.molcel.2010.08.011]
 - 32 **Gonzalez I**, Munita R, Agirre E, Dittmer TA, Gysling K, Misteli T, Lucio RF. A lncRNA regulates alternative splicing via establishment of a splicing-specific chromatin signature. *Nat Struct Mol Biol* 2015; **22**: 370-376 [PMID: 25849144 DOI: 10.1038/nsmb.3005]
 - 33 **Krystal GW**, Armstrong BC, Battey JF. N-myc mRNA forms an RNA-RNA duplex with endogenous antisense transcripts. *Mol Cell Biol* 1990; **10**: 4180-4191 [PMID: 1695323]
 - 34 **Li K**, Blum Y, Verma A, Liu Z, Pramanik K, Leigh NR, Chun CZ, Samant GV, Zhao B, Garnaas MK, Horswill MA, Stanhope SA, North PE, Miao RQ, Wilkinson GA, Affolter M, Ramchandran R. A noncoding antisense RNA in tie-1 locus regulates tie-1 function in vivo. *Blood* 2010; **115**: 133-139 [PMID: 19880500 DOI: 10.1182/blood-2009-09-242180]
 - 35 **Beltran M**, Puig I, Peña C, García JM, Alvarez AB, Peña R, Bonilla F, de Herreros AG. A natural antisense transcript regulates Zeb2/Sip1 gene expression during Snail1-induced epithelial-mesenchymal transition. *Genes Dev* 2008; **22**: 756-769 [PMID: 18347095 DOI: 10.1101/gad.455708]
 - 36 **Cai X**, Cullen BR. The imprinted H19 noncoding RNA is a primary microRNA precursor. *RNA* 2007; **13**: 313-316 [PMID: 17237358 DOI: 10.1261/rna.351707]
 - 37 **Carrieri C**, Cimatti L, Biagioli M, Beugnot A, Zucchelli S, Fedele S, Pesce E, Ferrer I, Collavin L, Santoro C, Forrest AR, Carninci P, Biffo S, Stupka E, Gustincich S. Long non-coding antisense RNA controls Uchl1 translation through an embedded SINEB2 repeat. *Nature* 2012; **491**: 454-457 [PMID: 23064229 DOI: 10.1038/nature11508]
 - 38 **Yoon JH**, Abdelmohsen K, Srikantan S, Yang X, Martindale JL, De S, Huarte M, Zhan M, Becker KG, Gorospe M. LincRNA-p21 suppresses target mRNA translation. *Mol Cell* 2012; **47**: 648-655 [PMID: 22841487 DOI: 10.1016/j.molcel.2012.06.027]
 - 39 **Yuan JH**, Yang F, Wang F, Ma JZ, Guo YJ, Tao QF, Liu F, Pan W, Wang TT, Zhou CC, Wang SB, Wang YZ, Yang Y, Yang N, Zhou WP, Yang GS, Sun SH. A long noncoding RNA activated by TGF- β promotes the invasion-metastasis cascade in hepatocellular carcinoma. *Cancer Cell* 2014; **25**: 666-681 [PMID: 24768205 DOI: 10.1016/j.ccr.2014.03.010]
 - 40 **Gong C**, Maquat LE. lncRNAs transactivate STAU1-mediated mRNA decay by duplexing with 3' UTRs via Alu elements. *Nature* 2011; **470**: 284-288 [PMID: 21307942 DOI: 10.1038/nature09701]
 - 41 **Liu XH**, Sun M, Nie FQ, Ge YB, Zhang EB, Yin DD, Kong R, Xia R, Lu KH, Li JH, De W, Wang KM, Wang ZX. Lnc RNA HOTAIR functions as a competing endogenous RNA to regulate HER2 expression by sponging miR-331-3p in gastric cancer. *Mol Cancer* 2014; **13**: 92 [PMID: 24775712 DOI: 10.1186/1476-4598-13-92]
 - 42 **Song B**, Guan Z, Liu F, Sun D, Wang K, Qu H. Long non-coding RNA HOTAIR promotes HLA-G expression via inhibiting miR-152 in gastric cancer cells. *Biochem Biophys Res Commun* 2015; **464**: 807-813 [PMID: 26187665 DOI: 10.1016/j.bbrc.2015.07.040]

- 43 **Ge XS**, Ma HJ, Zheng XH, Ruan HL, Liao XY, Xue WQ, Chen YB, Zhang Y, Jia WH. HOTAIR, a prognostic factor in esophageal squamous cell carcinoma, inhibits WIF-1 expression and activates Wnt pathway. *Cancer Sci* 2013; **104**: 1675-1682 [PMID: 24118380 DOI: 10.1111/cas.12296]
- 44 **Lv XB**, Lian GY, Wang HR, Song E, Yao H, Wang MH. Long noncoding RNA HOTAIR is a prognostic marker for esophageal squamous cell carcinoma progression and survival. *PLoS One* 2013; **8**: e63516 [PMID: 23717443 DOI: 10.1371/journal.pone.0063516]
- 45 **Kogo R**, Shimamura T, Mimori K, Kawahara K, Imoto S, Sudo T, Tanaka F, Shibata K, Suzuki A, Komune S, Miyano S, Mori M. Long noncoding RNA HOTAIR regulates polycomb-dependent chromatin modification and is associated with poor prognosis in colorectal cancers. *Cancer Res* 2011; **71**: 6320-6326 [PMID: 21862635 DOI: 10.1158/0008-5472.CAN-11-1021]
- 46 **Svoboda M**, Slysokova J, Schneiderova M, Makovicky P, Bielick L, Levy M, Lipska L, Hemmelova B, Kala Z, Protivankova M, Vycital O, Liska V, Schwarzova L, Vodickova L, Vodicka P. HOTAIR long non-coding RNA is a negative prognostic factor not only in primary tumors, but also in the blood of colorectal cancer patients. *Carcinogenesis* 2014; **35**: 1510-1515 [PMID: 24583926 DOI: 10.1093/carcin/bgu055]
- 47 **Arita T**, Ichikawa D, Konishi H, Komatsu S, Shiozaki A, Shoda K, Kawaguchi T, Hirajima S, Nagata H, Kubota T, Fujiwara H, Okamoto K, Otsuji E. Circulating long non-coding RNAs in plasma of patients with gastric cancer. *Anticancer Res* 2013; **33**: 3185-3193 [PMID: 23898077]
- 48 **Shao Y**, Ye M, Jiang X, Sun W, Ding X, Liu Z, Ye G, Zhang X, Xiao B, Guo J. Gastric juice long noncoding RNA used as a tumor marker for screening gastric cancer. *Cancer* 2014; **120**: 3320-3328 [PMID: 24986041 DOI: 10.1002/cncr.28882]
- 49 **Ji Q**, Liu X, Fu X, Zhang L, Sui H, Zhou L, Sun J, Cai J, Qin J, Ren J, Li Q. Resveratrol inhibits invasion and metastasis of colorectal cancer cells via MALAT1 mediated Wnt/ β -catenin signal pathway. *PLoS One* 2013; **8**: e78700 [PMID: 24244343 DOI: 10.1371/journal.pone.0078700]
- 50 **Deng Q**, He B, Gao T, Pan Y, Sun H, Xu Y, Li R, Ying H, Wang F, Liu X, Chen J, Wang S. Up-regulation of 91H promotes tumor metastasis and predicts poor prognosis for patients with colorectal cancer. *PLoS One* 2014; **9**: e103022 [PMID: 25058480 DOI: 10.1371/journal.pone.0103022]
- 51 **Hu Y**, Chen HY, Yu CY, Xu J, Wang JL, Qian J, Zhang X, Fang JY. A long non-coding RNA signature to improve prognosis prediction of colorectal cancer. *Oncotarget* 2014; **5**: 2230-2242 [PMID: 24809982 DOI: 10.18632/oncotarget.1895]
- 52 **Alaiyan B**, Ilyayev N, Stojadinovic A, Izadjoo M, Roistacher M, Pavlov V, Tzivin V, Halle D, Pan H, Trink B, Gure AO, Nissan A. Differential expression of colon cancer associated transcript1 (CCAT1) along the colonic adenoma-carcinoma sequence. *BMC Cancer* 2013; **13**: 196 [PMID: 23594791 DOI: 10.1186/1471-2407-13-196]
- 53 **He X**, Tan X, Wang X, Jin H, Liu L, Ma L, Yu H, Fan Z. C-Myc-activated long noncoding RNA CCAT1 promotes colon cancer cell proliferation and invasion. *Tumour Biol* 2014; **35**: 12181-12188 [PMID: 25185650 DOI: 10.1007/s13277-014-2526-4]
- 54 **Ling H**, Spizzo R, Atlasi Y, Nicoloso M, Shimizu M, Redis RS, Nishida N, Gafà R, Song J, Guo Z, Ivan C, Barbarotto E, De Vries I, Zhang X, Ferracin M, Churchman M, van Galen JF, Beverloo BH, Shariati M, Haderk F, Estecio MR, Garcia-Manero G, Patijn GA, Götley DC, Bhardwaj V, Shureiqi I, Sen S, Multani AS, Welsh J, Yamamoto K, Taniguchi I, Song MA, Gallinger S, Casey G, Thibodeau SN, Le Marchand L, Tiirikainen M, Mani SA, Zhang W, Davuluri RV, Mimori K, Mori M, Sieuwerts AM, Martens JW, Tomlinson I, Negrini M, Berindan-Neagoe I, Foekens JA, Hamilton SR, Lanza G, Kopetz S, Fodde R, Calin GA. CCAT2, a novel noncoding RNA mapping to 8q24, underlies metastatic progression and chromosomal instability in colon cancer. *Genome Res* 2013; **23**: 1446-1461 [PMID: 23796952 DOI: 10.1101/gr.152942.112]
- 55 **Yue B**, Sun B, Liu C, Zhao S, Zhang D, Yu F, Yan D. Long non-coding RNA Fer-1-like protein 4 suppresses oncogenesis and exhibits prognostic value by associating with miR-106a-5p in colon cancer. *Cancer Sci* 2015; **106**: 1323-1332 [PMID: 26224446 DOI: 10.1111/cas.12759]
- 56 **Wu ZH**, Wang XL, Tang HM, Jiang T, Chen J, Lu S, Qiu GQ, Peng ZH, Yan DW. Long non-coding RNA HOTAIR is a powerful predictor of metastasis and poor prognosis and is associated with epithelial-mesenchymal transition in colon cancer. *Oncol Rep* 2014; **32**: 395-402 [PMID: 24840737 DOI: 10.3892/or.2014.3186]
- 57 **Qi P**, Xu MD, Ni SJ, Huang D, Wei P, Tan C, Zhou XY, Du X. Low expression of LOC285194 is associated with poor prognosis in colorectal cancer. *J Transl Med* 2013; **11**: 122 [PMID: 23680400 DOI: 10.1186/1479-5876-11-122]
- 58 **Xu C**, Yang M, Tian J, Wang X, Li Z. MALAT-1: a long non-coding RNA and its important 3' end functional motif in colorectal cancer metastasis. *Int J Oncol* 2011; **39**: 169-175 [PMID: 21503572 DOI: 10.3892/ijo.2011.1007]
- 59 **Yang MH**, Hu ZY, Xu C, Xie LY, Wang XY, Chen SY, Li ZG. MALAT1 promotes colorectal cancer cell proliferation/migration/invasion via PRKA kinase anchor protein 9. *Biochim Biophys Acta* 2015; **1852**: 166-174 [PMID: 25446987 DOI: 10.1016/j.bbdis.2014.11.013]
- 60 **Kan JY**, Wu DC, Yu FJ, Wu CY, Ho YW, Chiu YJ, Jian SF, Hung JY, Wang JY, Kuo PL. Chemokine (C-C Motif) Ligand 5 is Involved in Tumor-Associated Dendritic Cell-Mediated Colon Cancer Progression Through Non-Coding RNA MALAT-1. *J Cell Physiol* 2015; **230**: 1883-1894 [PMID: 25546229 DOI: 10.1002/jcp.24918]
- 61 **Yin DD**, Liu ZJ, Zhang E, Kong R, Zhang ZH, Guo RH. Decreased expression of long noncoding RNA MEG3 affects cell proliferation and predicts a poor prognosis in patients with colorectal cancer. *Tumour Biol* 2015; **36**: 4851-4859 [PMID: 25636452 DOI: 10.1007/s13277-015-3139-2]
- 62 **Qi P**, Xu MD, Ni SJ, Shen XH, Wei P, Huang D, Tan C, Sheng WQ, Zhou XY, Du X. Down-regulation of ncRAN, a long non-coding RNA, contributes to colorectal cancer cell migration and invasion and predicts poor overall survival for colorectal cancer patients. *Mol Carcinog* 2015; **54**: 742-750 [PMID: 24519959 DOI: 10.1002/mc.22137]
- 63 **Yan B**, Gu W, Yang Z, Gu Z, Yue X, Gu Q, Liu L. Downregulation of a long noncoding RNA-ncRUPAR contributes to tumor inhibition in colorectal cancer. *Tumour Biol* 2014; **35**: 11329-11335 [PMID: 25119598 DOI: 10.1007/s13277-014-2465-0]
- 64 **Li Y**, Li Y, Chen W, He F, Tan Z, Zheng J, Wang W, Zhao Q, Li J. NEAT expression is associated with tumor recurrence and unfavorable prognosis in colorectal cancer. *Oncotarget* 2015; **6**: 27641-27650 [PMID: 26314847 DOI: 10.18632/oncotarget.4737]
- 65 **Takahashi Y**, Sawada G, Kurashige J, Uchi R, Matsumura T, Ueo H, Takano Y, Eguchi H, Sudo T, Sugimachi K, Yamamoto H, Doki Y, Mori M, Mimori K. Amplification of PVT-1 is involved in poor prognosis via apoptosis inhibition in colorectal cancers. *Br J Cancer* 2014; **110**: 164-171 [PMID: 24196785 DOI: 10.1038/bjc.2013.698]
- 66 **Sun J**, Ding C, Yang Z, Liu T, Zhang X, Zhao C, Wang J. The long non-coding RNA TUG1 indicates a poor prognosis for colorectal cancer and promotes metastasis by affecting epithelial-mesenchymal transition. *J Transl Med* 2016; **14**: 42 [PMID: 26856330 DOI: 10.1186/s12967-016-0786-z]
- 67 **Gao T**, He B, Pan Y, Xu Y, Li R, Deng Q, Sun H, Wang S. Long non-coding RNA 91H contributes to the occurrence and progression of esophageal squamous cell carcinoma by inhibiting IGF2 expression. *Mol Carcinog* 2015; **54**: 359-367 [PMID: 24706416 DOI: 10.1002/mc.22106]
- 68 **Wu W**, Bhagat TD, Yang X, Song JH, Cheng Y, Agarwal R, Abraham JM, Ibrahim S, Bartenstein M, Hussain Z, Suzuki M, Yu Y, Chen W, Eng C, Greally J, Verma A, Meltzer SJ. Hypomethylation of noncoding DNA regions and overexpression of the long noncoding RNA, AFAP1-AS1, in Barrett's esophagus and esophageal adenocarcinoma. *Gastroenterology* 2013; **144**: 956-966.e4 [PMID: 23333711 DOI: 10.1053/j.gastro.2013.01.019]

- 69 **Zhang X**, Xu Y, He C, Guo X, Zhang J, He C, Zhang L, Kong M, Chen B, Zhu C. Elevated expression of CCAT2 is associated with poor prognosis in esophageal squamous cell carcinoma. *J Surg Oncol* 2015; **111**: 834-839 [PMID: 25919911 DOI: 10.1002/jso.23888]
- 70 **Gao T**, He B, Pan Y, Gu L, Chen L, Nie Z, Xu Y, Li R, Wang S. H19 DMR methylation correlates to the progression of esophageal squamous cell carcinoma through IGF2 imprinting pathway. *Clin Transl Oncol* 2014; **16**: 410-417 [PMID: 23943562 DOI: 10.1007/s12094-013-1098-x]
- 71 **Yang X**, Song JH, Cheng Y, Wu W, Bhagat T, Yu Y, Abraham JM, Ibrahim S, Ravich W, Roland BC, Khashab M, Singh VK, Shin EJ, Yang X, Verma AK, Meltzer SJ, Mori Y. Long non-coding RNA HNF1A-AS1 regulates proliferation and migration in oesophageal adenocarcinoma cells. *Gut* 2014; **63**: 881-890 [PMID: 24000294 DOI: 10.1136/gutjnl-2013-305266]
- 72 **Li X**, Wu Z, Mei Q, Li X, Guo M, Fu X, Han W. Long non-coding RNA HOTAIR, a driver of malignancy, predicts negative prognosis and exhibits oncogenic activity in oesophageal squamous cell carcinoma. *Br J Cancer* 2013; **109**: 2266-2278 [PMID: 24022190 DOI: 10.1038/bjc.2013.548]
- 73 **Chen FJ**, Sun M, Li SQ, Wu QQ, Ji L, Liu ZL, Zhou GZ, Cao G, Jin L, Xie HW, Wang CM, Lv J, De W, Wu M, Cao XF. Upregulation of the long non-coding RNA HOTAIR promotes esophageal squamous cell carcinoma metastasis and poor prognosis. *Mol Carcinog* 2013; **52**: 908-915 [PMID: 24151120 DOI: 10.1002/mc.21944]
- 74 **Wang X**, Li M, Wang Z, Han S, Tang X, Ge Y, Zhou L, Zhou C, Yuan Q, Yang M. Silencing of long noncoding RNA MALAT1 by miR-101 and miR-217 inhibits proliferation, migration, and invasion of esophageal squamous cell carcinoma cells. *J Biol Chem* 2015; **290**: 3925-3935 [PMID: 25538231 DOI: 10.1074/jbc.M114.596866]
- 75 **Ji Q**, Zhang L, Liu X, Zhou L, Wang W, Han Z, Sui H, Tang Y, Wang Y, Liu N, Ren J, Hou F, Li Q. Long non-coding RNA MALAT1 promotes tumour growth and metastasis in colorectal cancer through binding to SFPQ and releasing oncogene PTBP2 from SFPQ/PTBP2 complex. *Br J Cancer* 2014; **111**: 736-748 [PMID: 25025966 DOI: 10.1038/bjc.2014.383]
- 76 **Wu XS**, Wang XA, Wu WG, Hu YP, Li ML, Ding Q, Weng H, Shu YJ, Liu TY, Jiang L, Cao Y, Bao RF, Mu JS, Tan ZJ, Tao F, Liu YB. MALAT1 promotes the proliferation and metastasis of gallbladder cancer cells by activating the ERK/MAPK pathway. *Cancer Biol Ther* 2014; **15**: 806-814 [PMID: 24658096 DOI: 10.4161/cbt.28584]
- 77 **Xu C**, Shao Y, Xia T, Yang Y, Dai J, Luo L, Zhang X, Sun W, Song H, Xiao B, Guo J. lncRNA-AC130710 targeting by miR-129-5p is upregulated in gastric cancer and associates with poor prognosis. *Tumour Biol* 2014; **35**: 9701-9706 [PMID: 24969565 DOI: 10.1007/s13277-014-2274-5]
- 78 **Wang Y**, Liu X, Zhang H, Sun L, Zhou Y, Jin H, Zhang H, Zhang H, Liu J, Guo H, Nie Y, Wu K, Fan D, Zhang H, Liu L. Hypoxia-inducible lncRNA-AK058003 promotes gastric cancer metastasis by targeting γ -synuclein. *Neoplasia* 2014; **16**: 1094-1106 [PMID: 25499222 DOI: 10.1016/j.neo.2014.10.008]
- 79 **Park SM**, Park SJ, Kim HJ, Kwon OH, Kang TW, Sohn HA, Kim SK, Moo Noh S, Song KS, Jang SJ, Sung Kim Y, Kim SY. A known expressed sequence tag, BM742401, is a potent lincRNA inhibiting cancer metastasis. *Exp Mol Med* 2013; **45**: e31 [PMID: 23846333 DOI: 10.1038/emmm.2013.59]
- 80 **Xu TP**, Huang MD, Xia R, Liu XX, Sun M, Yin L, Chen WM, Han L, Zhang EB, Kong R, De W, Shu YQ. Decreased expression of the long non-coding RNA FENDRR is associated with poor prognosis in gastric cancer and FENDRR regulates gastric cancer cell metastasis by affecting fibronectin1 expression. *J Hematol Oncol* 2014; **7**: 63 [PMID: 25167886 DOI: 10.1186/s13045-014-0063-7]
- 81 **Hu Y**, Wang J, Qian J, Kong X, Tang J, Wang Y, Chen H, Hong J, Zou W, Chen Y, Xu J, Fang JY. Long noncoding RNA GAPLINC regulates CD44-dependent cell invasiveness and associates with poor prognosis of gastric cancer. *Cancer Res* 2014; **74**: 6890-6902 [PMID: 25277524 DOI: 10.1158/0008-5472.CAN-14-0686]
- 82 **Zhang EB**, Han L, Yin DD, Kong R, De W, Chen J. c-Myc-induced, long, noncoding H19 affects cell proliferation and predicts a poor prognosis in patients with gastric cancer. *Med Oncol* 2014; **31**: 914 [PMID: 24671855 DOI: 10.1007/s12032-014-0914-7]
- 83 **Li H**, Yu B, Li J, Su L, Yan M, Zhu Z, Liu B. Overexpression of lncRNA H19 enhances carcinogenesis and metastasis of gastric cancer. *Oncotarget* 2014; **5**: 2318-2329 [PMID: 24810858 DOI: 10.18632/oncotarget.1913]
- 84 **Hajjari M**, Behmanesh M, Sadeghizadeh M, Zeinoddini M. Up-regulation of HOTAIR long non-coding RNA in human gastric adenocarcinoma tissues. *Med Oncol* 2013; **30**: 670 [PMID: 23888369 DOI: 10.1007/s12032-013-0670-0]
- 85 **Endo H**, Shiroki T, Nakagawa T, Yokoyama M, Tamai K, Yamanami H, Fujiya T, Sato I, Yamaguchi K, Tanaka N, Iijima K, Shimosegawa T, Sugamura K, Satoh K. Enhanced expression of long non-coding RNA HOTAIR is associated with the development of gastric cancer. *PLoS One* 2013; **8**: e77070 [PMID: 24130837 DOI: 10.1371/journal.pone.0077070]
- 86 **Lee NK**, Lee JH, Park CH, Yu D, Lee YC, Cheong JH, Noh SH, Lee SK. Long non-coding RNA HOTAIR promotes carcinogenesis and invasion of gastric adenocarcinoma. *Biochem Biophys Res Commun* 2014; **451**: 171-178 [PMID: 25063030 DOI: 10.1016/j.bbrc.2014.07.067]
- 87 **Okugawa Y**, Toiyama Y, Hur K, Toden S, Saigusa S, Tanaka K, Inoue Y, Mohri Y, Kusunoki M, Boland CR, Goel A. Metastasis-associated long non-coding RNA drives gastric cancer development and promotes peritoneal metastasis. *Carcinogenesis* 2014; **35**: 2731-2739 [PMID: 25280565 DOI: 10.1093/carcin/bgu200]
- 88 **Zhao Y**, Guo Q, Chen J, Hu J, Wang S, Sun Y. Role of long non-coding RNA HULC in cell proliferation, apoptosis and tumor metastasis of gastric cancer: a clinical and in vitro investigation. *Oncol Rep* 2014; **31**: 358-364 [PMID: 24247585 DOI: 10.3892/or.2013.2850]
- 89 **Han Y**, Ye J, Wu D, Wu P, Chen Z, Chen J, Gao S, Huang J. LEIGC long non-coding RNA acts as a tumor suppressor in gastric carcinoma by inhibiting the epithelial-to-mesenchymal transition. *BMC Cancer* 2014; **14**: 932 [PMID: 25496320 DOI: 10.1186/1471-2407-14-932]
- 90 **Guo X**, Yang Z, Zhi Q, Wang D, Guo L, Li G, Miao R, Shi Y, Kuang Y. Long noncoding RNA OR3A4 promotes metastasis and tumorigenicity in gastric cancer. *Oncotarget* 2016; **7**: 30276-30294 [PMID: 26863570 DOI: 10.18632/oncotarget.7217]
- 91 **Wang B**, Li W, Liu H, Yang L, Liao Q, Cui S, Wang H, Zhao L. miR-29b suppresses tumor growth and metastasis in colorectal cancer via downregulating Tiam1 expression and inhibiting epithelial-mesenchymal transition. *Cell Death Dis* 2014; **5**: e1335 [PMID: 25032858 DOI: 10.1038/cddis.2014.304]
- 92 **Ji P**, Diederichs S, Wang W, Böing S, Metzger R, Schneider PM, Tidow N, Brandt B, Buerger H, Bulk E, Thomas M, Berdel WE, Serve H, Müller-Tidow C. MALAT-1, a novel noncoding RNA, and thymosin beta4 predict metastasis and survival in early-stage non-small cell lung cancer. *Oncogene* 2003; **22**: 8031-8041 [PMID: 12970751 DOI: 10.1038/sj.onc.1206928]
- 93 **Zhang F**, Luo Y, Shao Z, Xu L, Liu X, Niu Y, Shi J, Sun X, Liu Y, Ding Y, Zhao L. MicroRNA-187, a downstream effector of TGF β pathway, suppresses Smad-mediated epithelial-mesenchymal transition in colorectal cancer. *Cancer Lett* 2016; **373**: 203-213 [PMID: 26820227 DOI: 10.1016/j.canlet.2016.01.037]
- 94 **Xu Q**, Deng F, Qin Y, Zhao Z, Wu Z, Xing Z, Ji A, Wang QJ. Long non-coding RNA regulation of epithelial-mesenchymal transition in cancer metastasis. *Cell Death Dis* 2016; **7**: e2254 [PMID: 27277676 DOI: 10.1038/cddis.2016.149]
- 95 **Wei G**, Luo H, Sun Y, Li J, Tian L, Liu W, Liu L, Luo J, He J, Chen R. Transcriptome profiling of esophageal squamous cell carcinoma reveals a long noncoding RNA acting as a tumor suppressor. *Oncotarget* 2015; **6**: 17065-17080 [PMID: 26158411 DOI: 10.18632/oncotarget.4185]
- 96 **Liu YW**, Sun M, Xia R, Zhang EB, Liu XH, Zhang ZH, Xu TP, De W, Liu BR, Wang ZX. LincHOTAIR epigenetically

- silences miR34a by binding to PRC2 to promote the epithelial-to-mesenchymal transition in human gastric cancer. *Cell Death Dis* 2015; **6**: e1802 [PMID: 26136075 DOI: 10.1038/cddis.2015.150]
- 97 **Niinuma T**, Suzuki H, Nojima M, Noshio K, Yamamoto H, Takamaru H, Yamamoto E, Maruyama R, Nobuoka T, Miyazaki Y, Nishida T, Bamba T, Kanda T, Ajioka Y, Taguchi T, Okahara S, Takahashi H, Nishida Y, Hosokawa M, Hasegawa T, Tokino T, Hirata K, Imai K, Toyota M, Shinomura Y. Upregulation of miR-196a and HOTAIR drive malignant character in gastrointestinal stromal tumors. *Cancer Res* 2012; **72**: 1126-1136 [PMID: 22258453 DOI: 10.1158/0008-5472.CAN-11-1803]
 - 98 **Yang C**, Tang R, Ma X, Wang Y, Luo D, Xu Z, Zhu Y, Yang L. Tag SNPs in long non-coding RNA H19 contribute to susceptibility to gastric cancer in the Chinese Han population. *Oncotarget* 2015; **6**: 15311-15320 [PMID: 25944697 DOI: 10.18632/oncotarget.3840]
 - 99 **Han D**, Gao X, Wang M, Qiao Y, Xu Y, Yang J, Dong N, He J, Sun Q, Lv G, Xu C, Tao J, Ma N. Long noncoding RNA H19 indicates a poor prognosis of colorectal cancer and promotes tumor growth by recruiting and binding to eIF4A3. *Oncotarget* 2016; **7**: 22159-22173 [PMID: 26989025 DOI: 10.18632/oncotarget.8063]
 - 100 **Xia T**, Liao Q, Jiang X, Shao Y, Xiao B, Xi Y, Guo J. Long noncoding RNA associated-competing endogenous RNAs in gastric cancer. *Sci Rep* 2014; **4**: 6088 [PMID: 25124853 DOI: 10.1038/srep06088]

P- Reviewer: Lakatos PT, Mocellin S, Nakajima N, Voutsadakis IA
S- Editor: Gong ZM **L- Editor:** Ma JY **E- Editor:** Wang CH



Preoperative therapy in locally advanced esophageal cancer

Pankaj Kumar Garg, Jyoti Sharma, Ashish Jakhetiya, Aakanksha Goel, Manish Kumar Gaur

Pankaj Kumar Garg, Aakanksha Goel, Manish Kumar Gaur, Department of Surgery, University College of Medical Sciences and Guru Teg Bahadur Hospital, University of Delhi, Delhi 110095, India

Jyoti Sharma, Department of Surgical Oncology, Sawai Man Singh Medical College, Jaipur 302004, India

Ashish Jakhetiya, Department of Surgical Oncology, Dr BRA Institute Rotary Cancer Hospital, All India Institute of Medical Sciences, New Delhi 110029, India

Author contributions: Garg PK conceptualized the study; Garg PK and Sharma J searched the literature, analyzed the retrieved literature, and wrote the initial draft; Jakhetiya A, Goel A and Gaur MK provided critical inputs in literature search and analysis, and drafting the manuscript; all the authors read the final draft and approved it.

Conflict-of-interest statement: There is no conflict of interest associated with any of the author.

Open-Access: This article is an open-access article which was selected by an in-house editor and fully peer-reviewed by external reviewers. It is distributed in accordance with the Creative Commons Attribution Non Commercial (CC BY-NC 4.0) license, which permits others to distribute, remix, adapt, build upon this work non-commercially, and license their derivative works on different terms, provided the original work is properly cited and the use is non-commercial. See: <http://creativecommons.org/licenses/by-nc/4.0/>

Manuscript source: Invited manuscript

Correspondence to: Dr. Pankaj Kumar Garg, Associate Professor, Department of Surgery, University College of Medical Sciences and Guru Teg Bahadur Hospital, University of Delhi, Dilshad Garden, Delhi 110095, India. dr.pankajgarg@gmail.com
Telephone: +91-11-22592536
Fax: +91-11-22590495

Received: June 7, 2016
Peer-review started: June 11, 2016
First decision: July 29, 2016
Revised: August 23, 2016

Accepted: September 28, 2016
Article in press: September 28, 2016
Published online: October 21, 2016

Abstract

Esophageal cancer is an aggressive malignancy associated with dismal treatment outcomes. Presence of two distinct histopathological types distinguishes it from other gastrointestinal tract malignancies. Surgery is the cornerstone of treatment in locally advanced esophageal cancer (T2 or greater or node positive); however, a high rate of disease recurrence (systemic and loco-regional) and poor survival justifies a continued search for optimal therapy. Various combinations of multimodality treatment (preoperative/perioperative, or postoperative; radiotherapy, chemotherapy, or chemoradiotherapy) are being explored to lower disease recurrence and improve survival. Preoperative therapy followed by surgery is presently considered the standard of care in resectable locally advanced esophageal cancer as postoperative treatment may not be feasible for all the patients due to the morbidity of esophagectomy and prolonged recovery time limiting the tolerance of patient. There are wide variations in the preoperative therapy practiced across the centres depending upon the institutional practices, availability of facilities and personal experiences. There is paucity of literature to standardize the preoperative therapy. Broadly, chemoradiotherapy is the preferred neo-adjuvant modality in western countries whereas chemotherapy alone is considered optimal in the far East. The present review highlights the significant studies to assist in opting for the best evidence based preoperative therapy (radiotherapy, chemotherapy or chemoradiotherapy) for locally advanced esophageal cancer.

Key words: Esophageal cancer; Preoperative therapy; Multimodality treatment; Chemotherapy; Radiotherapy; Chemoradiotherapy

© The Author(s) 2016. Published by Baishideng Publishing Group Inc. All rights reserved.

Core tip: The literature suggests that preoperative chemoradiotherapy followed by surgery results in optimal outcome while managing locally advanced esophageal cancer; however, there is a need to compare preoperative chemoradiotherapy with chemotherapy alone to further refine the role of preoperative therapy. The standard of care continues to be debated due to difference of opinions and practices across the world and lack of any trial with head-to-head comparison between these two established treatment protocols.

Garg PK, Sharma J, Jakhetiya A, Goel A, Gaur MK. Preoperative therapy in locally advanced esophageal cancer. *World J Gastroenterol* 2016; 22(39): 8750-8759 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v22/i39/8750.htm> DOI: <http://dx.doi.org/10.3748/wjg.v22.i39.8750>

INTRODUCTION

The eighth most common cancer in the world is esophageal cancer. Esophageal cancer, being the sixth most common cause of death from cancer, has an overall ratio of mortality to incidence of 0.88^[1]. Esophageal cancer comprises of two distinct histological entities, *i.e.* squamous cell carcinoma (SCC) and adenocarcinoma. These two histological types differ in their epidemiology, etiopathogenesis, tumor biology, management and outcomes. SCC is common in Asia and Eastern Europe while adenocarcinoma is prevalent in North America and Western Europe. Unfortunately, majority of the patients present with locally advanced disease in esophageal cancer and survival is dismal regardless of histology.

Traditionally, surgical resection has been the mainstay of treatment with a potential to ensure loco-regional control as well as long-term survival. However, surgery alone fails to contend against the natural history of disease owing to the presence of occult micrometastasis and fatal distant and loco-regional disease relapse is common. Median survival after esophagectomy is 15 to 18 mo with a 5-year survival rate of 20% to 25%^[2]. Therefore, clinicians now are inclined towards use of some form of multidisciplinary treatment including surgery as standard of care for locally advanced esophageal cancer. Locally advanced esophageal cancer can be defined as those restricted to the esophagus or resectable periesophageal tissue (T2-T4) and/or lymphnode involvement (N1-N3) in the absence of distant metastasis^[3]. The optimal multimodality treatment is still controversial. Potential contentious issues exist regarding the (1) ideal approach-preoperative, perioperative, or postoperative and (2) ideal combination-radiotherapy, chemotherapy or concurrent chemoradiation. Though

various randomized and non-randomized trials have been conducted to address these issues, no standard guidelines have been established till date. This review will highlight the significant studies to assist in opting for the best evidence based multimodality management of esophageal cancer. We limit this review to preoperative therapy followed by surgery as it is presently considered the standard of care. The objectives of preoperative treatment are to downstage the tumor to achieve R0 resection, reduce local and distant disease relapses and thus improve survival.

PREOPERATIVE RADIOTHERAPY

High rate of local failure after curative resection led to conception of many studies in the '80s and '90s to evaluate the role of preoperative radiotherapy (RT) in esophageal cancer. Preoperative RT was envisaged to increase the resectability rates with negative circumferential margins, to lower the loco-regional recurrences and to improve survival. Five randomized control trials (RCTs) addressed this issue and compared preoperative RT followed by surgery to surgery alone. Table 1 displays the salient features of these RCTs^[4-8]. None of the studies reported significantly higher complete resections following preoperative RT. Only two studies reported loco-regional recurrences in the study arms: Wang *et al.*^[7] reported no difference whereas Gignoux *et al.*^[8] observed significantly lower loco-regional recurrences following preoperative RT and surgery compared to surgery alone (46% vs 67%). None of the RCTs reported significant improvement in survival. Nygaard *et al.*^[6] reported improvement in 3-year overall survival (OS) (21% vs 9%) among patients who underwent surgery following preoperative RT compared to patients who were operated upfront. Oesophageal Cancer Collaborative Group conducted a quantitative meta-analysis using updated data from these five RCTs comprising 1147 patients to assess whether preoperative radiotherapy improves OS and whether it is differentially effective in patients defined by age, sex and tumour location^[9] (Figure 1). In a group of patients with mostly squamous cell carcinoma, who had a median follow up of 9 years, the hazard ratio (HR) was found to be 0.89 (95%CI: 0.78-1.01). This suggested an overall reduction in the risk of death by 11% and an absolute survival benefit of 3% at 2 years and 4% at 5 years. It, however, failed to achieve conventional statistical significance ($P = 0.062$). There was no apparent difference in the magnitude of the benefit by sex, age or tumor location. The authors concluded that there is not enough evidence to suggest that preoperative radiotherapy improves the survival of patients with potentially resectable esophageal cancer. The effect of such preoperative radiotherapy regimens is likely to be modest, even if they do improve survival. The absolute improvement in survival is not expected to be more than 3% to 4%. To detect such an improvement (from

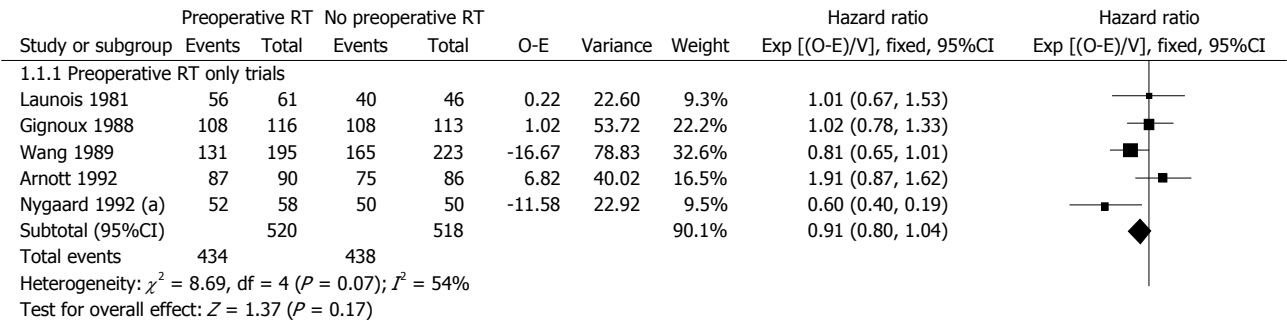


Figure 1 Forest plot comparing survival in esophageal cancer in patients who received preoperative radiotherapy followed by surgery vs surgery alone (Reproduced with permission from Arnott *et al*^[9]).

Table 1 Salient features of randomized controlled trials addressing the role of preoperative radiotherapy followed by surgery versus surgery alone in the management of esophageal cancer

Ref.	Study period	Treatment	No. of patients	Histology	Complete resection	Local recurrence rate	Operative mortality	5-yr OS	Conclusion
Launois <i>et al</i> ^[5] , 1981	1973-1976	40 Gy RT + Surgery	67	SCC	74%	NA	22.6%	9.5%	No significant benefit of pre-op RT
		Surgery	57	SCC	78%	NA	23.4%	11.5%	
Gignoux <i>et al</i> ^[8] , 1987	1976-1982	33 Gy RT + Surgery	NA	SCC	43%	46%	NA	11%	No significant benefit of pre-op RT
		Surgery	NA	SCC	55%	67%	NA	10%	
Wang <i>et al</i> ^[7] , 1989	1977-1985	40 Gy RT + Surgery	104	SCC	74%	41%	5%	5%	Higher pre-op RT dose or post-op RT required
		Surgery	102	SCC	65%	34%	6%	30%	
Arnott <i>et al</i> ^[4] , 1993	1979-1983	20 Gy RT + Surgery	90	SCC/AC	76%	NA	NA	9%	No benefit of low dose RT
		Surgery	86	SCC/AC	72%	NA	NA	17%	
Nygaard <i>et al</i> ^[6] , 1992	1983-1988	35 Gy RT + Surgery	NA	SCC	34%	NA	NA	21%	Beneficial effect of pre-op RT
		Surgery	NA	SCC	32%	NA	NA	9%	

SCC: Squamous cell cancer; AC: Adenocarcinoma; RT: Radiotherapy; NA: Not available; OS: Overall survival.

15% to 20%) reliably, trials or a meta-analysis of not less than 2000 patients (90% power, 5% significance level) would be needed^[10].

It can be inferred, based on available RCTs, that preoperative radiation therapy is unlikely to benefit esophageal cancer patients, both in terms of significantly lowering local failure rate and improving survival.

PREOPERATIVE CHEMOTHERAPY

Distant recurrence following curative resection in localized esophageal cancer constitutes significant proportion of disease relapse and invariably limits survival. Recurrence pattern analysis of 439 patients who underwent R0 resection highlighted that almost one fifth of the patients developed distant recurrence^[11]. Furthermore, autopsy study of 43 curatively resected cases of esophageal cancer revealed that 17 of the 27 patients who had disease recurrence were found to have haematogenous metastasis^[12]. Two studies showed that almost 80% of the patients had disseminated tumour cells in the bone marrow samples of ribs resected during esophagectomy for localized esophageal cancer^[13,14]. Presence of systemic micro-metastasis in a significant number of esophageal cancer patients and the pattern

of higher rates of distant recurrence leading to failure in curatively treated patients led to exploration of role of induction preoperative chemotherapy in esophageal cancer.

The enthusiasm to use preoperative chemotherapy arose due to its potential to exterminate micro-metastasis, to down-stage the tumor, thus enhancing resectability, to improve loco-regional control and to provide relief of dysphagia. The downside of giving preoperative chemotherapy is development of chemo-resistance and progression of disease in patients who do not respond to it. It is known that almost half of the patients are unresponsive to the presently employed chemotherapy^[2]. Moreover, delay in local treatment can further compromise the already marginal nutritional status of the patient when surgery is not the initial treatment.

The role of preoperative/perioperative chemotherapy followed by surgery compared to surgery alone has been addressed by 13 RCTs (Table 2)^[6,15-26]. A pooled random-effects meta-analysis of 10 RCTs comprising 2122 participants highlighted the lower risk of mortality among patients who were given preoperative chemotherapy compared to those treated with surgery alone (HR = 0.88, 95%CI: 0.80-0.96, $P = 0.003$)^[27] (Figure 2). Another updated meta-analysis of 10 RCTs revealed that the risk of all-cause mortality

Table 2 Salient features of randomized controlled trials addressing the role of preoperative/perioperative chemotherapy followed by surgery versus surgery alone in the management of esophageal cancer

Trials	Study period	Treatment	No. of patients	Histology	R0 resection	pCR	pN+	Median follow up	LRR	OS (%)	Conclusions
Roth <i>et al</i> ^[21] , 1988	1982-1986	Periop Cisplatin vindesine, bleomycin + S	19	SCC	35%	6%	NS	30 mo	NS	25 (3 yr)	Prolonged OS in responders in perioperative chemotherapy arm with acceptable toxicity and post-op complications
Nygaard <i>et al</i> ^[6] , 1992	1983-1988	Surgery	20	SCC	21%	-	NS	30 mo	NS	05 (3 yr)	No improvement in survival in chemotherapy arm
		Preop Cisplatin, Bleomycin + S	44	SCC	44%	NS	NS	NA	NS	03 (3 yr)	
Schlag <i>et al</i> ^[22] , 1992	1980's	Surgery	41	SCC	36%	-				09 (3 yr)	No influence on resectability or OS in chemotherapy arm. Rather, it results in Increase in side effects and postop mortality rate
		Preop FC + S	22	SCC	44%	6%	NA	NA	NS	NS	
		Surgery	24	SCC	45%	-	NA	NA	NS	NS	Better OS in control group. Poorly nourished patients may tolerate smaller dosages of chemotherapy
Maipang <i>et al</i> ^[19] , 1994	1988-1990	Preop Cisplatin Vindesine, Bleomycin + S	24	SCC	NS	0%	NS	NA	NS	31 (3 yr)	Significant downstaging and an increased likelihood of R0 resection in chemotherapy arm. No survival difference but responders fared better
Law <i>et al</i> ^[18] , 1997	1989-1995	Surgery	22	SCC		-		NA		36 (3 yr)	
		Preop FC + S	74	SCC	67%	6.7%	70	NA	12	44 (2 yr)	Significantly improved long term survival in patients with pathologic complete response following preoperative chemotherapy. Perioperative chemotherapy decreased tumor size and stage, and significantly improved PFS, OS
		Surgery	73	SCC	35%	-	88		30	31 (2 yr)	
Ancona <i>et al</i> ^[15] , 2001	1992-1997	Preop FC + S	47	SCC	90%	13%	NS	30 mo	32	34 (5 yr)	No improvement in OS in chemotherapy arm. Only R0 resection results in long-term survival, regardless of pre-op chemotherapy
		Surgery	47	SCC	87%	-		30 mo	34	22 (5 yr)	
Cunnigham <i>et al</i> ^[26] , 2006 (Magic trial)	1994-2002	Peri-op ECF + S	37/250	AC	69.3%	NA	NS	49	14.4	36.3 (5 yr)	Preop chemotherapy improves survival and should be considered as a standard of care
		Surgery	36/253	AC	66.4%	-		47	20.6	23 (5 yr)	
Kelsen <i>et al</i> ^[17] , (RTOG 8911, US Intergroup 113) 2007	1990-1995	Preop FC + S	213	SCC - 98, AC - 115	63%	2.5%	NS	8.8 yr	25	23 (3 yr)	Significant improvement in OS in chemotherapy arm
		Surgery	227	SCC - 106, AC - 121	59%	-			19	26 (3 yr)	
MRC OEO2 trial, 2009 Allum <i>et al</i> ^[25]	1992-1998	Preop FC + S	400	SCC - 123, AC - 265, Others - 12	60%	4%	58	5.9 yr	11.5	23 (5 yr)	Peri-op chemotherapy significantly increased R0 resection rate, DFS, and OS
		Surgery	402	SCC - 124, AC - 268, Others - 10	54%	-	68	6.1 yr	12.2	17 (5 yr)	
Ychou <i>et al</i> ^[23] , 2011	1995-2003	Peri-op FC + S	113	AC	84%	3%	67	8.8 yr	12	38 (5 yr)	Significant improvement in OS in chemotherapy arm
		Surgery	111	AC	73%	-	80		8	24 (5 yr)	
Boonstra <i>et al</i> ^[16] , 2011	1989-1996	Preop Cisplatin, Etoposide + S	85	SCC	71%	7%	43	15 mo	19	26 (5 yr)	Pre-op chemotherapy can be regarded as standard treatment
		Surgery	84	SCC	57%	-	46	14 mo	25	17 (5 yr)	
Ando <i>et al</i> ^[24] , 2012- JCOG 9907	2000-2006	Preop FC + S	164	SCC	96%	2%	65	62 mo	25	55 (5 yr)	
		Surgery	166	SCC	91%	-	76	NA	31	43 (5 yr)	

Periop: Perioperative; SCC: Squamous cell cancer; AC: Adenocarcinoma; RT: Radiotherapy; NA: Not available; OS: Overall survival; NS: Not stated; ECF: Epirubicin, cisplatin, 5-FU; S: Surgery.

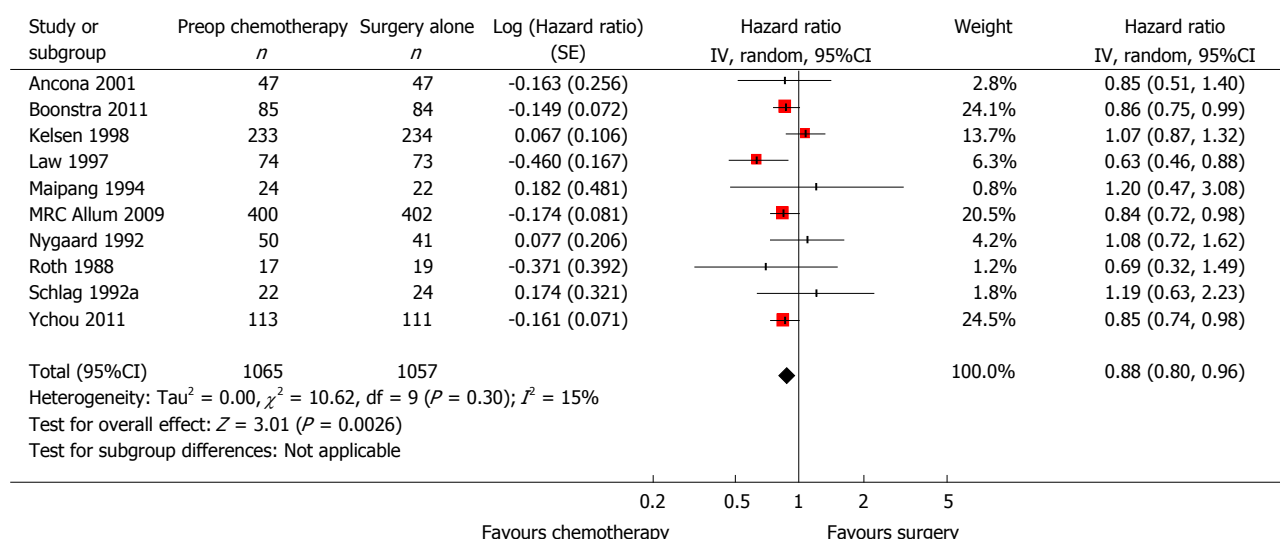


Figure 2 Forest plot comparing survival in esophageal cancer in patients who received preoperative chemotherapy followed by surgery vs surgery alone (Reproduced with permission from Kidane *et al*^[27]).

was significantly less for preoperative chemotherapy followed by surgery compared to surgery alone (pooled HR = 0.87, 95%CI: 0.79-0.96, $P = 0.005$). The absolute survival difference at 2 years was calculated as 5.1% (number needed to treat = 19). A subgroup analysis by histological type for these studies showed that the preoperative chemotherapy proved beneficial in adenocarcinoma (HR = 0.83, 95%CI: 0.71-0.95, $P = 0.01$) and not in squamous-cell carcinoma (HR = 0.92, 95%CI: 0.81-1.04, $P = 0.18$)^[28].

Though the objective of the present review is to analyze the role of preoperative therapy, it is worthwhile to discuss perioperative chemotherapy here as well. The role of perioperative chemotherapy (before and after the surgery) has been evaluated in lower esophageal adenocarcinoma in two RCTs. The British Medical Research Council conducted a phase III trial (MAGIC trial) to evaluate perioperative chemotherapy in the management of resectable gastro-esophageal adenocarcinoma. Though the proportion of patients with esophageal cancers was less (lower esophagus 14% and esophagogastric junction 11%), results of this trial definitely indicated benefit of perioperative chemotherapy. OS significantly improved from 23% to 36%. Only 104 of 250 patients (41.6%) randomized to perioperative chemotherapy arm in this study could complete all six cycles of chemotherapy, due to disease progression or early death, patient choice, postoperative complications, prior toxicity, lack of response to preoperative treatment, and worsening coexisting disease, thus acting as a limitation^[26]. Another recent FNCLCC/FFCD trial reported that perioperative chemotherapy improved 5-year OS from 24% to 38%. It must be noted that 75% of tumors in this trial were lower esophageal or junctional and among the 109 patients who received at least one cycle of preoperative chemotherapy, 54 patients (50%) received postoperative chemotherapy^[23]. These

two trials suggest that the approach of perioperative chemotherapy may be beneficial for patients with lower esophagus or junctional adenocarcinoma but only half of the patients are able to complete the planned treatment after surgery.

PREOPERATIVE CHEMORADIATION

High risk of both loco-regional and systemic failures following curative treatment, led to integration of all three treatment modalities namely surgery, chemotherapy and radiation in the management of esophageal cancer. As preoperative RT failed to meet the high expectations of providing additional loco-regional control compared to surgery alone in esophageal cancer, attempts were made to use chemo-radiotherapy (CRT) as a double-edged sword. While RT was expected to improve loco-regional control, simultaneously, chemotherapy was thought to eradicate the micrometastasis. Chemotherapy adds several benefits with its use along with preoperative RT by (1) containing micrometastasis and lowering systemic failure; (2) providing additive effect to radiation by acting against the different tumor cell populations; and (3) providing assistance to radiotherapy for control of loco-regional disease (spatial cooperation)^[29]. Spatial cooperation may exist at different levels: (1) with altered DNA repair or modification of the lesions induced by chemotherapy or radiation at the molecular level; (2) through cytokinetic cooperation arising from differential sensitivity of the various compartments of the cell cycle to the drug or radiation at the cellular level, notably; and (3) including re-oxygenation, increased drug uptake or inhibition of repopulation or angiogenesis at the tissue level^[30].

Table 3 displays the RCTs which compared preoperative CRT followed by surgery to surgery alone^[31-39]. The most promising trials which have

Table 3 Salient features of randomized controlled trials addressing the role of preoperative chemoradiotherapy followed by surgery *vs* surgery alone in the management of esophageal cancer

Trial	Study period	Treatment	No. of patients	Histology	Completed treatment	R0	pCR	pN+	LRR	Median survival (mo)	OS	Treatment related mortality	DFS median/proportions	Conclusion
Apinop <i>et al</i> ^[31] , 1994	1986-1992	FC + 40 Gy RT + Surgery	35	SCC	26	NA	26.9%	NA	NA	NA	NS	NS	NA	No statistically significant difference in OS, complication rate, mortality
Le Prise <i>et al</i> ^[32] , 1994	1988-1991	Surgery Sequential FC-20 Gy RT-FC + Surgery	34 41	SCC SCC	- 39	NA 51.0%	- NA	NA	NA	NA	NS 19.2 (3 yr)	NS 8.5%	NA 7.6 mo	No change in operative mortality or survival time
Walsh <i>et al</i> ^[33] , 1996	1990-1995	Surgery FC + 40 Gy RT + Surgery	45 58	SCC AC	42 53	36.0% NA	- 25%	NA	21.4% NA	10 32	13.8 (3 yr) 37 (3 yr)	7% 3%	5 mo NA	Multimodal treatment superior to surgery alone
Lee <i>et al</i> ^[34] , 2004	1999-2002	Surgery FC + 45.6 Gy RT + Surgery	55 51	AC SCC	54 35	NA 100%	- 43%	82 37	NA 22.8%	11 28.2	07 (3 yr) 55 (2 yr)	2% 8.5%	NA 49% (2 yr)	CRT induced high clinical and pathological response, but no statistically significant benefit in OS and DFS
Burmeister <i>et al</i> ^[35] , 2005	1994-2000	Surgery FC + 35 Gy RT + Surgery	50 128	SCC 45 SCC + 80 AC + 3 others	48 105	87.5% 80.0%	- 16%	78 43	10.8% 11%	27.3 22.2	57 (2 yr) NS	4.7%	51% (2 yr) 16 mo	No significant improvement in PFS or OS
Tepper <i>et al</i> ^[36] , 2008 (CALGB 9781)	1997-2000	Surgery FC + 50.4 Gy RT + Surgery	128 30	50 SCC + 78 AC 7 SCC + 23 AC	110 29	59.0% 84.6%	- 40%	67 12	14% 13.7%	19.3 53.7	NS 39 (5 yr)	5.4% 5 yr	12 mo 28% (5 yr)	Long-term survival advantage supports trimodality therapy as a standard of care
Lv <i>et al</i> ^[37] , 2010	1997-2004	2 Cis, Pacli+ 40 gy + Surgery	80	SCC	80	97.4%	NA	NA	11.3%	53	24.5 (10 yr)	3.4%	61.3% (3 yr)	Rational application of pre-op or post-op CRT can improve PFS, OS
Van Hagen <i>et al</i> ^[38] , 2012 (CROSS trials)	2004-2008	5 Pacli, Carbo + 41.4 Gy + Surgery	80 178	SCC 41 SCC + 134 AC + 3 other	80 168	80.0% 92.0%	- 29%	NA 13	35% 3.3%	36 49.4	12.5 (10 yr) 47 (5 yr)	0% 5.9%	49.3% (3 yr) not reached	Improved survival with acceptable adverse-event rates
Mariette <i>et al</i> ^[39] , 2014	2000-2009	2 Cis, 5FU + Surgery	188 98	43 SCC + 141 AC + 4 other 67 SCC + 30 AC + 1 other	186 84	69.0% 93.8%	- 33.3%	75 30.8	9.3% 22.1%	24 31.8	34 41 (5 yr)	6.9% 11.1%	24.2 mo 35.6% (5 yr)	No effect on R0 resection rate or survival but enhanced postoperative mortality
		Surgery	97	70 SCC + 27 AC	91	92.1%	-	52.8	28.9%	41.2	33.8	3.4%	27.7% (5 yr)	

Periop: Perioperative; SCC: Squamous cell cancer; AC: Adenocarcinoma; RT: Radiotherapy; NA: Not available; OS: Overall survival; NS: Not stated; ECF: Epirubicin, cisplatin, 5-FU; S: Surgery.

almost established the role of preoperative CRT are the Dutch chemoradiotherapy in Oesophageal Cancer and the Surgery Study (CROSS) trials^[29,38]. The investigators randomly assigned 368 patients with resectable esophageal cancers to either preoperative CRT followed by surgery or to surgery alone. The patients in preoperative CRT arm received weekly carboplatin and paclitaxel for 5 wk and concurrent radiotherapy (41.4 Gy in 23 fractions of 1.8 Gy each, with 5 fractions administered per week, starting on the first day of the first chemotherapy cycle) followed by surgery. The histopathological types were adenocarcinoma (75%), SCC (23%), and large-cell undifferentiated carcinoma (2%). R0 resection rates were significantly better in preoperative CRT arm compared to surgery alone (92% vs 69%, $P < 0.001$). More than one fourth of patients (29%) achieved pathological complete response following preoperative CRT. Though postoperative complications and in-hospital mortality were similar in the two groups, median OS was significantly better in preoperative CRT group (49.4 mo vs 24.0 mo, HR for survival, 0.657, 95%CI: 0.495-0.871, $P = 0.003$). However, the HR for esophageal adenocarcinoma was only marginally statistically significant (adjusted HR = 0.74, 95%CI: 0.54-1.02, $P = 0.07$). Long-term results of the study were recently published. These revealed that the survival advantage for preoperative CRT persisted after a median follow up of 84.1 mo for surviving patients (range 61.1-116.8, IQR: 70.7-96.6). Median OS was 48.6 mo (95%CI 32.1-65.1) in the preoperative chemoradiotherapy plus surgery group and 24.0 mo (14.2-33.7) in the surgery alone group (HR = 0.68, 95%CI: 0.53-0.88, log-rank $P = 0.003$). The improvement in survival was evident for both histological subtypes - median OS for patients with squamous cell carcinomas and adenocarcinoma were 81.6 mo (95%CI: 47.2-116.0) and 43.2 mo (95%CI: 24.9-61.4) respectively in the preoperative CRT group compared to 21.1 mo (95%CI: 15.4-26.7) and 27.1 mo (95%CI: 13.0-41.2) in the surgery alone group^[40]. Preoperative CRT led to significantly lower loco-regional recurrences (14% vs 34%, $P < 0.001$) and peritoneal carcinomatosis (4% vs 14%, $P < 0.001$)^[41].

In sharp contrast to the findings of the CROSS trial, the French trial (FFCD 9901) could not find any significant benefit of preoperative CRT. FFCD 9901 was a multicentre RCT which was conducted to assess improvement in outcomes for patients with stage I or II esophageal cancer with use of preoperative CRT. The investigators randomized 195 patients (in 30 centres) to either preoperative CRT followed by surgery ($n = 98$) or surgery alone ($n = 97$). CRT protocol was 45 Gy in 25 fractions over 5 wk with two courses of concomitant chemotherapy composed of fluorouracil 800 mg/m² and cisplatin 75 mg/m². Preoperative CRT did not improve R0 resection rates (93.8% vs 92.1%, $P = 0.749$); there was no difference in 3-year OS either (47.5% vs

53.0%, HR = 0.99, 95%CI: 0.69-1.40, $P = 0.94$). Moreover, significantly higher postoperative mortality was seen in patients who received preoperative CRT (11.1% vs 3.4%, $P = 0.049$). The authors concluded that preoperative CRT does not improve R0 resection rate or survival but enhances postoperative mortality in patients with stage I or II esophageal cancer^[39]. A number of factors can be attributed to these differences in survival outcomes between the French and Dutch studies: (1) small sample size in the FFCD 9901 trial reducing the statistical power of detecting a survival benefit; (2) different histological profiles in two studies (70% of patients in the French study were SCC compared with 23% in the Dutch study); and (3) larger number of patients with early-stage disease (fewer node-positive and T3 patients) in the French study compared to Dutch study^[42]. The CROSS trial investigators cautioned against coming to the conclusion that preoperative CRT is not beneficial in early-stage esophageal cancer, as one might, from the results of the FFCD 9901 trial. They highlighted that CROSS trial findings should still be considered for stage II cancers in view of its larger study population, more consistent inclusion rate, less toxic CRT regimen, more sophisticated radiation techniques and lower postoperative mortality rate^[43].

A meta-analysis which was designed to compare the role of preoperative CRT for esophageal carcinoma including 14 RCTs ($n = 1737$) concluded that it has the potential to improve the long-term survival and reduce locoregional cancer recurrence compared to surgery alone. Five-year survival was significantly better in preoperative CRT group compared to the surgery alone group (OR = 1.64, 95%CI: 1.28-2.12). The authors further reported that a complete pathological response to CRT was observed in 10%-45.5% of patients^[44].

In a meta-analysis of 12 trials which compared preoperative CRT followed by surgery versus surgery alone ($n = 1854$), the Australasian gastro-intestinal trials group reported that preoperative CRT led to significant reduction in all-cause mortality (HR = 0.78, 95%CI: 0.70-0.88, $P < 0.0001$). The beneficial effect was evident in both histological subtypes - the HR for squamous-cell carcinoma was 0.80 (95%CI: 0.68-0.93, $P = 0.004$) and for adenocarcinoma was 0.75 (95%CI: 0.59-0.95, $P = 0.02$). They further undertook the pooled analysis of two RCTs and highlighted that the preoperative CRT seemed to lower all-cause mortality compared to preoperative chemotherapy (HR for the overall indirect comparison 0.88, 95%CI: 0.76-1.01, $P = 0.07$). They concluded that there seemed to be strong evidence for survival benefit with the use of preoperative CRT or chemotherapy followed by surgery versus surgery alone in resectable esophageal cancer^[28].

Another meta-analysis which included 13 RCTs ($n = 1930$, resectable esophageal cancers) addressed the issue of postoperative complications following preoperative CRT compared to surgery alone^[45]. The

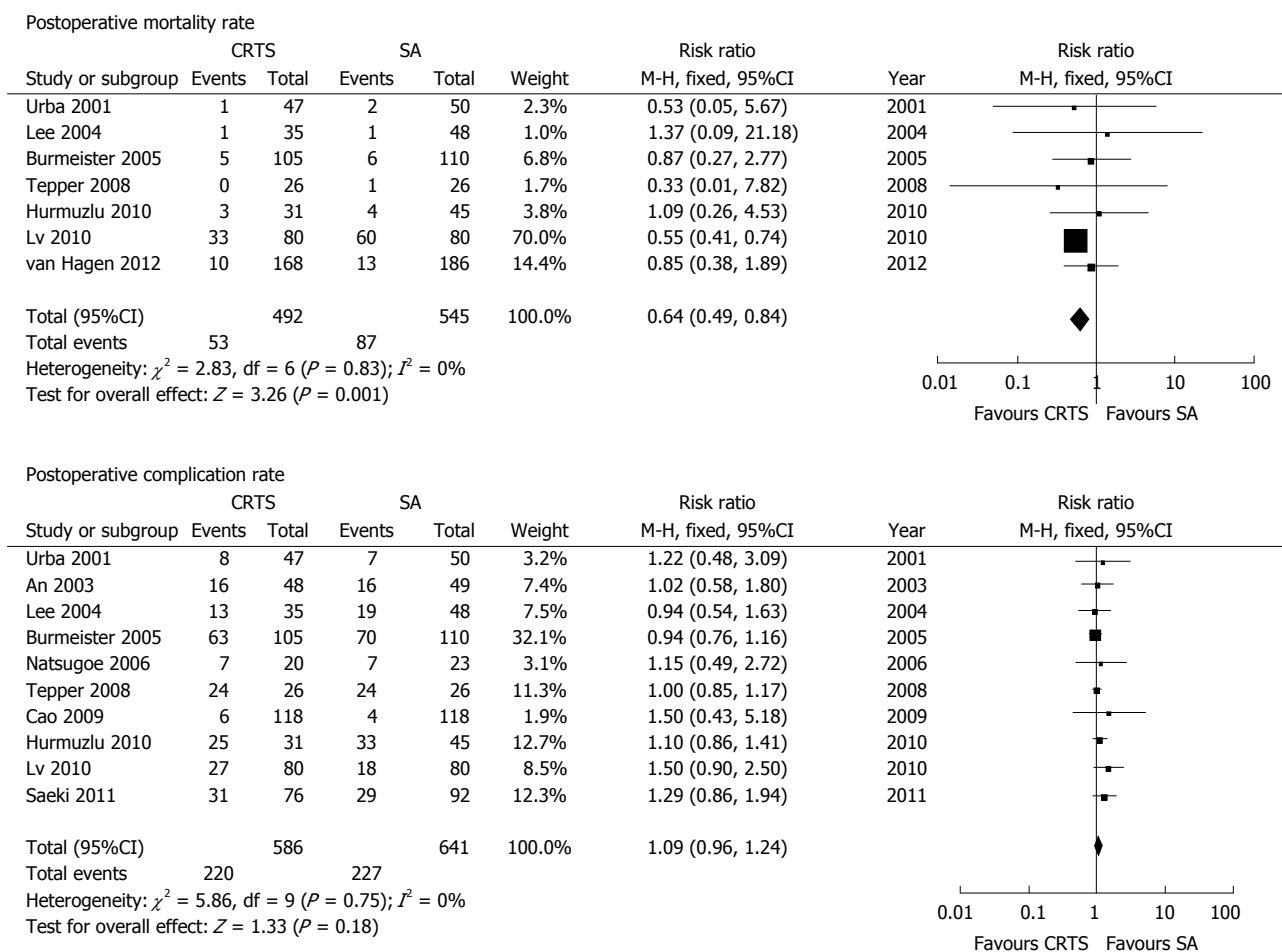


Figure 3 The forest plots of postoperative mortality and complications of chemoradiotherapy followed by surgery vs surgery alone using a fixed effects model (Reproduced from Deng *et al*^[48]).

authors reported that preoperative CRT did not lead to significantly higher postoperative complications compared to surgery alone (RR = 1.09, 95%CI: 0.96-1.24, $P = 0.18$) (Figure 3); indeed, preoperative CRT led to reduction in postoperative mortality (RR = 0.64, 95%CI: 0.49-0.84, $P = 0.001$). Moreover, there was a significant reduction in local recurrence (RR = 0.53, 95%CI: 0.39-0.73, $P < 0.000$) and distant recurrence (RR = 0.82, 95%CI: 0.68-0.98, $P = 0.03$). These results clear the apprehension that preoperative CRT may result in higher postoperative complications.

CONCLUSION

The literature suggests that preoperative chemoradiotherapy followed by surgery results in optimal outcome while managing locally advanced esophageal cancer; however, there is a need to compare preoperative chemoradiotherapy with chemotherapy alone to further refine the role of preoperative therapy. The standard of care continues to be debated due to difference of opinions and practices across the world and lack of any trial with head-to-head comparison between these two established treatment protocols.

REFERENCES

- 1 **Ferlay J**, Soerjomataram I, Ervik M, Dikshit R, Eser S, Mathers C, Rebelo M, Parkin DM, Forman D, Bray F. GLOBOCAN 2012 v1.0, Cancer Incidence and Mortality Worldwide: IARC CancerBase No. 11 [Internet]. Lyon, France: International Agency for Research on Cancer; 2013 cited 2015-06-25. Available from: URL: <http://globocan.iarc.fr>
- 2 **Posner MC**, Minsky BD, Ilson DH. Cancer of the Esophagus. In: DeVita VT, Lawrence TS, Rosenberg SA, editors. DeVita, Hellman, and Rosenberg's Cancer: Principles & Practice of Oncology. 10th ed. Philadelphia: Wolters Kluwer, 2015: 574-612
- 3 **Keditsu KK**, Jiwnani S, Karimundackal G, Pramesh CS. Multimodality management of esophageal cancer. *Indian J Surg Oncol* 2013; **4**: 96-104 [PMID: 24426708 DOI: 10.1007/s13193-013-0216-0]
- 4 **Arnett SJ**, Duncan W, Kerr GR, Walbaum PR, Cameron E, Jack WJ, Mackillop WJ. Low dose preoperative radiotherapy for carcinoma of the oesophagus: results of a randomized clinical trial. *Radiother Oncol* 1992; **24**: 108-113 [PMID: 1496141 DOI: 10.1016/0167-8140(92)90287-5]
- 5 **Launois B**, Delarue D, Campion JP, Kerbaol M. Preoperative radiotherapy for carcinoma of the esophagus. *Surg Gynecol Obstet* 1981; **153**: 690-692 [PMID: 6794167]
- 6 **Nygaard K**, Hagen S, Hansen HS, Hatlevoll R, Hultborn R, Jakobsen A, Mäntyla M, Modig H, Munck-Wikland E, Rosengren B. Pre-operative radiotherapy prolongs survival in operable esophageal carcinoma: a randomized, multicenter study of pre-operative radiotherapy and chemotherapy. The second

- Scandinavian trial in esophageal cancer. *World J Surg* 1992; **16**: 1104-1109; discussion 1110 [PMID: 1455880 DOI: 10.1007/BF02067069]
- 7 **Wang M**, Gu XZ, Yin WB, Huang GJ, Wang LJ, Zhang DW. Randomized clinical trial on the combination of preoperative irradiation and surgery in the treatment of esophageal carcinoma: report on 206 patients. *Int J Radiat Oncol Biol Phys* 1989; **16**: 325-327 [PMID: 2646253 DOI: 10.1016/0360-3016(89)90323-4]
 - 8 **Gignoux M**, Roussel A, Paillot B, Gillet M, Schlag P, Favre JP, Dalesio O, Buyse M, Duez N. The value of preoperative radiotherapy in esophageal cancer: results of a study of the E.O.R.T.C. *World J Surg* 1987; **11**: 426-432 [PMID: 3630187]
 - 9 **Arnott SJ**, Duncan W, Gignoux M, Hansen HS, Launois B, Nygaard K, Parmar MK, Rousell A, Spiliopoulos G, Stewart G, Tierney JF, Wang M, Rhugang Z; Oesophageal Cancer Collaborative Group. Preoperative radiotherapy for esophageal carcinoma. *Cochrane Database Syst Rev* 2005; **(4)**: CD001799 [PMID: 16235286 DOI: 10.1002/14651858.CD001799.pub2]
 - 10 **Arnott SJ**, Duncan W, Gignoux M, Girling DJ, Hansen HS, Launois B, Nygaard K, Parmar MK, Rousell A, Spiliopoulos G, Stewart LA, Tierney JF, Wang M, Rhugang Z. Preoperative radiotherapy for esophageal carcinoma. Oesophageal Cancer Collaborative Group. *Cochrane Database Syst Rev* 2000; **(2)**: CD001799 [PMID: 10796823 DOI: 10.1002/14651858.CD001799]
 - 11 **Mariette C**, Balon JM, Piessen G, Fabre S, Van Seuningen I, Triboulet JP. Pattern of recurrence following complete resection of esophageal carcinoma and factors predictive of recurrent disease. *Cancer* 2003; **97**: 1616-1623 [PMID: 12655517 DOI: 10.1002/cncr.11228]
 - 12 **Katayama A**, Mafune K, Tanaka Y, Takubo K, Makuuchi M, Kaminishi M. Autopsy findings in patients after curative esophagectomy for esophageal carcinoma. *J Am Coll Surg* 2003; **196**: 866-873 [PMID: 12788422 DOI: 10.1016/S1072-7515(03)00116-9]
 - 13 **Bonavina L**, Soligo D, Quirici N, Bossolasco P, Cesana B, Lemberthenghi Delilieri G, Peracchia A. Bone marrow-disseminated tumor cells in patients with carcinoma of the esophagus or cardia. *Surgery* 2001; **129**: 15-22 [PMID: 11150029 DOI: 10.1067/msy.2001.109503]
 - 14 **O'sullivan GC**, Sheehan D, Clarke A, Stuart R, Kelly J, Kiely MD, Walsh T, Collins JK, Shanahan F. Micrometastases in esophagogastric cancer: high detection rate in resected rib segments. *Gastroenterology* 1999; **116**: 543-548 [PMID: 10029612]
 - 15 **Ancona E**, Ruol A, Santi S, Merigliano S, Sileni VC, Koussis H, Zaninotto G, Bonavina L, Peracchia A. Only pathologic complete response to neoadjuvant chemotherapy improves significantly the long term survival of patients with resectable esophageal squamous cell carcinoma: final report of a randomized, controlled trial of preoperative chemotherapy versus surgery alone. *Cancer* 2001; **91**: 2165-2174 [PMID: 11391598]
 - 16 **Boonstra JJ**, Kok TC, Wijnhoven BP, van Heijl M, van Berge Henegouwen MI, Ten Kate FJ, Siersema PD, Dinjens WN, van Lanschot JJ, Tilanus HW, van der Gaast A. Chemotherapy followed by surgery versus surgery alone in patients with resectable oesophageal squamous cell carcinoma: long-term results of a randomized controlled trial. *BMC Cancer* 2011; **11**: 181 [PMID: 21595951 DOI: 10.1186/1471-2407-11-181]
 - 17 **Kelsen DP**, Winter KA, Gunderson LL, Mortimer J, Estes NC, Haller DG, Ajani JA, Kocha W, Minsky BD, Roth JA, Willett CG; Radiation Therapy Oncology Group; USA Intergroup. Long-term results of RTOG trial 8911 (USA Intergroup 113): a random assignment trial comparison of chemotherapy followed by surgery compared with surgery alone for esophageal cancer. *J Clin Oncol* 2007; **25**: 3719-3725 [PMID: 17704421 DOI: 10.1200/JCO.2006.10.4760]
 - 18 **Law S**, Fok M, Chow S, Chu KM, Wong J. Preoperative chemotherapy versus surgical therapy alone for squamous cell carcinoma of the esophagus: a prospective randomized trial. *J Thorac Cardiovasc Surg* 1997; **114**: 210-217 [PMID: 9270638 DOI: 10.1016/S0022-5223(97)70147-8]
 - 19 **Maipang T**, Vasinanukorn P, Petpichetchian C, Chamroonkul S, Geater A, Chansawwaang S, Kuapanich R, Panjapiyakul C, Watanaarepornchai S, Punperk S. Induction chemotherapy in the treatment of patients with carcinoma of the esophagus. *J Surg Oncol* 1994; **56**: 191-197 [PMID: 7518020 DOI: 10.1002/jso.2930560314]
 - 20 **Medical Research Council Oesophageal Cancer Working Group**. Surgical resection with or without preoperative chemotherapy in oesophageal cancer: a randomised controlled trial. *Lancet* 2002; **359**: 1727-1733 [PMID: 12049861 DOI: 10.1016/S0140-6736(02)08651-8]
 - 21 **Roth JA**, Pass HI, Flanagan MM, Graeber GM, Rosenberg JC, Steinberg S. Randomized clinical trial of preoperative and postoperative adjuvant chemotherapy with cisplatin, vindesine, and bleomycin for carcinoma of the esophagus. *J Thorac Cardiovasc Surg* 1988; **96**: 242-248 [PMID: 2456424]
 - 22 **Schlag PM**. Randomized trial of preoperative chemotherapy for squamous cell cancer of the esophagus. The Chirurgische Arbeitsgemeinschaft Fuer Onkologie der Deutschen Gesellschaft Fuer Chirurgie Study Group. *Arch Surg* 1992; **127**: 1446-1450 [PMID: 1365692]
 - 23 **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]
 - 24 **Ando N**, Kato H, Igaki H, Shinoda M, Ozawa S, Shimizu H, Nakamura T, Yabusaki H, Aoyama N, Kurita A, Ikeda K, Kanda T, Tsujinaka T, Nakamura K, Fukuda H. A randomized trial comparing postoperative adjuvant chemotherapy with cisplatin and 5-fluorouracil versus preoperative chemotherapy for localized advanced squamous cell carcinoma of the thoracic esophagus (JCOG9907). *Ann Surg Oncol* 2012; **19**: 68-74 [PMID: 21879261 DOI: 10.1245/s10434-011-2049-9]
 - 25 **Allum WH**, Stenning SP, Bancewicz J, Clark PI, Langley RE. Long-term results of a randomized trial of surgery with or without preoperative chemotherapy in esophageal cancer. *J Clin Oncol* 2009; **27**: 5062-5067 [PMID: 19770374 DOI: 10.1200/JCO.2009.22.2083]
 - 26 **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]
 - 27 **Kidane B**, Coughlin S, Vogt K, Malthaner R. Preoperative chemotherapy for resectable thoracic esophageal cancer. In: *Cochrane Database of Systematic Reviews* [Internet]. John Wiley & Sons, Ltd, 2015 [DOI: 10.1002/14651858.CD001556.pub3]
 - 28 **Sjoquist KM**, Burmeister BH, Smithers BM, Zalcberg JR, Simes RJ, Barbour A, Gebski V. Survival after neoadjuvant chemotherapy or chemoradiotherapy for resectable oesophageal carcinoma: an updated meta-analysis. *Lancet Oncol* 2011; **12**: 681-692 [PMID: 21684205 DOI: 10.1016/S1470-2045(11)70142-5]
 - 29 **van Heijl M**, van Lanschot JJ, Koppert LB, van Berge Henegouwen MI, Muller K, Steyerberg EW, van Dekken H, Wijnhoven BP, Tilanus HW, Richel DJ, Busch OR, Bartelsman JF, Koning CC, Offerhaus GJ, van der Gaast A. Neoadjuvant chemoradiation followed by surgery versus surgery alone for patients with adenocarcinoma or squamous cell carcinoma of the esophagus (CROSS). *BMC Surg* 2008; **8**: 21 [PMID: 19036143 DOI: 10.1186/1471-2482-8-21]
 - 30 **Hennequin C**, Favaudon V. Biological basis for chemoradiotherapy interactions. *Eur J Cancer* 2002; **38**: 223-230 [PMID: 11803139]
 - 31 **Apinop C**, Puttisak P, Preecha N. A prospective study of combined therapy in esophageal cancer. *Hepatogastroenterology* 1994; **41**: 391-393 [PMID: 7959579]

- 32 **Le Prise E**, Etienne PL, Meunier B, Maddern G, Ben Hassel M, Gedouin D, Boutin D, Campion JP, Launois B. A randomized study of chemotherapy, radiation therapy, and surgery versus surgery for localized squamous cell carcinoma of the esophagus. *Cancer* 1994; **73**: 1779-1784 [PMID: 8137201 DOI: 10.1002/1097-0142(19940401)73:7<1779::AID>]
- 33 **Walsh TN**, Noonan N, Hollywood D, Kelly A, Keeling N, Hennessy TP. A comparison of multimodal therapy and surgery for esophageal adenocarcinoma. *N Engl J Med* 1996; **335**: 462-467 [PMID: 8672151 DOI: 10.1056/NEJM199608153350702]
- 34 **Lee JL**, Park SI, Kim SB, Jung HY, Lee GH, Kim JH, Song HY, Cho KJ, Kim WK, Lee JS, Kim SH, Min YI. A single institutional phase III trial of preoperative chemotherapy with hyperfractionation radiotherapy plus surgery versus surgery alone for resectable esophageal squamous cell carcinoma. *Ann Oncol* 2004; **15**: 947-954 [PMID: 15151953 DOI: 10.1093/annonc/mdh219]
- 35 **Burmeister BH**, Smithers BM, Gebiski V, Fitzgerald L, Simes RJ, Devitt P, Ackland S, Gotley DC, Joseph D, Millar J, North J, Walpole ET, Denham JW; Trans-Tasman Radiation Oncology Group; Australasian Gastro-Intestinal Trials Group. Surgery alone versus chemoradiotherapy followed by surgery for resectable cancer of the oesophagus: a randomised controlled phase III trial. *Lancet Oncol* 2005; **6**: 659-668 [PMID: 16129366 DOI: 10.1016/S1470-2045(05)70288-6]
- 36 **Tepper J**, Krasna MJ, Niedzwiecki D, Hollis D, Reed CE, Goldberg R, Kiel K, Willett C, Sugarbaker D, Mayer R. Phase III trial of trimodality therapy with cisplatin, fluorouracil, radiotherapy, and surgery compared with surgery alone for esophageal cancer: CALGB 9781. *J Clin Oncol* 2008; **26**: 1086-1092 [PMID: 18309943 DOI: 10.1200/JCO.2007.12.9593]
- 37 **Lv J**, Cao XF, Zhu B, Ji L, Tao L, Wang DD. Long-term efficacy of perioperative chemoradiotherapy on esophageal squamous cell carcinoma. *World J Gastroenterol* 2010; **16**: 1649-1654 [PMID: 20355244 DOI: 10.3748/wjg.v16.i13.1649]
- 38 **van Hagen P**, Hulshof MC, van Lanschot JJ, Steyerberg EW, van Berge Henegouwen MI, Wijnhoven BP, Richel DJ, Nieuwenhuijzen GA, Hospers GA, Bonenkamp JJ, Cuesta MA, Blaisse RJ, Busch OR, ten Kate FJ, Creemers GJ, Punt CJ, Plukker JT, Verheul HM, Spillenaar Bilgen EJ, van Dekken H, van der Slangen MJ, Rozema T, Biermann K, Beukema JC, Piet AH, van Rij CM, Reinders JG, Tilanus HW, van der Gaast A. Preoperative chemoradiotherapy for esophageal or junctional cancer. *N Engl J Med* 2012; **366**: 2074-2084 [PMID: 22646630 DOI: 10.1056/NEJMoa1112088]
- 39 **Mariette C**, Dahan L, Mornex F, Maillard E, Thomas PA, Meunier B, Boige V, Pezet D, Robb WB, Le Brun-Ly V, Bosset JF, Mabrut JY, Triboulet JP, Bedenne L, Seitz JF. Surgery alone versus chemoradiotherapy followed by surgery for stage I and II esophageal cancer: final analysis of randomized controlled phase III trial FFCD 9901. *J Clin Oncol* 2014; **32**: 2416-2422 [PMID: 24982463 DOI: 10.1200/JCO.2013.53.6532]
- 40 **Shapiro J**, van Lanschot JJ, Hulshof MC, van Hagen P, van Berge Henegouwen MI, Wijnhoven BP, van Laarhoven HW, Nieuwenhuijzen GA, Hospers GA, Bonenkamp JJ, Cuesta MA, Blaisse RJ, Busch OR, Ten Kate FJ, Creemers GJ, Punt CJ, Plukker JT, Verheul HM, Bilgen EJ, van Dekken H, van der Slangen MJ, Rozema T, Biermann K, Beukema JC, Piet AH, van Rij CM, Reinders JG, Tilanus HW, Steyerberg EW, van der Gaast A. Neoadjuvant chemoradiotherapy plus surgery versus surgery alone for oesophageal or junctional cancer (CROSS): long-term results of a randomised controlled trial. *Lancet Oncol* 2015; **16**: 1090-1098 [PMID: 26254683 DOI: 10.1016/S1470-2045(15)00040-6]
- 41 **Oppedijk V**, van der Gaast A, van Lanschot JJ, van Hagen P, van Os R, van Rij CM, van der Slangen MJ, Beukema JC, Rütten H, Spruit PH, Reinders JG, Richel DJ, van Berge Henegouwen MI, Hulshof MC. Patterns of recurrence after surgery alone versus preoperative chemoradiotherapy and surgery in the CROSS trials. *J Clin Oncol* 2014; **32**: 385-391 [PMID: 24419108 DOI: 10.1200/JCO.2013.51.2186]
- 42 **Czito BG**, Palta M, Willett CG. Results of the FFCD 9901 trial in early-stage esophageal carcinoma: is it really about neoadjuvant therapy? *J Clin Oncol* 2014; **32**: 2398-2400 [PMID: 24982460 DOI: 10.1200/JCO.2014.55.7231]
- 43 **Shapiro J**, van Lanschot JJ, Hulshof MC, van der Gaast A. Effectiveness of neoadjuvant chemoradiotherapy for early-stage esophageal cancer. *J Clin Oncol* 2015; **33**: 288-289 [PMID: 25452442 DOI: 10.1200/JCO.2014.59.2428]
- 44 **Lv J**, Cao XF, Zhu B, Ji L, Tao L, Wang DD. Effect of neoadjuvant chemoradiotherapy on prognosis and surgery for esophageal carcinoma. *World J Gastroenterol* 2009; **15**: 4962-4968 [PMID: 19842230 DOI: 10.3748/wjg.15.4962]
- 45 **Deng J**, Wang C, Xiang M, Liu F, Liu Y, Zhao K. Meta-analysis of postoperative efficacy in patients receiving chemoradiotherapy followed by surgery for resectable esophageal carcinoma. *Diagn Pathol* 2014; **9**: 151 [PMID: 25030066 DOI: 10.1186/1746-1596-9-151]

P- Reviewer: Garcia-Olmo D, Osawa S, Schmidt T

S- Editor: Gong ZM **L- Editor:** A **E- Editor:** Wang CH



Basic Study

Oral administration of a non-absorbable plant cell-expressed recombinant anti-TNF fusion protein induces immunomodulatory effects and alleviates nonalcoholic steatohepatitis

Yaron Ilan, Ami Ben Ya'acov, Yehudit Shabbat, Svetlana Gingis-Velitski, Einat Almon, Yoseph Shaaltiel

Yaron Ilan, Ami Ben Ya'acov, Yehudit Shabbat, Gastroenterology and Liver Units, Department of Medicine, Hadassah Hebrew University Medical Center, Jerusalem IL91120, Israel

Svetlana Gingis-Velitski, Einat Almon, Yoseph Shaaltiel, Protalix Biotherapeutics, Carmiel 20100, Israel

Author contributions: All authors contributed to the conception, experiments, writing and final approval of the manuscript.

Supported by Protalix Biotherapeutics and The Roman-Epstein Liver Research Foundation (in part).

Conflict-of-interest statement: Ilan Y is a consultant for Protalix, Gingis-Velitski S, Almon E, Shaaltiel Y are employees of Protalix. The study was supported in part by Protalix, Israel.

Open-Access: This article is an open-access article which was selected by an in-house editor and fully peer-reviewed by external reviewers. It is distributed in accordance with the Creative Commons Attribution Non Commercial (CC BY-NC 4.0) license, which permits others to distribute, remix, adapt, build upon this work non-commercially, and license their derivative works on different terms, provided the original work is properly cited and the use is non-commercial. See: <http://creativecommons.org/licenses/by-nc/4.0/>

Manuscript source: Unsolicited manuscript

Correspondence to: Yaron Ilan, MD, Gastroenterology and Liver Units, Department of Medicine, Hadassah Hebrew University Medical Center, Ein-Karem, POB 1200, Jerusalem IL91120, Israel. ilan@hadassah.org.il
Telephone: +972-2-6778231

Received: May 25, 2016

Peer-review started: May 27, 2016

First decision: July 13, 2016

Revised: July 21, 2016

Accepted: August 5, 2016

Article in press: August 5, 2016

Published online: October 21, 2016

Abstract

AIM

To evaluate the immunomodulatory effect of oral administration of PRX-106 in the high-fat diet model.

METHODS

For 22 wk, C57BL/6 HFD-fed mice received daily oral treatments with BY-2 cells expressing recombinant anti-tumor necrosis factor alpha fusion protein (PRX-106). Mice were followed for serum liver enzyme and triglyceride levels, liver histology and intrahepatic and systemic FACS.

RESULTS

The orally administered non-absorbable PRX-106 was biologically active. Altered distribution of CD4+CD25+FoxP3+ between the liver and spleen and an increase in the intrasplenic-to-intrahepatic CD4+CD25+FoxP3+ ratio and a decrease in the intrasplenic-to-intrahepatic CD8+CD25+FoxP3+ ratio were observed. An increase in intrahepatic NKT cells and a decrease in the intrasplenic-to-intrahepatic NKT ratio were noted. Assessment of the CD4-to-CD8 ratios showed sequestration of CD8+ lymphocytes in the liver. These effects were associated with a decrease in serum triglyceride levels, decrease in the aspartate aminotransferase levels, serum glucose levels, and HOMA-IR score. A decrease in hepatic triglycerides content was observed in the high dose-treated mice.

CONCLUSION

Orally administered PRX-106 shows biological activity and exerts an immunomodulatory effect, alleviating liver

damage. The data suggest that PRX-106 may provide an oral immunotherapy for nonalcoholic steatohepatitis.

Key words: Nonalcoholic steatohepatitis; Nonalcoholic fatty liver disease; Tumor necrosis alpha; Enbrel; Diabetes

© **The Author(s) 2016.** Published by Baishideng Publishing Group Inc. All rights reserved.

Core tip: The BY-2 plant cell-expressed recombinant anti-tumor necrosis factor alpha (TNF) fusion protein (PRX-106) that consists of the soluble form of the human TNF receptor fused to the Fc component of a human IgG1 domain was orally administered in high-fat diet model. Altered distribution of CD4+CD25+FoxP3+ and a decrease in the intrasplenic-to-intrahepatic CD8+CD25+FoxP3+ ratio were observed. These effects were associated with a decrease in serum triglyceride levels, decrease in the aspartate aminotransferase levels, serum glucose levels, and HOMA-IR score. A decrease in hepatic triglycerides content was observed in the high dose-treated mice. The data suggest that PRX-106 may provide an oral immunotherapy for nonalcoholic steatohepatitis.

Ilan Y, Ben Ya'acov A, Shabbat Y, Gingis-Velitski S, Almon E, Shaaltiel Y. Oral administration of a non-absorbable plant cell-expressed recombinant anti-TNF fusion protein induces immunomodulatory effects and alleviates nonalcoholic steatohepatitis. *World J Gastroenterol* 2016; 22(39): 8760-8769 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v22/i39/8760.htm> DOI: <http://dx.doi.org/10.3748/wjg.v22.i39.8760>

INTRODUCTION

A chronic inflammatory state and dysfunction of metabolic-inflammatory signaling are involved in the development of various aspects of non-alcoholic steatohepatitis (NASH)^[1,2]. Several downstream signaling pathways, which provide the crosstalk between inflammatory and metabolic signaling, have been identified. The pro-inflammatory axis consisting of the nuclear transcription factor NF-kappa B and its upstream kinase IKK-beta is one pathway responsible for nutritionally induced inflammation^[3]. c-Jun N-terminal kinase and I kappa beta kinase (I kappa K) have been identified as mediators of insulin resistance (IR)^[4,5]. These pathways are activated along with other signals by tumor necrosis factor alpha (TNF- α)^[6].

The importance of TNF in human and animal fatty liver diseases, which were induced both by genetic manipulation and by overnutrition, has been demonstrated^[7]. Neutralization of TNF- α activity improves IR and fatty liver disease in animals. Adiponectin is a potent TNF-neutralizing and anti-inflammatory adipocytokine. Both *in vitro* and *in vivo* studies showed

its importance in counteracting inflammation and IR^[8,9]. Some of the anti-inflammatory effects of adiponectin are mediated by suppression of TNF synthesis and the promotion of anti-inflammatory cytokines, including IL-10 and the IL-1 receptor antagonist^[7].

The NLRP6 and NLRP3 inflammasomes and the effector protein IL-18 negatively regulate NASH progression^[10]. Inflammasome deficiency-associated changes in the configuration of the gut microbiota are associated with exacerbated hepatic steatosis and inflammation through influx of TLR4 and TLR9 agonists into the portal circulation, leading to the enhanced hepatic TNF- α expression that drives NASH progression^[10]. A recent meta-analysis showed a difference in the TNF- α -238 genotype distribution between nonalcoholic fatty liver disease (NAFLD) patients and controls, suggesting that a polymorphism at position-238 is a risk factor for NAFLD^[11]. Increased gut permeability along with bacterial translocation (BT) and increased lipopolysaccharide (LPS) levels have been described in patients with NASH^[12,13]. BT at mesenteric lymph nodes leads to lymphocyte activation^[14]. The TNF-alpha mRNA expression in liver tissue is significantly higher in patients with NASH and correlates with the increase in the plasma levels of LPS binding protein.

The development of biological agents that target TNF have markedly changed the therapeutic approach to inflammatory diseases^[15]. Pentoxifylline, an anti-TNF- α agent was shown to reduce aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels and to improve liver histological scores in patients with NAFLD/NASH^[16]. Parenteral administration of recombinant anti-TNF proteins lowers disease activity and, in some patients, induces remission. A report described a NASH patient who experienced rapid normalization of liver biochemistry during treatment for an associated rheumatoid arthritis with the humanized anti-TNF-alpha antibody adalimumab^[17].

Etanercept is a recombinant, dimeric, soluble tumor necrosis factor receptor fusion protein that blocks only soluble TNF but not membrane-bound TNF. Parenteral administration of etanercept is being used for rheumatoid arthritis, juvenile rheumatoid arthritis, psoriatic arthritis, psoriasis, and ankylosing spondylitis^[15,18]. Parenterally administered TNF antagonists are generally well tolerated but carry a risk of side effects. Areas of concern include opportunistic and non-opportunistic infections, vaccination, neurological complications, hepatotoxicity, hematological side effects, malignancies, infusion reactions and autoimmunity. Contraindications, such as heart failure and acute infectious diseases, are also of concern^[19]. The immunosuppressive capacity of these agents necessitates a rigorous long-term safety follow-up, and the potential risks of their use should always be taken into consideration^[19,20].

Oral delivery of therapeutic proteins is a major goal when developing new therapeutic modalities. The BY-2 plant cell-expressed recombinant anti-TNF fusion protein

(PRX-106) consisting of the soluble form of the human TNF receptor (TNFR) fused to the Fc component of a human IgG1 domain can be orally administered, and PRX-106 has an amino acid sequence that is identical to that of Enbrel™.

The aim of the present study was to determine the immunomodulatory effect of oral administration of plant cells expressing PRX-106 in an animal model of NASH.

MATERIALS AND METHODS

Animals

Male C57BL/6 mice (11-12-wk-old) were obtained from Harlan Laboratories (Jerusalem, Israel) and maintained in the Animal Core of the Hadassah-Hebrew University Medical School. Mice were administered standard laboratory chow and water *ad libitum* and kept on a 12 h light/dark cycle. Animal experiments were carried out according to the guidelines of the Hebrew University-Hadassah Institutional Committee for the Care and Use of Laboratory Animals and with the committee's approval. Mice were fed a high-fat diet (HFD, Harlan, TD88137; 42% of the calories are from fat) from day 0 until their sacrifice at 24 wk.

Experimental groups

Four groups of C57BL/6 mice, $n = 10$ each, were orally fed three times a week for 24 wk with one of the following at a volume of 35 μ L: phosphate-buffered saline (PBS, group A), BY-2 cells at 28.8 mg of BY- (mock cells, group B), 2.88 mg (0.5 μ g TNF) of BY+ (group C), or 2.88 mg (10 μ g anti-TNF) of BY+ (group D). Fresh preparations were made before each administration.

Assessment of the effect of oral PRX-106 on the systemic immune system

The immunomodulatory effect of PRX-106 was determined by FACS analysis and serum cytokines.

Isolation of splenocytes and hepatic lymphocytes: Spleens and livers were kept in RPMI-1640 supplemented with FCS. Spleens were crushed through a 70 μ m nylon cell strainer^[21] and centrifuged (1250 rpm for 7 min) to remove debris. Red blood cells were lysed with 1 mL of cold 155 mmol/L ammonium chloride lysis buffer and immediately centrifuged (1250 rpm for 3 min). The splenocytes were then washed and suspended in 1 mL of RPMI + FCS. Any remaining connective tissue was removed. The viability, as assessed using trypan blue staining, was above 90%. For the isolation of hepatic lymphocytes, livers were first crushed through a stainless mesh (size 60, Sigma), and the cell suspension was placed in a 50 mL tube for 5 min so that the cell debris could form a pellet. The cell suspension was slowly underlaid with 10 mL of Lymphoprep (Ficoll, Axis-Shield PoC

AS, Oslo, Norway) in a 50 mL tube. The tubes were then centrifuged at 1800 rpm for 18 min. Cells at the interface were collected and transferred to a new tube that was centrifuged again at 1800 rpm for 10 min. The resulting pellet of hepatocyte-depleted lymphocytes was suspended in a final volume of 250 μ L, approximately 1×10^6 intrahepatic lymphocytes were recovered per mouse liver.

Flow cytometry: Flow cytometry was performed on splenocytes and hepatic lymphocytes, which were suspended in 1 mL of FACS buffer (PBS + 1% BSA + 0.1% sodium azide). The cells were stained with the antibodies for CD8, CD4, CD25, Foxp3, CD3, and NK 1.1. Flow cytometry was performed using LSR-II, and and FSC express software.

Cytokine measurement: Serum TNF- α levels were measured in each animal using commercial kits, according to the manufacturer's instructions (Quantikine, R&D Systems, Minneapolis, MN, United States).

Assessment of the effect of oral PRX-106 on the lipid profile, glucose intolerance and liver damage

Liver enzymes: Serum was obtained from individual mice. The serum AST and ALT levels were determined using an automatic analyzer.

Triglyceride levels in the serum were measured using standard kits.

Fasting serum glucose and insulin levels were measured on day 1 and at week 24.

Glucose tolerance test: Glucose tolerance test (GTT) was performed on all animals in all groups at weeks 8 and 24.

Histological examination of the liver: Paraffin-embedded liver sections were prepared from each mouse. The livers were cut into 4-5 μ m thin slices and stained with hematoxylin-eosin. A blinded pathologist examined the tissues using light microscopy to score for morphological and histopathological changes that are characteristic of NAS. Trichrome staining was used to evaluate liver fibrosis. The maximal score for steatosis (= 3) was assigned for greater than 66%. The maximal score for lobular inflammation (= 3) was assigned for > 4 foci/ $\times 200$, and hepatocyte ballooning (= 2) was assigned for many cells/prominent ballooning. The maximal NAS score is a simple arithmetic combination of all three features (3 + 3 + 2 = 8).

Hepatic triglycerides content: Accumulation of intracellular triglycerides (TGs) within the liver was quantified using a modification of the Folch method. TGs were extracted from aliquots of snap-frozen livers and then assayed. Triglyceride determination was performed spectrophotometrically using a GPO-Trinder

kit (Sigma, Rehovot, Israel), and the levels were normalized to the protein content in the homogenate.

Statistical analysis

Statistical analysis was performed using Student's *t* test. A *P* value less than 0.05 was considered significant.

RESULTS

Oral administration of non-absorbable PRX-106 exerted an immunomodulatory effect

Oral administration of PRX-106 altered the distribution of CD4+CD25+FoxP3+ Tregs. Figure 1A shows the significant decrease in intrahepatic Tregs in the high dose-treated mice from the 3.53% value in the controls to 2.48% (*P* < 0.05). Figure 1B shows the significant effect on intrasplenic Tregs only for BY2 (-) treated mice from 18.4% to 13.73% (*P* < 0.01). A reduction trend was noted for the high dose-treated group (15.49%). The intrasplenic-to-intrahepatic CD4+CD25+FoxP3+ Treg ratio increased significantly in the high dose PRX-106-treated mice, as seen in Figure 1C, from 5.12 in controls to 6.25 (*P* < 0.05). The data indicate an effect of PRX106 on the redistribution of CD4+CD25+FoxP3+ Tregs.

Oral administration of PRX-106 also altered the distribution of CD8+CD25+FoxP3+ Tregs. Figure 1D shows a significant increase in intrahepatic CD8+CD25+FoxP3+ Tregs in the BY2 (-) and high dose-treated mice. The levels increased to 0.6% and 0.86%, respectively, compared with 0.21% in the controls (*P* < 0.05). Figure 1E shows no statistically significant effect on intrasplenic CD8+CD25+FoxP3+ Tregs. The intrasplenic-to-intrahepatic CD8+CD25+FoxP3+ Treg ratio was significantly altered by the treatments, as shown in Figure 1F. The ratio was 9.8 in the controls and decreased to 2.4 and 4 in the BY2 (-)- and high dose-treated mice while increasing to 16.2 in the low dose-treated mice (*P* < 0.005 compared with controls).

Oral administration of PRX-106 altered the distribution of NKT (CD3+NK1.1+) lymphocytes. Figure 1G shows an increase in intrahepatic NKT cells in the high dose-treated mice, with levels increasing 3.58% compared 2.31% in controls (*P* < 0.05). Figure 1H shows a significant decrease in the intrasplenic NKT cells in the BY2(-)-treated mice, in which the levels decreased to 0.42% compared with 0.82% in controls (*P* < 0.05). The intrasplenic-to-intrahepatic NKT ratio significantly decreased in all treated groups, as shown in Figure 1I. The levels decreased to 0.15, 0.24 and 0.16 for the BY2(-)-treated and low dose and high dose PRX106-treated mice, respectively, compared with 0.35 in the controls (*P* < 0.05 vs controls).

To determine the effect of treatment on lymphocyte trapping in the liver, the CD4/CD8 lymphocyte ratio was calculated. The splenic CD4/CD8 ratio was 1.62, 1.48, 1.56 and 1.46 for the controls, BY2 (-)-treated and low and high dose PRX106-treated

mice, respectively. The hepatic CD4/CD8 ratio was 0.73, 0.72, 0.82, and 0.68 for the controls, BY2(-)-treated and low and high dose PRX106-treated mice, respectively. Figure 1J shows the ratio between the splenic and hepatic CD4/CD8 ratios. For all treated groups, a decrease in the ratio was found: 2.06, 1.9, and 2.15 for the BY2(-)-treated and the low and high dose PRX106-treated mice compared with 2.2 for the controls (*P* < 0.005 for low dose PRX106 compared with controls). The data suggest that the treatment is associated with sequestration of CD8+ lymphocytes in the liver.

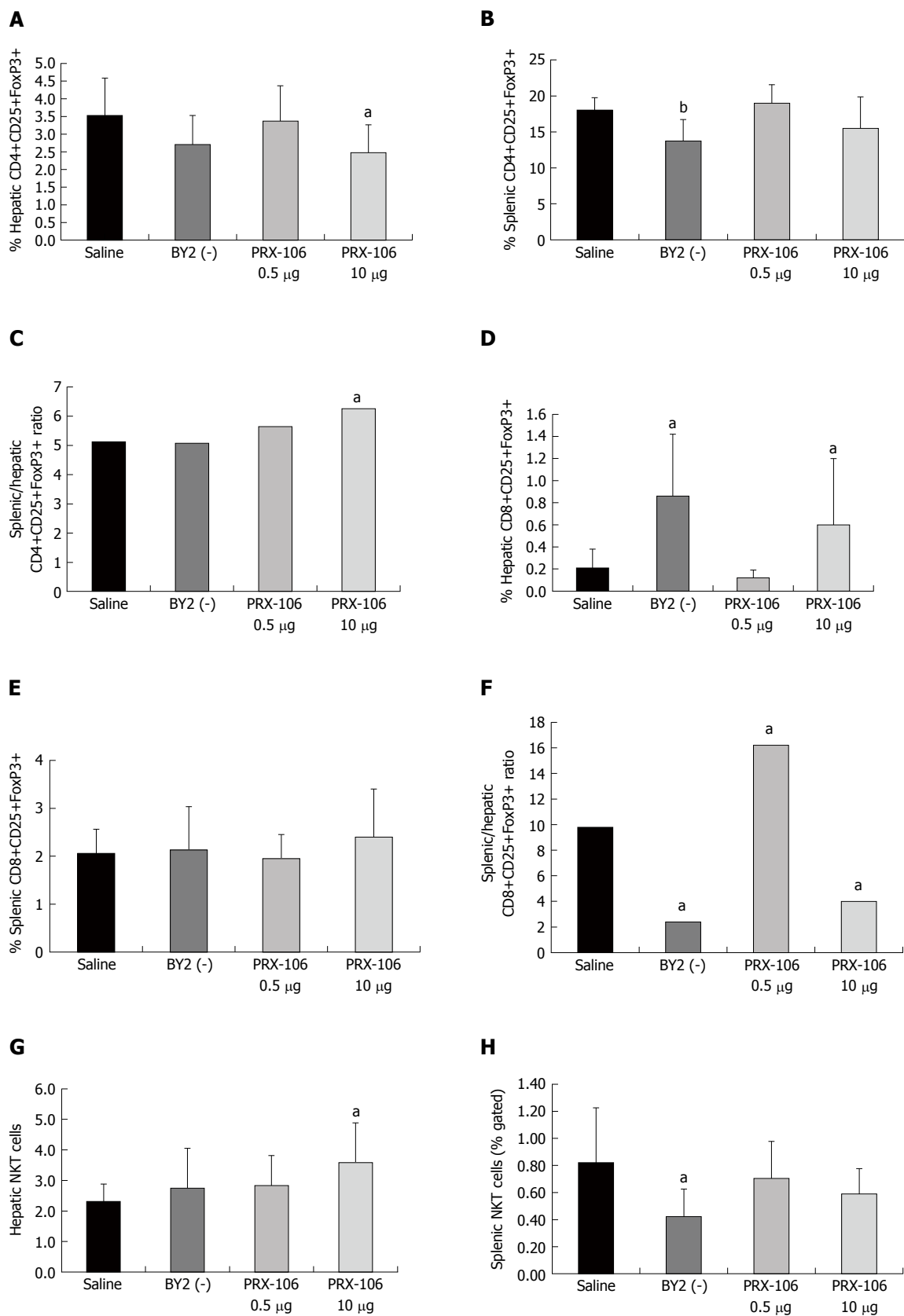
Oral administration of PRX-106 was associated with a mild increase in serum TNF- α levels. The levels increased to 11.53, 13.85, and 10.25 pg/mL for BY2 (-)-treated and low and high dose PRX106-treated mice, respectively, compared with 7.07 pg/mL in the controls (*P* < 0.05 for low dose PRX106 compared with controls). The data support the notion that the observed anti-inflammatory effect is not associated with a reduction in TNF- α levels.

Oral administration of non-absorbable PRX-106 exerted a beneficial effect on the liver, glucose and lipid metabolism in NASH

During the 24 wk of the experiment, the weight doubled for the mice in all groups. The average weight gain was 82.5%, 83.3%, 81.8%, and 89.1% for the controls, BY2(-)-treated and low and high dose PRX106-treated mice, respectively (*P* = NS). The data support the notion that the beneficial effect on the liver, glucose, and lipid metabolism was independent of weight.

Both dosages of PRX-106 exerted a beneficial effect on the serum TGs at week 24, as shown in Figure 2A. The serum triglyceride levels decreased to 186 and 124 mg/dL for the low and high PRX106-treated groups compared with 260 mg/dL for untreated controls (*P* < 0.01 for high dose vs controls). Figure 2B shows that oral administration of the high dose of PRX-106 decreased the AST levels at week 24. The AST levels were 297 compared with 496 IU for the high dose PRX106 vs the controls, respectively (*P* = 0.06). No significant effects on the ALT levels were observed.

A beneficial effect of oral PRX106 on fasting glucose levels was noted. At week 5, a significant decrease in serum glucose levels was observed for the lower dose-treated PRX 106 with levels of 88, 76 and 86 mg% for the BY2(-)-treated and low and high dose PRX106-treated mice, respectively, compared with 104 mg% in the controls (*P* < 0.05 for low dose vs controls). Figure 3A shows the beneficial effect of the treatments at the end of trial, with a reduction in the glucose levels to 102, 103, and 102 mg% for the BY2(-)-treated and low and high dose PRX106-treated mice, respectively, compared with 119 mg% in controls. Figure 3B shows the beneficial effect of the treatment on the delta of



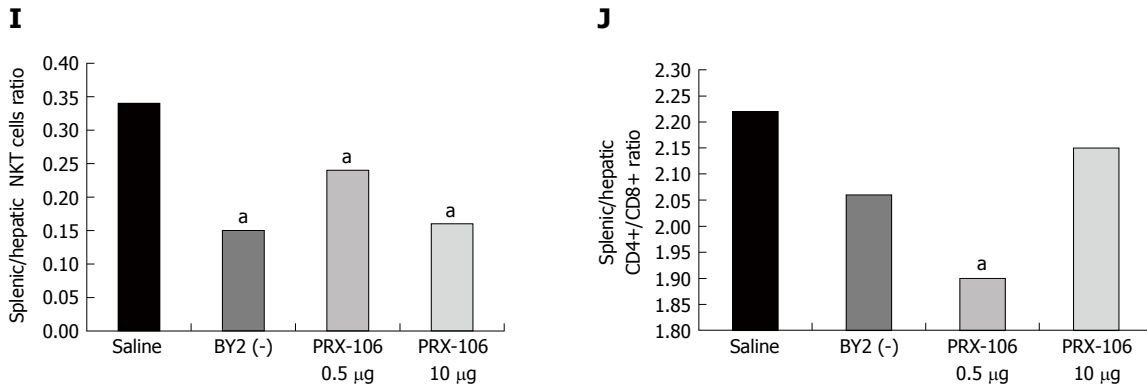


Figure 1 FACS analysis was performed on lymphocytes harvested from the spleens and livers of all mice from the experimental and control groups. The results were compared for the following subsets of cells: A: Intrahepatic CD4+CD25+FoxP3+ lymphocytes; B: Intrasplenic CD4+CD25+FoxP3+ lymphocytes; C: The intrasplenic-to-intrahepatic CD4+CD25+FoxP3+ ratio was calculated; D: Intrahepatic CD8+CD25+FoxP3+ lymphocytes; E: Intrasplenic CD8+CD25+FoxP3+ lymphocytes; F: The intrasplenic-to-intrahepatic CD8+CD25+FoxP3+ ratio was calculated; G: Intrahepatic NKT lymphocytes; H: Intrasplenic NKT lymphocytes; I: The intrasplenic-to-intrahepatic NKT ratio was calculated; J: To determine the effect of the treatment on the lymphocyte trapping in the liver, the CD4/CD8 lymphocyte ratio was calculated in the spleen and in the liver. The ratio between the splenic and hepatic CD4/CD8 ratios was calculated. ^a $P < 0.05$, ^b $P < 0.01$.

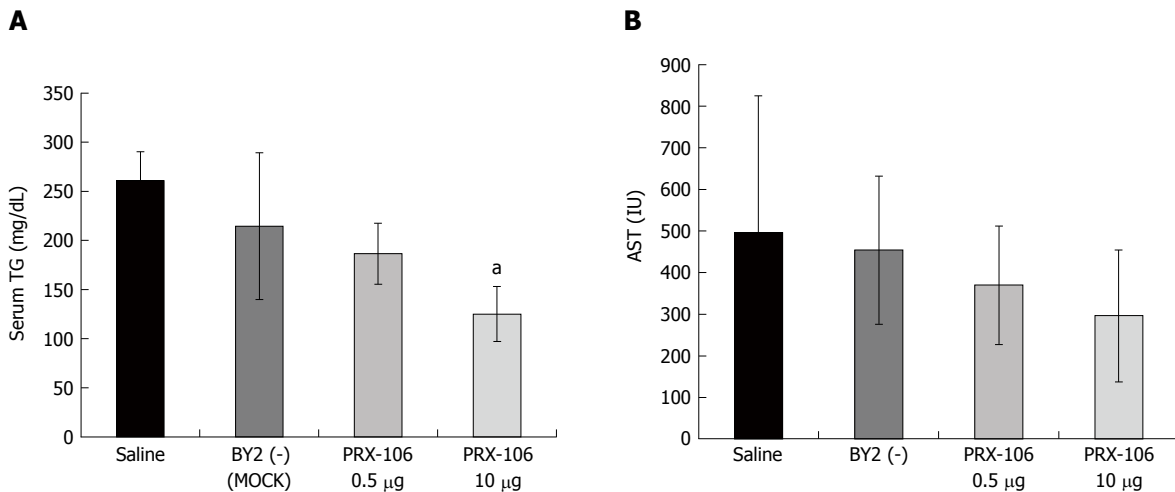


Figure 2 Serum levels of triglycerides (A) and aspartate aminotransferase (B) were measured in all mice from the experimental and control groups. AST: Aspartate aminotransferase. ^a $P < 0.05$.

the increase in fasting insulin levels between week 0 and 24. A trend for a reduction in the average increase in insulin levels between weeks 0 and 24 was noted in all treated mice. The average change in insulin levels was 2.69, 0.92, and 2.77 pg/mL for BY2 (-)-treated and low and high dose PRX106-treated mice, respectively, compared with 3.06 pg/mL in controls. Figure 3C shows the effect of oral PRX106 on the HOMA-IR. A reduction trend for a reduction to 1.09 was observed for mice in the low dose PRX106-treated group compared with the 1.25 value in the controls. No significant differences were noted between groups in the oral GTT performed at weeks 7 and 22 of the study.

A decrease in hepatic TG content was observed in the high dose-treated mice, as shown in Figure 4. The triglyceride content was reduced to 23.54% per g liver for the high dose PRX-106-treated mice compared with 33.98% for the controls ($P = 0.03$).

DISCUSSION

Orally administered plant cells expressing recombinant anti-TNF fusion protein show biological activity and exert an immunomodulatory effect, alleviating the liver damage in the HFD model.

The pathogenesis of NASH involves a number of immune mechanisms^[22]. Immunomodulatory treatments have been suggested to play a role in alleviating the disease^[1]. The results of the present study show that oral administration of non-absorbable PRX-106 exerted an immunomodulatory effect. Oral administration of PRX-106 was associated with an increase in the intrasplenic-to-intrahepatic CD4+CD25+FoxP3+ ratio, suggesting that the drug generates a signal in the gut that promotes regulatory cells in the periphery, which can account for its anti-inflammatory effects^[23]. Resident Treg cells in adipose tissue modulate metabolism and glucose homeostasis^[24]. Interactions

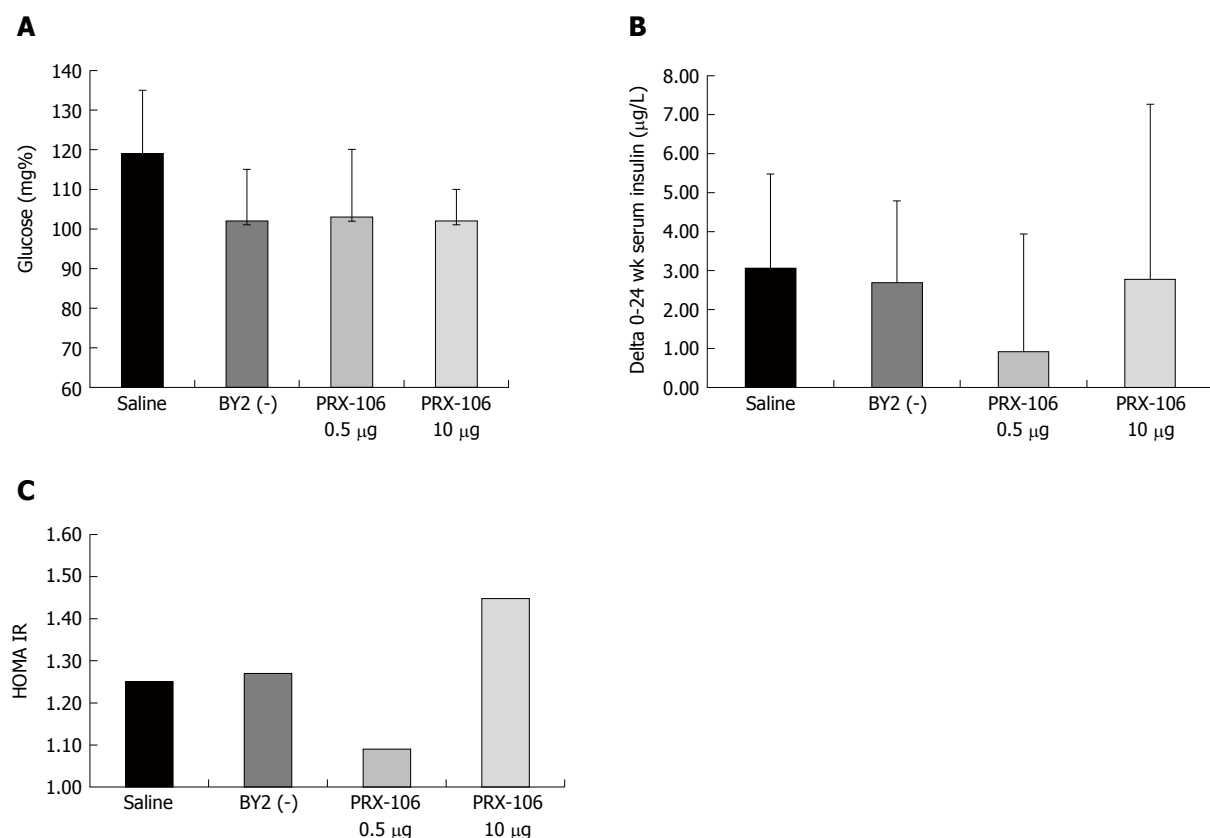


Figure 3 A beneficial effect of oral PRX106 on fasting glucose levels was noted. Serum glucose levels were measured at the end of the study in all mice (A); the delta between the average serum insulin levels on day 0 and the end of trial was calculated (B); HOMA-IR was calculated for all mice at the end of the study (C).

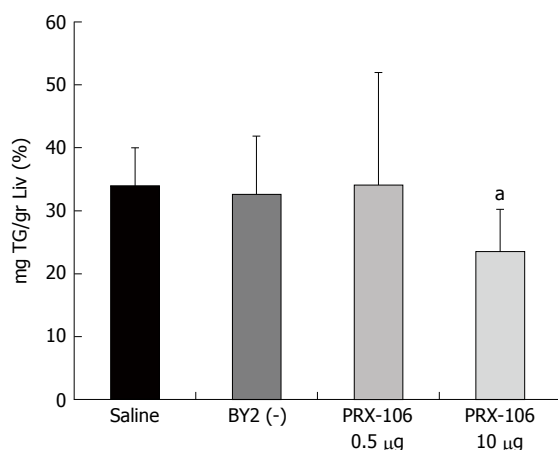


Figure 4 The liver triglyceride content was measured and calculated per liver weight to determine the percentage of fat in the mouse livers. ^a*P* = 0.03.

among leptin, Treg cells and adipose tissue are potential targets for therapeutic interventions^[25]. The Treg frequency is lower in TNF- α transgenic mice than in wild-type mice^[26]. In humans, anti-TNF α therapy has a clinical effect associated with promotion of Treg number and function. Anti-TNF α therapy increased the Treg proportion and suppressed effector T cells in patients with arthritis^[27].

Oral administration of PRX-106 also altered the distribution of CD8+CD25+FoxP3+ Tregs. CD8+CD25+FoxP3+ Tregs were suggested to be

important in the induction of the systemic anti-inflammatory effects mediated by several immunomodulatory agents^[28,29] and to suppress inflammation in several immune-mediated disorders^[30]. The data suggest a dose dependency of the observed immune system effect, which may underlie some of the differences noted in the clinical effects.

Oral administration of PRX-106 altered the distribution of NKT (CD3+NK1.1+) lymphocytes. The intrasplenic-to-intrahepatic NKT ratio significantly decreased in all treated groups. NKT cells play a

regulatory role that helps to prevent diet-induced obesity and metabolic dysfunction^[31]. A reduction in the number and an altered function of intrahepatic NKT lymphocytes have been reported in leptin-deficient ob/ob mice, a murine model for NASH^[32-35]. NKT cells were suggested to be important in the regulation of immune-mediated disorders^[36,37], and alteration of their distribution was shown to be relevant in immunomodulation^[38-42]. Our results further support a regulatory role for NKT cells in the crosstalk between metabolism and the immune system^[31].

The ratio between the splenic and hepatic CD4/CD8 ratios decreased for all treated groups, supporting the notion that the treatment is associated with sequestration of CD8+ lymphocytes in the liver. The liver is a site at which apoptotic CD8+ cells accumulate during the clearance phase of peripheral immune responses^[43]. It serves as a "graveyard" for T cells activated in the periphery^[44]. The liver was shown to be an important site for CD8+ accumulation during tolerance induction in a process that was independent of NK cells^[45]. Our results support the ability of PRX-106 to promote CD8 lymphocytes in the liver during an active systemic inflammatory process.

Oral administration of PRX-106 was associated with a mild increase in serum TNF- α levels, suggesting that the anti-inflammatory effects are independent of this cytokine. PRX-106 is not absorbed, and its immunomodulatory effect is therefore associated with biological activity in the gut. A similar effect was reported for the oral administration of the non-absorbable anti-CD3^[46-49] and delayed release 6 mercaptopurine^[50], all of which generate a similar signal in the gut which alters the systemic immune system.

Oral administration of PRX106 was associated with a reduction in the serum triglyceride, glucose, insulin, and AST levels. A decrease in hepatic TG content was observed in the high dose-treated mice. Taken together, our data suggest that the profound immunomodulatory effect of oral PRX106 is associated with improvement in the metabolic syndrome in the HFD model.

Etanercept is a TNFR2-Fc fusion protein that blocks only soluble TNF but not membrane-bound TNF^[51]. Parenteral administration of this compound has been successfully used in the treatment of rheumatoid arthritis and several other immunomodulatory treatments. The data of the present study support an immunomodulatory effect of orally administered PRX-106. Several immunomodulatory agents exert a different effect on the systemic immune system when administered orally compared with during parenteral administration^[52-56]. Their local effect on the gut is different than when the drug is administered parentally. These compounds use the inherent ability of the immune system of the gut to systemically promote Tregs^[54,55] and to exert potent anti-inflammatory effects^[54,55]. These effects are not associated with generalized immune suppression.

Adjuvants were suggested to be important for augmenting the effect of orally administered

immunomodulatory agents^[48,49,56]. The plant cell wall, which is composed of cellulose, serves to protect the active molecule from acid-base changes in the stomach. The data of the present study suggest that the plant cell wall also serves as an immune adjuvant in the gut. Oral administration of mock cells, BY2 (-), which included vehicle alone, did exert some immunomodulatory effects.

Previous studies showed that oral administration of BY-2 cells expressing PRX-106 alleviated immune-mediated liver injury in the Concanavalin A immune-mediated hepatitis model^[57]. Similarly, in the TNBS colitis model, oral administration of BY-2 plant cells expressing PRX-106 resulted in a decrease in the weight loss associated with immune-mediated colitis, along with improvement in bowel histology^[58].

In summary, orally administered plant cells expressing recombinant anti-TNF fusion protein show biological activity when administered orally. It alters the systemic immune environment and affects both the intrahepatic and splenic regulatory T lymphocyte and NKT cell subsets. These changes were associated with the observed beneficial effects on the metabolic syndrome in the HFD model. As a non-absorbable agent, PRX106 may serve as a potent immunomodulatory agent that lacks immunosuppressive properties.

COMMENTS

Background

The BY-2 plant cell-expressed recombinant anti-TNF fusion protein (PRX-106) that consists of the soluble form of the human TNF receptor (TNFR) fused to the Fc component of a human IgG1 domain was orally administered. The aim of the study was to evaluate the immunomodulatory effect of oral administration of PRX-106 in the high-fat diet (HFD) model of non-alcoholic steatohepatitis (NASH).

Research frontiers

PRX-106 consisting of the soluble form of the human TNFR fused to the Fc component of a human IgG1 domain can be orally administered, and PRX-106 has an amino acid sequence that is identical to that of EnbrelTM.

Innovations and breakthroughs

For 22 wk, C57BL/6 HFD-fed mice received daily oral treatments with BY-2 cells expressing PRX-106. Orally administered PRX-106 shows biological activity and exerts an immunomodulatory effect, alleviating liver damage. The data suggest that PRX-106 may provide an oral immunotherapy for NASH. The orally administered non-absorbable PRX-106 was biologically active. Altered distribution of CD4+CD25+FoxP3+ between the liver and spleen and an increase in the intrasplenic-to-intrahepatic CD4+CD25+FoxP3+ ratio and a decrease in the intrasplenic-to-intrahepatic CD8+CD25+FoxP3+ ratio were observed. An increase in intrahepatic NKT cells and a decrease in the intrasplenic-to-intrahepatic NKT ratio were noted. Assessment of the CD4-to-CD8 ratios showed sequestration of CD8+ lymphocytes in the liver. These effects were associated with a decrease in serum triglyceride levels, decrease in the aspartate aminotransferase levels, serum glucose levels, and HOMA-IR score. A decrease in hepatic TG content was observed in the high dose-treated mice.

Applications

PRX-106 is biologically active when administered orally. It alters the systemic immune environment and affects both the intrahepatic and splenic regulatory

T lymphocyte and NKT cell subsets. These changes were associated with the observed beneficial effects on the metabolic syndrome in the HFD model of NASH. As a non-absorbable agent, PRX106 may serve as a safe and potent immunomodulatory agent that lacks immunosuppressive properties.

Terminology

A chronic inflammatory state and dysfunction of metabolic-inflammatory signaling are involved in the development of various aspects of NASH. Oral delivery of therapeutic proteins is a major goal when developing new therapeutic modalities.

Peer-review

This is a pre-clinical trial that shows the potential beneficial effect of oral administration of PRX-106 in a model for NASH. Further clinical trials are required for showing its effects in patients with type 2 diabetes and NASH.

REFERENCES

- Ilan Y. Immune therapy for nonalcoholic steatohepatitis: are we there yet? *J Clin Gastroenterol* 2013; **47**: 298-307 [PMID: 23442833 DOI: 10.1097/MCG.0b013e31827873dc]
- Hotamisligil GS. Inflammation and metabolic disorders. *Nature* 2006; **444**: 860-867 [PMID: 17167474 DOI: 10.1038/nature05485]
- Cai D. NFkappaB-mediated metabolic inflammation in peripheral tissues versus central nervous system. *Cell Cycle* 2009; **8**: 2542-2548 [PMID: 19633416 DOI: 10.4161/cc.8.16.9386]
- Sztajnkrycer MJ, Bond GR. Chronic acetaminophen overdosing in children: risk assessment and management. *Curr Opin Pediatr* 2001; **13**: 177-182 [PMID: 11317062 DOI: 10.1097/00008480-200104000-00016]
- Hotamisligil GS. Inflammation and endoplasmic reticulum stress in obesity and diabetes. *Int J Obes (Lond)* 2008; **32** Suppl 7: S52-S54 [PMID: 19136991 DOI: 10.1038/ijo.2008.238]
- Hotamisligil GS. Inflammatory pathways and insulin action. *Int J Obes Relat Metab Disord* 2003; **27** Suppl 3: S53-S55 [PMID: 14704746 DOI: 10.1038/sj.ijo.0802502]
- Tilg H. The role of cytokines in non-alcoholic fatty liver disease. *Dig Dis* 2010; **28**: 179-185 [PMID: 20460908 DOI: 10.1159/000282083]
- Andrade-Oliveira V, Câmara NO, Moraes-Vieira PM. Adipokines as drug targets in diabetes and underlying disturbances. *J Diabetes Res* 2015; **2015**: 681612 [PMID: 25918733 DOI: 10.1155/2015/681612]
- Gu P, Xu A. Interplay between adipose tissue and blood vessels in obesity and vascular dysfunction. *Rev Endocr Metab Disord* 2013; **14**: 49-58 [PMID: 23283583 DOI: 10.1007/s11154-012-9230-8]
- Henao-Mejia J, Elinav E, Jin C, Hao L, Mehal WZ, Strowig T, Thaiss CA, Kau AL, Eisenbarth SC, Jurczak MJ, Camporez JP, Shulman GI, Gordon JI, Hoffman HM, Flavell RA. Inflammation-mediated dysbiosis regulates progression of NAFLD and obesity. *Nature* 2012; **482**: 179-185 [PMID: 22297845 DOI: 10.1038/nature10809]
- Wang JK, Feng ZW, Li YC, Li QY, Tao XY. Association of tumor necrosis factor- α gene promoter polymorphism at sites -308 and -238 with non-alcoholic fatty liver disease: a meta-analysis. *J Gastroenterol Hepatol* 2012; **27**: 670-676 [PMID: 22097889 DOI: 10.1111/j.1440-1746.2011.06978.x]
- Almeida J, Galhenage S, Yu J, Kurtovic J, Riordan SM. Gut flora and bacterial translocation in chronic liver disease. *World J Gastroenterol* 2006; **12**: 1493-1502 [PMID: 16570339 DOI: 10.3748/wjg.v12.i10.1493]
- Szabo G, Bala S, Petrasek J, Gattu A. Gut-liver axis and sensing microbes. *Dig Dis* 2010; **28**: 737-744 [PMID: 21525758 DOI: 10.1159/000324281]
- Úbeda M, Muñoz L, Borrero MJ, Díaz D, Francés R, Monserrat J, Lario M, Lledó L, Such J, Álvarez-Mon M, Albillos A. Critical role of the liver in the induction of systemic inflammation in rats with preascitic cirrhosis. *Hepatology* 2010; **52**: 2086-2095 [PMID: 21105108 DOI: 10.1002/hep.23961]
- Wiedmann MW, Mössner J, Baerwald C, Pierer M. TNF alpha inhibition as treatment modality for certain rheumatologic and gastrointestinal diseases. *Endocr Metab Immune Disord Drug Targets* 2009; **9**: 295-314 [PMID: 19594416 DOI: 10.2174/187153009789044347]
- Li W, Zheng L, Sheng C, Cheng X, Qing L, Qu S. Systematic review on the treatment of pentoxifylline in patients with non-alcoholic fatty liver disease. *Lipids Health Dis* 2011; **10**: 49 [PMID: 21477300 DOI: 10.1186/1476-511X-10-49]
- Schramm C, Schneider A, Marx A, Lohse AW. Adalimumab could suppress the activity of non alcoholic steatohepatitis (NASH). *Z Gastroenterol* 2008; **46**: 1369-1371 [PMID: 19053005 DOI: 10.1055/s-2008-1027411]
- Hoy SM, Scott LJ. Etanercept: a review of its use in the management of ankylosing spondylitis and psoriatic arthritis. *Drugs* 2007; **67**: 2609-2633 [PMID: 18034593 DOI: 10.2165/00003495-200767170-00009]
- Stallmach A, Hagel S, Bruns T. Adverse effects of biologics used for treating IBD. *Best Pract Res Clin Gastroenterol* 2010; **24**: 167-182 [PMID: 20227030 DOI: 10.1016/j.bpg.2010.01.002]
- Rongioletti F, Burlando M, Parodi A. Adverse effects of biological agents in the treatment of psoriasis. *Am J Clin Dermatol* 2010; **11** Suppl 1: 35-37 [PMID: 20586505 DOI: 10.2165/1153420-S0-000000000-00000]
- Falcone M, Facciotti F, Ghidoli N, Monti P, Olivieri S, Zaccagnino L, Bonifacio E, Casorati G, Sanvito F, Sarvetnick N. Up-regulation of CD1d expression restores the immunoregulatory function of NKT cells and prevents autoimmune diabetes in nonobese diabetic mice. *J Immunol* 2004; **172**: 5908-5916 [PMID: 15128771 DOI: 10.4049/jimmunol.172.10.5908]
- Valenti L, Fracanzani AL, Fargion S. The immunopathogenesis of alcoholic and nonalcoholic steatohepatitis: two triggers for one disease? *Semin Immunopathol* 2009; **31**: 359-369 [PMID: 19440711 DOI: 10.1007/s00281-009-0152-9]
- Singer BD, King LS, D'Alessio FR. Regulatory T cells as immunotherapy. *Front Immunol* 2014; **5**: 46 [PMID: 24575095 DOI: 10.3389/fimmu.2014.00046]
- Shalev I, Schmelzle M, Robson SC, Levy G. Making sense of regulatory T cell suppressive function. *Semin Immunol* 2011; **23**: 282-292 [PMID: 21592823 DOI: 10.1016/j.smim.2011.04.003]
- Matarese G, Proccaccini C, De Rosa V, Horvath TL, La Cava A. Regulatory T cells in obesity: the leptin connection. *Trends Mol Med* 2010; **16**: 247-256 [PMID: 20493774 DOI: 10.1016/j.molmed.2010.04.002]
- Biton J, Semerano L, Delavallée L, Lemeiter D, Laborie M, Grouard-Vogel G, Boissier MC, Bessis N. Interplay between TNF and regulatory T cells in a TNF-driven murine model of arthritis. *J Immunol* 2011; **186**: 3899-3910 [PMID: 21346237 DOI: 10.4049/jimmunol.1003372]
- Huang Z, Yang B, Shi Y, Cai B, Li Y, Feng W, Fu Y, Luo L, Wang L. Anti-TNF- α therapy improves Treg and suppresses Tef in patients with rheumatoid arthritis. *Cell Immunol* 2012; **279**: 25-29 [PMID: 23059810 DOI: 10.1016/j.cellimm.2012.09.001]
- Tsai YG, Lee CY, Lin TY, Lin CY. CD8⁺ Treg cells associated with decreasing disease activity after intravenous methylprednisolone pulse therapy in lupus nephritis with heavy proteinuria. *PLoS One* 2014; **9**: e81344 [PMID: 24475019 DOI: 10.1371/journal.pone.0081344]
- Notley CA, McCann FE, Inglis JJ, Williams RO. ANTI-CD3 therapy expands the numbers of CD4⁺ and CD8⁺ Treg cells and induces sustained amelioration of collagen-induced arthritis. *Arthritis Rheum* 2010; **62**: 171-178 [PMID: 20039431 DOI: 10.1002/art.25058]
- Correale J, Villa A. Role of CD8⁺ CD25⁺ Foxp3⁺ regulatory T cells in multiple sclerosis. *Ann Neurol* 2010; **67**: 625-638 [PMID: 20437560]
- Martin-Murphy BV, You Q, Wang H, De La Houssaye BA, Reilly TP, Friedman JE, Ju C. Mice lacking natural killer T cells are more susceptible to metabolic alterations following high fat diet feeding. *PLoS One* 2014; **9**: e80949 [PMID: 24465369 DOI: 10.1371/journal.pone.0080949]
- Guebre-Xabier M, Yang S, Lin HZ, Schwenk R, Krzych U, Diehl AM. Altered hepatic lymphocyte subpopulations in obesity-related

- murine fatty livers: potential mechanism for sensitization to liver damage. *Hepatology* 2000; **31**: 633-640 [PMID: 10706553 DOI: 10.1002/hep.510310313]
- 33 **Krzych U**, Schwenk R, Guebre-Xabier M, Sun P, Palmer D, White K, Chalom I. The role of intrahepatic lymphocytes in mediating protective immunity induced by attenuated *Plasmodium berghei* sporozoites. *Immunol Rev* 2000; **174**: 123-134 [PMID: 10807512 DOI: 10.1034/j.1600-0528.2002.00013h.x]
 - 34 **Osman Y**, Kawamura T, Naito T, Takeda K, Van Kaer L, Okumura K, Abo T. Activation of hepatic NKT cells and subsequent liver injury following administration of alpha-galactosylceramide. *Eur J Immunol* 2000; **30**: 1919-1928 [PMID: 10940881 DOI: 10.1002/1521-4141(200007)30:7<1919::AID-IMMU1919>3.0.CO;2-3]
 - 35 **Takeda K**, Hayakawa Y, Van Kaer L, Matsuda H, Yagita H, Okumura K. Critical contribution of liver natural killer T cells to a murine model of hepatitis. *Proc Natl Acad Sci USA* 2000; **97**: 5498-5503 [PMID: 10792025 DOI: 10.1073/pnas.040566697]
 - 36 **Vivier E**, Ugolini S, Blaise D, Chabannon C, Brossay L. Targeting natural killer cells and natural killer T cells in cancer. *Nat Rev Immunol* 2012; **12**: 239-252 [PMID: 22437937 DOI: 10.1038/nri3174]
 - 37 **Zakka LR**, Fradkov E, Keskin DB, Tabansky I, Stern JN, Ahmed AR. The role of natural killer cells in autoimmune blistering diseases. *Autoimmunity* 2012; **45**: 44-54 [PMID: 21923616 DOI: 10.3109/08916934.2011.606446]
 - 38 **Adar T**, Ben Ya'acov A, Lalazar G, Lichtenstein Y, Nahman D, Mizrahi M, Wong V, Muller B, Rawlin G, Ilan Y. Oral administration of immunoglobulin G-enhanced colostrum alleviates insulin resistance and liver injury and is associated with alterations in natural killer T cells. *Clin Exp Immunol* 2012; **167**: 252-260 [PMID: 22236001 DOI: 10.1111/j.1365-2249.2011.04511.x]
 - 39 **El Haj M**, Ben Ya'acov A, Lalazar G, Ilan Y. Potential role of NKT regulatory cell ligands for the treatment of immune mediated colitis. *World J Gastroenterol* 2007; **13**: 5799-5804 [PMID: 17990345 DOI: 10.3748/wjg.v13.i44.5799]
 - 40 **Menachem Y**, Trop S, Kolker O, Shibolet O, Alper R, Nagler A, Ilan Y. Adoptive transfer of NK 1.1+ lymphocytes in immune-mediated colitis: a pro-inflammatory or a tolerizing subgroup of cells? *Microbes Infect* 2005; **7**: 825-835 [PMID: 15893498 DOI: 10.1016/j.micinf.2005.03.019]
 - 41 **Shibolet O**, Kalish Y, Klein A, Alper R, Zolotarov L, Thalenfeld B, Engelhardt D, Rabbani E, Ilan Y. Adoptive transfer of ex vivo immune-programmed NKT lymphocytes alleviates immune-mediated colitis. *J Leukoc Biol* 2004; **75**: 76-86 [PMID: 14557387 DOI: 10.1189/jlb.0703351]
 - 42 **Trop S**, Nagler A, Ilan Y. Role of NK1.1+ and AsGm-1+ cells in oral immunoregulation of experimental colitis. *Inflamm Bowel Dis* 2003; **9**: 75-86 [PMID: 12769441 DOI: 10.1097/00054725-200303000-00001]
 - 43 **Crispe IN**, Dao T, Klugewitz K, Mehal WZ, Metz DP. The liver as a site of T-cell apoptosis: graveyard, or killing field? *Immunol Rev* 2000; **174**: 47-62 [PMID: 10807506 DOI: 10.1034/j.1600-0528.2002.017412.x]
 - 44 **Holz LE**, McCaughan GW, Benseler V, Bertolino P, Bowen DG. Liver tolerance and the manipulation of immune outcomes. *Inflamm Allergy Drug Targets* 2008; **7**: 6-18 [PMID: 18473895 DOI: 10.2174/187152808784165225]
 - 45 **Shibolet O**, Alper R, Zolotarov L, Trop S, Thalenfeld B, Engelhardt D, Rabbani E, Ilan Y. The role of intrahepatic CD8+ T cell trapping and NK1.1+ cells in liver-mediated immune regulation. *Clin Immunol* 2004; **111**: 82-92 [PMID: 15093555 DOI: 10.1016/j.clim.2003.12.001]
 - 46 **Lalazar G**, Mizrahi M, Turgeman I, Adar T, Ben Ya'acov A, Shabat Y, Nimer A, Hemed N, Zolotarova L, Lichtenstein Y, Lisovoder N, Samira S, Shalit I, Ellis R, Ilan Y. Oral Administration of OKT3 MAb to Patients with NASH, Promotes Regulatory T-cell Induction, and Alleviates Insulin Resistance: Results of a Phase IIa Blinded Placebo-Controlled Trial. *J Clin Immunol* 2015; **35**: 399-407 [PMID: 25876706 DOI: 10.1007/s10875-015-0160-6]
 - 47 **Halota W**, Ferenci P, Koziellewicz D, Dybowska D, Lisovoder N, Samira S, Shalit I, Ellis R, Ilan Y. Oral anti-CD3 immunotherapy for HCV-nonresponders is safe, promotes regulatory T cells and decreases viral load and liver enzyme levels: results of a phase-2a placebo-controlled trial. *J Viral Hepat* 2015; **22**: 651-657 [PMID: 25412903 DOI: 10.1111/jvh.12369]
 - 48 **Ilan Y**, Maron R, Tukpah AM, Maioli TU, Murugaiyan G, Yang K, Wu HY, Weiner HL. Induction of regulatory T cells decreases adipose inflammation and alleviates insulin resistance in ob/ob mice. *Proc Natl Acad Sci USA* 2010; **107**: 9765-9770 [PMID: 20445103 DOI: 10.1073/pnas.0908771107]
 - 49 **Ilan Y**, Zigmund E, Lalazar G, Dembinsky A, Ben Ya'acov A, Hemed N, Kasis I, Axelrod E, Zolotarov L, Klein A, El Haj M, Gandhi R, Baecher-Allan C, Wu H, Murugaiyan G, Kivisakk P, Farez MF, Quintana FJ, Khoury SJ, Weiner HL. Oral administration of OKT3 monoclonal antibody to human subjects induces a dose-dependent immunologic effect in T cells and dendritic cells. *J Clin Immunol* 2010; **30**: 167-177 [PMID: 19756989 DOI: 10.1007/s10875-009-9323-7]
 - 50 **Israeli E**, Goldin E, Fishman S, Konikoff F, Lavy A, Chowers Y, Melzer E, Lahat A, Mahamid M, Shirin H, Nussinson E, Segol O, Ya'acov AB, Shabbat Y, Ilan Y. Oral administration of non-absorbable delayed release 6-mercaptopurine is locally active in the gut, exerts a systemic immune effect and alleviates Crohn's disease with low rate of side effects: results of double blind Phase II clinical trial. *Clin Exp Immunol* 2015; **181**: 362-372 [PMID: 25846055 DOI: 10.1111/cei.12640]
 - 51 **Mudter J**, Neurath MF. Apoptosis of T cells and the control of inflammatory bowel disease: therapeutic implications. *Gut* 2007; **56**: 293-303 [PMID: 16956919 DOI: 10.1136/gut.2005.090464]
 - 52 **Weiner HL**, da Cunha AP, Quintana F, Wu H. Oral tolerance. *Immunol Rev* 2011; **241**: 241-259 [PMID: 21488901 DOI: 10.1111/j.1600-065X.2011.01017.x]
 - 53 **Wu HY**, Maron R, Tukpah AM, Weiner HL. Mucosal anti-CD3 monoclonal antibody attenuates collagen-induced arthritis that is associated with induction of LAP+ regulatory T cells and is enhanced by administration of an emulsome-based Th2-skewing adjuvant. *J Immunol* 2010; **185**: 3401-3407 [PMID: 20720210 DOI: 10.4049/jimmunol.1000836]
 - 54 **Mizrahi M**, Ilan Y. The gut mucosa as a site for induction of regulatory T-cells. *Curr Pharm Des* 2009; **15**: 1191-1202 [PMID: 19355960 DOI: 10.2174/138161209787846784]
 - 55 **Castro-Sánchez P**, Martín-Villa JM. Gut immune system and oral tolerance. *Br J Nutr* 2013; **109** Suppl 2: S3-11 [PMID: 23360879 DOI: 10.1017/S0007114512005223]
 - 56 **Ilan Y**. Oral tolerance: can we make it work? *Hum Immunol* 2009; **70**: 768-776 [PMID: 19559742 DOI: 10.1016/j.humimm.2009.06.018]
 - 57 **Shaaltiel Y**, BYa A, Shabat Y, Zolotarov L, Gingis-Velitski S, Almon E, Aviezer D, Ilan Y. Oral administration of a plant cell expressed recombinant anti-TNF fusion protein in biologically active in the gut and alleviates immune mediated hepatitis. *Hepatology* 2013; **751** Suppl 58: 564A
 - 58 **Shaaltiel Y**, BYaA, Shabbat Y, Zolotarov L, Gingis-Velitski S, Almon E, Aviezer D, Ilan Y. A novel method for anti-TNF based-oral immunotherapy: Oral administration of a plant cell-expressed recombinant anti-TNF fusion protein for treating of Crohn's disease. *Gastroenterology* 2014; **5** Suppl 1: S901 [DOI: 10.1016/S0016-5085(14)63276-5]

P- Reviewer: Peltec S, Sherif ZA, Strom SC **S- Editor:** Yu J
L- Editor: A **E- Editor:** Zhang FF



Basic Study

Predicting malignant transformation of esophageal squamous cell lesions by combined biomarkers in an endoscopic screening program

Hao Zhang, Hao Li, Qing Ma, Fang-Yan Yang, Tao-Yu Diao

Hao Zhang, Department of Gastroenterology, The Third Hospital of Jinan, Jinan 250132, Shandong Province, China

Hao Li, Tumor Center, Qilu Hospital of Shandong University, Jinan 250012, Shandong Province, China

Qing Ma, Shandong Provincial Hospital Affiliated to Shandong University, Jinan 250021, Shandong Province, China

Fang-Yan Yang, Department of Epidemiology and Biostatistics, West China School of Public Health, Sichuan University, Chengdu 610041, Sichuan Province, China

Tao-Yu Diao, Institute of Basic Medicine, Shandong Academy of Medical Sciences, Jinan 250062, Shandong Province, China

Author contributions: Zhang H, Li H and Ma Q contributed equally to this study; Li H made a substantial contribution to conception and design; Zhang H, Ma Q, Yang FY and Diao TY performed the experiments and data analysis; and Li H interpreted the data.

Supported by the National Natural Science Foundation of China, No. 30571601.

Institutional review board statement: The study was reviewed and approved by the Institutional Review Board of Shandong Academy of Medical Sciences.

Informed consent statement: All participants gave their written informed consent.

Conflict-of-interest statement: There are no conflicts of interest to declare for all authors of the manuscript.

Data sharing statement: No additional data are available.

Open-Access: This article is an open-access article which was selected by an in-house editor and fully peer-reviewed by external reviewers. It is distributed in accordance with the Creative Commons Attribution Non Commercial (CC BY-NC 4.0) license, which permits others to distribute, remix, adapt, build upon this

work non-commercially, and license their derivative works on different terms, provided the original work is properly cited and the use is non-commercial. See: <http://creativecommons.org/licenses/by-nc/4.0/>

Manuscript source: Unsolicited manuscript

Correspondence to: Hao Li, MD, Vice Professor of Medicine, Tumor Center, Qilu Hospital of Shandong University, No. 107 Wenhuxi Road, Jinan 250012, Shandong Province, China. haoli2003611@163.com
Telephone: +86-531-82169873
Fax: +86-531-82169873

Received: June 19, 2016

Peer-review started: June 20, 2016

First decision: July 29, 2016

Revised: August 23, 2016

Accepted: September 14, 2016

Article in press: September 14, 2016

Published online: October 21, 2016

Abstract

AIM

To determine the association of p53, carcinoembryonic antigen (CEA) and CA19-9 protein expression with esophageal carcinogenesis.

METHODS

An iodine staining endoscopic screening program of esophageal lesions was carried out in the high-incidence area of Feicheng County, China. Seventy-seven patients with basal cell hyperplasia (BCH), 247 with low-grade dysplasia (LGD), 51 with high-grade dysplasia (HGD), 134 with invasive cancer, and 80 normal controls diagnosed by mucous membrane biopsy pathology were enrolled. Immunohistochemical detection of p53, CEA and CA19-9 proteins was performed. In the ROC

curve analysis, the expression of a single biomarker and the expression of a combination of biomarkers were used to predict the risk of these four esophageal lesions.

RESULTS

The positive rates of p53 protein expression in invasive cancer, HGD, LGD, BCH and the normal control groups were 53.0%, 52.9%, 35.6%, 27.3% and 20.0%, respectively; the positive rates of CA19-9 protein expression were 44.0%, 33.3%, 16.5%, 9.2% and 6.2%, respectively; the positive rates of CEA protein expression were 74.6%, 60.8%, 23.3%, 23.7% and 16.2%, respectively. The positive rates of the combined expression of the three biomarkers were 84.3%, 76.5%, 47.6%, 42.9% and 27.5%, respectively. In the receiver operating characteristic curves of the combination of the three biomarkers, the specificity was 88.8% for the normal controls, and the sensitivity was 58.2% for invasive cancer, 25.5% for HGD, 11.2% for LGD, and 6.5% for BCH.

CONCLUSION

p53, CEA and CA19-9 protein expression was correlated with esophageal carcinogenesis, and testing for the combination of these biomarkers is useful for identifying high-risk patients with precancerous lesions.

Key words: Esophageal squamous cell cancer; Esophageal squamous cell dysplasia; p53; Carcinoembryonic antigen; CA19-9; Immunohistochemistry; Prediction

© **The Author(s) 2016.** Published by Baishideng Publishing Group Inc. All rights reserved.

Core tip: Immunohistochemical detection of p53, carcinoembryonic antigen (CEA) and CA19-9 proteins was carried out in patients with basal cell hyperplasia, low-grade dysplasia, high-grade dysplasia, invasive cancer, and normal controls from an area with a high incidence of esophageal lesions. Our data suggest that p53, CEA, and CA19-9 protein expression correlated with the stages of esophageal carcinogenesis. In an endoscopic screening program, the expression of these three biomarkers will be a useful panel for identifying high-risk patients with precancerous lesions, and the results will provide a basis for targeted prevention in a high-incidence area of esophageal carcinoma.

Zhang H, Li H, Ma Q, Yang FY, Diao TY. Predicting malignant transformation of esophageal squamous cell lesions by combined biomarkers in an endoscopic screening program. *World J Gastroenterol* 2016; 22(39): 8770-8778 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v22/i39/8770.htm> DOI: <http://dx.doi.org/10.3748/wjg.v22.i39.8770>

INTRODUCTION

Esophageal squamous cell carcinoma (ESCC) is one

of the most lethal malignancies^[1]. Due to the lack of effective clinical methods for early detection, most patients are at an advanced stage at the time of diagnosis^[2-4]. ESCC is the fourth most common cause of cancer death in China^[5,6].

The pathogenesis of ESCC involves a stepwise progression from basal cell hyperplasia (BCH) to low-grade dysplasia (LGD), high-grade dysplasia (HGD), carcinoma *in situ*, and finally invasive carcinoma. Esophageal dysplasia is a precancerous lesion^[7-11], but the development from dysplasia to carcinoma is by no means inevitable^[12]. Long-term epidemiological studies have indicated that severe-dysplasia is related to the risk of ESCC development, but LGD transforms into HGD only in a small number of patients, and can revert to normal mucosa or does not transform into malignant lesions in the majority of patients^[13-16].

Endoscopic screening is effective in early esophageal cancer and to ascertain the stages of esophageal carcinogenesis^[17-21]. Current management guidelines promote endoscopic screening in individuals with esophageal dysplasia, thus it is very important to identify biomarkers to screen high-risk subjects who should undergo endoscopic examination.

The accumulation of p53 protein appears in the very early stage of esophageal carcinogenesis and culminates in malignant transformation, and increased p53 expression observed on immunostaining is associated with a higher risk of histological progression^[22-27]. Carcinoembryonic antigen (CEA) is normally produced during fetal development and is used as a classic tumor marker^[28,29]. Carbohydrate antigen sialyl Lewis a (CA19-9) is associated with cancers of the colon, stomach, pancreas and bile duct, but it is also associated with noncancerous conditions^[30-33]. As far as we know there are few studies on CEA and CA19-9 protein expression in biopsy tissue from precancerous lesions of the esophagus in a high-incidence area.

Therefore, we determined whether CEA and CA19-9 protein expression is also related to the early stage of esophageal carcinogenesis transition, whether there are associations between these three biomarkers, and whether the combined expression of these biomarkers may lead to a more thorough understanding of the evolving process of ESCC. The aim was to prove a relationship between the three biomarkers and the stages in the transition to esophageal carcinoma, and to evaluate the combination of these protein biomarkers in predicting the malignant transition of LGD and HGD in an endoscopic screening program.

MATERIALS AND METHODS

Subjects

An iodine staining endoscopic screening program of esophageal lesions in the high-incidence area of Feicheng County was carried out from January 2004 to December 2007. A questionnaire was used to interview all subjects to obtain basic information. The endoscopic

screening test was performed in a small mobile car in villages. For persons with a non-staining area of the mucosa, random 4-quadrant biopsy specimens were obtained. Histopathologic diagnosis was carried out by two pathologists independently. The ethics committee of Shandong Academy of Medical Sciences approved the study protocol and all participants gave their written informed consent.

In this study, subjects with liver diseases and cardiovascular diseases were excluded. Immunohistochemical analysis was performed to determine the expression of p53, CEA and CA19-9 in histological sections of endoscopic biopsies from 603 persons who had free iodine staining regions of the esophagus. Based on pathological diagnosis of the biopsies, BCH was diagnosed in 77 persons, LGD in 247 (mild dysplasia in 167 and moderate dysplasia in 80), HGD in 51 (severe dysplasia in 35 and carcinoma *in situ* in 16), and early invasive carcinoma in 134. Eighty persons had no abnormal lesions and acted as normal controls. The data of 14 persons were excluded from the analysis due to failure of the immunohistochemical test (5 for p53, 4 for CEA and 5 for CA19-9).

Immunohistochemistry

The immunohistochemical detection of p53, CEA and CA19-9 proteins was performed using enzyme-linked immunosorbent assay (ELISA) kits (Zymed Laboratories, Inc., South San Francisco, CA, United States).

Reviewing and scoring of the sections

The stained sections were reviewed and scored independently by two investigators using an Olympus microscope. Sections stained for p53, CEA and CA19-9 protein expression were scored from 0 to 4: 0, negative (no staining); 1, weakly positive (positive cells were $\leq 10\%$); 2, positive (positive cells were $> 10\%$ but $\leq 25\%$); 3, strongly positive (positive cells were $> 25\%$ but $\leq 50\%$); and 4, very strongly positive (positive cells were $> 50\%$). In the multinomial logistic model analysis, each biomarker was used as a dependent variable and we defined 0 as negative and 1 as positive in order to avoid a zero number in one or more groups.

Statistical analysis

The χ^2 and Kruskal-Wallis H tests were used in the univariate analysis. The Spearman correlation test was performed to determine the association between the three biomarkers. Odds ratios (ORs) were calculated in the multinomial logistic model analysis. Receiver operating characteristic (ROC) curve analysis was used to discriminate the sensitivity and specificity between each lesion group and the normal control group for positive expression of the three biomarkers. All statistical analyses were performed using SPSS (version 17.0), and $P < 0.05$ (two-sided) was accepted

as statistically significant.

RESULTS

p53, CEA and CA19-9 protein expression

The univariate analysis indicated that there were significant differences in age, school year, income per year-person, alcohol drinking, and smoking among the five groups, therefore these five variables were adjusted in the multinomial logistic analysis as potential confounding factors. Table 1 shows the characteristics of the variables in the five groups.

The positive rates of p53 protein expression in the ESCC, HGD, LGD, BCH and normal control groups were 53.0%, 52.9%, 35.6%, 27.3% and 20.0%, respectively; the positive rates of CA19-9 protein expression were 44.0%, 33.3%, 16.5%, 9.2% and 6.2%, respectively; the positive rates of CEA protein expression were 74.6%, 60.8%, 23.3%, 23.7% and 16.2%, respectively (Table 2). The differences in the expression of the three biomarkers in the five groups were significant, and there were also significant linear trends in the increase in positive ratios with the transformation from normal to carcinoma.

The positive rates of the combined expression of the three biomarkers (if the expression of any one of the three biomarkers was positive, the combined expression was counted as positive; if there was an overlap in expression, the score of the strongest expression was taken as the score of the combination) were 84.3%, 76.5%, 47.6%, 42.9% and 27.5% in the five groups, respectively.

Correlations between the three biomarkers and their correlations with other factors

The correlation coefficients of p53 and CA19-9, p53 and CEA, and CA19-9 and CEA were 0.325, 0.374 and 0.503, respectively (all $P < 0.01$). The correlation coefficients of p53, CA19-9 and CEA with the degree of esophageal lesions were 0.287, 0.326 and 0.455, respectively (all $P < 0.01$). Both p53 and CEA, but not CA 19-9, had significant correlations with age (both $P < 0.05$). The three biomarkers had no significant correlations with school year, alcohol drinking index, smoking index and family history of esophageal cancer (Table 3).

Relationships of the three biomarkers with the stages of transformation from basal squamous cell hyperplasia to invasive carcinoma

The normal control group was regarded as the baseline (OR = 1.0), and both the ORs and 95% CIs of the three biomarkers in the other four groups were calculated using multinomial logistic models. The positive protein expression of p53, CEA and CA19-9 was significantly associated with the four esophageal lesions. As shown in Table 4, almost all ORs (95%CIs) of p53, CEA and CA19-9 increased with the stages

Table 1 Distribution of selected variables in the esophageal basal cell hyperplasia, low-grade dysplasia, high-grade dysplasia, esophageal squamous cell cancer and control groups¹ *n* (%)

Variables	ESCC	HGD	LGD	BCH	Normal control
Gender					
Male	89 (66.4)	32 (62.7)	146 (59.1)	48 (62.3)	40 (50.0)
Female	45 (3.6)	19 (37.3)	101 (40.9)	29 (37.7)	40 (50.0)
Age (yr)					
40-50	52 (38.8)	16 (31.4)	60 (24.3)	20 (26.0)	7 (8.8)
50-60	60 (44.8)	25 (49.0)	128 (51.8)	34 (44.2)	29 (36.3)
≥ 60	22 (16.4)	10 (19.6)	59 (23.9)	23 (29.9)	44 (55.0)
School year (yr)					
≤ 6	79 (59.0)	20 (39.3)	105 (42.5)	32 (41.6)	23 (28.8)
7-11	50 (37.3)	22 (43.1)	98 (39.7)	30 (39.0)	36 (45.0)
≥ 12	5 (3.7)	9 (17.6)	44 (17.8)	15 (19.5)	21 (26.3)
Income per year-person (\$)					
< 150	60 (44.8)	22 (43.1)	101 (40.9)	26 (33.8)	8 (10.0)
150-350	56 (41.8)	17 (33.3)	92 (37.2)	24 (31.2)	21 (26.3)
≥ 350	18 (13.4)	12 (23.5)	54 (21.9)	27 (35.1)	51 (63.8)
Family history of esophageal cancer					
Yes	19 (14.2)	12 (23.5)	50 (20.2)	16 (20.8)	8 (10.0)
No	115 (85.8)	39 (76.5)	197 (79.8)	61 (79.2)	72 (90.0)
Smoking index ²					
≥ 450	52 (38.8)	23 (45.1)	69 (27.8)	31 (40.0)	14 (17.7)
< 450	25 (18.7)	4 (7.8)	40 (16.3)	12 (16.0)	16 (20.3)
None	57 (42.5)	24 (47.1)	138 (55.9)	34 (44.0)	49 (62.0)
Alcohol drinking index ³					
≥ 120	56 (42.0)	16 (31.4)	77 (31.0)	22 (28.0)	6 (7.5)
< 120	28 (20.6)	11 (21.6)	44 (18.0)	17 (22.7)	29 (36.3)
None	50 (37.4)	24 (47.1)	126 (51.0)	38 (49.3)	45 (56.3)

¹In the five groups, χ^2 test values for gender, age, school year, family history of esophageal cancer, income per year-person, smoking index, and alcohol drinking index were 6.046 ($P = 0.196$), 52.858 ($P < 0.01$), 45.436 ($P < 0.01$), 76.476 ($P < 0.01$), 7.137 ($P = 0.129$), 21.682 ($P = 0.006$), and 34.085 ($P < 0.01$), respectively; ²Smoking index = cigarette/day \times number of smoking years; ³Alcohol drinking ≥ 120 g/d represents heavy drinking. ESCC: Esophageal squamous cell cancer; HGD: High-grade dysplasia; LGD: Low-grade dysplasia; BCH: Basal cell hyperplasia.

of transition. The strongest relationship was seen in the ESCC group. BCH had no significant association with the positive protein expression of the three biomarkers. However, the combination of the three biomarkers had significant relationships with all four esophageal lesions.

ROC curves and the possibility of predicting the malignant development of the esophageal lesions based on the positive expression of the three biomarkers

In the ROC curve analysis, we used a single biomarker and the combination of the three biomarkers to predict the risk of the four esophageal lesions. As shown in Table 5, the areas of ROC curves for the combination of the three biomarkers were 0.837, 0.740, 0.590 and 0.562 in the ESCC, HGD, LGD and BCH groups, respectively. Statistical significance was found in the ESCC, HGD and LGD groups, but not in the BCH group.

When the positive expression score of 10%-25% was taken as the cut-off value, the sensitivities and specificities of the four ROC curves were determined and are shown in Table 6 and Figure 1. In the ROC curves of the combination of the three biomarkers, the specificity was 88.8% for the normal control group, the sensitivity was 58.2% for the ESCC group, 25.5% for the HGD group, 11.2% for the LGD group, and 6.5%

for the BCH group.

DISCUSSION

Wild-type p53 suppresses cell proliferation in normal tissues^[34], and p53 overexpression is regarded as a potential tumor prognostic factor^[35-39]. Increased p53 expression in the pathogenesis of ESCC or adenocarcinoma indicates that p53 overexpression is involved in the initial steps of esophageal carcinogenesis and contributes to the development of precancerous lesions. van Dekken *et al.*^[40] studied histologic sections of endoscopic biopsies from patients with Barrett's esophagus, and found a significant trend for p53 protein overexpression during malignant progression. Kim *et al.*^[23] reported that positively stained p53 protein was observed in 87% of ESCC, 80% of esophageal dysplasia, and was not observed in normal mucosa. Bellini *et al.*^[26] reported that positive p53 immunohistochemistry progressively increased with pathology severity: Chagas disease (7.7%), chagasic megaesophagus (26.1%), chronic esophagitis (52.2%) and ESCC (100%). We also found that p53 expression showed a significant linear trend with the transition from normal to cancer.

An increase in CEA or CA19-9 level is associated with a more advanced tumor stage^[32,41], and their

Table 2 Distribution of p53, CA19-9 and carcinoembryonic antigen expression in the five groups¹ *n* (%)

Protein expression	ESCC	HGD	LGD	BCH	Normal control
p53					
Negative	63 (47.0)	24 (47.1)	159 (64.4)	56 (72.7)	64 (80.0)
Positive					
1%-10%	18 (13.4)	17 (33.3)	62 (25.1)	17 (22.1)	11 (13.8)
10%-25%	20 (14.9)	8 (15.7)	20 (8.1)	3 (3.9)	5 (6.3)
25%-50%	24 (17.9)	2 (3.9)	6 (2.4)	1 (1.3)	0 (0)
≥ 50%	9 (6.8)	0 (0)	0 (0)	0 (0)	0 (0)
CA19-9					
Negative	75 (56.0)	34 (66.7)	206 (83.5)	70 (90.8)	75 (93.8)
Positive					
1%-10%	43 (32.1)	15 (29.4)	40 (16.1)	6 (7.9)	4 (5.0)
10%-25%	13 (9.7)	1 (2.0)	1 (0.4)	0 (0)	1 (1.3)
≥ 25%	3 (2.2)	1 (2.0)	0 (0)	1 (1.3)	0 (0)
CEA					
Negative	34 (25.4)	20 (39.2)	189 (76.7)	59 (76.3)	67 (83.8)
Positive					
1%-10%	47 (35.1)	27 (52.9)	56 (22.5)	16 (21.1)	7 (8.8)
10%-25%	31 (23.2)	4 (7.8)	2 (0.8)	1 (1.3)	6 (7.5)
25%-50%	19 (14.2)	0 (0)	0 (0)	1 (1.3)	0 (0)
≥ 50%	3 (2.2)	0 (0)	0 (0)	0 (0)	0 (0)
Three biomarkers combined ²					
Negative	21 (15.7)	12 (23.5)	129 (52.4)	44 (57.1)	58 (72.5)
Positive					
1%-10%	35 (26.1)	26 (51.0)	90 (36.4)	28 (36.4)	13 (16.3)
10%-25%	30 (22.4)	10 (19.6)	22 (8.8)	4 (5.2)	9 (11.3)
25%-50%	36 (26.8)	3 (5.9)	6 (2.4)	1 (1.3)	0 (0)
≥ 50%	12 (9.0)	0 (0)	0 (0)	0 (0)	0 (0)

¹In the five groups, the Kruskal-Wallis *H* test values of the differences in positive protein expression ratios of p53, CA19-9, CEA, and the three biomarkers combined were 50.279 ($P < 0.001$), 68.660 ($P < 0.001$), 160.453 ($P < 0.001$), and 142.393 ($P < 0.001$), respectively. The values of the linear-by-linear association test were 68.737 ($P < 0.001$), 61.076 ($P < 0.001$), 128.738 ($P < 0.001$), and 141.591 ($P < 0.001$), respectively; ²If the expression of any one of the three biomarkers was positive, the combined expression was counted as positive; if there was an overlap in expression, the score of the strongest expression was taken as the score of the combination. ESCC: Esophageal squamous cell cancer; HGD: High-grade dysplasia; LGD: Low-grade dysplasia; BCH: Basal cell hyperplasia.

Table 3 The correlation coefficients for p53, CA19-9, CEA and selected variables

Variables	Spearman's correlation coefficient		
	p53	CA19-9	CEA
Age	-0.081 ¹	-0.065	-0.093 ¹
School year	0.197	0.139	0.213
Alcohol drinking index	-0.050	-0.056	-0.021
Smoking index	-0.038	-0.050	-0.003
Family history of esophageal cancer	0.020	-0.078	-0.035
Degree of esophageal lesions ³	0.287 ²	0.326 ²	0.455 ²
p53	1	0.325 ²	0.374 ²
CA19-9		1.000	0.503 ²
CEA			1.000

¹Correlation is significant at the 0.05 level (2-tailed); ²Correlation is significant at the 0.01 level (2-tailed); ³The variable definitions for the degree of esophageal lesions were: 1, normal control; 2, basal cell hyperplasia; 3, low-grade dysplasia; 4, high-grade dysplasia; 5, invasive squamous cell cancer.

combination may provide more information for diagnosis and prognosis. Bagaria *et al*^[42] reported that with the specificity set at 100%, the sensitivity for esophageal cancer was 28%, 18% and 42% for CEA, CA19-9 and their combination, respectively.

Scarpa *et al*^[43] reported that the two biomarkers should be considered when evaluating candidates for esophagectomy.

Setoyama *et al*^[44] found that CEA mRNA expression was positively related to tumor depth and lymph node metastasis in ESCC patients. In another study, CEA mRNA was expressed in the blood, even though CEA and CA19-9 were normal in patients with relapse^[45]. From these studies, we suggest that the serum level of CEA or CA19-9 protein expression in patients with early stages of carcinoma may be too low to be detected. In contrast, CEA or CA19-9 protein expression in biopsies from patients with the initial stages of esophageal carcinogenesis can easily be detected by immunohistochemistry. In the present study, the main finding was that the positive expression of CEA and CA19-9 proteins increased with the severity of BCH, dysplasia and carcinoma of the esophagus. These results are useful in understanding the mechanism of the evolution of esophageal cancer at the molecular level of protein expression.

Positive protein expression of p53, CEA and CA19-9 was associated with esophageal carcinogenesis, and a moderate association was found between the three biomarkers in the present study. Interestingly, we discovered that when the positive expressions of the

Table 4 The associations of the positive expression of p53, CA19-9 and CEA proteins with basal cell hyperplasia, low-grade dysplasia, high-grade dysplasia, and esophageal squamous cell cancer [OR (95%CI)]

Variables	ESCC	HGD	LGD	BCH
p53				
Model 1 ¹	4.51 (2.38-8.59)	4.50 (2.07-9.78)	2.21 (1.21-4.06)	1.50 (0.71-3.15)
Model 2 ²	4.52 (2.17-4.92)	4.88 (2.10-11.33)	2.31 (1.17-4.56)	1.60 (0.72-3.57)
CA19-9				
Model 1 ¹	15.15 (7.45-30.84)	7.99 (3.53-18.10)	1.57 (0.81-3.04)	1.60 (0.72-3.54)
Model 2 ²	23.57 (9.27-56.86)	12.01 (4.61-31.32)	2.05 (0.91-4.63)	1.99 (0.81-4.93)
CEA				
Model 1 ¹	11.80 (4.49-31.05)	7.50 (2.56-22.00)	2.96 (1.13-7.76)	1.52 (0.46-5.02)
Model 2 ²	17.40 (4.76-63.59)	11.06 (2.80-43.71)	3.80 (1.06-13.66)	1.81 (0.41-7.96)
Three biomarkers combined ³				
Model 1 ¹	14.19 (7.21-27.91)	8.57 (3.80-19.30)	2.40 (1.38-4.15)	1.98 (1.02-3.85)
Model 2 ²	16.64 (7.68-36.05)	10.11 (4.20-24.32)	2.59 (1.38-4.86)	2.15 (1.04-4.45)

¹In the regression model, ORs were calculated without adjustments for any variables; ²In the regression model, ORs were calculated after adjustment for age, school year, income per year-person, smoking, alcohol drinking, and family history of esophageal cancer; ³If the expression of any one of the three biomarkers was positive, the combined expression was counted as positive; if there was an overlap in expression, the score of the strongest expression was taken as the score of the combination. ESCC: Esophageal squamous cell cancer; HGD: High-grade dysplasia; LGD: Low-grade dysplasia; BCH: Basal cell hyperplasia.

Table 5 Area of receiver operating characteristic curves for p53, CA19-9, CEA and the three biomarkers combined in the four esophageal lesions

Protein expression	Area	SE	95%CI	Z value	P value
p53					
ESCC	0.696	0.0357	0.629-0.756	5.483	0.001
HGD	0.669	0.0495	0.581-0.749	3.416	0.006
LGD	0.578	0.0356	0.523-0.632	2.197	0.028
BCH	0.533	0.0461	0.452-0.613	0.725	0.468
CA19-9					
ESCC	0.650	0.0359	0.625-0.751	5.288	< 0.001
HGD	0.638	0.0509	0.549-0.720	2.714	0.007
LGD	0.554	0.0363	0.498-0.609	1.484	0.138
BCH	0.515	0.0464	0.433-0.595	0.317	0.751
CEA					
ESCC	0.802	0.0293	0.742-0.853	10.302	< 0.001
HGD	0.712	0.0479	0.260-0.788	4.423	< 0.001
LGD	0.532	0.0369	0.476-0.558	0.872	0.383
BCH	0.531	0.0463	0.449-0.611	0.668	0.504
Three biomarkers combined ¹					
ESCC	0.837	0.0260	0.780-0.884	212.656	< 0.001
HGD	0.740	0.0460	0.656-0.813	5.220	< 0.001
LGD	0.590	0.0352	0.535-0.644	2.586	0.010
BCH	0.562	0.0458	0.0481-0.641	1.360	0.174

¹If the expression of any one of the three biomarkers was positive, the combined expression was counted as positive; if there was an overlap in expression, the score of the strongest expression was taken as the score of the combination. ESCC: Esophageal squamous cell cancer; HGD: High-grade dysplasia; LGD: Low-grade dysplasia; BCH: Basal cell hyperplasia.

three biomarkers were combined, the specificity for diagnosis of esophageal lesions was 88.8%, which was the target level for screening high-risk individuals, and the sensitivities markedly increased with severity of the esophageal lesions.

However, the identification of patients with dysplasia who will then develop malignant lesions is very important. It is known that p53, CA19-9 and CEA proteins are secreted by malignant cells, and the simultaneous positive expression of these biomarkers in benign pathological lesions of the esophagus

may possibly indicate that these lesions will become malignant. Our results indicate that an endoscopic screening program to detect these three biomarkers is beneficial to identify high-risk individuals with esophageal diseases. This information will be useful for doctors to plan the follow-up interval for endoscopic biopsy surveillance, and to decide on appropriate treatment.

It is difficult to obtain a biopsy from the free iodine staining area exactly at the site of the lesion, thus there may be some misclassification of diagnosis, and

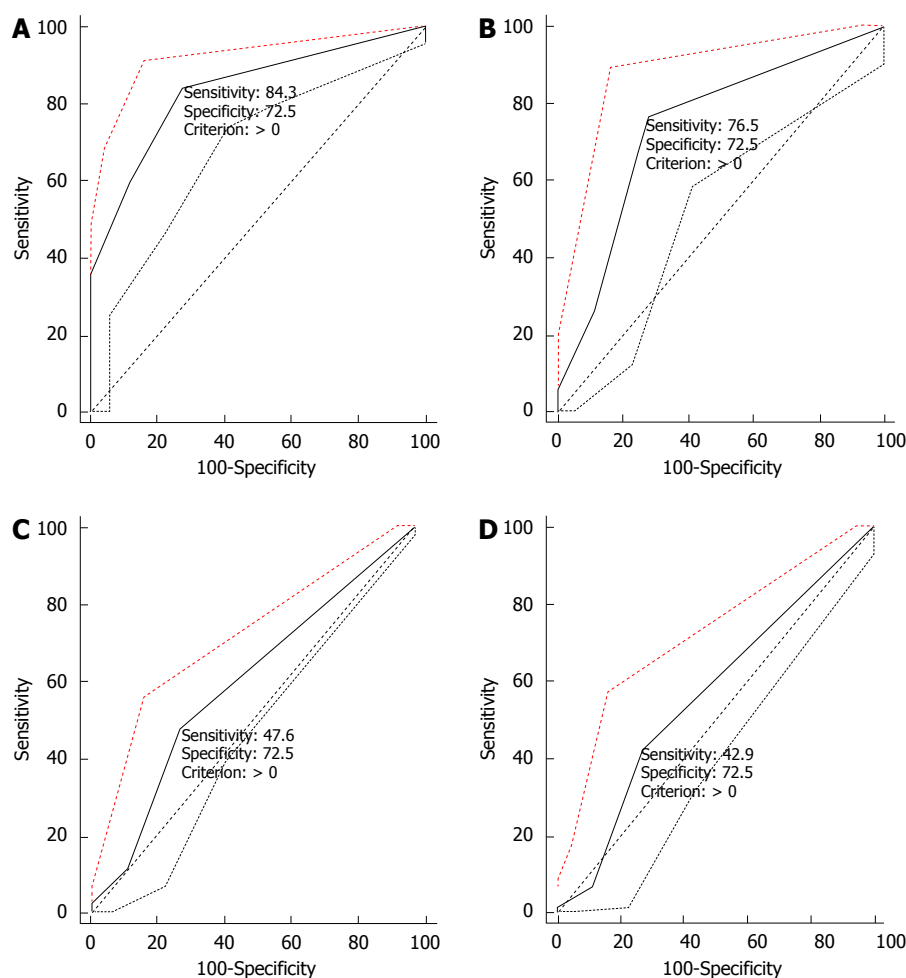


Figure 1 Curves for predicting esophageal lesions based on positive expression of the three biomarkers. These ROC curves are for ESCC (A), HGD (B), LGD (C), and BCH (D), respectively. Note: If the expression of any one of the three biomarkers was positive, the combined expression was counted as positive; if there was an overlap in expression, the score of the strongest expression was taken as the score of the combination. ESCC: Esophageal squamous cell cancer; HGD: High-grade dysplasia; LGD: Low-grade dysplasia; BCH: Basal cell hyperplasia; ROC: Receiver operating characteristic curve.

Table 6 Diagnostic values of predicting the four esophageal lesions based on the positive expression of the three biomarkers combined at the cut off value of 10%-25%¹ (%)

Group	Sensitivity	95%CI	+ PV	95%CI
ESCC	58.2	49.4-66.7	89.7	81.3-95.1
HGD	25.5	14.3-39.6	59.1	36.4-79.3
LGD	11.2	7.6-15.8	75.7	58.8-88.2
BCH	6.5	2.2-14.5	35.7	12.9-64.8

¹If the expression of any one of the three biomarkers was positive, the combined expression was counted as positive; if there was an overlap in expression, the score of the strongest expression was taken as the score of the combination. The specificity was 88.8% for the normal control group, with a 95%CI of 79.7%-94.7%. ESCC: Esophageal squamous cell cancer; HGD: High-grade dysplasia; LGD: Low-grade dysplasia; BCH: Basal cell hyperplasia.

the controls may not have normal esophageal mucosa. Therefore, the expression rates of the three biomarkers were much higher in the controls. It is possible that the predictive values of the three biomarkers for the stages of ESCC may have been underestimated.

In addition, the expression of other biomarkers, such as p21^[46], Ki-67, ProExC^[47] and cyclin D1^[48], is associated with ESCC, thus the relatively low sensitivity of the three biomarkers combined in the present study may have been influenced by other mechanisms involved in ESCC carcinogenesis.

ACKNOWLEDGMENTS

We appreciate Dr. Guo-Qing Wang and Gui-Qi Wang of the Cancer Institute and Hospital, Chinese Academy of Medical Science for guidance on the endoscopic screening of esophageal diseases in Feicheng County.

COMMENTS

Background

Esophageal squamous cell carcinoma (ESCC) is one of the most lethal malignancies, and the pathogenesis involves a stepwise progression from basal cell hyperplasia (BCH) to low-grade dysplasia (LGD), high-grade dysplasia (HGD), carcinoma *in situ*, and finally invasive carcinoma. It is very important to identify biomarkers to screen high-risk subjects.

Research frontiers

The accumulation of p53 protein appears in the very early stage of esophageal carcinogenesis and culminates in malignant transformation. There are few studies on carcinoembryonic antigen (CEA) and CA19-9 protein expression in biopsy tissue from precancerous lesions of the esophagus in a high-incidence area. Therefore, the authors determined whether the combined expression of these three biomarkers would lead to a more thorough understanding of the development of ESCC.

Innovations and breakthroughs

The positive protein expression of p53, CEA and CA19-9 was associated with esophageal carcinogenesis. When the positive expressions of the three biomarkers were combined, the specificity achieved the target level for screening high-risk individuals, and the sensitivities markedly increased with the severity of esophageal lesions.

Applications

The results of this study indicate that an endoscopic screening program to determine these three biomarkers is beneficial to identify high-risk individuals with esophageal diseases. Information on the expression of these three biomarkers will be useful for doctors to plan the follow-up interval for endoscopic biopsy surveillance, and to decide on appropriate treatment.

Terminology

CA19-9 is a high-molecular-mass mucin glycoprotein complex and is used as a tumor marker for cancers of the colon, stomach, pancreas and bile duct.

Peer-review

The authors revealed that the combined assessment of p53, CEA and CA19-9 could predict the malignant potential of esophageal lesions. The concept of the study is clinically relevant.

REFERENCES

- 1 Taylor PR, Abnet CC, Dawsey SM. Squamous dysplasia--the precursor lesion for esophageal squamous cell carcinoma. *Cancer Epidemiol Biomarkers Prev* 2013; **22**: 540-552 [PMID: 23549398 DOI: 10.1158/1055-9965.EPI-12-1347]
- 2 Shimada H, Nabeya Y, Okazumi S, Matsubara H, Shiratori T, Gunji Y, Kobayashi S, Hayashi H, Ochiai T. Prediction of survival with squamous cell carcinoma antigen in patients with resectable esophageal squamous cell carcinoma. *Surgery* 2003; **133**: 486-494 [PMID: 12773976]
- 3 Roshandel G, Khoshnia M, Sotoudeh M, Merat S, Etemadi A, Nickmanesh A, Norouzi A, Pourshams A, Poustchi H, Semnani S, Ghasemi-Kebria F, Noorbakhsh R, Abnet C, Dawsey SM, Malekzadeh R. Endoscopic screening for precancerous lesions of the esophagus in a high risk area in Northern Iran. *Arch Iran Med* 2014; **17**: 246-252 [PMID: 24724600]
- 4 Chen JW, Xie JD, Ling YH, Li P, Yan SM, Xi SY, Luo RZ, Yun JP, Xie D, Cai MY. The prognostic effect of perineural invasion in esophageal squamous cell carcinoma. *BMC Cancer* 2014; **14**: 313 [PMID: 24886020 DOI: 10.1186/1471-2407-14-313]
- 5 Li LD, Lu FZ, Zhang SW. Analysis of cancer mortality rates and distribution in China, 1990-92. *Zhonghua Zhongliu Zazhi* 1996; **18**: 403-407
- 6 Daly JM, Karnell LH, Menck HR. National Cancer Data Base report on esophageal carcinoma. *Cancer* 1996; **78**: 1820-1828 [PMID: 8859198]
- 7 Shimizu M, Ban S, Odze RD. Squamous dysplasia and other precursor lesions related to esophageal squamous cell carcinoma. *Gastroenterol Clin North Am* 2007; **36**: 797-811, v-vi [PMID: 17996791]
- 8 Kuwano H, Watanabe M, Sadanaga N, Ikebe M, Mori M, Sugimachi K. Squamous epithelial dysplasia associated with squamous cell carcinoma of the esophagus. *Cancer Lett* 1993; **72**: 141-147 [PMID: 8402583]
- 9 Schlemper RJ, Dawsey SM, Itabashi M, Iwashita A, Kato Y, Koike M, Lewin KJ, Riddell RH, Shimoda T, Sipponen P, Stolte M, Watanabe H. Differences in diagnostic criteria for esophageal squamous cell carcinoma between Japanese and Western pathologists. *Cancer* 2000; **88**: 996-1006 [PMID: 10699887]
- 10 Rubio CA, Liu FS, Zhao HZ. Histological classification of intraepithelial neoplasias and microinvasive squamous carcinoma of the esophagus. *Am J Surg Pathol* 1989; **13**: 685-690 [PMID: 2751041]
- 11 Li QD, Li H, Wang MS, Diao TY, Zhou ZY, Fang QX, Yang FY, Li QH. Multi-susceptibility genes associated with the risk of the development stages of esophageal squamous cell cancer in Feicheng County. *BMC Gastroenterol* 2011; **11**: 74 [PMID: 21672255 DOI: 10.1186/1471-230X-11-74]
- 12 Qiu SL, Yang GR. Precursor lesions of esophageal cancer in high-risk populations in Henan Province, China. *Cancer* 1988; **62**: 551-557 [PMID: 3390795]
- 13 Wang LD, Yang HH, Fan ZM, Lü XD, Wang JK, Liu XL, Sun Z, Jiang YN, He X, Zhou Q. Cytological screening and 15 years' follow-up (1986-2001) for early esophageal squamous cell carcinoma and precancerous lesions in a high-risk population in Anyang County, Henan Province, Northern China. *Cancer Detect Prev* 2005; **29**: 317-322 [PMID: 16118042]
- 14 Dawsey SM, Lewin KJ, Wang GQ, Liu FS, Nieberg RK, Yu Y, Li JY, Blot WJ, Li B, Taylor PR. Squamous esophageal histology and subsequent risk of squamous cell carcinoma of the esophagus. A prospective follow-up study from Linxian, China. *Cancer* 1994; **74**: 1686-1692 [PMID: 8082069]
- 15 Wang GQ. Clinical preventive strategies to decrease incidence and death rates of esophageal cancer in high-risk areas. *Linchuang Zhongliuxue Zazhi* 1999; **21**: 223 (in Chinese)
- 16 Wang LD, Zhou Q, Feng CW, Liu B, Qi YJ, Zhang YR, Gao SS, Fan ZM, Zhou Y, Yang CS, Wei JP, Zheng S. Intervention and follow-up on human esophageal precancerous lesions in Henan, northern China, a high-incidence area for esophageal cancer. *Gan To Kagaku Ryoho* 2002; **29** Suppl 1: 159-172 [PMID: 11890101]
- 17 Ishihara R, Takeuchi Y, Chatani R, Kidu T, Inoue T, Hanaoka N, Yamamoto S, Higashino K, Uedo N, Iishi H, Tatsuta M, Tomita Y, Ishiguro S. Prospective evaluation of narrow-band imaging endoscopy for screening of esophageal squamous mucosal high-grade neoplasia in experienced and less experienced endoscopists. *Dis Esophagus* 2010; **23**: 480-486 [PMID: 20095991 DOI: 10.1111/j.1442-2050.2009.01039.x]
- 18 Domper Arnal MJ, Ferrández Arenas Á, Lanás Arbeloa Á. Esophageal cancer: Risk factors, screening and endoscopic treatment in Western and Eastern countries. *World J Gastroenterol* 2015; **21**: 7933-7943 [PMID: 26185366 DOI: 10.3748/wjg.v21.i26.7933]
- 19 Yang J, Wei WQ, Niu J, Liu ZC, Yang CX, Qiao YL. Cost-benefit analysis of esophageal cancer endoscopic screening in high-risk areas of China. *World J Gastroenterol* 2012; **18**: 2493-2501 [PMID: 22654446 DOI: 10.3748/wjg.v18.i20.2493]
- 20 Roshandel G, Nourouzi A, Pourshams A, Semnani S, Merat S, Khoshnia M. Endoscopic screening for esophageal squamous cell carcinoma. *Arch Iran Med* 2013; **16**: 351-357 [PMID: 23725069]
- 21 Wang GQ, Liu YY, Hao CQ, Lai SQ, Wang GQ, Lu N, Yang L. A comparative study of endoscopic image stained by iodine and histopathology in early esophageal cancer and precancerous lesions (dysplasia). *Zhonghua Zhongliu Zazhi* 2004; **26**: 342-344 [PMID: 15312343]
- 22 Bennett WP, Hollstein MC, He A, Zhu SM, Resau JH, Trump BF, Metcalf RA, Welsh JA, Midgley C, Lane DP. Archival analysis of p53 genetic and protein alterations in Chinese esophageal cancer. *Oncogene* 1991; **6**: 1779-1784 [PMID: 1923503]
- 23 Kim SG, Hong SJ, Kwon KW, Jung SW, Kim WY, Jung IS, Ko BM, Ryu CB, Kim YS, Moon JH, Kim JO, Cho JY, Lee JS, Lee MS, Shim CS, Kim BS. The expression of p53, p16, cyclin D1 in esophageal squamous cell carcinoma and esophageal dysplasia. *Korean J Gastroenterol* 2006; **48**: 269-276 [PMID: 17060721]
- 24 Lin DC, Du XL, Wang MR. Protein alterations in ESCC and

- clinical implications: a review. *Dis Esophagus* 2009; **22**: 9-20 [PMID: 18564170 DOI: 10.1111/j.1442-2050.2008.00845.x]
- 25 **Kerkhof M**, Steyerberg EW, Kusters JG, van Dekken H, van Vuuren AJ, Kuipers EJ, Siersema PD. Aneuploidy and high expression of p53 and Ki67 is associated with neoplastic progression in Barrett esophagus. *Cancer Biomark* 2008; **4**: 1-10 [PMID: 18334729]
 - 26 **Bellini MF**, Leite KR, Cury PM, Silva AE. p53, p16 and p115 proteins expressions in chronic esophagitis and Chagas disease. *Anticancer Res* 2008; **28**: 3793-3799 [PMID: 19189666]
 - 27 **Alcolea MP**, Greulich P, Wabik A, Frede J, Simons BD, Jones PH. Differentiation imbalance in single oesophageal progenitor cells causes clonal immortalization and field change. *Nat Cell Biol* 2014; **16**: 615-622 [PMID: 24814514 DOI: 10.1038/ncb2963]
 - 28 **Lukaszewicz-Zajac M**, Mroczko B, Kozłowski M, Nikliński J, Ludański J, Siewko M, Szmitkowski M. Comparative evaluation of serum C-reactive protein (CRP) levels in the different histological subtypes of esophageal cancer (squamous cell carcinoma and adenocarcinoma of esophagus). *J Clin Lab Anal* 2012; **26**: 73-81 [PMID: 22467322 DOI: 10.1002/jcla.21486]
 - 29 **Sanders DS**, Kerr MA. Lewis blood group and CEA related antigens; coexpressed cell-cell adhesion molecules with roles in the biological progression and dissemination of tumours. *Mol Pathol* 1999; **52**: 174-178 [PMID: 10694936]
 - 30 **O'Brien DP**, Sandanayake NS, Jenkinson C, Gentry-Maharaj A, Apostolidou S, Fourkala EO, Camuzeaux S, Blyuss O, Gunu R, Dawney A, Zaikin A, Smith RC, Jacobs IJ, Menon U, Costello E, Pereira SP, Timms JF. Serum CA19-9 is significantly upregulated up to 2 years before diagnosis with pancreatic cancer: implications for early disease detection. *Clin Cancer Res* 2015; **21**: 622-631 [PMID: 24938522 DOI: 10.1158/1078-0432.CCR-14-0365]
 - 31 **Yu J**, Zhang S, Zhao B. Differences and correlation of serum CEA, CA19-9 and CA72-4 in gastric cancer. *Mol Clin Oncol* 2016; **4**: 441-449 [PMID: 26998301 DOI: 10.3892/mco.2015.712]
 - 32 **Grotowski M**. Antigens (CEA and CA 19-9) in diagnosis and prognosis colorectal cancer. *Pol Merkur Lekarski* 2002; **12**: 77-80 [PMID: 11957811]
 - 33 **Kannagi R**. Carbohydrate antigen sialyl Lewis a--its pathophysiological significance and induction mechanism in cancer progression. *Chang Gung Med J* 2007; **30**: 189-209 [PMID: 17760270]
 - 34 **Indinnimeo M**, Reale MG, Cicchini C, Stazi A, Fiori E, Izzo P. CEA, TPA, CA 19-9, SCC and CYFRA at diagnosis and in the follow-up of anal canal tumors. *Int Surg* 1997; **82**: 275-279 [PMID: 9372374]
 - 35 **Qian Y**, Chen X. Senescence regulation by the p53 protein family. *Methods Mol Biol* 2013; **965**: 37-61 [PMID: 23296650 DOI: 10.1007/978-1-62703-239-1_3]
 - 36 **Kandioler D**, Schoppmann SF, Zwrtek R, Kappel S, Wolf B, Mittlböck M, Kührer I, Hejna M, Pluschnig U, Ba-Ssalamah A, Wrba F, Zacherl J. The biomarker TP53 divides patients with neoadjuvantly treated esophageal cancer into 2 subgroups with markedly different outcomes. A p53 Research Group study. *J Thorac Cardiovasc Surg* 2014; **148**: 2280-2286 [PMID: 25135238 DOI: 10.1016/j.jtcvs.2014.06.079]
 - 37 **Yokoyama A**, Tanaka Y, Yokoyama T, Mizukami T, Matsui T, Maruyama K, Omori T. p53 protein accumulation, iodine-unstained lesions, and alcohol dehydrogenase-1B and aldehyde dehydrogenase-2 genotypes in Japanese alcoholic men with esophageal dysplasia. *Cancer Lett* 2011; **308**: 112-117 [PMID: 21601984 DOI: 10.1016/j.canlet.2011.04.020]
 - 38 **Nicolopoulou-Stamati P**, Tsiplis A, Chelidonis G, Patsouris E, Athanassiadou P, Gonidi M, Athanassiadou AM. Prognostic value of COX-2, P53, and EZH-2 evaluated by quantitative image analysis in premalignant and malignant breast lesions. *Diagn Cytopathol* 2015; **43**: 294-300 [PMID: 25355039 DOI: 10.1002/dc.23217]
 - 39 **Apostolou G**, Apostolou N, Biteli M, Kavantzis N, Patsouris E, Athanassiadou P. Utility of Ki-67, p53, Bcl-2, and Cox-2 biomarkers for low-grade endometrial cancer and disordered proliferative/benign hyperplastic endometrium by imprint cytology. *Diagn Cytopathol* 2014; **42**: 134-142 [PMID: 23729350 DOI: 10.1002/dc.23010]
 - 40 **van Dekken H**, Hop WC, Tilanus HW, Haringsma J, van der Valk H, Wink JC, Vissers KJ. Immunohistochemical evaluation of a panel of tumor cell markers during malignant progression in Barrett esophagus. *Am J Clin Pathol* 2008; **130**: 745-753 [PMID: 18854267 DOI: 10.1309/AJCP031THGVEUIDH]
 - 41 **Sato H**, Usuda N, Kuroda M, Hashimoto S, Maruta M, Maeda K. Significance of serum concentrations of E-selectin and CA19-9 in the prognosis of colorectal cancer. *Jpn J Clin Oncol* 2010; **40**: 1073-1080 [DOI: 10.1093/jjco/hyq095]
 - 42 **Bagaria B**, Sood S, Sharma R, Lalwani S. Comparative study of CEA and CA19-9 in esophageal, gastric and colon cancers individually and in combination (ROC curve analysis). *Cancer Biol Med* 2013; **10**: 148-157 [PMID: 24379990 DOI: 10.7497/j.issn.2095-3941.2013.03.005]
 - 43 **Scarpa M**, Noaro G, Saadeh L, Cavallin F, Cagol M, Alfieri R, Plebani M, Castoro C. Esophageal cancer management: preoperative CA19.9 and CEA serum levels may identify occult advanced adenocarcinoma. *World J Surg* 2015; **39**: 424-432 [PMID: 25326423 DOI: 10.1007/s00268-014-2835-1]
 - 44 **Setoyama T**, Natsugoe S, Okumura H, Matsumoto M, Uchikado Y, Ishigami S, Owaki T, Takao S, Aikou T. Carcinoembryonic antigen messenger RNA expression in blood predicts recurrence in esophageal cancer. *Clin Cancer Res* 2006; **12**: 5972-5977 [PMID: 17062668]
 - 45 **Mataki Y**, Takao S, Maemura K, Mori S, Shinchi H, Natsugoe S, Aikou T. Carcinoembryonic antigen messenger RNA expression using nested reverse transcription-PCR in the peripheral blood during follow-up period of patients who underwent curative surgery for biliary-pancreatic cancer: longitudinal analyses. *Clin Cancer Res* 2004; **10**: 3807-3814 [PMID: 15173089]
 - 46 **Liu J**, Hu Y, Hu W, Xie X, Ela Bella A, Fu J, Rao D. Expression and prognostic relevance of p21WAF1 in stage III esophageal squamous cell carcinoma. *Dis Esophagus* 2012; **25**: 67-71 [PMID: 21689205 DOI: 10.1111/j.1442-2050.2011.01217.x]
 - 47 **Wang WC**, Wu TT, Chandan VS, Lohse CM, Zhang L. Ki-67 and ProExC are useful immunohistochemical markers in esophageal squamous intraepithelial neoplasia. *Hum Pathol* 2011; **42**: 1430-1437 [PMID: 21420715 DOI: 10.1016/j.humpath.2010.12.009]
 - 48 **Hussain S**, M Y, Thakur N, Salam I, Singh N, Mir MM, Bhat MA, Siddiqi MA, Das BC, Bharadwaj M. Association of cyclin D1 gene polymorphisms with risk of esophageal squamous cell carcinoma in Kashmir Valley: a high risk area. *Mol Carcinog* 2011; **50**: 487-498 [PMID: 21268129 DOI: 10.1002/mc.20732]

P- Reviewer: Garg P, Kuribayashi S, La Mazza A

S- Editor: Gong ZM L- Editor: Webster JR E- Editor: Wang CH



Retrospective Study

Macrophage colony-stimulating factor expressed in non-cancer tissues provides predictive powers for recurrence in hepatocellular carcinoma

Hiroshi Kono, Hideki Fujii, Shinji Furuya, Michio Hara, Kazuyoshi Hirayama, Yoshihiro Akazawa, Yuuki Nakata, Masato Tsuchiya, Naohiro Hosomura, Chao Sun

Hiroshi Kono, Hideki Fujii, Shinji Furuya, Michio Hara, Kazuyoshi Hirayama, Yoshihiro Akazawa, Yuuki Nakata, Masato Tsuchiya, Naohiro Hosomura, Chao Sun, First Department of Surgery, Faculty of Medicine, University of Yamanashi, Chuo, Yamanashi 409-3898, Japan

Author contributions: Kono H conducted this experiment; Fujii H organized this experiment; Furuya S collected samples; Hara M collected samples; Hirayama K analyzed the data; Akazawa Y analyzed the data; Nakata Y analyzed the data; Tsuchiya M collected samples; Hosomura N and Sun C collected samples.

Institutional review board statement: This study was reviewed and approved by the University of Yamanashi, Faculty of Medicine IRB (Prof Zentaro Yamagata).

Informed consent statement: All involved persons (subjects or legally authorized representatives) gave their informed consent (written or verbal, as appropriate) prior to study inclusion.

Conflict-of-interest statement: There are no conflicts-of-interest on this study.

Data sharing statement: Participants gave informed consent for data sharing.

Open-Access: This article is an open-access article which was selected by an in-house editor and fully peer-reviewed by external reviewers. It is distributed in accordance with the Creative Commons Attribution Non Commercial (CC BY-NC 4.0) license, which permits others to distribute, remix, adapt, build upon this work non-commercially, and license their derivative works on different terms, provided the original work is properly cited and the use is non-commercial. See: <http://creativecommons.org/licenses/by-nc/4.0/>

Manuscript source: Unsolicited manuscript

Correspondence to: Hiroshi Kono, MD, PhD, First Department of Surgery, Faculty of Medicine, University of Yamanashi, 1110 Shimokato, Chuo, Yamanashi 409-3898,

Japan. hkouno@yamanashi.ac.jp
Telephone: +81-55-2737390
Fax: +81-55-2736751

Received: November 10, 2015

Peer-review started: November 11, 2015

First decision: December 21, 2015

Revised: January 22, 2016

Accepted: March 18, 2016

Article in press: March 18, 2016

Published online: October 21, 2016

Abstract

AIM

To investigate the role of macrophage colony-stimulating factor (M-CSF) in patients with hepatocellular carcinoma (HCC) after surgery.

METHODS

Expression of M-CSF, distribution of M2 macrophages (M ϕ s), and angiogenesis were assessed in the liver, including tumors and peritumoral liver tissues. The prognostic power of these factors was assessed. Mouse isolated hepatic M ϕ s or monocytes were cultured with media containing M-CSF. The concentration of vascular endothelial growth factor (VEGF) in media was assessed. Furthermore, the role of the M-CSF-matured hepatic M ϕ s on proliferation of the vascular endothelial cell (VEC) was investigated.

RESULTS

A strong correlation between the expressions of M-CSF and CD163 was observed in the peritumoral area. Also, groups with high density of M-CSF, CD163 or CD31 showed a significantly shorter time to recurrence (TTR) than low density groups. Multivariate analysis revealed

the expression of M-CSF or hepatic M2M ϕ s in the peritumoral area as the most crucial factor responsible for shorter TTR. Moreover, the expression of M-CSF and hepatic M2M ϕ s in the peritumoral area had better predictable power of overall survival. Values of VEGF in culture media were significantly greater in the hepatic M ϕ s compared with the monocytes. Proliferation of the VEC was greatest in the cells co-cultured with hepatic M ϕ s when M-CSF was present in media.

CONCLUSION

M-CSF increases hepatocarcinogenesis, most likely by enhancing an angiogenic factor derived from hepatic M ϕ and could be a useful target for therapy against HCC.

Key words: M2 macrophage; Vascular endothelial growth factor; Vascular endothelial cell; Monocyte; Angiogenesis

© The Author(s) 2016. Published by Baishideng Publishing Group Inc. All rights reserved.

Core tip: This study was designed to investigate the role of macrophage colony-stimulating factor (M-CSF) in patients with hepatocellular carcinoma (HCC) after resection. Groups with high density of M-CSF, CD163 or CD31 showed a significantly shorter time to recurrence (TTR) than low density groups. Multivariate analysis revealed the expression of M-CSF or hepatic M2M ϕ s in the peritumoral area as the most crucial factor responsible for shorter TTR. The expression of M-CSF and hepatic M2M ϕ s in the peritumoral area had better power for the prediction of overall survival. Thus, M-CSF could be a useful target for therapy against HCC.

Kono H, Fujii H, Furuya S, Hara M, Hirayama K, Akazawa Y, Nakata Y, Tsuchiya M, Hosomura N, Sun C. Macrophage colony-stimulating factor expressed in non-cancer tissues provides predictive powers for recurrence in hepatocellular carcinoma. *World J Gastroenterol* 2016; 22(39): 8779-8789 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v22/i39/8779.htm> DOI: <http://dx.doi.org/10.3748/wjg.v22.i39.8779>

INTRODUCTION

Hepatocellular carcinoma (HCC) is a globally common cancer, resulting over one million deaths every year^[1]. The etiology of HCC is strongly associated with liver cirrhosis due to alcohol abuse and hepatitis viral infection, exposure to aflatoxin B1 and various metabolic liver diseases^[2]. The mechanisms of HCC are mainly still unknown.

Although liver resection improves overall survival in patients with HCC^[3], a high rate of postoperative recurrence is still one critical problem, including intrahepatic metastasis (IM) or multicentric (MC) recurrence. Biomarkers, which derived from carcinoma cells or tumor-associated fibrotic tissues, have

been studied^[4]; however, results have not been well elucidated. Previously, the oncogenesis of HCC has been predominantly investigated in terms of oncogenic factors above mentioned. Alternatively, chronic inflammation is also strongly linked to mechanisms of initiation or progression of carcinoma by increasing production of reactive oxygen species and inflammatory cytokines from inflammatory cells, such as hepatic macrophages (M ϕ), "Kupffer cells (KC)", and infiltrating neutrophils into the liver^[5,6]. Previously, we reported a relationship between chronic inflammation caused by hepatitis viral infection and DNA damage due to oxidative stress in patients with HCC. Furthermore, it was also reported that the period of post-operative recurrence was much shorter in patients with high oxidative stress compared with patients who had low oxidative stress in HCV-infected liver^[7]. In addition to the oncogenic factors, the microenvironment is also an important factor as the soil for initiation and progression of HCC. Budhu *et al*^[8] reported that metastasis of HCC was associated with an immune system in the liver, suggesting that liver tissues surrounding tumors have an effect on prognosis of HCC.

Macrophages, one of the predominant infiltrating cell types into the tumor^[9], are attracted by chemokines^[4]. When M ϕ s are activated, they can eliminate malignant cells or draw out reactions causing tissue destruction^[10]. Although the significant role of macrophage colony-stimulating factors (M-CSF) and tumor-infiltrating M ϕ s in IM of HCC was already reported by Budhu *et al*^[8], the postoperative prognostic significance has not been cleared. Results from the most recent report suggest expression of M-CSF was associated with poor survival after curative liver resection in patients with HCC, emphasizing the important role of the microenvironment in the intrahepatic recurrence of HCC^[11]. M-CSF can also induce production of Th2 cytokines^[12,13] and some growth factors by the monocyte, which are essential for cell growth and invasion of tumor^[14]. Indeed, M-CSF induces vascular endothelial growth factor (VEGF) production and angiogenesis in monocytes. Furthermore, suppression of M-CSF decreased the infiltration of M ϕ s and suppressed tumor progression. On the other hand, hyper expression of M-CSF or treatment of recombinant M-CSF enhanced infiltration of M ϕ s, which is associated with growth of tumor cells and angiogenesis^[15-17].

Based on these results, it was hypothesized that M-CSF expression in non-tumoral liver tissues may enhance recruitment of M2M ϕ and angiogenesis, and enhance growth of tumor cells. This may increase the rate of recurrence of HCC, leading to a poor overall survival. Furthermore, in this study, the role of M-CSF on production of VEGF by the hepatic M ϕ s and the proliferation of vascular endothelial cells (VEC) were investigated *in vitro*.

Table 1 Clinicopathological features of the patients

Clinicopathological features	
Patients	n = 77
Gender	male : female = 38 : 10
Age (yr)	mean = 66.6 (44-85)
Virus infection	HCV : HBV : NBNC = 72 : 4 : 1
UICC-TNM classification	I : II : IIIA : IIIB = 41 : 32 : 3 : 1
Tumor number	single : multiple : unknown = 48 : 28 : 1
Tumor size (cm)	mean = 3.0 (1.0-9.5)
Portal vein invasion	yes : no : unknown = 7 : 68 : 2
Tumor differentiation	well : mod : poor : unknown = 18 : 44 : 13 : 2
Number of platelet ($\times 10^4$)	mean = 13.4 (3.8-28.1)
Alanine aminotransferase (ALT) (U/L)	mean = 50.9 (16-207)
Total bilirubin (mg/dL)	mean = 0.9 (0.3-1.9)
Prothrombin activity (%)	mean = 77.1 (33.0-104.0)
Indocyanine green R15 (%)	mean = 19.1 (6.0-44.4)
Alpha-fetoprotein (ng/mL)	mean = 389.6 (1.8-602.6)

From July 2000 to June 2008, consecutive patients in the University of Yamanashi Hospital (Yamanashi, Japan) who (1) were diagnosed as having HCC and (2) underwent curative resection surgery, were enrolled in this study.

MATERIALS AND METHODS

Patients

This is a retrospective single-center, open-label study. From July 2000 to June 2008, consecutive patients in the University of Yamanashi Hospital (Yamanashi, Japan) who (1) were diagnosed as having HCC; and (2) underwent curative resection were enrolled in this study (Table 1).

Follow-up and postoperative treatment

All patients were observed until December 2013. The presence and identification of the hepatitis virus was determined by one or more of the following techniques: (1) the presence of anti-HCV and anti-HBV reactive serum proteins; (2) reverse transcription-PCR for serum HCV-RNA; or (3) the branched DNA-HCV probe assay. Following liver resection patients returned to the ambulatory care clinic for additional tests monthly. Serum α -fetoprotein levels were measured every month. In addition, ultrasonography and computed tomography (CT) of the liver were performed every 2 and 4 mo, respectively. Informed consent was obtained from all subjects who participated in the study, and the study was approved by the Institutional Board on Ethics for Human Science at the University of Yamanashi. All available clinical data for the patients enrolled in this study are summarized in Table 1. Overall survival (OS) was defined as the interval between the dates of surgery and death. Disease-free survival (DFS) was defined as the interval between the dates of surgery and recurrence; if recurrence was not diagnosed, patients were censored on the date of death or the last follow-up.

Animals

For the *in vitro* study, male C57BL/6 mice (8-10 wk of age, obtained from Jackson Laboratories, Bar Harbor,

ME), were housed in a clean, temperature-controlled environment with a 12-h light-dark cycle and were given free access to regular laboratory chow diet and water for several days. All animals received humane care, and the study protocols were approved by the Committee of Laboratory Animals at University of Yamanashi according to institutional guidelines.

Liver tissues

Sections of tumor and surrounding liver tissues were collected at the time of the operation. Then, sections were fixed in formalin (10%), dehydrated in absolute ethanol and embedded in paraffin. Serial sections (5 μ m thick) were prepared for further immunohistochemical analysis.

Immunohistochemical detection of M-CSF, CD68, CD163, and CD31

Paraffin-embedded serial sections of liver tissues were stained immunohistochemically with anti-M-CSF (Santa Cruz Biotechnology, Santa Cruz, CA), anti-CD68 (DAKO, Kyoto, Japan), anti-CD163 (Abcam, Cambridge, United Kingdom), or anti-CD31 (Abcam) antibodies. Briefly, after deparaffinization and rehydration, the antigen retrieval procedure was applied by heating the slides in 0.1 mol/L citrate buffer (pH 6.0) for 10 min. The sections were then incubated first with 0.3% H₂O₂ in distilled water for 5 min to block endogenous peroxidase and then incubated with one of the following antibodies: monoclonal mouse antihuman CD68 (diluted to 1:200), CD163 (diluted to 1:200), CD31 (diluted to 1:200), and M-CSF (diluted to 1:100) at 4 °C overnight. Biotinylated secondary antibody conjugated with avidin-biotin horseradish peroxidase (Dako Envision kit/HRP; Dako, Kyoto, Japan) and 3',3'-diaminobenzidine tetrahydrochloride were used for standard avidin biotinylated peroxidase detection. Quantitation of cells that stained positive for CD68, CD163, CD31 and M-CSF was performed using image analysis software (Scion Image; Scion Corp., Frederick, MD) by evaluating five different fields (magnification: $\times 400$) for areas positively stained and expressed as a percentage of the total area.

Effects of M-CSF on production of vascular endothelial growth factor by the isolated Kupffer cells and the monocytes from mice *in vitro*

Kupffer cells were isolated by counterflow centrifugal elution as described in our previous work with some modifications^[18]. Briefly, nonparenchymal cells were isolated by collagenase digestion and differential centrifugation using Nycodenz (Nycomed Pharma AS, Oslo, Norway) as described elsewhere^[19]. Furthermore, the peripheral blood monocytes were isolated by the centrifugation method using magnetic beads EasySep (Tokyo, Japan). These cells were incubated in media containing recombinant mouse M-CSF (100 ng/mL) (Sigma) for a designated time period (from 1 to 5 d). In another set of experiments, isolated KCs

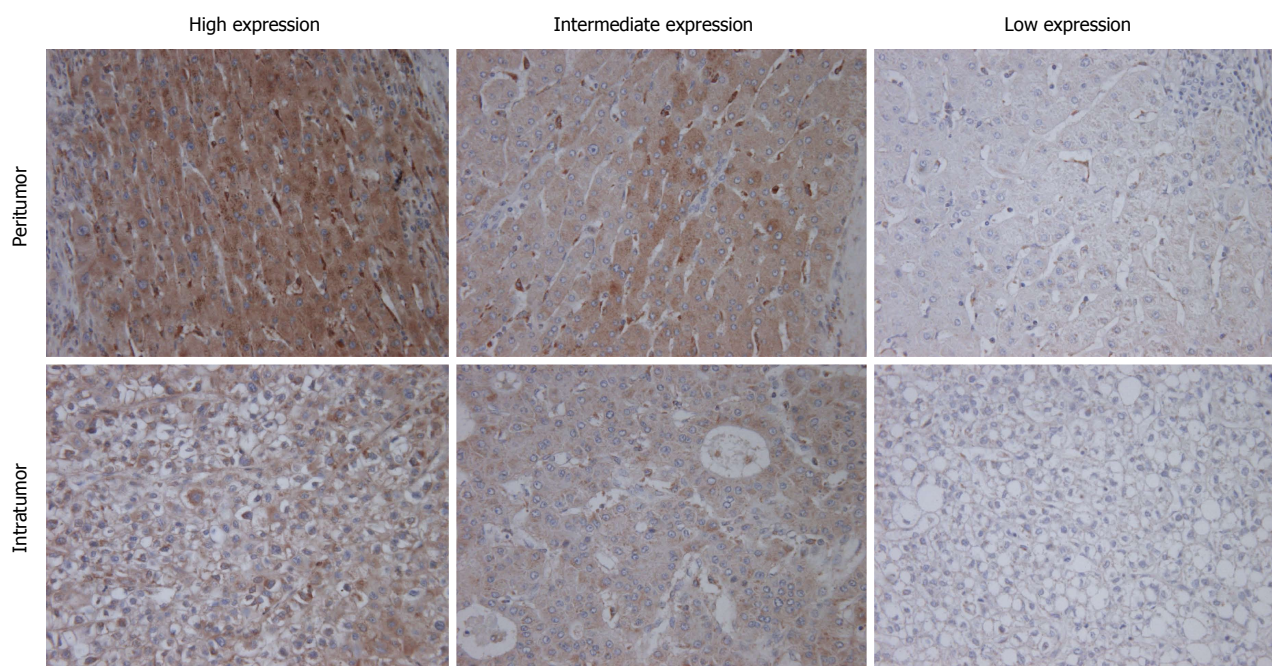


Figure 1 Immunohistochemical staining for macrophage colony-stimulating factor. Immunohistochemical staining for M-CSF in intratumoral and peritumoral tissues in the liver was performed as described in Patients and Methods. Representative photomicrographs are shown. Original magnification, $\times 400$.

were incubated with different doses of M-CSF (0, 1 ng, 10 ng, and 100 ng/mL) in media for 5 d. The concentration of VEGF in media was measured by an ELISA kit (R&D Systems, Minneapolis, MN).

Effects of hepatic macrophages and M-CSF on proliferation of the vascular endothelial cells

The effects of M-CSF and the KCs on the proliferation of the VECs were investigated. The KCs were isolated by collagenase digestion and the centrifugation method above mentioned, and the VECs were isolated from the aorta. The VECs were seeded on the bottom chamber of a trans-well chamber (Corning Inc., Corning, NY) and the hepatic macrophages were seeded on the upper chamber. These cells were incubated with the media containing M-CSF (mouse recombinant M-CSF; 100 ng/mL) (Sigma, St. Louis, MO) for 4 d and proliferation of the VECs was assessed.

Statistical analysis

Analysis was performed using SPSS 13.0 for Windows (SPSS Inc., Chicago IL). The Pearson χ^2 test or Fisher's exact test was used to compare qualitative variables, and quantitative variables were analyzed by the *t* test or Pearson's correlation test. Kaplan-Meier analysis was used to determine the survival (time to recurrence and overall survival). Receiver operating characteristic (ROC) curve analysis was used to determine the predictive value of the parameters. For the univariate analysis, linear regression, or Log-rank test statistical procedures were used to assess which endpoints could be used for predicting the prognosis of HCC patients after surgery. For the multivariate analysis, the Cox

proportional hazard model was used to calculate hazard ratio and the *P* value of each parameter. *P* < 0.05 was considered statistically significant.

RESULTS

The clinical characteristics of HCC patients in this study are shown in Table 1. Experiments were performed using resected liver specimens including HCC and tumor free non-cancerous liver tissue by surgery.

Expression of M-CSF in intratumoral and peritumoral tissues in patients with HCC

Immunohistochemical staining for M-CSF was performed as described in the Patients and Methods section. Immunohistochemical staining for M-CSF was observed in the cytoplasm of both tumor cells and hepatocytes (Figure 1). On the other hand, most of the non-parenchymal cells were negatively stained.

The expression of M-CSF in intratumoral tissues was not correlated with DFS and overall survival rate (OS) (data not shown), consistent with the previous report^[11]. On the other hand, in peritumoral tissues, DFS and OS were significantly shorter in the high expression group than in the low expression group (Figures 2 and 3), consistent with the previous reports^[11].

Expression of CD68, CD163, and CD31 in liver tissues of patients with HCC

Immunohistochemical staining for CD68 (a marker of the M1M ϕ s and/or the monocytes), CD163 (a marker of the M2M ϕ s), and CD31 (a marker of the vascular

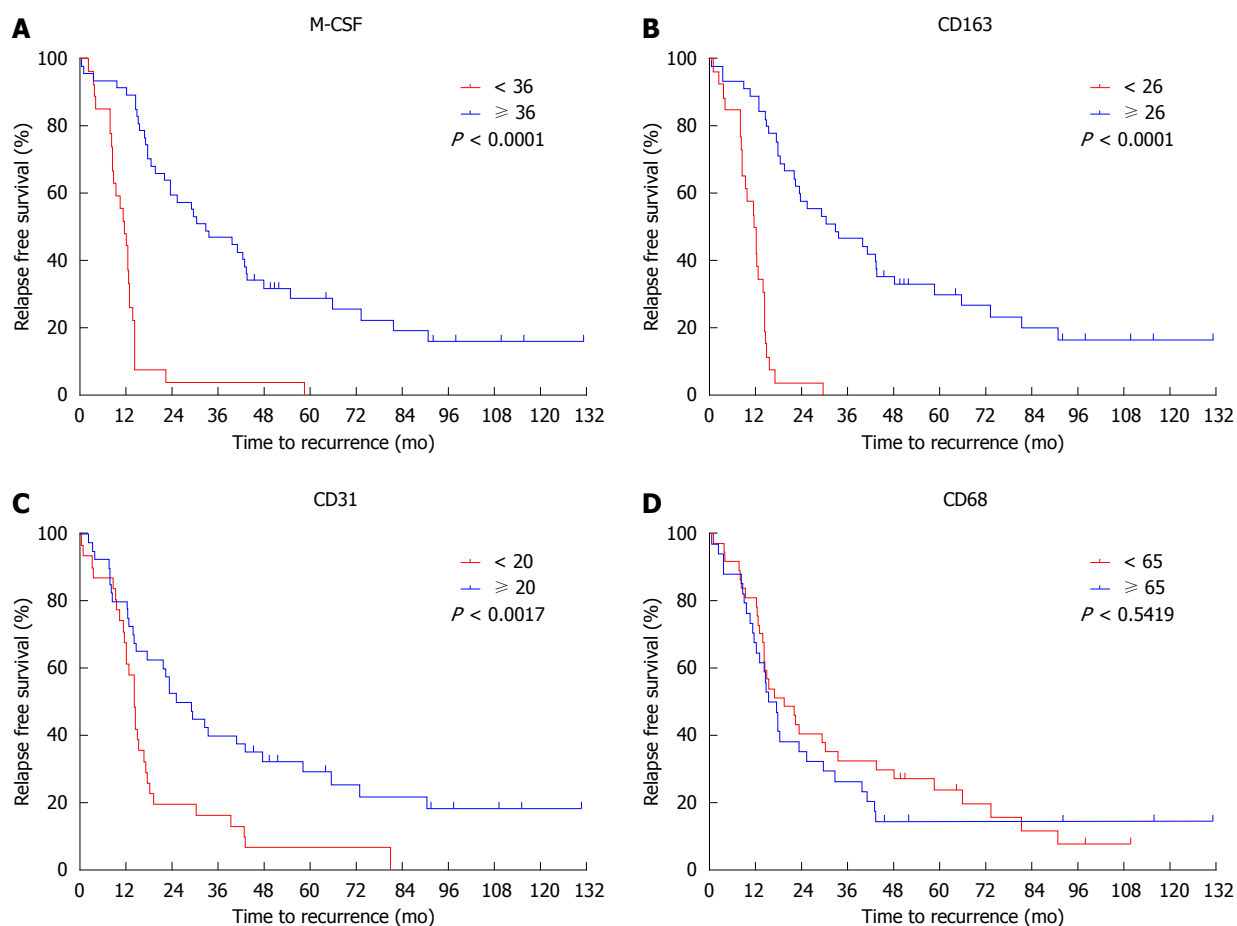


Figure 2 Cumulative disease-free survival curves of patients with high or low peritumoral features. Disease free survival curves of patients with high or low expression of CD68, CD163, CD31 and macrophage colony-stimulating factor (M-CSF) in peritumoral liver tissues are shown. A: M-CSF; B: CD163; C: CD31; D: CD68. The intratumoral features were not associated with disease-free survival.

endothelial cells) was performed as described in the Patients and Methods section. A positive correlation was observed between the expression of M-CSF and CD31 in the peritumoral liver tissues, but not between M-CSF and CD163 (Figure 4), or CD68 (data not shown).

To determine the cutoff value of the M-CSF positive area, an ROC curve was drawn using recurrence within one year. Patients with a high expression of CD31 or CD163 in peritumoral tissues showed significantly poorer DFS compared with patients with low expression of these markers (Figures 2 and 3). Furthermore, patients with high expression of CD163 in peritumoral tissues showed significantly poorer OS compared with patients with low expression of these markers. Although positive staining for CD68 was detected in peritumoral tissues, the number of CD68-positive cells was not correlated with DFS (Figure 2) or OS (data not shown).

Prognostic factors

According to the ROC curve, the ideal cutoff value of the densities of M-CSF, CD163, and CD31 were 36, 26, and 20, respectively. The median DFS was 12.3 mo for patients with high expression of M-CSF in peritumoral

tissues, which was significantly shorter compared with the median DFS for patients with low expression of M-CSF (43.3 mo). Furthermore, the median survival times for the high CD163 density and the low density group were 12.2 and 32.0 mo, respectively. The median survival times for the high CD31 density and the low density group were 13.6 and 37.3 mo, respectively.

M-CSF density, CD163 index, and CD31 index in peritumoral tissues are predictable factors for prognosis in patients with HCC

The univariate analysis of the prognostic value for various factors assessed in the present study is shown in Table 2.

In clinicopathologic factors, the size of the tumor, the clinical disease stage, serum ALT and AFP levels are useful to assess recurrence of HCC after surgery. In the present study, there were significant differences in the univariate analysis of DFS between the UICC-TNM Stages (Stage I vs Stages II and III) (Table 2). Furthermore, there were significant differences in the univariate analysis of OS between the tumor number (single vs multiple) and the fibrosis score (1-3 vs 4). In multivariate analysis of the prognostic power, the

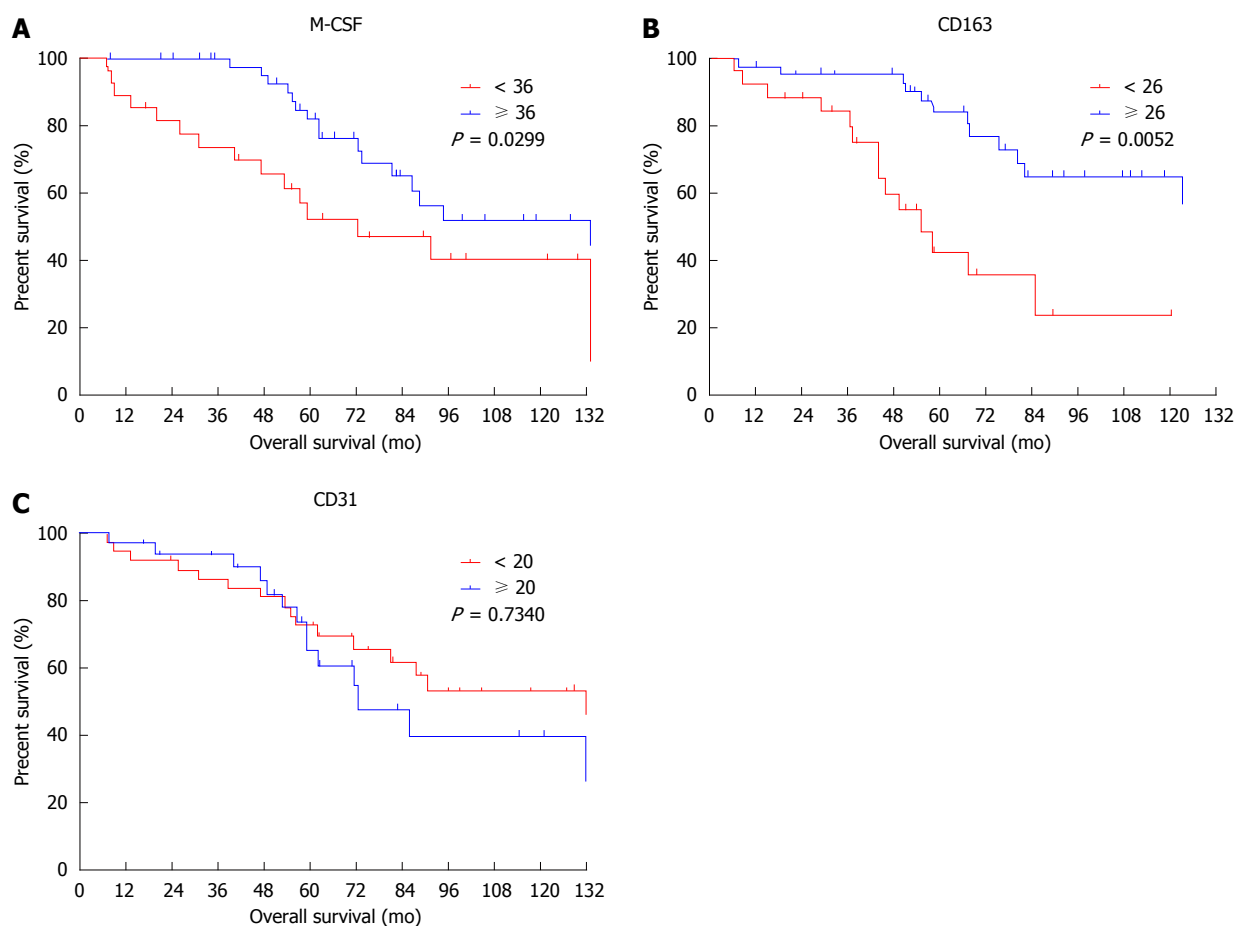


Figure 3 Cumulative overall survival curves of patients with high or low macrophage colony-stimulating factor expression in peritumoral features. Overall survival curves of patients with high or low expression of macrophage colony-stimulating factor (M-CSF) in peritumoral liver tissues are shown. A: M-CSF; B: CD163; C: CD31. The intratumoral features were not associated with overall survival.

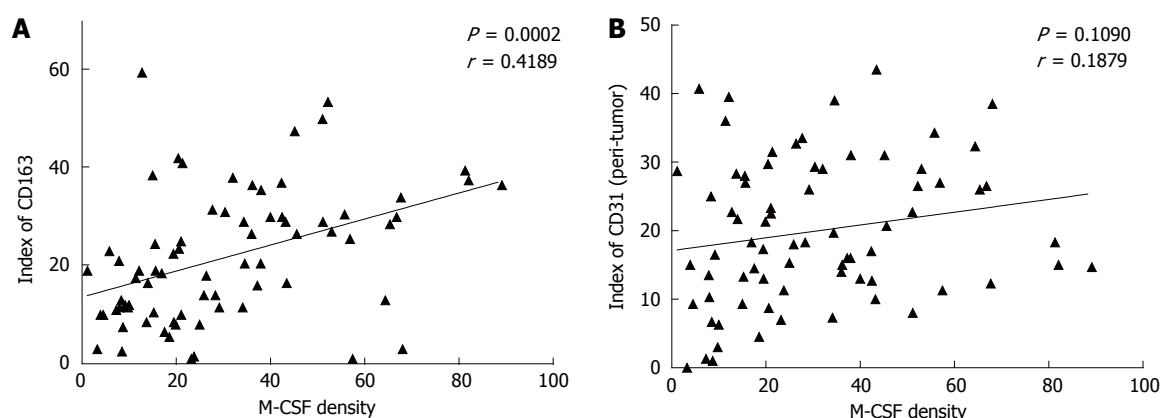


Figure 4 Correlation between the expression of macrophage colony-stimulating factor and CD163, or CD31 in the non-tumoral liver tissues. Immunohistochemical staining for macrophage colony-stimulating factor (M-CSF), CD163, and CD31 was performed in non-cancerous liver tissue collected from patients as described in detail in the Patients and Methods section. A (between M-CSF and CD163) and B (between M-CSF and CD31) show correlations between markers assessed in this study in each of the patients with HCC.

fibrosis score was a factor for predicting the length of overall survival in patients with HCC (Table 2).

M-CSF density, CD163 index, and CD31 index in peritumoral liver tissues appear to be risk factors for DFS by univariate analysis (Table 2). When each of these markers was included in the multivariate analysis, M-CSF density and CD163 index were

found to be the only significant predictors for DFS. Alternatively, peritumoral M-CSF density, CD163 index, and CD31 index appear to be risk factors for OS by univariate analysis. Furthermore, when each of these markers was included in the multivariate analysis, the CD163 index was found to be the only significant predictor for OS.

Table 2 Univariate and Multivariate analyses of factors associated with recurrence and survival

Factors	Time to recurrence				Overall survival			
	Univariate	Multivariate			Univariate	Multivariate		
	P value	Hazard ratio	95%CI	P value	P value	Hazard ratio	95%CI	P value
Age: ≥ 60 yr <i>vs</i> < 60 yr	0.240			NA	0.523			NA
Gender: female <i>vs</i> male	0.760			NA	0.111			NA
Indx of α -SMA: ≥ 12 <i>vs</i> < 12	0.751			NS	0.423			NS
Indx of CD163: ≥ 26 <i>vs</i> < 26	< 0.001	4.517	1.879-10.857	0.001	< 0.001	1.086	1.039-1.135	< 0.001
M-CSF density: ≥ 36 <i>vs</i> < 36	< 0.001	4.826	2.045-11.389	< 0.001	0.037			NS
Indx of CD31: ≥ 20 <i>vs</i> < 20	0.002			NS	0.020			NS
AFP: ≥ 100 <i>vs</i> < 100	0.232			NA	0.785			NA
AFP-L3%: ≥ 10 <i>vs</i> < 10	0.827			NA	0.900			NA
PIVKA-II: ≥ 100 <i>vs</i> < 100	0.841			NA	0.325			NA
Tumor size: ≥ 2 cm <i>vs</i> < 2 cm	0.185			NA	0.847			NA
Tumor number: multiple <i>vs</i> single	0.447			NA	0.038			NS
Tumor differentiation: well <i>vs</i> mod. <i>vs</i> poor	0.379			NA	0.745			NA
Fibrosis score: 1, 2, 3 <i>vs</i> 4	0.755			NA	0.015			0.002
TNM stage: I <i>vs</i> II <i>vs</i> IIIa	0.020			NS	0.173			NA

Tables are shown univariate and multivariate analysis of prognostic markers associated with the disease-free and the overall survival.

Production of VEGF by isolated Kupffer cells and monocytes from mice

The production of VEGF by isolated KCs increased in a dose- and time-dependent manner after incubation with M-CSF in media (Figure 5). Furthermore, the production by isolated monocytes also increased in a time dependent manner by stimulation with M-CSF (Figure 5). Although the production of VEGF increased in both isolated KCs and monocytes incubated with M-CSF, production was significantly greater in the KC compared with the monocytes (Figure 5).

Proliferation of isolated vascular endothelial cells isolated

Cell proliferation of isolated VECs markedly increased in cells treated with M-CSF compared with those that were not treated with M-CSF in media (Figure 6). Importantly, among the groups studied, the proliferation was greatest in the M-CSF-treated VECs co-cultured with the KCs.

DISCUSSION

M-CSF is involved in progression of hepatocellular carcinoma after curative resection

It was previously reported that overexpression of M-CSF is observed in tumor tissues in various human cancers and is related with poor survival^[20-25]. It was also reported that M-CSF is predominantly detected in peritumoral liver tissues^[8]. Furthermore, it was reported from this laboratory that the incidence of chemically-induced HCC was reduced in M-CSF deficient mice (KO) compared with their littermates (WT) by inhibiting the expression of M2 M ϕ and angiogenesis in peritumor liver tissues^[26]. In the present study, the high expression of M-CSF in peritumoral tissues was correlated with a high incidence of HCC, including MC recurrence and IM,

and poor prognosis after curative resection (Figures 2 and 4), consistent with the previous report^[11]. Thus, M-CSF is involved in hepatic carcinogenesis and its progression. Based on these reports, this study investigated whether M-CSF induces hepatic carcinogenesis, most likely by enhancing angiogenesis by M-CSF-induced hepatic macrophages.

In the present study, expression of M-CSF differed in intratumoral tissues and peritumoral liver tissues, suggesting that the expression level of M-CSF differs in each individual. Importantly, the level of M-CSF expression did not correlate with any pathophysiologic factors. Therefore, the expression of M-CSF may be associated with other genetic factors in each patient^[11]. Indeed, Okamoto *et al.*^[27] reported that a specific gene profile in non-tumoral tissue predicted MC recurrence or IM of HCC. Alternatively, placental^[28,29] and endothelial cells^[30] excreted M-CSF under hypoxic conditions. Based on this result, distribution of M-CSF may be caused by hypoxia in hepatocytes due to compression by the primary tumor. A tumor itself also enhances acute and chronic inflammation, causes cytokine production and attracts M ϕ s to peritumoral liver tissues. The previous study indicated that implanted or spontaneous tumors induced vascular endothelial growth factor in peritumoral tissue that was much greater than in the intratumoral tissue^[31]. Furthermore, angiogenesis was greater in peritumoral tissues than in the intratumoral tissues in chemically-induced HCC in mice^[26]. Taken together, the expression of M-CSF in peritumoral hepatocytes may be associated with the genetic heterogeneity in each individual or inflammatory factors in the liver.

Role of the hepatic microenvironment in initiation and progression of HCC

The high rate of IM and MC recurrence after complete resection indicated that the non-tumoral hepatic

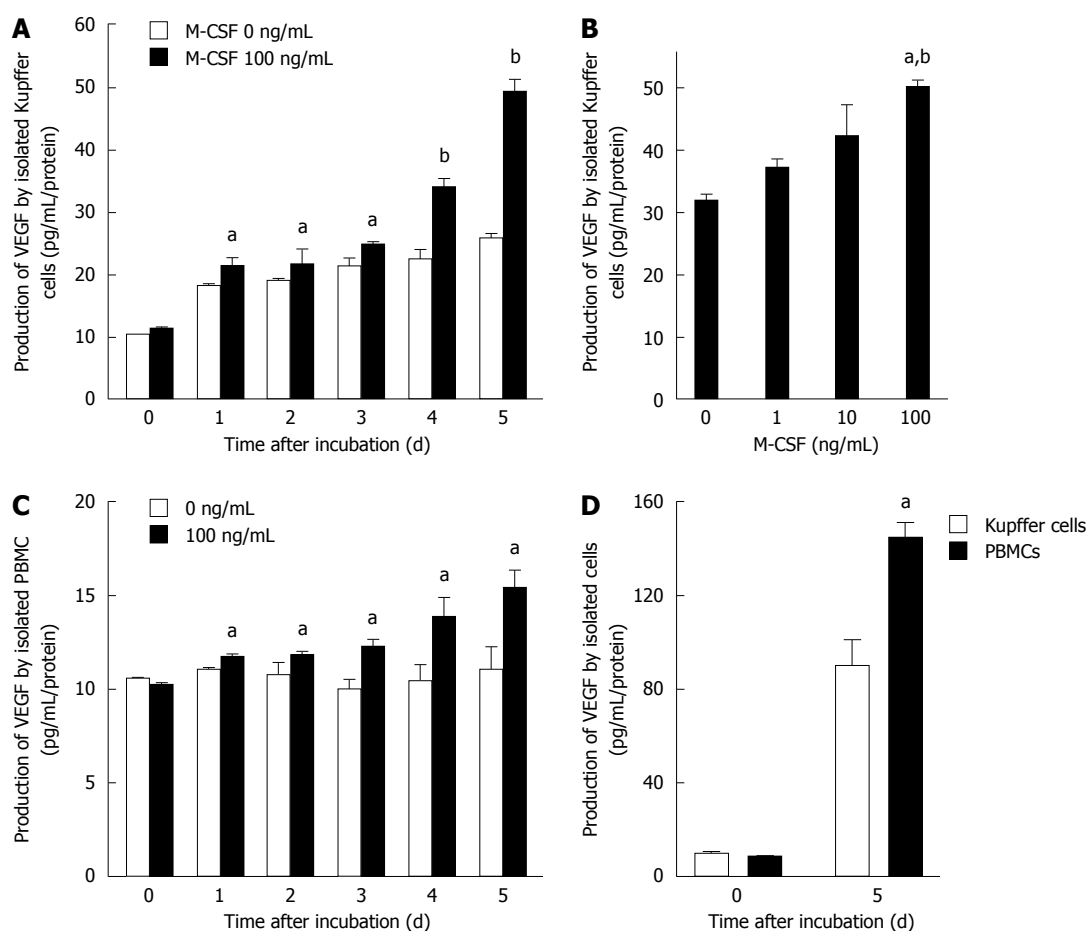


Figure 5 Production of vascular endothelial growth factor by isolated Kupffer cells and peripheral blood monocytes. Production of vascular endothelial growth factor (VEGF) by isolated Kupffer cells and peripheral blood monocytes was determined as described in the Methods. A: Kupffer cells were cultured with or without macrophage colony-stimulating factor (M-CSF) in media for designated experimental periods ($n = 6$). $^aP < 0.05$ vs with Kupffer cells without M-CSF stimulation; and $^bP < 0.01$ vs with Kupffer cells without M-CSF stimulation by ANOVA with Bonferroni's post-hoc test; B: Kupffer cells were treated with different doses of M-CSF in media, and cultured for 5 d ($n = 6$). $^aP < 0.05$ vs with Kupffer cells without M-CSF stimulation; and $^bP < 0.01$ vs with Kupffer cells with 10 ng/mL of M-CSF in media by ANOVA with Bonferroni's post-hoc test; C: Peripheral blood monocytes were isolated and were cultured for designated experimental periods. The concentration of VEGF in media was then determined as described in the Methods ($n = 6$). $^aP < 0.05$ vs with peripheral blood monocytes without M-CSF stimulation by ANOVA with Bonferroni's post-hoc test; D: Isolated Kupffer cells or PBMC were incubated with 100 ng/mL of M-CSF in media for 5 d. The concentration of VEGF was determined as described in Materials and Methods ($n = 6$). $^aP < 0.05$ vs with PBMC by ANOVA with Bonferroni's post-hoc test.

microenvironment plays a key role in tumor initiation and progression. Previous investigations of causes of HCC were mainly focused on factors of tumor initiation as "seeds." On the other hand, few studies that focus on hepatic microenvironmental factors as "soil" were reported. Indeed, Ezaki *et al.*^[32,33] reported that the hyper expression of thymidine phosphorylase in the peritumoral liver tissues was correlated with the higher incidence of postoperative recurrence of HCC. Furthermore, Yu *et al.*^[34] reported that vascular density was higher in peritumoral tissues compared with that in intratumoral tissue, leading to increased expression of VEGF and hypoxia inducible factor-1 in peritumoral liver tissues. Moreover, it was reported that immunological features in the peritumoral tissue predicted venous metastases in HCC^[35]. In this study, the high density of M-CSF in peritumoral liver tissues was correlated with a high incidence of HCC and prognosis, consistent with the previous report^[11] (Figures 2 and 3). Taken together, it is concluded

that the peritumoral microenvironment, as "the soil," is also important in understanding the mechanism of incidence of HCC. Furthermore, previous studies and this study suggest that postoperative adjuvant therapies could target not only the subclinical carcinoma cells, but also the microenvironment in the liver.

Role of M-CSF induced macrophage in the progression of HCC

The union of M-CSF expression, population of M2M ϕ s and angiogenesis in peritumoral tissues had a better predictable power for prognosis in HCC (Figures 2 and 3, and Table 2). The number of M ϕ s was greater in peritumoral tissue than in intratumoral tissue, consistent with results in previous reported studies^[35,36]. In the present study, expression of M-CSF, M2M ϕ s and angiogenesis in the peritumoral liver tissue was correlated with DFS after surgery (Figure 2). The role of microenvironments in intratumoral tissues and

Group	1	2	3	4
M-CSF (100 ng/mL)	-	+	-	+
KC (2×10^5 cell/well)	-	-	+	+
VEC (1.5×10^5 cell/well)	+	+	+	+

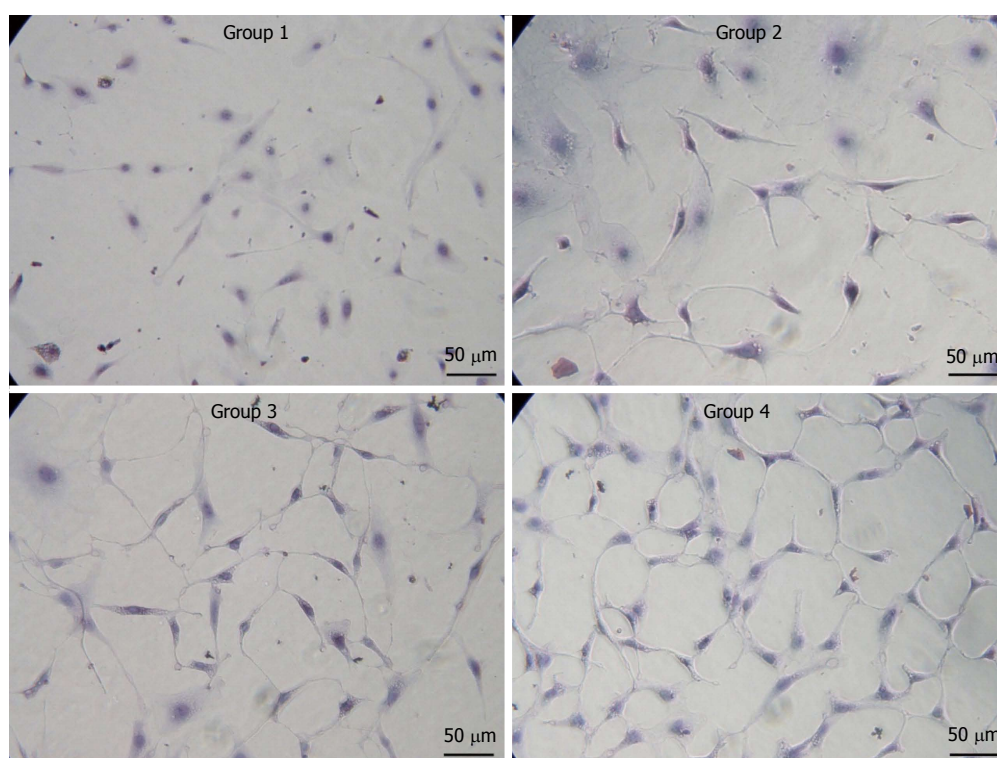


Figure 6 Proliferation of isolated vascular endothelial cells. Isolated vascular endothelial cells (VECs) were co-cultured with or without the isolated Kupffer cells in media containing of macrophage colony-stimulating factor (M-CSF). Cell proliferation of VECs was determined as described in the Patients and Methods ($n = 5$). Treatments of each group are shown in the table. Representative photomicrographs are shown. Original magnification, $\times 400$.

peritumoral tissues may be different in initiation and progression of HCC. Expression of M-CSF, CD31 and M2M ϕ s in intratumoral tissues may be involved in promoting the dissemination of the cancer cells^[37-39]. M-CSF and M2M ϕ s in the peritumoral tissues are involved in accelerating colonization and growth of disseminated tumor cells, leading to micrometastasis, since M-CSF and M2 M ϕ in the tumor is eliminated by operation. Therefore, as the defense to inhibit growth of the tumor, the peritumoral liver tissue, which supplies M-CSF to the tumor^[31], plays a pivotal role by providing a fruitful soil for micrometastasis of HCC as "seeds". The role of the M ϕ s in providing a fertile soil as the metastasis niche has also been reported^[40]. In this study, the number of M2M ϕ s in the peritumoral tissues was correlated with the expression of M-CSF in the peritumoral tissues and was linked with DFS and OS (Figures 2 and 3). Thus, M-CSF-induced M2M ϕ s may be involved in DFS. In addition to this result, the number of M2M ϕ s and the expression of angiogenic factors were also positively correlated. Importantly, VEGF production by isolated hepatic M ϕ was increased by M-CSF stimulation (Figure 5) and proliferation of isolated VEC was greatest in the cells incubated with M-CSF-stimulated hepatic M ϕ in the presence of M-CSF

in media (Figure 6). Thus, M-CSF-induced M2M ϕ s could be involved in progression of HCC by inducing angiogenesis.

Clinical applications and conclusion

Undoubtedly, M-CSF and M2M ϕ could be suitable targets for adjuvant therapy after surgery, since in the present study the expression of M-CSF and the number of M2M ϕ s increased in the peritumoral tissues, hepatic M ϕ s incubated with M-CSF in media produced VEGF, and the proliferation was greatest in the M-CSF-treated VECs co-cultured with the KCs among the groups studied. Thus, this study indicates that M-CSF expressed in the peritumoral liver tissue could predict the recurrence of patients with HCC after surgery and also indicates that the remnant liver may play an important role in recurrence and metastasis. Therefore, evaluation of M-CSF is a useful result that can be easily assessed in specimens collected during surgery.

COMMENTS

Background

Expression of macrophage colony-stimulating factor (M-CSF) was associated

with hepatocellular carcinoma (HCC) progression, disease recurrence, and poor survival after hepatectomy, highlighting the importance of the peritumoral tissue environment in the intrahepatic recurrence of HCC.

Research frontiers

In this study, the authors investigated the relationship between the M-CSF-matured hepatic macrophage and the prognosis of HCC after resection.

Innovations and breakthroughs

In this manuscript, the major point is that vascular endothelial growth factor derived from the M-CSF-matured hepatic macrophage plays a pivotal role in the prognosis of HCC.

Applications

M-CSF or its receptor could be a new molecular target for therapy against HCC.

Terminology

The present study indicates that M-CSF expressed in peritumoral liver tissues can predict recurrence in patients with HCC after surgery and also implies that the remnant liver may play a key role in recurrence and metastasis. Therefore, evaluation of M-CSF is a useful result that can be easily assessed in specimens collected during liver tumor resection.

Peer-review

In this paper, the authors aimed at investigating the role of M-CSF in patients with HCC after resection. They analyze the prognostic value of MCSF, CD163, CD31 and other clinicopathologic parameters for patients' disease-free survival (DFS) with HCC and find M-CFS in non-cancer tissues can act as a predictor for patients DFS after resection.

REFERENCES

- 1 Inoue H, Seitz HK. Viruses and alcohol in the pathogenesis of primary hepatic carcinoma. *Eur J Cancer Prev* 2001; **10**: 107-110 [PMID: 11263585 DOI: 10.1097/00008469-200102000-00016]
- 2 Kountouras J, Lygidakis NJ. New epidemiological data on liver oncogenesis. *Hepatogastroenterology* 2000; **47**: 855-861 [PMID: 10919047]
- 3 Tang ZY, Ye SL, Liu YK, Qin LX, Sun HC, Ye QH, Wang L, Zhou J, Qiu SJ, Li Y, Ji XN, Liu H, Xia JL, Wu ZQ, Fan J, Ma ZC, Zhou XD, Lin ZY, Liu KD. A decade's studies on metastasis of hepatocellular carcinoma. *J Cancer Res Clin Oncol* 2004; **130**: 187-196 [PMID: 14685850 DOI: 10.1007/s00432-003-0511-1]
- 4 Wiktor-Jedrzejczak W, Gordon S. Cytokine regulation of the macrophage (M phi) system studied using the colony stimulating factor-1-deficient op/op mouse. *Physiol Rev* 1996; **76**: 927-947 [PMID: 8874489]
- 5 Koike K, Miyoshi H. Oxidative stress and hepatitis C viral infection. *Hepatol Res* 2006; **34**: 65-73 [PMID: 16364681 DOI: 10.1016/j.hepres.2005.11.001]
- 6 Llovet JM, Burroughs A, Bruix J. Hepatocellular carcinoma. *Lancet* 2003; **362**: 1907-1917 [PMID: 14667750 DOI: 10.1016/S0140-6736(03)14964-1]
- 7 Maki A, Kono H, Gupta M, Asakawa M, Suzuki T, Matsuda M, Fujii H, Rusyn I. Predictive power of biomarkers of oxidative stress and inflammation in patients with hepatitis C virus-associated hepatocellular carcinoma. *Ann Surg Oncol* 2007; **14**: 1182-1190 [PMID: 17195915 DOI: 10.1245/s10434-006-9049-1]
- 8 Budhu A, Forgues M, Ye QH, Jia HL, He P, Zanetti KA, Kammula US, Chen Y, Qin LX, Tang ZY, Wang XW. Prediction of venous metastases, recurrence, and prognosis in hepatocellular carcinoma based on a unique immune response signature of the liver microenvironment. *Cancer Cell* 2006; **10**: 99-111 [PMID: 16904609 DOI: 10.1016/j.ccr.2006.06.016]
- 9 Mantovani A, Bottazzi B, Colotta F, Sozzani S, Ruco L. The origin and function of tumor-associated macrophages. *Immunol Today* 1992; **13**: 265-270 [PMID: 1388654 DOI: 10.1016/0167-5699(92)90008-U]
- 10 Balkwill F, Mantovani A. Inflammation and cancer: back to Virchow? *Lancet* 2001; **357**: 539-545 [PMID: 11229684 DOI: 10.1016/S0140-6736(00)04046-0]
- 11 Zhu XD, Zhang JB, Zhuang PY, Zhu HG, Zhang W, Xiong YQ, Wu WZ, Wang L, Tang ZY, Sun HC. High expression of macrophage colony-stimulating factor in peritumoral liver tissue is associated with poor survival after curative resection of hepatocellular carcinoma. *J Clin Oncol* 2008; **26**: 2707-2716 [PMID: 18509183 DOI: 10.1200/JCO.2007.15.6521]
- 12 Verreck FA, de Boer T, Langenberg DM, Hoeve MA, Kramer M, Vaisberg E, Kastelein R, Kolk A, de Waal-Malefyt R, Ottenhoff TH. Human IL-23-producing type 1 macrophages promote but IL-10-producing type 2 macrophages subvert immunity to (myco)bacteria. *Proc Natl Acad Sci USA* 2004; **101**: 4560-4565 [PMID: 15070757 DOI: 10.1073/pnas.0400983101]
- 13 Smith W, Feldmann M, Londei M. Human macrophages induced in vitro by macrophage colony-stimulating factor are deficient in IL-12 production. *Eur J Immunol* 1998; **28**: 2498-2507 [PMID: 9710227 DOI: 10.1002/(SICI)1521-4141(199808)28]
- 14 Zins K, Abraham D, Sioud M, Aharinejad S. Colon cancer cell-derived tumor necrosis factor-alpha mediates the tumor growth-promoting response in macrophages by up-regulating the colony-stimulating factor-1 pathway. *Cancer Res* 2007; **67**: 1038-1045 [PMID: 17283136 DOI: 10.1158/0008-5472.CAN-06-2295]
- 15 Lin EY, Pollard JW. Tumor-associated macrophages press the angiogenic switch in breast cancer. *Cancer Res* 2007; **67**: 5064-5066 [PMID: 17545580 DOI: 10.1158/0008-5472.CAN-07-0912]
- 16 Sica A, Schioppa T, Mantovani A, Allavena P. Tumour-associated macrophages are a distinct M2 polarised population promoting tumour progression: potential targets of anti-cancer therapy. *Eur J Cancer* 2006; **42**: 717-727 [PMID: 16520032 DOI: 10.1016/j.ejca.2006.01.003]
- 17 Paulus P, Stanley ER, Schäfer R, Abraham D, Aharinejad S. Colony-stimulating factor-1 antibody reverses chemoresistance in human MCF-7 breast cancer xenografts. *Cancer Res* 2006; **66**: 4349-4356 [PMID: 16618760 DOI: 10.1158/0008-5472.CAN-05-3523]
- 18 Kono H, Fujii H, Asakawa M, Yamamoto M, Maki A, Matsuda M, Rusyn I, Matsumoto Y. Functional heterogeneity of the kupffer cell population is involved in the mechanism of gadolinium chloride in rats administered endotoxin. *J Surg Res* 2002; **106**: 179-187 [PMID: 12127824 DOI: 10.1006/jsre.2002.6434]
- 19 Kono H, Enomoto N, Connor HD, Wheeler MD, Bradford BU, Rivera CA, Kadiiska MB, Mason RP, Thurman RG. Medium-chain triglycerides inhibit free radical formation and TNF-alpha production in rats given enteral ethanol. *Am J Physiol Gastrointest Liver Physiol* 2000; **278**: G467-G476 [PMID: 10712267]
- 20 Kacinski BM. CSF-1 and its receptor in ovarian, endometrial and breast cancer. *Ann Med* 1995; **27**: 79-85 [PMID: 7742005 DOI: 10.3109/07853899509031941]
- 21 Pollard JW. Tumour-educated macrophages promote tumour progression and metastasis. *Nat Rev Cancer* 2004; **4**: 71-78 [PMID: 14708027 DOI: 10.1038/nrc1256]
- 22 Smith HO, Anderson PS, Kuo DY, Goldberg GL, DeVictoria CL, Boocock CA, Jones JG, Runowicz CD, Stanley ER, Pollard JW. The role of colony-stimulating factor 1 and its receptor in the etiopathogenesis of endometrial adenocarcinoma. *Clin Cancer Res* 1995; **1**: 313-325 [PMID: 9815987]
- 23 Mroczko B, Groblewska M, Wereszczyńska-Siemiakowska U, Okulczyk B, Kedra B, Łaszewicz W, Dąbrowski A, Szmikowski M. Serum macrophage-colony stimulating factor levels in colorectal cancer patients correlate with lymph node metastasis and poor prognosis. *Clin Chim Acta* 2007; **380**: 208-212 [PMID: 17368603 DOI: 10.1016/j.cca.2007.02.037]
- 24 Mroczko B, Groblewska M, Wereszczyńska-Siemiakowska U, Kedra B, Konopko M, Szmikowski M. The diagnostic value of G-CSF measurement in the sera of colorectal cancer and adenoma patients. *Clin Chim Acta* 2006; **371**: 143-147 [PMID: 16603145 DOI: 10.1016/j.cca.2006.02.037]

- 25 **Groblewska M**, Mroczo B, Wereszczyńska-Siemiatkowska U, Myśliwiec P, Kedra B, Szmitkowski M. Serum levels of granulocyte colony-stimulating factor (G-CSF) and macrophage colony-stimulating factor (M-CSF) in pancreatic cancer patients. *Clin Chem Lab Med* 2007; **45**: 30-34 [PMID: 17243911 DOI: 10.1515/CCLM.2007.025]
- 26 **Hara M**, Kono H, Furuya S, Hirayama K, Tsuchiya M, Fujii H. Macrophage colony-stimulating factor plays a pivotal role in chemically induced hepatocellular carcinoma in mice. *Hepatol Res* 2014; **44**: 798-811 [PMID: 23710613 DOI: 10.1111/hepr.12174]
- 27 **Okamoto M**, Utsunomiya T, Wakiyama S, Hashimoto M, Fukuzawa K, Ezaki T, Hanai T, Inoue H, Mori M. Specific gene-expression profiles of noncancerous liver tissue predict the risk for multicentric occurrence of hepatocellular carcinoma in hepatitis C virus-positive patients. *Ann Surg Oncol* 2006; **13**: 947-954 [PMID: 16788756 DOI: 10.1245/ASO.2006.07.018]
- 28 **Hayashi M**, Ohkura T, Inaba N. Elevation of serum macrophage colony-stimulating factor before the clinical manifestations of preeclampsia. *Am J Obstet Gynecol* 2003; **189**: 1356-1360 [PMID: 14634568 DOI: 10.1067/S0002-9378(03)00674-4]
- 29 **Hayashi M**, Ohkura T, Inaba N. Increased levels of serum macrophage colony-stimulating factor before the onset of preeclampsia. *Horm Metab Res* 2003; **35**: 588-592 [PMID: 14605992 DOI: 10.1055/s-2003-43504]
- 30 **Krishnaswamy G**, Kelley J, Yerra L, Smith JK, Chi DS. Human endothelium as a source of multifunctional cytokines: molecular regulation and possible role in human disease. *J Interferon Cytokine Res* 1999; **19**: 91-104 [PMID: 10090394 DOI: 10.1089/107999099314234]
- 31 **Fukumura D**, Xavier R, Sugiura T, Chen Y, Park EC, Lu N, Selig M, Nielsen G, Taksir T, Jain RK, Seed B. Tumor induction of VEGF promoter activity in stromal cells. *Cell* 1998; **94**: 715-725 [PMID: 9753319 DOI: 10.1016/S0092-8674(00)81731-6]
- 32 **Ezaki T**, Ikegami T, Ishida T, Aimitsu S, Mori M, Fujihara M. Significance of thymidine phosphorylase in HCC with chronic liver disease for long-term postoperative recurrence. *J Surg Oncol* 2003; **83**: 173-19; discussion 179 [PMID: 12827687 DOI: 10.1002/jso.10259]
- 33 **Ezaki T**, Ikegami T, Maeda T, Yamada T, Ishida T, Hashizume M, Maehara Y. Prognostic value of thymidine phosphorylase activity in liver tissue adjacent to hepatocellular carcinoma. *Int J Clin Oncol* 2005; **10**: 171-176 [PMID: 15990964 DOI: 10.1007/s10147-005-0488-7]
- 34 **Yu D**, Zhuang L, Sun X, Chen J, Yao Y, Meng K, Ding Y. Particular distribution and expression pattern of endoglin (CD105) in the liver of patients with hepatocellular carcinoma. *BMC Cancer* 2007; **7**: 122 [PMID: 17608955 DOI: 10.1186/1471-2407-7-122]
- 35 **Liu K**, He X, Lei XZ, Zhao LS, Tang H, Liu L, Lei BJ. Pathomorphological study on location and distribution of Kupffer cells in hepatocellular carcinoma. *World J Gastroenterol* 2003; **9**: 1946-1949 [PMID: 12970881 DOI: 10.3748/wjg.v9.i9.1946]
- 36 **Cervello M**, Foderà D, Florena AM, Soresi M, Tripodo C, D' Alessandro N, Montalto G. Correlation between expression of cyclooxygenase-2 and the presence of inflammatory cells in human primary hepatocellular carcinoma: possible role in tumor promotion and angiogenesis. *World J Gastroenterol* 2005; **11**: 4638-4643 [PMID: 16094702 DOI: 10.3748/wjg.v11.i30.4638]
- 37 **Mehlen P**, Puisieux A. Metastasis: a question of life or death. *Nat Rev Cancer* 2006; **6**: 449-458 [PMID: 16723991 DOI: 10.1038/nrc1886]
- 38 **Bockhorn M**, Jain RK, Munn LL. Active versus passive mechanisms in metastasis: do cancer cells crawl into vessels, or are they pushed? *Lancet Oncol* 2007; **8**: 444-448 [PMID: 17466902 DOI: 10.1016/S1470-2045(07)70140-7]
- 39 **Gupta GP**, Massagué J. Cancer metastasis: building a framework. *Cell* 2006; **127**: 679-695 [PMID: 17110329 DOI: 10.1016/j.cell.2006.11.001]
- 40 **Kaplan RN**, Riba RD, Zacharoulis S, Bramley AH, Vincent L, Costa C, MacDonald DD, Jin DK, Shido K, Kerns SA, Zhu Z, Hicklin D, Wu Y, Port JL, Altorki N, Port ER, Ruggero D, Shmelkov SV, Jensen KK, Rafii S, Lyden D. VEGFR1-positive haematopoietic bone marrow progenitors initiate the pre-metastatic niche. *Nature* 2005; **438**: 820-827 [PMID: 16341007 DOI: 10.1038/nature04186]

P- Reviewer: Xu Y **S- Editor:** Qi Y **L- Editor:** O'Neill M
E- Editor: Wang CH



Retrospective Study

Slow-pull and different conventional suction techniques in endoscopic ultrasound-guided fine-needle aspiration of pancreatic solid lesions using 22-gauge needles

Jia-Ying Chen, Qing-Yu Ding, Yang Lv, Wen Guo, Fa-Chao Zhi, Si-De Liu, Tian-Ming Cheng

Jia-Ying Chen, Qing-Yu Ding, Wen Guo, Fa-Chao Zhi, Si-De Liu, Tian-Ming Cheng, Guangdong Provincial Key Laboratory of Gastroenterology, Department of Gastroenterology, Nanfang Hospital, Southern Medical University, Guangzhou 510515, Guangdong Province, China

Yang Lv, Department of Gastroenterology, Shenzhen Hospital of Southern Medical University, Shenzhen 518000, Guangdong Province, China

Tian-Ming Cheng, Hui Qiao Medical Center, Nanfang Hospital, Southern Medical University, Guangzhou 510515, Guangdong Province, China

Author contributions: Chen JY and Cheng TM contributed equally to this work; Chen JY and Cheng TM designed the research, analyzed the data and drafted the manuscript; Ding QY and Lv Y collected the data; Guo W, Zhi FC and Liu SD revised the manuscript for important intellectual content; all authors read and approved the final version of the manuscript.

Institutional review board statement: The study was reviewed and approved by the Southern Medical University Institutional Review Board.

Informed consent statement: All patients in this study provided a signed informed consent statement.

Conflict-of-interest statement: Each author certifies that he or she has no commercial associations that might pose a conflict of interest in connection with the submitted article.

Data sharing statement: No additional data are available.

Open-Access: This article is an open-access article which was selected by an in-house editor and fully peer-reviewed by external reviewers. It is distributed in accordance with the Creative Commons Attribution Non Commercial (CC BY-NC 4.0) license, which permits others to distribute, remix, adapt, build upon this work non-commercially, and license their derivative works on different terms, provided the original work is properly cited and the use is non-commercial. See: <http://creativecommons.org/licenses/by-nc/4.0/>

Manuscript source: Unsolicited manuscript

Correspondence to: Dr. Tian-Ming Cheng, Hui Qiao Medical Center, Nanfang Hospital, Southern Medical University, Guangzhou 510515, Guangdong Province, China. chengtm@smu.edu.cn
Telephone: +86-020-61642261
Fax: +86-020-61642494

Received: August 22, 2016

Peer-review started: August 23, 2016

First decision: September 12, 2016

Revised: September 19, 2016

Accepted: September 28, 2016

Article in press: September 28, 2016

Published online: October 21, 2016

Abstract

AIM

To evaluate the cytological diagnostic capacity and sample quality of the slow-pull technique and compare them with different suction techniques.

METHODS

From July 2010 to December 2015, 102 patients with pancreatic solid lesions who underwent endoscopic ultrasound-guided fine-needle aspiration (EUS-FNA) with 22-gauge needles were retrospectively evaluated. EUS-FNA diagnosis was based on a cytological examination, and final diagnosis was based on a comprehensive standard of cytological diagnosis, surgical pathology and clinical or imaging follow-up. Cytological specimens were characterized for cellularity and blood contamination. The cytological diagnostic capacity and sample quality of the slow-pull technique and suction techniques with 5-mL/10-mL/20-mL syringes were analyzed.

RESULTS

Of all of the EUS-FNA procedures, the slow-pull technique and suction techniques with 5-mL/10-mL/20-mL syringes were used in 31, 19, 34 and 18 procedures, respectively. There were significant differences between these four suction techniques in terms of cytological diagnostic accuracy (90.3% *vs* 63.2% *vs* 58.8% *vs* 55.6%, $P = 0.019$), sensitivity (88.2% *vs* 41.7% *vs* 40.0% *vs* 36.4%, $P = 0.009$) and blood contamination (score ≥ 2 for 29.0% *vs* 52.6% *vs* 70.6% *vs* 72.2%, $P = 0.003$). The accuracy and sensitivity of the slow-pull technique were significantly higher than those of the suction techniques using 5-mL ($P = 0.03$, $P = 0.014$), 10-mL ($P = 0.005$; $P = 0.006$) and 20-mL syringes ($P = 0.01$, $P = 0.01$). Blood contamination was significantly lower in the slow-pull technique than in the suction techniques with 10-mL ($P = 0.001$) and 20-mL syringes ($P = 0.007$).

CONCLUSION

The slow-pull technique may increase the cytological diagnostic accuracy and sensitivity with slight blood contamination during EUS-FNA when using 22-gauge needles for solid pancreatic masses.

Key words: Endoscopic ultrasound-guided fine-needle aspiration; Pancreatic solid lesion; Slow-pull technique; Suction; Negative pressure; Cytology

© The Author(s) 2016. Published by Baishideng Publishing Group Inc. All rights reserved.

Core tip: Endoscopic ultrasound-guided fine-needle aspiration (EUS-FNA) is an essential technique for obtaining tissue diagnoses for pancreatic masses, and application of suction is one of the potential influencing factors of EUS-FNA. The slow-pull technique has recently emerged as a new sampling technique in EUS-FNA of pancreatic masses. We found that the slow-pull technique using 22-gauge needles may increase the cytological diagnostic accuracy and sensitivity and result in only slight blood contamination in EUS-FNA of pancreatic solid lesions.

Chen JY, Ding QY, Lv Y, Guo W, Zhi FC, Liu SD, Cheng TM. Slow-pull and different conventional suction techniques in endoscopic ultrasound-guided fine-needle aspiration of pancreatic solid lesions using 22-gauge needles. *World J Gastroenterol* 2016; 22(39): 8790-8797 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v22/i39/8790.htm> DOI: <http://dx.doi.org/10.3748/wjg.v22.i39.8790>

INTRODUCTION

Endoscopic ultrasound-guided fine-needle aspiration (EUS-FNA) was first reported in 1992 and currently is applied as an essential technique to obtain tissue diagnoses for pancreatic masses^[1-3]. A recent meta-

analysis study concluded that the pooled sensibility and specificity of pancreatic EUS-FNA were 86.8% and 95.8%, respectively^[4]. Although the diagnostic accuracy is generally high, the optimal method for EUS-FNA has not been established. Various factors are potential influencing factors, such as the experience of the endosonographer, the size and location of the target lesion, the size and type of needle, the presence of a stylet, the application of suction, and the availability of onsite cytopathology^[5-7].

Application of suction during EUS-FNA has been controversial because it may result in damage to the cell structure and contamination of the blood while increasing cell quantity^[8-10]. Different levels of negative pressure were applied to seek a balanced point between specimen quantity and quality. Some studies suggested that low or no suction reduced the contamination from blood and improved specimen quality^[11-15]. Some suggested that high negative pressure obtained more tissue specimens for histological examination and improved diagnostic accuracy^[16,17]. However, there is no consensus on the optimal suction technique. The slow-pull technique has been recently introduced as a new sampling technique in EUS-FNA of pancreatic solid lesions^[18,19]. Different from conventional suction techniques using a syringe, the slow-pull technique provides minimum negative pressure by removing the stylet from the needle slowly and continuously^[20]. Several studies have found that the slow-pull technique could obtain high-quality specimens with unsubstantial blood contamination when combined with a novel core biopsy needle (EchoTip ProCore, Cook Medical, Bloomington, IN, United States)^[21-23]. These studies mainly focused on the biopsy needles; however, regular needles, such as 22-gauge needles, and cytological examinations are more frequently used in most institutions.

Therefore, to evaluate the diagnostic value of the slow-pull technique and to explore the optimal suction technique, we retrospectively analyzed the cytological diagnostic capacity and specimen quality of the slow-pull technique and different conventional suction techniques during EUS-FNA of pancreatic solid lesions using 22-gauge needles.

MATERIALS AND METHODS

Patients

We retrospectively analyzed all patients who underwent EUS-FNA for solid pancreatic lesions at our institution between July 2010 and December 2015. Inclusion criteria were as follows: primary pancreatic solid lesions, usage of 22-gauge needles, application of cytological examination, and a ≥ 6 -mo radiologic or clinical follow-up in patients diagnosed with benign lesions. Exclusion criteria were as follows: pancreatic cystic lesion or extra-pancreatic lesion, usage of 19-gauge or other needles, combination use

of different suction techniques, and lack of follow-up data. This study was conducted in accordance with the Declaration of Helsinki.

EUS-FNA procedure

All procedures were performed by one of two experienced endosonographers. Patients were conscious but sedated with intravenous propofol or a combination of intravenous meperidine and diazepam in the left lateral position. A curved linear array echoendoscope (EG-530UR; Fujinon Medical Systems, or GF-UE260; Olympus Medical Systems) was used to assess the pancreatic lesion. Once an optimal puncture route was determined, EUS-FNA was then performed with a curvilinear echoendoscope (EG-530UT; Fujinon Medical Systems, or GF-UCT260; Olympus Medical Systems) and a 22-gauge needle (Echotip Ultra; Wilson-Cook, Tokyo, Japan, or Expect; Boston Scientific, Natick, MA, United States); the stylet was inserted into the target lesion guided by real-time EUS imaging. In the slow-pull technique, the stylet was slowly withdrawn from the needle while 10-20 to-and-fro movements within the target lesion were performed. In conventional suction techniques, the stylet is completely removed before suction using a 5-mL/10-mL/20-mL syringe that is applied while 10-20 to-and-fro movements are performed within the target lesion.

After EUS-FNA, the aspirated material was completely expelled onto glass slides by reinsertion of the stylet and flushing with air. The aspirated materials were smeared on glass slides and fixed in an absolute alcohol solution for cytological examinations. Smeared slides were prepared by endosonographers trained in the appropriate slide preparation techniques. No on-site cytopathology examination was performed at our institution. EUS-FNA diagnosis was based on cytological examination.

Cytological specimen analysis

All cytological reports were retrospectively reviewed. Diagnosis of cytological examination was recorded in reports and categorized into the following five groups: benign or negative for malignant, atypical, suspicious for malignant, malignant, and inadequate for diagnosis. Cytological specimens were characterized for cellularity and bloodiness, and the semiquantitative scores were routinely recorded in the pathological reports. Cellularity was graded into 4 levels: 0, none; 1, few aggregates; 2, fair cellularity; 3, abundant cellularity. All types of cells, including tumor cells, were calculated in the cellularity scores. The contamination from blood was also graded in 4 levels: 0, none; 1, little; 2, moderate; 3, abundant.

Final diagnosis

The composite standard for each lesion was based on the EUS-FNA diagnosis, surgical pathology and a

clinical/imaging follow-up. Lesions were considered benign if surgical pathology confirmed a benign condition or if a lack of deterioration was noted on a ≥ 6 -mo follow-up. Lesions were considered malignant if surgical pathological diagnosis or EUS-FNA diagnosis (based on cytological examination with a compatible clinical course) was positive for malignancy or if there was clinical progression or an increase in the lesion size (or both) during follow-up.

Statistical analysis

All statistical analyses were processed using SPSS software (version 21.0; IBM SPSS Statistics for Windows, Armonk, NY, United States). Categorical variables are presented as frequencies, and continuous variables are presented as medians and ranges. We calculated descriptive statistics for sensitivity, specificity, accuracy, positive predictive value (PPV), and negative predictive value (NPV). We considered the diagnoses of malignant, atypical and suspicious as true positive, negative for malignant as true negative, and inadequate as false negative. Statistical analysis was undertaken using the χ^2 test, Fisher's exact test and the Kruskal-Wallis *H* test in univariate analyses. Logistic regression analysis was performed in a multivariate analysis, using lesion size (≤ 30 mm vs > 30 mm), endoscopist (endoscopist 2 vs endoscopist 1), lesion location (body or tail vs uncinate or head), needle passes (≤ 3 vs > 3) and suction techniques (slow-pull vs 5-mL vs 10-mL vs 20-mL) as potential predictive factors. Statistical tests were considered significant when the corresponding 2-sided *P* value was less than 0.05.

RESULTS

Patient characteristics

From July 2010 to December 2015, 139 patients underwent EUS-FNA for pancreatic lesions. A total of 37 patients were excluded: 9 patients with a pancreatic cystic lesion, 9 patients on whom a 19-gauge needle was used, 1 patient on whom a 25-gauge needle was used, 16 patients on whom combinations of different suction techniques were used, and 2 patients who were not followed-up. Finally, 102 patients with pancreatic solid lesions were included. The baseline characteristics and final diagnoses are listed in Table 1. The final diagnoses were malignant in 58 cases (56.9%) and benign in 44 cases (43.1%). Final diagnoses were confirmed from EUS-FNA specimens for 19 cases and surgically resected specimens for 30 cases; the remaining 53 cases were confirmed from the radiologic or clinical follow-up. The mean follow-up was 12 months (range: 1-24 mo). Of the 102 procedures, adverse events occurred in 6 cases (5.88%). Four patients developed mild pancreatitis, and 2 patients developed fever, but all of these adverse events were successfully treated with conservative therapy.

Table 1 Patient characteristics and final diagnosis of endoscopic ultrasound-guided fine-needle aspiration (*n* = 102)

Characteristic	Value
Median age (range), yr	53 (19-82)
Sex, male:female, <i>n</i>	67:35
Median tumor size (range), mm	34 (8-89)
Endoscopist, endoscopist 1:endoscopist 2, <i>n</i>	45:57
Location, uncinete or head:body or tail, <i>n</i>	59:43
Median number of passes, <i>n</i>	3 (1-5)
Final diagnosis, <i>n</i>	
Malignant	58
Pancreatic cancer	53
Neuroendocrine tumor, malignant	2
Solid-pseudopapillary neoplasm, malignant	3
Benign	44
Chronic pancreatitis	23
Autoimmune pancreatitis	7
Nonspecific inflammation	10
Cystadenoma, benign	2
Neuroendocrine tumor, benign	1
Benign lymphangioma	1

Cytological diagnostic capacity and sample quality

Cytological examinations were performed in all procedures. Of these cases, 61 were diagnosed as benign lesions, 19 as malignant lesions, 10 as atypical lesions, and 7 as suspicious lesions. The remaining 5 cases were inadequate for diagnosis: 3 of which were eventually confirmed as malignant masses from surgically resected specimens, and 2 were confirmed as benign masses from clinical follow-up. Of all of the EUS-FNA procedures, the slow-pull technique and suction techniques with 5-mL/10-mL/20-mL syringes were used in 31, 19, 34 and 18 procedures, respectively. There were no significant differences between these four suction techniques in terms of patient age, sex, endoscopists, lesion location, and number of passes; only tumor size showed a significant difference ($P = 0.031$) (Table 2).

We compared the cytological diagnostic capacities of the four suction techniques. The cytological diagnostic capacities and cytological specimen qualities of the different suction methods are shown in Table 3. The cytological diagnostic accuracy (90.3% vs 63.2% vs 58.8% vs 55.6%, $P = 0.019$), sensitivity (88.2% vs 41.7% vs 40.0% vs 36.4%, $P = 0.009$) and blood contamination (score ≥ 2 for 29.0% vs 52.6% vs 70.6% vs 72.2%, $P = 0.003$) of the four suction methods were statistically significantly different (Figure 1). Thus, cytological diagnostic accuracy, sensitivity and blood contamination between the slow-pull technique and the conventional suction techniques were further compared (Table 4). The cytological diagnostic accuracy and sensitivity of the slow-pull technique were significantly higher than suction techniques with 5-mL ($P = 0.03$, $P = 0.014$), 10-mL ($P = 0.005$, $P = 0.006$) and 20-mL syringes ($P = 0.01$, $P = 0.01$), and the blood contamination with the slow-pull technique was lower than that in the suction

Table 2 Baseline characteristics of the different suction techniques

	Slow-pull (<i>n</i> = 31)	5-mL (<i>n</i> = 19)	10-mL (<i>n</i> = 34)	20-mL (<i>n</i> = 18)	<i>P</i> value ¹
Median age (range), yr	56 (20-82)	54 (38-71)	51 (19-77)	49 (26-73)	0.949
Sex, male: female, <i>n</i>	17:14	11:8	25:9	14:4	0.238
Median lesion size (range), mm	25 (8-72)	38 (10-65)	36 (17-89)	35 (16-62)	0.031 ²
Endoscopist, endoscopist 1: endoscopist 2, <i>n</i>	13:18	11:8	11:23	10:8	0.223
Location, uncinate or head:body or tail, <i>n</i>	19:12	11:8	16:18	13:5	0.348
Median number of passes, <i>n</i>	3 (1-4)	3 (2-5)	3 (1-5)	3 (2-5)	0.280

¹ χ^2 test for categorical variables and Kruskal-Wallis *H* test for continuous variables; ²Statistically significant.

technique with the 10-mL ($P = 0.001$) and 20-mL syringes ($P = 0.007$).

Univariate and multivariate analyses

Both univariate and multivariate analyses were performed to define factors associated with the cytological diagnostic accuracy of EUS-FNA (Table 5). Suction techniques were significant factors in both the univariate ($P = 0.019$) and multivariate analyses [$P = 0.005$, odds ratio (OR) (95%CI) = 1.91 (1.21-3.00)].

DISCUSSION

There is still some dispute regarding the role of suction during EUS-FNA. Generally, the suction technique with a 10-mL syringe is used to increase the specimen cellularity. However, this procedure may also increase the risk of blood contamination and structural damage. The value of suction in EUS-FNA was first evaluated for lymph node sampling. In an experimental study, Bhutani *et al*^[11] found that continuous suction with smaller syringes (5-10 mL) provided optimal cellularity and better specimen quality in EUS-FNA of mediastinal lymph nodes. Subsequently, Wallace *et al*^[12] performed a randomized controlled trial comparing sampling techniques with or without suction in EUS-FNA of lymph nodes and concluded that the technique with suction increased the cellularity but worsened the specimen bloodiness. Different from lymph nodes, pancreatic lesions are rich in fibrous tissue, with fewer parenchymal cells, which increases the complexity and difficulty involved in obtaining a precise diagnosis for pancreatic lesions^[24-26]. Therefore, the European

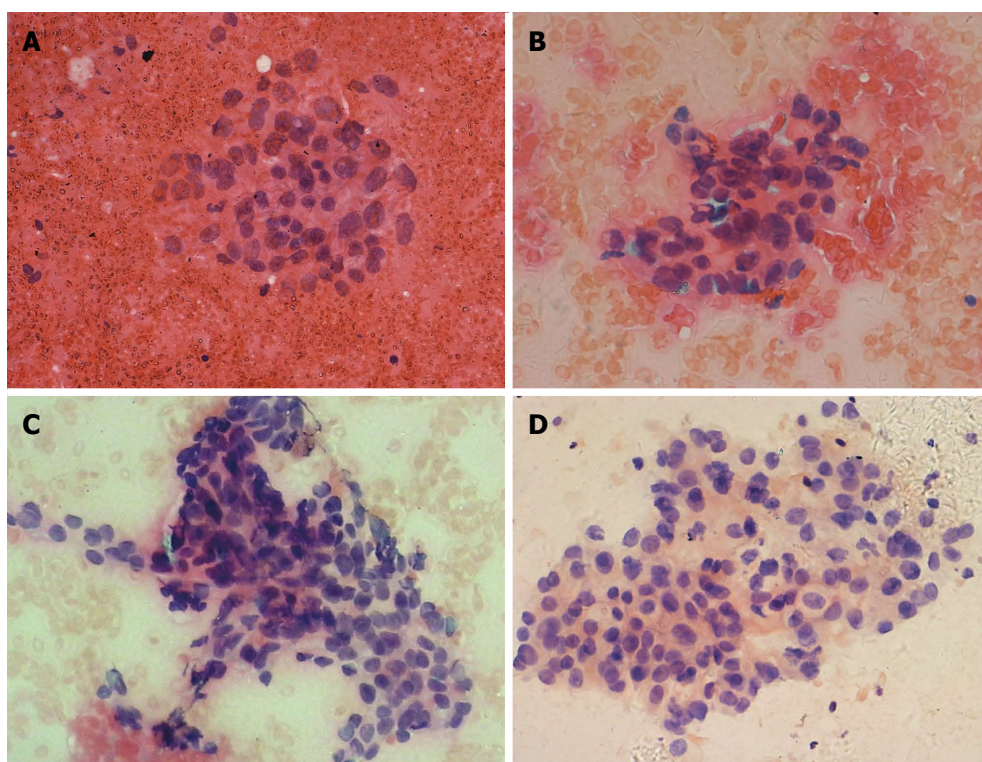


Figure 1 Cytological assessments of the samples obtained by endoscopic ultrasound-guided fine-needle aspiration using four suction techniques (HE, orig. mag. $\times 40$). A: The suction technique with a 20-mL syringe received a score of 2 for cellularity and a score of 3 for blood contamination; B: The suction technique with a 10-mL syringe received a score of 2 for cellularity and a score of 3 for blood contamination; C: The suction technique with a 5-mL syringe received a score of 3 for cellularity and a score of 2 for blood contamination; D: The slow-pull technique received a score of 3 for cellularity and a score of 1 for blood contamination.

Table 3 Cytological diagnostic capacity and specimen quality of the different suction techniques

	Slow-pull ($n = 31$)	5-mL ($n = 19$)	10-mL ($n = 34$)	20-mL ($n = 18$)	P value ¹
Cytological diagnostic capacity					
Accuracy	28/31 (90.3%)	12/19 (63.2%)	20/34 (58.8%)	10/18 (55.6%)	0.019 ²
Sensitivity	15/17 (88.2%)	5/12 (41.7%)	8/20 (40.0%)	4/11 (36.4%)	0.009 ²
Specificity	13/14 (92.9%)	7/7 (100%)	12/14 (85.7%)	6/7 (85.7%)	0.914
PPV	15/16 (93.8%)	5/5 (100%)	8/10 (80.0%)	4/5 (80.0%)	0.542
NPV	13/15 (86.7%)	7/14 (50.0%)	12/24 (50.0%)	6/13 (46.2%)	0.079
Cytological specimen quality					
Cellularity score ≥ 2	22/31 (71.0%)	11/19 (57.9%)	20/34 (58.8%)	13/18 (72.2%)	0.598
Blood contamination score ≥ 2	9/31 (29.0%)	10/19 (52.6%)	24/34 (70.6%)	13/18 (72.2%)	0.003 ²

¹Fisher's exact test for "specificity" and "PPV"; χ^2 test for the rest; ²Statistically significant. PPV: Positive predictive value; NPV: Negative predictive value.

Table 4 Comparisons between the slow-pull technique and conventional suction techniques in terms of cytological diagnostic accuracy, sensitivity and blood contamination

	5-mL	10-mL	20-mL
	<i>vs</i> slow-pull, P value ¹		
Accuracy	0.03 ²	0.005 ²	0.010 ²
Sensitivity	0.014 ²	0.006 ²	0.010 ²
Blood contamination score ≥ 2	0.135	0.001 ²	0.007 ²

¹Fisher's exact test; ²Statistically significant.

Society of Gastrointestinal Endoscopy technical guideline recommended using suction for EUS-FNA of solid masses/cystic lesions but not for EUS-FNA

of lymph nodes^[27]. However, in a recent randomized controlled trial, Lee *et al*^[28] found that using suction during EUS-FNA worsened specimen bloodiness, while the diagnostic yield and cellularity were improved.

In recent years, the slow-pull technique has been applied to EUS-FNA of pancreatic solid lesions, but its efficacy has not yet been clarified. In a retrospective study, Nakai *et al*^[18] reported that the slow-pull technique was associated with less contamination from blood and potentially increased the diagnostic yield in comparison to the suction technique. Both 22- and 25-gauge needles were used in this study, and different needles may impact the diagnostic yield. Therefore, Kin *et al*^[19] performed a prospective study to evaluate the value of the slow-pull technique

Table 5 Univariate and multivariate analyses of factors affecting cytological diagnostic accuracy

Variable		Accuracy	Univariate <i>P</i> value ¹	Multivariate <i>P</i> value ² OR (95%CI)	
Lesion size	≤ 30 mm	74.4% (32/43)	0.282	0.603	1.29 (0.50-3.36)
	> 30 mm	64.4% (38/59)			
Endoscopist	Endoscopist 2	71.9% (41/57)	0.419	0.367	1.52 (0.61-3.74)
	Endoscopist 1	64.4% (29/45)			
Location	Body or tail	69.8% (30/43)	0.832	0.687	1.21 (0.48-3.04)
	Uncinate or head	67.8% (40/59)			
Needle passes	≤ 3	69.7% (46/66)	0.753	0.233	1.81 (0.68-4.79)
	> 3	66.7% (24/36)			
Suction techniques	Slow-pull	90.3% (28/31)	0.019 ³	0.005 ³	1.91 (1.21-3.00)
	5-mL	63.2% (12/19)			
	10-mL	58.8% (20/34)			
	20-mL	55.6% (10/18)			

¹ χ^2 test; ²Logistic regression analysis; ³Statistically significant.

with 22-gauge needles in EUS-FNA and found that this technique could obtain adequate, high-quality, and unsubstantially blood-contaminated samples. However, the sample size in this study was so limited that it could not compare the diagnostic efficacy or sample quality between the slow-pull technique and traditional suction techniques. To provide more evidence for endosonographers when choosing a suction technique, our study not only analyzed the cytological diagnostic capacity and sample quality of the slow-pull technique with 22-gauge needles but also continued the comparison with different traditional suction techniques.

When comparing the cytological diagnostic capacities and specimen qualities of the four different suction techniques, we found that the degree of negative pressure, the level of blood contamination and the diagnostic accuracy were closely related. With the rise of negative pressure, the cytological diagnostic accuracy tended to decrease, and the blood contamination increased, possibly because the needle is filled with blood tissue while using a higher degree of negative pressure, which increases the puncture difficulty and adds blood contamination. In a randomized controlled trial with 90 pancreatic lesions, Kudo *et al.*^[17] showed that EUS-FNA with high negative pressure obtained more contamination from blood than low negative pressure. Therefore, application of a lower degree of negative pressure during EUS-FNA may help decrease the blood contamination.

The slow-pull technique is a new suction technique with a very weak suction force. An experimental study showed that the suction force produced by the slow-pull technique with a 22-gauge needle was less than 2.0 kPa, which is significantly lower than those of the suction techniques with 10-mL and 20-mL syringes^[29]. Such a slight suction force can help obtain enough specimens with minimal blood contamination. Our research showed that the cytological diagnostic accuracy and sensitivity of the slow-pull technique were 90.3% and 88.2%, respectively, which were significantly higher than the conventional suction

techniques with 5-mL/10-mL/20-mL syringes. When evaluating the quality of cytological specimens, we found that blood contamination scores of 2 or 3 when using the slow-pull technique were noted 29.0% of the time, which was significantly lower than the blood contamination scores noted when using conventional suction techniques with 10-mL and 20-mL syringes. Therefore, the slow-pull technique has a unique advantage in improving the cytological diagnostic capacity and specimen quality of EUS-FNA for pancreatic masses.

There were some limitations in the present study. First, biases are inevitable in a retrospective analysis, and the lesion sizes were different in these four suction techniques. The lesion size associated with the slow-pull technique was smaller, which might affect the diagnostic accuracy. However, there was no significant difference in the cytological diagnostic accuracy between different lesion sizes. Moreover, suction techniques were still statistically significant factors in multivariate analysis after adjusting for tumor size. Second, on-site cytological evaluation was not available in our study, although it is beneficial for EUS-FNA^[30,31]. On-site cytopathological evaluation could reduce the need for repeat punctures and could improve the specimen quality rate, but it could also prolong the procedure time and increase the working time of the pathologists. Therefore, many institutions, like ours, have not adopted on-site cytological evaluation^[32-34].

In conclusion, our retrospective study showed that the slow-pull technique might increase the cytological diagnostic accuracy and sensitivity with slight blood contamination during EUS-FNA for solid pancreatic masses using 22-gauge needles. Additional studies should be performed with a prospective randomized design to better understand the efficacy of the slow-pull technique.

COMMENTS

Background

Endoscopic ultrasound-guided fine-needle aspiration (EUS-FNA) has become

an essential technique to obtain tissue diagnoses for pancreatic masses. However, application of suction during EUS-FNA is controversial because it may result in damage to the cell structure and contamination of the blood while increasing cell quantity.

Research frontiers

The slow-pull technique has recently been introduced as a new sampling technique in EUS-FNA of pancreatic solid lesions. In contrast to conventional suction techniques using a syringe, the slow-pull technique provides minimum negative pressure by slowly and continuously removing the stylet from the needle. Such a slight suction force may help obtain sufficient specimens with minimal blood contamination.

Innovations and breakthroughs

The cytological diagnostic accuracy and sensitivity of the slow-pull technique were 90.3% and 88.2%, respectively, which were significantly higher than those of the conventional suction techniques with 5-mL/10-mL/20-mL syringes. Blood contamination was lower in the slow-pull technique than in the suction techniques with 10-mL and 20-mL syringes.

Applications

The study supports the idea that the slow-pull technique using 22-gauge needles may increase the cytological diagnostic accuracy and sensitivity, and result in only slight blood contamination in EUS-FNA of pancreatic solid lesions.

Terminology

Slow-pull technique: A new sampling technique that can provide minimum negative pressure by slowly and continuously removing the stylet from the needle.

Peer-review

This is an interesting study about the slow-pull and different conventional suction techniques in EUS-FNA of pancreatic solid lesions using 22-gauge needles. EUS-FNA using a slow-pull technique has recently emerged as a new method to obtain tissue diagnosis for pancreatic diseases. However, the optimal suction technique has not been clearly established, and the efficacy of the slow-pull technique remains unclear. In this study, the authors evaluated the cytological diagnostic capacity and sample quality of the slow-pull technique and compare it with different conventional suction techniques.

REFERENCES

- Vilmann P**, Jacobsen GK, Henriksen FW, Hancke S. Endoscopic ultrasonography with guided fine needle aspiration biopsy in pancreatic disease. *Gastrointest Endosc* 1992; **38**: 172-173 [PMID: 1568614 DOI: 10.1016/S0016-5107(92)70385-X]
- Weston BR**, Bhutani MS. Optimizing Diagnostic Yield for EUS-Guided Sampling of Solid Pancreatic Lesions: A Technical Review. *Gastroenterol Hepatol* (N Y) 2013; **9**: 352-363 [PMID: 23935542]
- Cosgrove ND**, Yan L, Siddiqui A. Preoperative endoscopic ultrasound-guided fine needle aspiration for diagnosis of pancreatic cancer in potentially resectable patients: Is this safe? *Endosc Ultrasound* 2015; **4**: 81-84 [PMID: 26020040 DOI: 10.4103/2303-9027.156708]
- Puli SR**, Bechtold ML, Buxbaum JL, Eloubeidi MA. How good is endoscopic ultrasound-guided fine-needle aspiration in diagnosing the correct etiology for a solid pancreatic mass?: A meta-analysis and systematic review. *Pancreas* 2013; **42**: 20-26 [PMID: 23254913 DOI: 10.1097/MPA.0b013e3182546e79]
- Hijioka S**, Hara K, Mizuno N, Imaoka H, Bhatia V, Mekky MA, Yoshimura K, Yoshida T, Okuno N, Hieda N, Tajika M, Tanaka T, Ishihara M, Yatabe Y, Shimizu Y, Niwa Y, Yamao K. Diagnostic performance and factors influencing the accuracy of EUS-FNA of pancreatic neuroendocrine neoplasms. *J Gastroenterol* 2016; **51**: 923-930 [PMID: 26768605 DOI: 10.1007/s00535-016-1164-6]
- Berzosa M**, Villa N, El-Serag HB, Sejal DV, Patel KK. Comparison of endoscopic ultrasound guided 22-gauge core needle with standard 25-gauge fine-needle aspiration for diagnosing solid pancreatic lesions. *Endosc Ultrasound* 2015; **4**: 28-33 [PMID: 25789281 DOI: 10.4103/2303-9027.151320]
- Petrone MC**, Arcidiacono PG. Basic technique in endoscopic ultrasound-guided fine needle aspiration for solid lesions: How many passes? *Endosc Ultrasound* 2014; **3**: 22-27 [PMID: 24949407 DOI: 10.4103/2303-9027.124310]
- Varadarajulu S**, Fockens P, Hawes RH. Best practices in endoscopic ultrasound-guided fine-needle aspiration. *Clin Gastroenterol Hepatol* 2012; **10**: 697-703 [PMID: 22475740 DOI: 10.1016/j.cgh.2012.03.017]
- Wani S**. Basic techniques in endoscopic ultrasound-guided fine-needle aspiration: Role of a stylet and suction. *Endosc Ultrasound* 2014; **3**: 17-21 [PMID: 24949406 DOI: 10.4103/2303-9027.123008]
- Tadic M**, Stoos-Veic T, Kusec R. Endoscopic ultrasound guided fine needle aspiration and useful ancillary methods. *World J Gastroenterol* 2014; **20**: 14292-14300 [PMID: 25339816 DOI: 10.3748/wjg.v20.i39.14292]
- Bhutani MS**, Suryaprasad S, Moezzi J, Seabrook D. Improved technique for performing endoscopic ultrasound guided fine needle aspiration of lymph nodes. *Endoscopy* 1999; **31**: 550-553 [PMID: 10533740 DOI: 10.1055/s-1999-125]
- Wallace MB**, Kennedy T, Durkalski V, Eloubeidi MA, Etamad R, Matsuda K, Lewin D, Van Velse A, Hennessey W, Hawes RH, Hoffman BJ. Randomized controlled trial of EUS-guided fine needle aspiration techniques for the detection of malignant lymphadenopathy. *Gastrointest Endosc* 2001; **54**: 441-447 [PMID: 11577304 DOI: 10.1067/mge.2001.117764]
- Mohammad Alizadeh AH**, Hadizadeh M, Padashi M, Shahbaazi S, Molaei M, Shariatpanahi ZV. Comparison of two techniques for endoscopic ultrasonography fine-needle aspiration in solid pancreatic mass. *Endosc Ultrasound* 2014; **3**: 174-178 [PMID: 25184124 DOI: 10.4103/2303-9027.138790]
- Gimeno-Garcia AZ**, Elwassief A, Paquin SC, Gariépy G, Sahai AV. Randomized controlled trial comparing stylet-free endoscopic ultrasound-guided fine-needle aspiration with 22-G and 25-G needles. *Dig Endosc* 2014; **26**: 467-473 [PMID: 24877242 DOI: 10.1111/den.12204]
- Paquin SC**, Sahai AV. Techniques for EUS-guided FNA cytology. *Gastrointest Endosc Clin N Am* 2014; **24**: 71-81 [PMID: 24215761 DOI: 10.1016/j.giec.2013.08.007]
- Larghi A**, Noffsinger A, Dye CE, Hart J, Waxman I. EUS-guided fine needle tissue acquisition by using high negative pressure suction for the evaluation of solid masses: a pilot study. *Gastrointest Endosc* 2005; **62**: 768-774 [PMID: 16246694 DOI: 10.1016/j.gie.2005.05.014]
- Kudo T**, Kawakami H, Hayashi T, Yasuda I, Mukai T, Inoue H, Katanuma A, Kawakubo K, Ishiwatari H, Doi S, Yamada R, Maguchi H, Isayama H, Mitsuhashi T, Sakamoto N; Japan EUS-FNA Negative Pressure Suction Study Group. High and low negative pressure suction techniques in EUS-guided fine-needle tissue acquisition by using 25-gauge needles: a multicenter, prospective, randomized, controlled trial. *Gastrointest Endosc* 2014; **80**: 1030-7.e1 [PMID: 24890422 DOI: 10.1016/j.gie.2014.04.012]
- Nakai Y**, Isayama H, Chang KJ, Yamamoto N, Hamada T, Uchino R, Mizuno S, Miyabayashi K, Yamamoto K, Kawakubo K, Kogure H, Sasaki T, Hirano K, Tanaka M, Tada M, Fukayama M, Koike K. Slow pull versus suction in endoscopic ultrasound-guided fine-needle aspiration of pancreatic solid masses. *Dig Dis Sci* 2014; **59**: 1578-1585 [PMID: 24429514 DOI: 10.1007/s10620-013-3019-9]
- Kin T**, Katanuma A, Yane K, Takahashi K, Osanai M, Takaki R, Matsumoto K, Gon K, Matsumori T, Tomonari A, Maguchi H, Shinohara T, Nojima M. Diagnostic ability of EUS-FNA for pancreatic solid lesions with conventional 22-gauge needle using the slow pull technique: a prospective study. *Scand J Gastroenterol* 2015; **50**: 900-907 [PMID: 25732902 DOI: 10.3109/00365521.2014.983155]
- Matsubayashi H**, Matsui T, Yabuuchi Y, Imai K, Tanaka M,

- Kakushima N, Sasaki K, Ono H. Endoscopic ultrasonography guided-fine needle aspiration for the diagnosis of solid pancreaticobiliary lesions: Clinical aspects to improve the diagnosis. *World J Gastroenterol* 2016; **22**: 628-640 [PMID: 26811612 DOI: 10.3748/wjg.v22.i2.628]
- 21 **Wang J**, Wu X, Yin P, Guo Q, Hou W, Li Y, Wang Y, Cheng B. Comparing endoscopic ultrasound (EUS)-guided fine needle aspiration (FNA) versus fine needle biopsy (FNB) in the diagnosis of solid lesions: study protocol for a randomized controlled trial. *Trials* 2016; **17**: 198 [PMID: 27071386 DOI: 10.1186/s13063-016-1316-2]
 - 22 **Iwashita T**, Nakai Y, Samarasekera JB, Park DH, Zhang Z, Gu M, Lee JG, Chang KJ. High single-pass diagnostic yield of a new 25-gauge core biopsy needle for EUS-guided FNA biopsy in solid pancreatic lesions. *Gastrointest Endosc* 2013; **77**: 909-915 [PMID: 23433596 DOI: 10.1016/j.gie.2013.01.001]
 - 23 **Iglesias-Garcia J**, Poley JW, Larghi A, Giovannini M, Petrone MC, Abdulkader I, Monges G, Costamagna G, Arcidiacono P, Biermann K, Rindi G, Bories E, Doglioni C, Bruno M, Dominguez-Muñoz JE. Feasibility and yield of a new EUS histology needle: results from a multicenter, pooled, cohort study. *Gastrointest Endosc* 2011; **73**: 1189-1196 [PMID: 21420083 DOI: 10.1016/j.gie.2011.01.053]
 - 24 **Bachem MG**, Schünemann M, Ramadani M, Siech M, Beger H, Buck A, Zhou S, Schmid-Kotsas A, Adler G. Pancreatic carcinoma cells induce fibrosis by stimulating proliferation and matrix synthesis of stellate cells. *Gastroenterology* 2005; **128**: 907-921 [PMID: 15825074 DOI: 10.1053/j.gastro.2004.12.036]
 - 25 **Dumonceau JM**, Polkowski M, Larghi A, Vilman P, Giovannini M, Frossard JL, Heresbach D, Pujol B, Fernández-Esparrach G, Vazquez-Sequeiros E, Ginès A; European Society of Gastrointestinal Endoscopy. Indications, results, and clinical impact of endoscopic ultrasound (EUS)-guided sampling in gastroenterology: European Society of Gastrointestinal Endoscopy (ESGE) Clinical Guideline. *Endoscopy* 2011; **43**: 897-912 [PMID: 21842456 DOI: 10.1055/s-0030-1256754]
 - 26 **Iglesias-Garcia J**, Lariño-Noia J, Domínguez-Muñoz JE. When to puncture, when not to puncture: Pancreatic masses. *Endosc Ultrasound* 2014; **3**: 91-97 [PMID: 24955338 DOI: 10.4103/2303-9027.123007]
 - 27 **Polkowski M**, Larghi A, Weynand B, Boustière C, Giovannini M, Pujol B, Dumonceau JM; European Society of Gastrointestinal Endoscopy (ESGE). Learning, techniques, and complications of endoscopic ultrasound (EUS)-guided sampling in gastroenterology: European Society of Gastrointestinal Endoscopy (ESGE) Technical Guideline. *Endoscopy* 2012; **44**: 190-206 [PMID: 22180307 DOI: 10.1055/s-0031-1291543]
 - 28 **Lee JK**, Choi JH, Lee KH, Kim KM, Shin JU, Lee JK, Lee KT, Jang KT. A prospective, comparative trial to optimize sampling techniques in EUS-guided FNA of solid pancreatic masses. *Gastrointest Endosc* 2013; **77**: 745-751 [PMID: 23433878 DOI: 10.1016/j.gie.2012.12.009]
 - 29 **Katanuma A**, Itoi T, Baron TH, Yasuda I, Kin T, Yane K, Maguchi H, Yamazaki H, Sano I, Minami R, Manabu SY, Ikarashi S, Osanai M, Takahashi K. Bench-top testing of suction forces generated through endoscopic ultrasound-guided aspiration needles. *J Hepatobiliary Pancreat Sci* 2015; **22**: 379-385 [PMID: 25557010 DOI: 10.1002/jhbp.201]
 - 30 **Klapman JB**, Logrono R, Dye CE, Waxman I. Clinical impact of on-site cytopathology interpretation on endoscopic ultrasound-guided fine needle aspiration. *Am J Gastroenterol* 2003; **98**: 1289-1294 [PMID: 12818271 DOI: 10.1111/j.1572-0241.2003.07472.x]
 - 31 **Iglesias-Garcia J**, Lariño-Noia J, Abdulkader I, Domínguez-Muñoz JE. Rapid on-site evaluation of endoscopic-ultrasound-guided fine-needle aspiration diagnosis of pancreatic masses. *World J Gastroenterol* 2014; **20**: 9451-9457 [PMID: 25071339 DOI: 10.3748/wjg.v20.i28.9451]
 - 32 **Hébert-Magee S**. Basic technique for solid lesions: Cytology, core, or both? *Endosc Ultrasound* 2014; **3**: 28-34 [PMID: 24949408 DOI: 10.4103/2303-9027.123010]
 - 33 **O'Connor K**, Cheriyan DG, Li-Chang HH, Kaloger SE, Garrett J, Byrne MF, Weiss AA, Donnellan F, Schaeffer DF. Gastrointestinal Endoscopic Ultrasound-Guided Fine-Needle Aspiration Biopsy Specimens: Adequate Diagnostic Yield and Accuracy Can Be Achieved without On-Site Evaluation. *Acta Cytol* 2015; **59**: 305-310 [PMID: 26339900 DOI: 10.1159/000439398]
 - 34 **Harada R**, Kato H, Fushimi S, Iwamuro M, Inoue H, Muro S, Sakakihara I, Noma Y, Yamamoto N, Horiguchi S, Tsutsumi K, Okada H, Yamamoto K. An expanded training program for endosonographers improved self-diagnosed accuracy of endoscopic ultrasound-guided fine-needle aspiration cytology of the pancreas. *Scand J Gastroenterol* 2014; **49**: 1119-1123 [PMID: 24896656 DOI: 10.3109/00365521.2014.915051]

P- Reviewer: Hatta W, Zimmerman M **S- Editor:** Gong ZM
L- Editor: Filipodia **E- Editor:** Wang CH



Retrospective Study

Cyclooxygenase-2 expression is associated with initiation of hepatocellular carcinoma, while prostaglandin receptor-1 expression predicts survival

Hao-Jie Yang, Jing-Hang Jiang, Yu-Ting Yang, Xiang-Di Yang, Zhe Guo, Ya-Peng Qi, Feng-Hua Zeng, Ke-Lan Zhang, Neng-Zhi Chen, Bang-De Xiang, Le-Qun Li

Hao-Jie Yang, Jing-Hang Jiang, Ya-Peng Qi, Bang-De Xiang, Le-Qun Li, Department of Hepatobiliary Surgery, Affiliated Tumor Hospital of Guangxi Medical University, Nanning 530021, Guangxi Zhuang Autonomous Region, China

Hao-Jie Yang, Feng-Hua Zeng, Ke-Lan Zhang, Neng-Zhi Chen, Department of General Surgery, The First People's Hospital of Changde, Changde 415000, Hunan Province, China

Jing-Hang Jiang, Department of General Surgery, Second People's Hospital of Jing Men, Jingmen 448000, Hubei Province, China

Yu-Ting Yang, Department of Chemotherapy, Affiliated Tumor Hospital of Guangxi Medical University, Nanning 530021, Guangxi Zhuang Autonomous Region, China

Xiang-Di Yang, Department of Oncology, Chenzhou No.1 People's Hospital, Chenzhou 423000, Hunan Province, China

Zhe Guo, Department of Thyroid and Breast Surgery, Central Hospital of Wuhan, Wuhan 430000, Hubei Province, China

Author contributions: Yang HJ, Jiang JH and Yang YT contributed equally to this work; Xiang BD and Li LQ designed the research; Yang HJ, Jiang JH, Yang YT and Qi YP performed the research; Yang HJ, Yang YT, Zhang KL and Chen NZ evaluated the clinic records and performed the statistical analyses; Yang HJ wrote the manuscript; all authors have read and approved the final manuscript.

Supported by National Natural Science Foundation of China, No. 81260331; and Key Laboratory for High-Incidence Tumor Prevention and Treatment, Ministry of Education, No. GKE2015-ZZ05.

Institutional review board statement: The study was reviewed and approved by the Tumor Hospital of Guangxi Medical

University Institutional Review Board.

Informed consent statement: All study participants, or their legal guardian, provided informed written consent prior to study enrollment.

Conflict-of-interest statement: No potential conflicts of interest relevant to this article were reported.

Data sharing statement: Technical appendix, statistical code, and dataset are available from the corresponding author at cdsdyrmmy01@163.com. Participants gave informed consent for data sharing.

Open-Access: This article is an open-access article which was selected by an in-house editor and fully peer-reviewed by external reviewers. It is distributed in accordance with the Creative Commons Attribution Non Commercial (CC BY-NC 4.0) license, which permits others to distribute, remix, adapt, build upon this work non-commercially, and license their derivative works on different terms, provided the original work is properly cited and the use is non-commercial. See: <http://creativecommons.org/licenses/by-nc/4.0/>

Manuscript source: Unsolicited manuscript

Correspondence to: Dr. Bang-De Xiang, Professor, Department of Hepatobiliary Surgery, Affiliated Tumor Hospital of Guangxi Medical University, 71 Hedi Road, Nanning 530021, Guangxi Zhuang Autonomous Region, China. yhj894924067@163.com
Telephone: +86-771-5310045
Fax: +86-771-5312000

Received: June 15, 2016
Peer-review started: June 16, 2016
First decision: July 29, 2016
Revised: August 15, 2016
Accepted: August 30, 2016
Article in press: August 30, 2016
Published online: October 21, 2016

Abstract

AIM

To determine whether cyclooxygenase-2 (COX-2) and prostaglandin E1 receptor (EP₁) contribute to disease and whether they help predict prognosis.

METHODS

We retrospectively reviewed the records of 116 patients with hepatocellular carcinoma (HCC) who underwent surgery between 2008 and 2011 at our hospital. Expression of COX-2 and EP₁ receptor was examined by immunohistochemistry of formalin-fixed, paraffin-embedded tissues using polyclonal antibodies. Possible associations between immunohistochemical scores and survival were determined.

RESULTS

Factors associated with poor overall survival (OS) were alpha-fetoprotein > 400 ng/mL, tumor size \geq 5 cm, and high EP₁ receptor expression, but not high COX-2 expression. Disease-free survival was not significantly different between patients with low or high levels of COX-2 or EP₁. COX-2 immunoreactivity was significantly higher in well-differentiated HCC tissues (Edmondson grade I - II) than in poorly differentiated tissues (Edmondson grade III - IV) ($P = 0.003$). EP₁ receptor immunoreactivity was significantly higher in poorly differentiated tissue than in well-differentiated tissue ($P = 0.001$).

CONCLUSION

COX-2 expression appears to be linked to early HCC events (initiation), while EP₁ receptor expression may participate in tumor progression and predict survival.

Key words: Cyclooxygenase-2; Hepatocellular carcinoma; Liver resection; Prognosis; Prostaglandin E1 receptor

© The Author(s) 2016. Published by Baishideng Publishing Group Inc. All rights reserved.

Core tip: We retrospectively reviewed the records of 116 patients with hepatocellular carcinoma (HCC) who underwent surgery between 2008 and 2011 at our hospital. Our results suggest that the factors associated with poor overall survival were alpha-fetoprotein > 400 ng/mL, tumor size \geq 5 cm, and high prostaglandin E1 (EP₁) receptor expression, but not high cyclooxygenase-2 (COX-2) expression. Disease-free survival did not differ significantly between patients with low or high levels of COX-2 or EP₁. COX-2 immunoreactivity was significantly higher in well-differentiated HCC tissues (Edmondson grade I - II) than in poorly differentiated tissues (Edmondson grade III - IV) ($P = 0.003$). EP₁ receptor immunoreactivity was significantly higher in poorly differentiated tissue than in well-differentiated tissue ($P = 0.001$).

while prostaglandin receptor-1 expression predicts survival. *World J Gastroenterol* 2016; 22(39): 8798-8805 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v22/i39/8798.htm> DOI: <http://dx.doi.org/10.3748/wjg.v22.i39.8798>

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most aggressive tumors and the third most frequent cause of cancer-related death in the world^[1,2]. Although early diagnosis and treatment of HCC have improved substantially, prognosis remains unsatisfactory. HCC often involves highly malignant tumors that respond poorly or not at all to adjuvant systemic and local therapies. This highlights the need for new approaches to prevent and treat the disease.

Two molecules that may be involved in HCC at different stages, and that therefore may be useful for understanding the pathogenesis and progression of the disease, are cyclooxygenase-2 (COX-2) and prostaglandin E1 receptor (EP₁ receptor). These molecules have already been shown to play important roles in the onset of various cancers, including HCC^[3-9].

COX-2 inhibits apoptosis and increases proliferation in various type of tumors^[10-12]. It also triggers the production of vascular endothelial growth factor and activates metalloproteinases, substantially altering the tumor microenvironment of various cancers^[13-15]. The precise role of COX-2 in HCC remains unclear. Its expression decreases with extent of de-differentiation, and it does not appear to be associated with prognosis^[16-19]. It may be involved in HCC initiation, although direct evidence of this is lacking.

COX-2 catalyzes the conversion of arachidonic acid to prostaglandin E2, which promotes the progression of various types of tumors by binding to the G-protein-coupled EP₁ receptor. This led us to wonder whether EP₁ receptor expression might correlate with HCC progression and might even serve as a prognostic indicator of survival. In fact, in a mouse model of chemically induced colon cancer, administration of selective EP₁ receptor antagonists or knockout of the EP₁ receptor gene led to nearly 60% fewer precancerous lesions and a lower overall colon cancer incidence^[20,21]. EP₁ receptor antagonists have also been reported to block the progression of other types of tumor^[22,23], including HCC^[18,24].

To begin to clarify the potential roles of COX-2 and EP₁ receptor in HCC, and to determine the potential prognostic value of EP₁ receptor expression, we examined relative expression levels in tissues taken from HCC patients treated at our hospital, and correlated these levels with survival.

MATERIALS AND METHODS

This research was approved by the Ethics Committee of the Tumor Hospital of Guangxi Medical University,

Yang HJ, Jiang JH, Yang YT, Yang XD, Guo Z, Qi YP, Zeng FH, Zhang KL, Chen NZ, Xiang BD, Li LQ. Cyclooxygenase-2 expression is associated with initiation of hepatocellular carcinoma,

and patients provided informed consent for their data and tissue to be used for research purposes when they were admitted for treatment at our hospital.

Patients

This study was a retrospective analysis of HCC patients treated by curative hepatectomy at the Tumor Hospital of Guangxi Medical University between May 2008 and May 2011. To be included in our study, patients needed to have pathology-confirmed HCC and no history of antitumor therapies before hepatic resection. They also needed to satisfy the following curative hepatectomy criteria: (1) the tumor removed by hepatectomy was solitary; (2) the surgery margin was greater than 1 cm; (3) there was no residual tumor^[25], portal tumor thromboses^[26] or extrahepatic metastases based on post-surgical imaging; and (4) patients with high levels of alpha-fetoprotein (AFP) before surgery had normal levels within two months after surgery. Patients were excluded from the study if they had multiple tumors, extrahepatic metastases or macroscopic intrahepatic metastases adjacent to the primary tumor.

Follow-up

All HCC patients were followed up 1 mo after resection, then at 3-mo intervals in the first year, and then at 3-6 mo intervals thereafter until 60 mo after resection or death. During each follow-up visit, routine investigations including AFP level, liver function, chest X-ray, ultrasound, CT or MRI were conducted.

Immunohistochemistry of COX-2 and EP₁ receptor

Tumor specimens were fixed in 10% formalin, embedded in paraffin, cut into 3- μ m sections, deparaffinized with xylene and rehydrated by decreasing concentrations of ethanol. Antigen retrieval was performed for 10 min at 95 °C in citrate buffer (pH 6.0) in a microwave oven. Sections were immersed in 3% hydrogen peroxide for 15 min to block endogenous peroxidases, then incubated at 37 °C for 1 h with rabbit anti-human COX-2 polyclonal antibody (1:400; Abcam, United Kingdom) or rabbit anti-human EP₁ receptor polyclonal antibody (1:200; Abcam). The sections were rinsed in phosphate-buffered saline (PBS), incubated with biotinylated anti-rabbit immunoglobulin for 20 min at room temperature, and then rinsed again with PBS. The sections were incubated with anti-horseradish peroxidase conjugate for 10 min, rinsed with PBS, and incubated with diaminobenzidine for 10 min. Finally, the sections were counterstained with hematoxylin. As a negative control, tissues were treated as described above, except they were incubated with PBS instead of primary antibodies.

Immunohistochemical staining results were independently evaluated by three authors (Hao-Jie Yang, Zhe Guo and Yu-Ting Yang) and an experienced hepatopathologist (Chun-Jun Li) from the Department of Pathology of Guangxi Tumor Hospital. The per-

centages of cells positive for COX-2 or EP₁ receptor as well as relative staining intensity were determined. Percentages of positive cells were categorized as follows: 0 (no positive tumor cells), 1 (1%-25% positive), 2 (26%-50% positive), 3 (51%-75% positive), and 4 (76%-100% positive). Staining intensity was categorized as follows: 0 (no staining), 1 (weak, light yellow), 2 (moderate, yellow-brown), and 3 (strong, brown)^[27]. The scores for positive cell percentages and for staining intensity were multiplied together to yield a single immunohistochemical staining index from 0 to 12. Sections with an index of 0-5 were defined as showing low expression, while those with an index of 6-12 were defined as showing high expression.

Statistical analysis

All statistical analyses were performed using SPSS 19.0 (IBM, United States). Inter-group differences in categorical variables were assessed for significance using the χ^2 test; differences in continuous variables were assessed using the Mann-Whitney *U* test or *t*-test. OS and DFS were analyzed using the Kaplan-Meier method, and differences between curves were assessed for significance using the log-rank test. Multivariate Cox proportional hazards modeling was used to identify independent prognostic factors. The threshold for significance was defined as *P* < 0.05.

RESULTS

Patient characteristics

During the study, 748 patients with HCC were scheduled for hepatectomy in my center. Of these, 221 (29.5%) were excluded as they had received initial HCC treatment in other hospitals. Of the remaining 527 patients, 161 (30.5%) had solitary nodular tumors without portal tumor thromboses or extrahepatic metastases. We excluded 33 (20.4%) because they underwent only transarterial chemoembolization, local ablation therapy, or ethanol injection, and we excluded 12 (7.4%) as they lacked complete follow-up data. In total, 116 (72%) patients were included in the final analysis (93 men, 23 women) with a median age of 67 (range, 39-83) (Table 1). Immunohistochemistry showed low COX-2 expression in 62 patients (53.4%) and low EP₁ receptor expression in 73 (62.9%).

EP₁ receptor expression is a prognostic predictor of OS

OS was significantly lower among patients with high expression of EP₁ receptor than among those with low expression (Figure 1). OS did not differ significantly between patients with low or high COX-2 expression. DFS did not differ significantly between patients with low or high expression of COX-2 or EP₁ receptor.

The Cox hazards model showed 3 independent predictors of poor OS: tumor size \geq 5 cm, high expression of EP₁ receptor and AFP \geq 400 ng/mL (Table 2).

Table 1 Clinicopathological characteristics of 116 hepatocellular carcinoma patients treated by curative resection, stratified by COX-2 and EP₁ receptor expression

Variable	COX-2		P value	EP ₁ receptor		P value
	Low expression (n = 62)	High expression (n = 54)		Low expression (n = 73)	High expression (n = 43)	
Gender, M/F	48/14	45/9	0.426	60/13	33/10	0.477
Age (yr)	46.9 ± 10.8	47.9 ± 11.5	0.308	46.6 ± 11.1	47.3 ± 11.2	0.486
HbsAg						
Negative	10	10	0.734	11	9	0.420
Positive	52	44		62	34	
Liver cirrhosis						
No	10	10	0.734	14	6	0.472
Yes	52	44		59	37	
AFP (ng/mL)						
< 400	41	44	0.062	56	29	0.276
≥ 400	21	10		17	14	
Edmondson grade						
I - II	26	37	0.004	46	17	0.014
III-IV	36	17		27	26	
Child-Pugh class						
A	43	48	0.773	60	31	0.201
B	11	14		13	12	
Tumor capsule						
Complete	37	34	0.717	44	27	0.788
Incomplete	25	20		29	16	
Tumor size (cm)	5.7 (3-7)	6.6 (4-8.6)	0.134	5 (3.5-7)	6 (3.7-8.5)	0.207
Albumin (g/L)	41.1 ± 4.4	40.4 ± 4.6	0.434	41.5 ± 4.3	40.2 ± 4.5	0.134
Platelet count (10 ⁹ /L)	167.9 (107.3-205.3)	186.6 (136.3-230)	0.136	178.9 (107.3-205.3)	186.6 (136.3-230)	0.833
AST (U/L)	56.6 (28.3-61)	55.6 (27-60)	0.924	38 (27-60)	44 (30.5-61.5)	0.737
ALT (U/L)	54.8 (28-56)	62.7 (24.8-57.9)	0.539	37(27-56)	38(27-55)	0.917
Total bilirubin (μmol/L)	14.08 (8.9-18.4)	14.3 (9.4-15.3)	0.912	11.9(9.35-16.6)	13.4(9-17.7)	0.580

AFP: Alpha-fetoprotein; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; HBsAg: Hepatitis B surface antigen.

Table 2 Multivariable analysis to identify predictors of overall survival in 116 hepatocellular carcinoma patients

Factors	Hazard ratio	95%CI	P value
AFP ≥ 400 ng/mL	1.691	1.094-2.614	0.018
Incomplete tumor capsule	0.979	0.659-1.454	0.915
Tumor size ≥ 5 cm	1.582	1.027-2.438	0.038
Edmondson grade III-IV	1.149	0.663-1.992	0.621
EP ₁ receptor expression	2.318	1.190-4.516	0.014

AFP: Alpha-fetoprotein.

COX-2 and EP₁ receptor expression correlates with tumor differentiation

COX-2 immunoreactivity was significantly higher in well-differentiated HCC tissues (Edmondson grade I - II) than in poorly differentiated tissues (Edmondson grade III-IV) ($P = 0.003$). EP₁ receptor immunoreactivity was significantly higher in poorly differentiated tissue than in well-differentiated tissue (Figure 2; $P = 0.001$). Figure 3 shows representative examples of different staining results in tissues of different histology grade.

DISCUSSION

HCC is one of the most aggressive tumors and has a poor prognosis. Some patients can undergo curative resection, but this treatment is associated with a high

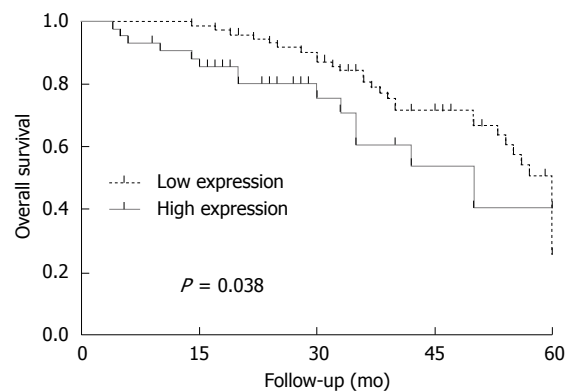


Figure 1 Overall survival of hepatocellular carcinoma patients stratified by low or high expression of EP₁ receptor.

rate of intrahepatic recurrence. Most patients with HCC are ineligible for resection because their disease has already reached an advanced stage by the time it is diagnosed. These patients are treated with local or systemic adjuvant modalities that provide only short-term regression, stabilization, or symptomatic control. Therefore, new therapeutic strategies are needed to improve long-term survival.

Our results indicate that the EP₁ receptor is involved in HCC progression, suggesting that it may be a reasonable therapeutic target. High expression of the EP₁ receptor was associated with poor prognosis in our

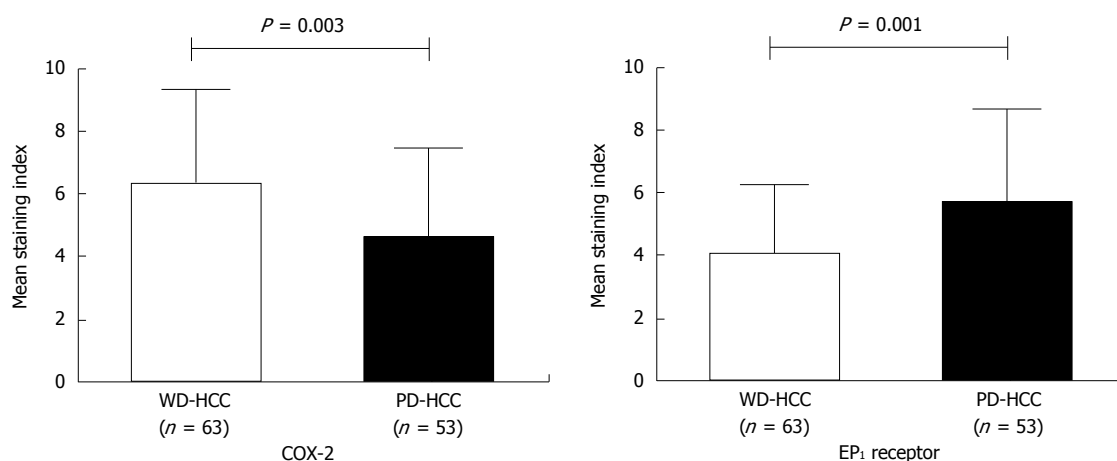
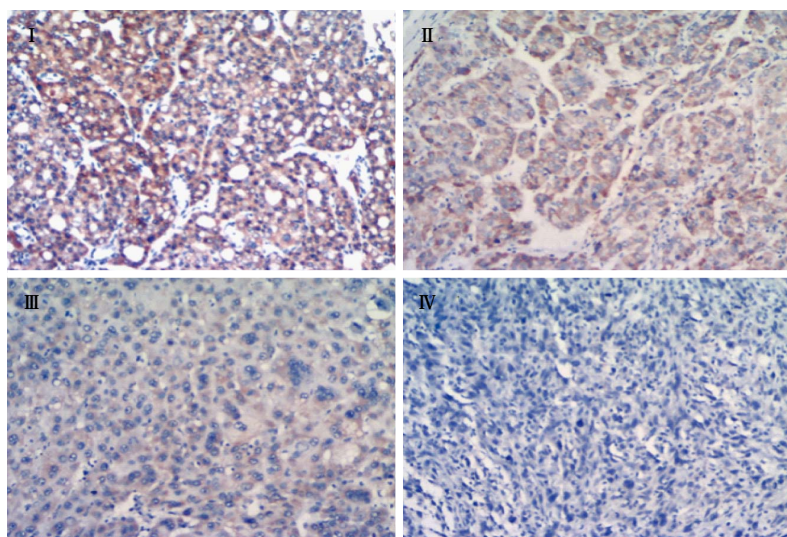


Figure 2 Cyclooxygenase-2 and prostaglandin E1 receptor immunoreactivity scores in hepatocellular carcinoma tissues at different histological grades. Cyclooxygenase-2 (COX-2) expression was higher in well-differentiated tissue (Edmondson grade I - II), while EP₁ receptor expression was higher in poorly-differentiated tissue (Edmondson grade III-IV). WD: Well-differentiated; PD: Poorly differentiated; HCC: Hepatocellular carcinoma.

COX-2



EP₁ receptor

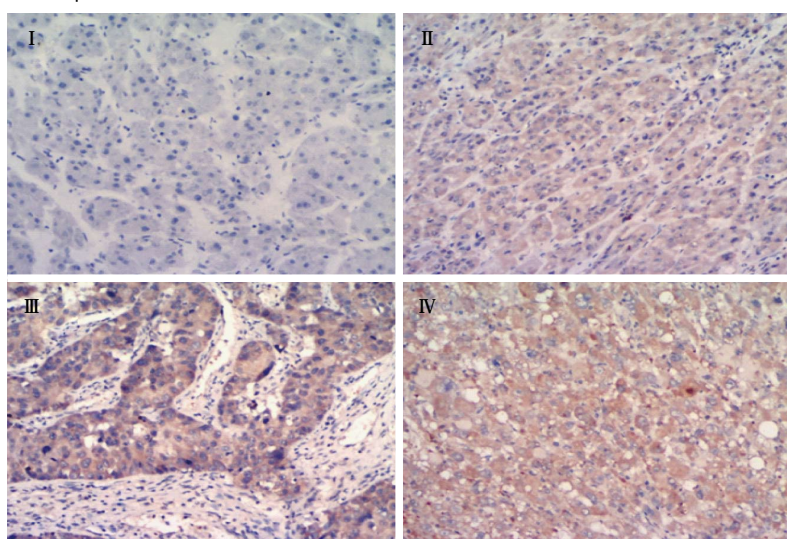


Figure 3 Representative micrographs showing different intensities of immunohistochemical stain against cyclooxygenase-2 and prostaglandin E1 receptor in tissues with different histological grades (Edmondson grade I -IV). Magnification, × 100. Cyclooxygenase-2 (COX-2) expression level decreased with lower grade of differentiation, while EP₁ receptor expression increased with lower grade of differentiation.

patients, and expression was significantly higher in poorly differentiated tissue than in well-differentiated tissue. The observed correlation between higher expression and poorer differentiation is consistent with a previous study^[18]. These results suggest that targeting the EP₁ receptor may provide a more selective approach to treating HCC than using COX inhibitors to block prostaglandin E₂ synthesis, which increases the risk of cardiovascular events^[28].

Although studies have associated COX-2 expression with differentiation, invasion and metastasis in HCC^[3,18,24], it has not been linked with survival. In the present study, we failed to find an association between COX-2 expression and survival when patients were dichotomized into groups with low or high expression. While it is possible that a more quantitative approach may identify associations between COX-2 levels and survival, we believe it is more likely that expression of COX-2 may be important only during initiation of HCC, whereas the EP₁ receptor, which is the downstream target of prostaglandin E₂ generated by COX-2, may be involved in disease progression. This may explain why we observed a different relationship between the expression of COX-2 and EP₁ receptor: patients with high expression of one showed low expression of the other. This may also explain why we found that expression of the EP₁ receptor, but not the COX-2 receptor, predicted OS in our cohort.

Several factors may help to explain why high expression of the EP₁ receptor predicts poor survival. The receptor enhances tumor cell proliferation, invasion and migration^[4,6,29,30], as well as adaptation to hypoxic conditions^[6,31]. The receptor has also been reported to inhibit immune function and promote tumor progression^[32]. The EP₁ receptor can even induce prostaglandin E₂ production by binding to the receptor of Fas ligand^[32-34].

This study has some limitations. Firstly, the authors used only an HE method, therefore more accurate and quantitative methods, such as Western blot, polymerase chain reaction *etc.*, should be applied in future studies. Secondly, it is known that other cytokines, except COX-2 and EP₁, have been reported to be strongly associated with HCC. These cytokines may be involved in different aspects of the pathogenesis of HCC and should be explored as a network pattern in future studies.

In conclusion, our results suggest that COX-2 expression correlates with an early event during the initiation of HCC, while EP₁ receptor expression plays an important role in tumor progression and predicts OS.

COMMENTS

Background

Hepatocellular carcinoma (HCC) is one of the most aggressive tumors and the third most frequent cause of cancer-related death in the world. Although early diagnosis and treatment of HCC have improved substantially, prognosis

remains unsatisfactory. HCC often involves highly malignant tumors that respond poorly or not at all to adjuvant systemic and local therapies. This highlights the need for new approaches to prevent and treat the disease.

Research frontiers

Two molecules that may be involved in HCC at different stages, and that therefore may be useful for understanding the pathogenesis and progression of the disease, are cyclooxygenase-2 (COX-2) and prostaglandin E₁ receptor (EP₁ receptor). These molecules have already been shown to play important roles in the onset of various cancers, including HCC.

Innovations and breakthroughs

The authors retrospectively reviewed the records of 116 patients with HCC who underwent surgery between 2008 and 2011 at their hospital, and found that COX-2 expression appears to be linked to early HCC events (initiation), while EP₁ receptor expression may participate in tumor progression and predict survival.

Applications

These results suggest that targeting the EP₁ receptor may provide a more selective approach to treating HCC than using COX inhibitors to block prostaglandin E₂ synthesis, which increases the risk of cardiovascular events.

Terminology

Percentages of positive cells were categorized as follows: 0 (no positive tumor cells), 1 (1%-25% positive), 2 (26%-50% positive), 3 (51%-75% positive), and 4 (76%-100% positive). Staining intensity was categorized as follows: 0 (no staining), 1 (weak, light yellow), 2 (moderate, yellow-brown), and 3 (strong, brown). The scores for positive cell percentages and for staining intensity were multiplied together to yield a single immunohistochemical staining index from 0 to 12. Sections with an index of 0-5 were defined as showing low expression, while those with an index of 6-12 were defined as showing high expression.

Peer-review

The paper is a good study on COX-2 and EP₁ receptor immunoreactivity in patients with HCC. The investigators shown that COX-2 immunoreactivity was higher in well-differentiated HCC tissues and EP₁ receptor immunoreactivity was significantly higher in poorly differentiated tissue than in well-differentiated tissue.

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 **Yang HJ**, Guo Z, Yang YT, Jiang JH, Qi YP, Li JJ, Li LQ, Xiang BD. Blood neutrophil-lymphocyte ratio predicts survival after hepatectomy for hepatocellular carcinoma: A propensity score-based analysis. *World J Gastroenterol* 2016; **22**: 5088-5095 [PMID: 27275101 DOI: 10.3748/wjg.v22.i21.5088]
- 3 **Guo Z**, Jiang JH, Zhang J, Yang HJ, Yang FQ, Qi YP, Zhong YP, Su J, Yang RR, Li LQ, Xiang BD. COX-2 Promotes Migration and Invasion by the Side Population of Cancer Stem Cell-Like Hepatocellular Carcinoma Cells. *Medicine* (Baltimore) 2015; **94**: e1806 [PMID: 26554780 DOI: 10.1097/MD.0000000000001806]
- 4 **Zhang H**, Cheng S, Zhang M, Ma X, Zhang L, Wang Y, Rong R, Ma J, Xia S, Du M, Shi F, Wang J, Yang Q, Bai X, Leng J. Prostaglandin E₂ promotes hepatocellular carcinoma cell invasion through upregulation of YB-1 protein expression. *Int J Oncol* 2014; **44**: 769-780 [PMID: 24378923 DOI: 10.3892/ijo.2013.2234]
- 5 **Jin J**, Chang Y, Wei W, He YF, Hu SS, Wang D, Wu YJ. Prostanoid EP₁ receptor as the target of (-)-epigallocatechin-3-gallate in suppressing hepatocellular carcinoma cells in vitro. *Acta Pharmacol Sin* 2012; **33**: 701-709 [PMID: 22555372 DOI: 10.1038/aps.2012.13]
- 6 **Kim SH**, Park YY, Kim SW, Lee JS, Wang D, DuBois RN. ANGPTL4 induction by prostaglandin E₂ under hypoxic

- conditions promotes colorectal cancer progression. *Cancer Res* 2011; **71**: 7010-7020 [PMID: 21937683 DOI: 10.1158/0008-5472.CAN-11-1262]
- 7 **Bai XM**, Jiang H, Ding JX, Peng T, Ma J, Wang YH, Zhang L, Zhang H, Leng J. Prostaglandin E2 upregulates survivin expression via the EP1 receptor in hepatocellular carcinoma cells. *Life Sci* 2010; **86**: 214-223 [PMID: 20035770 DOI: 10.1016/j.lfs.2009.12.009]
 - 8 **Han C**, Michalopoulos GK, Wu T. Prostaglandin E2 receptor EP1 transactivates EGFR/MET receptor tyrosine kinases and enhances invasiveness in human hepatocellular carcinoma cells. *J Cell Physiol* 2006; **207**: 261-270 [PMID: 16331686 DOI: 10.1002/jcp.20560]
 - 9 **Hull MA**, Ko SC, Hawcroft G. Prostaglandin EP receptors: targets for treatment and prevention of colorectal cancer? *Mol Cancer Ther* 2004; **3**: 1031-1039 [PMID: 15299086]
 - 10 **Zhang P**, Luo HS, Li M, Tan SY. Artesunate inhibits the growth and induces apoptosis of human gastric cancer cells by downregulating COX-2. *Onco Targets Ther* 2015; **8**: 845-854 [PMID: 25945055 DOI: 10.2147/OTT.S81041]
 - 11 **Qian M**, Yang X, Li Z, Jiang C, Song D, Yan W, Liu T, Wu Z, Kong J, Wei H, Xiao J. P50-associated COX-2 extragenic RNA (PACER) overexpression promotes proliferation and metastasis of osteosarcoma cells by activating COX-2 gene. *Tumour Biol* 2016; **37**: 3879-3886 [PMID: 26476537 DOI: 10.1007/s13277-015-3838-8]
 - 12 **Lv P**, Zhang P, Li X, Chen Y. Micro ribonucleic acid (RNA)-101 inhibits cell proliferation and invasion of lung cancer by regulating cyclooxygenase-2. *Thorac Cancer* 2015; **6**: 778-784 [PMID: 26557918 DOI: 10.1111/1759-7714.12283]
 - 13 **Lee JH**, Piao MS, Choi JY, Yun SJ, Lee JB, Lee SC. Up-regulation of cyclooxygenase 2 and matrix metalloproteinases-2 and -9 in cutaneous squamous cell carcinoma: active role of inflammation and tissue remodeling in carcinogenesis. *Ann Dermatol* 2013; **25**: 145-151 [PMID: 23717003 DOI: 10.5021/ad.2013.25.2.145]
 - 14 **Mohammad MA**, Zeeneldin AA, Abd Elmageed ZY, Khalil EH, Mahdy SM, Sharada HM, Sharawy SK, Abdel-Wahab AH. Clinical relevance of cyclooxygenase-2 and matrix metalloproteinases (MMP-2 and MT1-MMP) in human breast cancer tissue. *Mol Cell Biochem* 2012; **366**: 269-275 [PMID: 22527932 DOI: 10.1007/s11010-012-1305-z]
 - 15 **Li Y**, Li S, Sun D, Song L, Liu X. Expression of 15-hydroxy-prostaglandin dehydrogenase and cyclooxygenase-2 in non-small cell lung cancer: Correlations with angiogenesis and prognosis. *Oncol Lett* 2014; **8**: 1589-1594 [PMID: 25202373 DOI: 10.3892/ol.2014.2371]
 - 16 **Yang Y**, Zhu J, Gou H, Cao D, Jiang M, Hou M. Clinical significance of Cox-2, Survivin and Bcl-2 expression in hepatocellular carcinoma (HCC). *Med Oncol* 2011; **28**: 796-803 [PMID: 20401641 DOI: 10.1007/s12032-010-9519-y]
 - 17 **Yildirim Y**, Ozyilkan O, Bilezikci B, Akcali Z, Haberal M. Lack of influence of cyclooxygenase-2 expression in hepatocellular carcinomas on patient survival. *Asian Pac J Cancer Prev* 2008; **9**: 295-298 [PMID: 18712978]
 - 18 **Breinig M**, Rieker R, Eiteneuer E, Wertenbruch T, Haugg AM, Helmke BM, Schirmacher P, Kern MA. Differential expression of E-prostanoid receptors in human hepatocellular carcinoma. *Int J Cancer* 2008; **122**: 547-557 [PMID: 17918156 DOI: 10.1002/ijc.23098]
 - 19 **Koga H**, Sakisaka S, Ohishi M, Kawaguchi T, Taniguchi E, Sasatomi K, Harada M, Kusaba T, Tanaka M, Kimura R, Nakashima Y, Nakashima O, Kojiro M, Kurohiji T, Sata M. Expression of cyclooxygenase-2 in human hepatocellular carcinoma: relevance to tumor dedifferentiation. *Hepatology* 1999; **29**: 688-696 [PMID: 10051469 DOI: 10.1002/hep.510290355]
 - 20 **Watanabe K**, Kawamori T, Nakatsugi S, Ohta T, Ohuchida S, Yamamoto H, Maruyama T, Kondo K, Narumiya S, Sugimura T, Wakabayashi K. Inhibitory effect of a prostaglandin E receptor subtype EP(1) selective antagonist, ONO-8713, on development of azoxymethane-induced aberrant crypt foci in mice. *Cancer Lett* 2000; **156**: 57-61 [PMID: 10840160 DOI: 10.1016/S0304-3835(00)00440-7]
 - 21 **Watanabe K**, Kawamori T, Nakatsugi S, Ohta T, Ohuchida S, Yamamoto H, Maruyama T, Kondo K, Ushikubi F, Narumiya S, Sugimura T, Wakabayashi K. Role of the prostaglandin E receptor subtype EP1 in colon carcinogenesis. *Cancer Res* 1999; **59**: 5093-5096 [PMID: 10537280]
 - 22 **Kawamori T**, Uchiya N, Nakatsugi S, Watanabe K, Ohuchida S, Yamamoto H, Maruyama T, Kondo K, Sugimura T, Wakabayashi K. Chemopreventive effects of ONO-8711, a selective prostaglandin E receptor EP(1) antagonist, on breast cancer development. *Carcinogenesis* 2001; **22**: 2001-2004 [PMID: 11751431 DOI: 10.1093/carcin/22.12.2001]
 - 23 **Tober KL**, Wilgus TA, Kusewitt DF, Thomas-Ahner JM, Maruyama T, Oberyshyn TM. Importance of the EP(1) receptor in cutaneous UVB-induced inflammation and tumor development. *J Invest Dermatol* 2006; **126**: 205-211 [PMID: 16417238 DOI: 10.1038/sj.jid.5700014]
 - 24 **Cusimano A**, Foderà D, Lampiasi N, Azzolina A, Notarbartolo M, Giannitrapani L, D'Alessandro N, Montalto G, Cervello M. Prostaglandin E2 receptors and COX enzymes in human hepatocellular carcinoma: role in the regulation of cell growth. *Ann N Y Acad Sci* 2009; **1155**: 300-308 [PMID: 19250221 DOI: 10.1111/j.1749-6632.2009.03701.x]
 - 25 **Fan ST**, Lo CM, Liu CL, Lam CM, Yuen WK, Yeung C, Wong J. Hepatectomy for hepatocellular carcinoma: toward zero hospital deaths. *Ann Surg* 1999; **229**: 322-330 [PMID: 10077043 DOI: 10.1097/0000658-199903000-00004]
 - 26 **Xia Y**, Qiu Y, Li J, Shi L, Wang K, Xi T, Shen F, Yan Z, Wu M. Adjuvant therapy with capecitabine postpones recurrence of hepatocellular carcinoma after curative resection: a randomized controlled trial. *Ann Surg Oncol* 2010; **17**: 3137-3144 [PMID: 20602260 DOI: 10.1245/s10434-010-1148-3]
 - 27 **Baker AM**, Bird D, Welti JC, Gourlaouen M, Lang G, Murray GI, Reynolds AR, Cox TR, Erler JT. Lysyl oxidase plays a critical role in endothelial cell stimulation to drive tumor angiogenesis. *Cancer Res* 2013; **73**: 583-594 [PMID: 23188504 DOI: 10.1158/0008-5472.CAN-12-2447]
 - 28 **Narumiya S**, FitzGerald GA. Genetic and pharmacological analysis of prostanoid receptor function. *J Clin Invest* 2001; **108**: 25-30 [PMID: 11435452 DOI: 10.1172/JCI13455]
 - 29 **Bai X**, Wang J, Zhang L, Ma J, Zhang H, Xia S, Zhang M, Ma X, Guo Y, Rong R, Cheng S, Shu W, Wang Y, Leng J. Prostaglandin E2 receptor EP1-mediated phosphorylation of focal adhesion kinase enhances cell adhesion and migration in hepatocellular carcinoma cells. *Int J Oncol* 2013; **42**: 1833-1841 [PMID: 23525457 DOI: 10.3892/ijo.2013.1859]
 - 30 **Yang SF**, Chen MK, Hsieh YS, Chung TT, Hsieh YH, Lin CW, Su JL, Tsai MH, Tang CH. Prostaglandin E2/EP1 signaling pathway enhances intercellular adhesion molecule 1 (ICAM-1) expression and cell motility in oral cancer cells. *J Biol Chem* 2010; **285**: 29808-29816 [PMID: 20647315 DOI: 10.1074/jbc.M110.108183]
 - 31 **Kaidi A**, Qualtrough D, Williams AC, Paraskeva C. Direct transcriptional up-regulation of cyclooxygenase-2 by hypoxia-inducible factor (HIF)-1 promotes colorectal tumor cell survival and enhances HIF-1 transcriptional activity during hypoxia. *Cancer Res* 2006; **66**: 6683-6691 [PMID: 16818642 DOI: 10.1158/0008-5472.CAN-06-0425]
 - 32 **O'Callaghan G**, Ryan A, Neary P, O'Mahony C, Shanahan F, Houston A. Targeting the EP1 receptor reduces Fas ligand expression and increases the antitumor immune response in an in vivo model of colon cancer. *Int J Cancer* 2013; **133**: 825-834 [PMID: 23390011 DOI: 10.1002/ijc.28076]
 - 33 **Zhang Y**, Liu Q, Zhang M, Yu Y, Liu X, Cao X. Fas signal promotes lung cancer growth by recruiting myeloid-derived suppressor cells via cancer cell-derived PGE2. *J Immunol*

2009; **182**: 3801-3808 [PMID: 19265159 DOI: 10.4049/jimmunol.0801548]

34 **O'Callaghan G**, Kelly J, Shanahan F, Houston A. Prostaglandin

E2 stimulates Fas ligand expression via the EP1 receptor in colon cancer cells. *Br J Cancer* 2008; **99**: 502-512 [PMID: 18648368 DOI: 10.1038/sj.bjc.6604490]

P- Reviewer: Alexopoulou A, de Oliveira CPMS, Mihaila RG
S- Editor: Gong ZM **L- Editor:** Webster JR **E- Editor:** Wang CH



Observational Study

Epidemiological study: Correlation between diet habits and constipation among elderly in Beijing region

Xiao-Jiao Yang, Mei Zhang, Hong-Ming Zhu, Zhe Tang, Dan-Dan Zhao, Bang-Yi Li, Amanda Gabriel

Xiao-Jiao Yang, Amanda Gabriel, McGill University, 845 Sherbrooke Street West, Montreal, Quebec H3A 0G4, Canada

Mei Zhang, Hong-Ming Zhu, Zhe Tang, Dan-Dan Zhao, Bang-Yi Li, Department of Gastroenterology, Xuanwu Hospital, Capital Medical University, Beijing 100053, China

Author contributions: Zhang M designed the research; Yang XJ performed data and statistical analysis, designed survey questions regarding diet habit and wrote the paper; Zhu HM and Tang Z performed the research; Zhao DD, Li BY and Gabriel A performed data collection and input.

Institutional review board statement: The study was reviewed and approved by the Department of Gastroenterology of Xuanwu Hospital, Capital Medical University.

Informed consent statement: All questionnaires were anonymous and verbal consent was obtained from all participants.

Conflict-of-interest statement: There are no conflicts of interest to report.

Data sharing statement: No additional data are available.

Open-Access: This article is an open-access article which was selected by an in-house editor and fully peer-reviewed by external reviewers. It is distributed in accordance with the Creative Commons Attribution Non Commercial (CC BY-NC 4.0) license, which permits others to distribute, remix, adapt, build upon this work non-commercially, and license their derivative works on different terms, provided the original work is properly cited and the use is non-commercial. See: <http://creativecommons.org/licenses/by-nc/4.0/>

Manuscript source: Invited manuscript

Correspondence to: Dr. Mei Zhang, Department of Gastroenterology, Xuanwu Hospital, Capital Medical University, No. 45 Changchun Street, Xuanwu District, Beijing 100053, China. zhang2955@sina.com
Telephone: +86-10-83198438

Fax: +86-10-83198438

Received: July 27, 2016

Peer-review started: July 28, 2016

First decision: August 15, 2016

Revised: August 19, 2016

Accepted: September 12, 2016

Article in press: September 12, 2016

Published online: October 21, 2016

Abstract

AIM

To investigate correlations between diet and prevalence of constipation among elderly people in Beijing.

METHODS

A total of 2776 (≥ 60 years) were selected in Beijing region for investigation. Data regarding constipation and diet habits was collected *via* hierarchical status, segmentation and random cluster sampling. Investigation included constipation-related demographic indicators and diet habits. Door-to-door questionnaires and surveys included daily staple food intakes, frequency of fish, egg, fruits and vegetables consumption. Constipation was defined according to the China Chronic Constipation Diagnosis and Treatment Guideline (2013), with the following constipation judgment indicators: decreased defecation frequency, dry and hard stool, and difficulty in defecation.

RESULTS

The prevalence of constipation among elderly people in Beijing region was 13%. There was a positive correlation between prevalence of constipation and age, but negative correlations between prevalence of constipation and staple food, fish and dietary fibres

(fruits and vegetables) intakes. These differences were all statistically significant.

CONCLUSION

The prevalence of elderly constipation in Beijing region is closely related to diet habits, and is significantly decreased by high staple foods intake, fish eating and high dietary fibres (fruits and vegetables) consumption.

Key words: Constipation; Elderly; Diet; Epidemiology; Prevalence; Factors

© **The Author(s) 2016.** Published by Baishideng Publishing Group Inc. All rights reserved.

Core tip: Because of high prevalence of constipation among elderly people, older populations are more susceptible to constipation related side effects. Many studies have concluded the significance of certain foods and dietary modification in treating constipation prior to any medical interventions. Benefits of frequent fish, dietary fibres consumption and large staple food intakes in alleviating symptoms of constipation were demonstrated in this study. Dietary modification should be promoted and emphasized as first line treatment to postpone or avoid drug use.

Yang XJ, Zhang M, Zhu HM, Tang Z, Zhao DD, Li BY, Gabriel A. Epidemiological study: Correlation between diet habits and constipation among elderly in Beijing region. *World J Gastroenterol* 2016; 22(39): 8806-8811 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v22/i39/8806.htm> DOI: <http://dx.doi.org/10.3748/wjg.v22.i39.8806>

INTRODUCTION

Constipation is a common gastrointestinal disease affecting all age groups, elderly individuals suffer particularly more than younger individuals, among which females have higher prevalence than males^[1,2]. High incidence of constipation in older populations not only accounts for change in organ functions with age, but diet habits play an essential role in prevention and treatment. Many studies have suggested that high dietary fiber intake can significantly reduce prevalence and alleviate symptoms of constipation^[3-6]. Constipation in older populations should firstly be treated with dietary modifications prior to any medical intervention to minimize side effects of certain drugs. Thus, we conducted an epidemiological study regarding diet habits and prevalence of constipation among elderly people in Beijing to further confirm the significance of diet in treating and alleviating symptoms of constipation without any potential side effects. This research is one of key parts of the special project, which funded by the National Health and Family Planning Commission of the People's Republic of

China. The project consists of two related programs: (1) the study on comprehensive geriatric health evaluation index system; and (2) Multidimensional longitudinal study of aging among the elderly in Beijing.

MATERIALS AND METHODS

Research subjects

According to 2015 Beijing sampling data of 5th population census in China, a stratified, segmented and randomized cluster sampling method was used to investigate 2776 elderly people (≥ 60 years) in urban (Xuanwu) and rural (Huairou and Daxing) areas of Beijing. Door-to-door questionnaires were used and collected, among which completed questionnaires regarding eggs and fish, fruits and vegetables were 2774 and 2486 respectively. Subjects' characteristics were the following: 1213 males (43.7%), 1563 females (56.3%), 1264 rural elderly (45.5%) and 1512 urban elderly (54.5%). Classification with regards to age: 60-64 years (510, 18.4%), 65-69 (485, 17.5%), 70-74 (551, 19.8%), 75-80 (607, 21.9%), > 80 (623, 22.4%).

Research method

Investigation was performed by uniformly trained professionals via door-to-door questionnaires in 2015 between July to September. Survey questions covered constipation-related demographic indicators and diet habits including daily staple food intake, frequency of fish, egg, fruits and vegetables consumption. Constipation was defined according to the China Chronic Constipation Diagnosis and Treatment Guideline (2013), with the following constipation judgment indicators: decreased defecation frequency, dry and hard stool, and difficulty in defecation. All questionnaires were anonymous and verbal consent was obtained from all participants.

Statistical analysis

EpiData was used to input data and establish database, processed data was converted to SPSS document. The *t* test was performed to analyze data and data was expressed in mean \pm SD; χ^2 test was performed, $P < 0.05$ was considered statistically significant.

RESULTS

Age, region and gender

Of 2776 subjects from both urban and rural areas of Beijing, prevalence of constipation increased with age. Of 510 elderly aged 60-65, 41 of which were constipated (prevalence 8.0%); 11.1% for 65 years old and above group; 11.3% for 70-75 group; 18.8% for 75-80 group; and 17.8% for > 80 group. The difference was statistically significant ($P < 0.001$, $\chi^2 = 41.338$). However, differences between females and males ($\chi^2 = 0.981$), urban and rural areas ($\chi^2 = 0.771$)

Table 1 Age, region (rural, urban) and gender in relation with constipation

Items	Cases	Prevalence	Constipation	χ^2
Gender				
Male	1213	158	13.0	0.981
Female	1563	224	14.3	
Region				
Urban	1512	216	14.3	0.771
Rural	1264	166	13.1	
Age ^a				
60-65	510	41	8.0	41.338
65-70	485	54	11.1	
70-75	551	62	11.3	
75-80	607	114	18.8	
> 80	623	111	17.8	
Total	2776			

^a $P < 0.001$.**Table 2** Correlation between daily staple food intakes and constipation

Staple food Intake	Cases	Constipation	Prevalence ^a
≤ 150 g	351	41	11.7%
200-300 g	1368	129	9.4%
350-450 g	530	47	8.9%
≥ 500 g	237	10	4.2%
Total	2486	227	9.1%

 $\chi^2 = 9.833$; ^a $P < 0.05$.

were not statistically significant ($P > 0.05$ for both) (Table 1).

Staple food

Of total 2486 subjects, 227 were constipated and total prevalence was 9.1%. Daily staple food intakes and prevalence of constipation indicated a negative correlation and the difference was statistically significant ($\chi^2 = 9.833$, $P < 0.05$). Prevalence of constipation was 11.7% for consumption of less than and equal to 150 g per day; 9.4% for consumption of 200-300 g per day; 8.9% for consumption of 350-450 g per day; 4.2% for consumption of greater than and equal to 500g per day (Table 2).

Fish and egg

Of total 2774 subjects, 2632 egg eaters had 359 cases of constipation (prevalence 13.6%); 142 subjects who did not consume eggs had 23 cases of constipation (prevalence 16.2%). The difference was not statistically significant with $P > 0.05$ ($\chi^2 = 0.742$). Of total 2774 subjects, 2257 fish eaters had 292 cases of constipation (prevalence 12.9%); 517 subjects who did not consume fish had 90 cases of constipation (prevalence 17.4%). The difference was statistically significant with $P < 0.01$ ($\chi^2 = 7.080$) (Table 3).

Fruits and vegetables

Of total 2486 subjects, 2431 frequent vegetables

Table 3 Correlation between fish, egg eating and constipation

Items	Cases	Constipation	Prevalence	χ^2
Egg eater	2632	359	13.6%	7.080
Rarely eat	142	23	16.2%	
Fish eater	2257	292	12.9% ^b	
Rarely eat	517	90	17.4% ^b	
Total	2774			

^b $P < 0.01$.**Table 4** Correlation between fruits and vegetables consumption and constipation

Items	Cases	Constipation	Prevalence ^a	χ^2
Vegetables				
Frequent	2431	218	9.0%	3.546
Rare	55	9	16.4%	
Fruits				
Frequent	1783	150	8.4%	3.921
Rare	703	77	11.0%	
Total	2486			

^a $P < 0.05$.

consumers had 218 cases of constipation (prevalence 9.0%); 9 out of 55 rare vegetable consumers were constipated (prevalence 16.4%). The difference was statistically significant with $P < 0.05$ ($\chi^2 = 3.546$). Of total 2486 subjects, 1783 frequent fruits consumers had 150 cases of constipation (prevalence 8.4%); 77 out of 703 rare fruits consumers were constipated (prevalence 11.0%). The difference was statistically significant with $P < 0.05$ ($\chi^2 = 3.921$) (Table 4).

DISCUSSION

This study has investigated the prevalence of constipation among elderly people in both urban and rural areas of Beijing. Our results show that the differences between females and males, living in urban or rural areas are not statistically significant ($P > 0.05$ for both) (Table 1). However, many other studies have indicated that females in general suffer more from constipation than males in all age groups^[1,2]. Age is one of the most important risk factors, our results ($P < 0.001$) are in accordance with other studies which have concluded the prevalence of constipation increased with age (Table 1)^[1,2]. The proportion of constipated elderly people increases drastically from 70-year-old group (11.3%) to 75-year-old group (18.8%), so dietary and lifestyle modifications are highly recommended as first line treatment in these age groups prior to any medical interventions. Ageing is a naturally occurring process which implies changes in organ functions with age, previous studies have pointed out that constipated older adults could have normal or delayed colonic transit, caused most commonly by distal colonic or anorectal dysfunction^[7-9]. This irreversible process can be partially compensated by establishing a healthy

intestinal microbiota. Spinzi *et al.*^[10] have suggested that the faecal flora changed markedly with age mostly by a fall in numbers of bifidobacteria. Nowadays, probiotics are commonly prescribed and widely available, probiotics not only promote the growth of bifidobacteria, but shorten bowel transit and soften stools most likely by increased short chain fatty acid concentration and decreased colonic pH^[11]. Although the causal relation between intestinal microbiome and constipation remains undefined, remarkable outcomes have been proven by the use of probiotics in alleviating constipation symptoms, probiotics supplement and fermented dairy products are highly suggested to be incorporated into diet due to lack of side effects and food and drug interactions.

Staple foods in China generally refer to rice and wheat products such as noodles and steamed buns. Our results indicate a clear negative correlation between amount of staple food consumed and prevalence of constipation among elderly populations (Table 2). By increasing staple food consumption from 150 g per day up to 500 g and more per day, the prevalence of constipation decreases from 11.7% to 4.2% ($P < 0.05$). A Japanese study has found out a clear dose-response relationship between increased intake of rice and a decreased prevalence of constipation among young female dietetic students^[12]. Also, increased intakes of rice at breakfast, lunch, and dinner were all associated with a decreased prevalence of constipation^[12]. The protective effect of rice on constipation has also been indicated in two previous studies conducted in Asian communities where rice is the main staple food^[13,14]. Two possible explanations can be speculated. (1) Elderly people are generally eating less than younger adults, so their caloric intake is also lower than young people. Towers *et al.*^[15] have concluded that there was a strong negative correlation between total transit time and number of calories as measured by the daily food diary ($r = -0.58$, $P < 0.0001$) and the diet questionnaire ($r = -0.28$, $P = 0.05$). Thus, slow transit times are associated with low caloric intake which potentially leads to constipation; and (2) rice and wheat products are main source of vitamins and minerals in China, some of constituents in rice and/or combinations of these constituents might exert a preventive effect on constipation^[8]. However, Western countries have different dieting habits where potato, bread and corns are considered staple foods. Protective effects of staple food indicated in our results cannot be directly applied to Western countries due to differences in eating habits, further investigation is required to confirm the beneficial effects of staple food in Western diets.

In this study, we have established a relationship between fish eating, egg eating and prevalence of constipation in older age groups. Although the prevalence of elderly who do not eat eggs is higher than egg-eaters, this result is not statistically significant ($P > 0.05$). However, a negative correlation between fish

eating and prevalence of constipation is demonstrated by our results (Table 3); elderly people who do not eat fish have higher prevalence than those fish eaters ($P < 0.01$). One study conducted on infants regarding diet and constipation has suggested that the easily digested proteins in breast milk, primarily whey, resulted in soft stools; breast-fed infants tended to have more frequent stools and soft in consistency^[16]. Nonetheless, less digestible casein proteins are believed to result in firmer stools, formula-fed infants tended to have more firmer stools and more frequent constipation problems^[16]. According to this result, we can speculate that certain proteins in fish might be more digestible than proteins found in eggs, so more digestible proteins promote the formation of soft stools among elderly people. Further study is required to confirm this hypothesis.

To date, many studies have agreed that higher dietary fibers (fruits and vegetables) consumption decreases prevalence of constipation in different age and gender groups. According to our study, elderly people who consume fruits and vegetables frequently have lower prevalence of constipation ($P < 0.05$) (Table 4). The mechanism of action of fiber on constipation includes: (1) fiber increases stool bulk and accelerates colon transit; (2) fermenting fiber produces short-chain fatty acids (butyrate, propionate, acetate, etc.), which increase osmotic load and accelerate colon transit; (3) short-chain fatty acids change the intraluminal microbiome (mass) directly or indirectly by decreasing luminal pH, which accelerates colon transit; and (4) fiber contains water. All these improve stool consistency and amount^[3-5]. Increasing soluble fiber intake by increasing fruits and vegetables consumption resulted in more frequent and softer stools^[6]. Meanwhile, fruits and vegetables contain relatively higher amount of water than other food groups, Murakami *et al.*^[12] have indicated that low intake of water from foods was independently associated with increasing prevalence of constipation^[12]. Therefore, frequent intake of fruits and vegetables is highly recommended to elderly people. Roma *et al.*^[17] have conducted a study on children in selected areas of Greece, and have observed that the mean daily fiber intake was lower in the parents of constipated children than in control parents. This implies that constipation is not "inherited" but "passed" in a family where eating habit is essential in both prevention and treatment of constipation. High prevalence of constipation among elderly is not only due to ageing processes, but dieting habit is also a key factor. Increase dietary fibers intake and modify eating pattern can both influence familial health status and potentially reduce incidences of constipation. Moreover, according to Sun Hwan Bae certain fruits such as pear, grape, plum, and apple with peel are useful in treating constipation due to their high fiber content^[18]. Green kiwifruits, prune, persimmon and banana are commonly consumed and available in Asia, green kiwifruits and prune have beneficial effects on constipation yet raw persimmon and banana

pose negative effects on constipated patients. Green kiwifruit significantly increases defecation frequency, stool volume, softness of bowel motion, and ease of defecation in adult clinical studies^[19]. Similarly, prune and Japanese apricot are beneficial to constipation; prune contains large amount of phenolic compounds which aid in laxative effect^[20], and Japanese apricot increases defecation frequency and contraction of the rat colon^[21]. Nevertheless, unripe persimmon contains high tannin concentration, tannin acid reduces intestinal secretions and inhibits peristalsis, and even healthy individuals experience painful defecation when ripe persimmons are eaten^[18], persimmons should be avoided by constipated elderly people. Also, unripe bananas contain 100-250 mg tannins/100 g and have high amylase-resistant starch content; they can cause or aggravate pre-existing constipation^[22]. Unripe banana should not be recommended to constipated elderly people, as many other sources of fibers are available. Additional examples in terms of which fruits can aggravate constipation require further study.

Our epidemiological investigation demonstrates that higher staple food (rice, wheat products) intake, frequent fruits and vegetables consumption, incorporation of fish into diet pose beneficial effect on constipation and reduce the prevalence of constipated patients. Increase dietary fibers intake from both fruits and vegetables and whole grains to alleviate symptoms of constipation is one of effective methods. A balanced diet should be promoted and emphasized as first line treatment prior to any medical intervention.

COMMENTS

Background

With an increasing trend of population aging in China, more elderly people will suffer from constipation. Aside from food abundance, more and more older people start to draw attention to healthy dieting to prevent various diseases. However, correlation between diet habits and morbidity of constipation is yet to be determined in China. The authors conducted an epidemiological study in Beijing with regards to diet habits and prevalence of constipation among elderly. China Chronic Constipation Diagnosis and Treatment Guideline (2013) were taken as standard reference with the following judgment indicators: decreased defecation frequency, dry and hard stool, and difficulty in defecation

Research frontiers

There has been little epidemiological study concerning correlation between diet habits and prevalence of constipation in Beijing among older population. This study has introduced significance of dietary modification in alleviating and preventing constipation prior to medical intervention. Further studies regarding dietary modification in relation to constipation should be emphasized and promoted

Innovations and breakthroughs

There has been no large sample epidemiological study of elderly constipation in the past decade in Beijing. There is little systemic research on epidemiology of constipation and diet habits. The authors carried out an epidemiological investigation regarding correlation between diet habits and prevalence of constipation among older population in Beijing.

Applications

The prevalence of elderly constipation in Beijing region is closely related to diet

habits, and is significantly decreased by high staple foods intake, fish eating and high dietary fibres (fruits and vegetables) consumption.

Terminology

Constipation was defined according to the China Chronic Constipation Diagnosis and Treatment Guideline (2013), with the following constipation judgment indicators: decreased defecation frequency, dry and hard stool, and difficulty in defecation.

Peer-review

The study "Epidemiological study: correlation between diet habits and constipation among elderly in Beijing region" is very interesting.

REFERENCES

- 1 **Harari D.** Constipation. In: Halter JB, Ouslander JG, Tinetti ME, editors. *Hazzard's Geriatric Medicine and Gerontology*. 6th ed. New York, USA: McGraw-Hill Companies, 2009: 1103-1122
- 2 **Harris LA.** Prevalence and ramifications of chronic constipation. *Manag Care Interface* 2005; **18**: 23-30 [PMID: 16127889]
- 3 **McRorie JW,** Daggy BP, Morel JG, Diersing PS, Miner PB, Robinson M. Psyllium is superior to docusate sodium for treatment of chronic constipation. *Aliment Pharmacol Ther* 1998; **12**: 491-497 [PMID: 9663731 DOI: 10.1046/j.1365-2036.1998.00336.x]
- 4 **Cummings JH,** Macfarlane GT. The control and consequences of bacterial fermentation in the human colon. *J Appl Bacteriol* 1991; **70**: 443-459 [PMID: 1938669]
- 5 **Topping DL,** Clifton PM. Short-chain fatty acids and human colonic function: roles of resistant starch and nonstarch polysaccharides. *Physiol Rev* 2001; **81**: 1031-1064 [PMID: 11427691]
- 6 **Loening-Baucke V,** Miele E, Staiano A. Fiber (glucmannan) is beneficial in the treatment of childhood constipation. *Pediatrics* 2004; **113**: e259-e264 [PMID: 14993586]
- 7 **Melkersson M,** Andersson H, Bosaeus I, Falkheden T. Intestinal transit time in constipated and non-constipated geriatric patients. *Scand J Gastroenterol* 1983; **18**: 593-597 [PMID: 6675181 DOI: 10.3109/00365528309181643]
- 8 **Wald A.** Constipation and fecal incontinence in the elderly. *Gastroenterol Clin North Am* 1990; **19**: 405-418 [PMID: 2194952 DOI: 10.4065/71.1.81]
- 9 **Eastwood HD.** Bowel transit studies in the elderly: radio-opaque markers in the investigation of constipation. *Gerontol Clin (Basel)* 1972; **14**: 154-159 [PMID: 4653950]
- 10 **Spinzi G,** Amato A, Imperiali G, Lenoci N, Mandelli G, Paggi S, Radaelli F, Terreni N, Terruzzi V. Constipation in the elderly: management strategies. *Drugs Aging* 2009; **26**: 469-474 [PMID: 19591521 DOI: 10.2165/00002512-200926060-00003]
- 11 **Miller LE,** Ouwehand AC. Probiotic supplementation decreases intestinal transit time: meta-analysis of randomized controlled trials. *World J Gastroenterol* 2013; **19**: 4718-4725 [PMID: 23922468 DOI: 10.3748/wjg.v19.i29.4718]
- 12 **Murakami K,** Okubo H, Sasaki S. Dietary intake in relation to self-reported constipation among Japanese women aged 18-20 years. *Eur J Clin Nutr* 2006; **60**: 650-657 [PMID: 16340942 DOI: 10.1038/sj.ejcn.1602365]
- 13 **Nakaji S,** Tokunaga S, Sakamoto J, Todate M, Shimoyama T, Umeda T, Sugawara K. Relationship between lifestyle factors and defecation in a Japanese population. *Eur J Nutr* 2002; **41**: 244-248 [PMID: 12474067 DOI: 10.1007/s00394-002-0380-4]
- 14 **Wong ML,** Wee S, Pin CH, Gan GL, Ye HC. Sociodemographic and lifestyle factors associated with constipation in an elderly Asian community. *Am J Gastroenterol* 1999; **94**: 1283-1291 [PMID: 10235208 DOI: 10.1111/j.1572-0241.1999.01078.x]
- 15 **Towers AL,** Burgio KL, Locher JL, Merkel IS, Safaeian M, Wald A. Constipation in the elderly: influence of dietary, psychological, and physiological factors. *J Am Geriatr Soc* 1994; **42**: 701-706 [PMID: 8014342]
- 16 **Georgieff MK.** Taking a rational approach to the choice of formula. *Contemp Pediatr* 2001; **18**: 112-130

- 17 **Roma E**, Adamidis D, Nikolara R, Constantopoulos A, Messaritakis J. Diet and chronic constipation in children: the role of fiber. *J Pediatr Gastroenterol Nutr* 1999; **28**: 169-174 [PMID: 9932850 DOI: 10.1097/00005176-199902000-00015]
- 18 **Bae SH**. Diets for constipation. *Pediatr Gastroenterol Hepatol Nutr* 2014; **17**: 203-208 [PMID: 25587519]
- 19 **Drummond L**, Gearry RB. Kiwifruit modulation of gastrointestinal motility. *Adv Food Nutr Res* 2013; **68**: 219-232 [PMID: 23394990 DOI: 10.1016/B978-0-12-394294-4.00012-2]
- 20 **Attaluri A**, Donahoe R, Valestin J, Brown K, Rao SS. Randomised clinical trial: dried plums (prunes) vs. psyllium for constipation. *Aliment Pharmacol Ther* 2011; **33**: 822-828 [PMID: 21323688 DOI: 10.1111/j.1365-2036.2011.04594.x]
- 21 **Na JR**, Oh KN, Park SU, Bae D, Choi EJ, Jung MA, Choi CY, Lee DW, Jun W, Lee KY, Kim YJ, Kim S. The laxative effects of Maesil (*Prunus mume* Siebold & Zucc.) on constipation induced by a low-fibre diet in a rat model. *Int J Food Sci Nutr* 2013; **64**: 333-345 [PMID: 23126362 DOI: 10.3109/09637486.2012.738648]
- 22 **Shiga TM**, Soares CA, Nascimento JR, Purgatto E, Lajolo FM, Cordenunsi BR. Ripening-associated changes in the amounts of starch and non-starch polysaccharides and their contributions to fruit softening in three banana cultivars. *J Sci Food Agric* 2011; **91**: 1511-1516 [PMID: 21445854 DOI: 10.1002/jsfa.4342]

P- Reviewer: Higgins PD, Thomas K, Tokunaga Y, Yamaoka Y

S- Editor: Qi Y **L- Editor:** A **E- Editor:** Wang CH



Observational Study

Comparison of magnetic resonance spectroscopy, proton density fat fraction and histological analysis in the quantification of liver steatosis in children and adolescents

Michele Di Martino, Lucia Pacifico, Mario Bezzi, Rossella Di Miscio, Beatrice Sacconi, Claudio Chiesa, Carlo Catalano

Michele Di Martino, Mario Bezzi, Rossella Di Miscio, Beatrice Sacconi, Carlo Catalano, Department of Radiological Sciences, Oncology and Anatomical Pathology, "Sapienza" University of Rome, 00161 Rome, Italy

Lucia Pacifico, Department of Pediatrics and Child Neuropsychiatry, "Sapienza" University of Rome, 00161 Rome, Italy

Claudio Chiesa, Institute of Translational Pharmacology, National Research Council, 00133 Rome, Italy

Author contributions: Di Martino M and Pacifico L contributed to study conception and design; Di Miscio R and Sacconi B contributed to data acquisition; Di Martino M, Pacifico L and Bezzi M contributed to data analysis and interpretation, and writing of article; Bezzi M, Chiesa C and Catalano C contributed to editing, reviewing and final approval of article.

Institutional review board statement: The study was reviewed and approved by the "Sapienza" University of Rome Institutional Review Board.

Informed consent statement: All study participants, or their legal guardian, provided informed written consent prior to study enrollment.

Conflict-of-interest statement: Nothing to disclosure.

Open-Access: This article is an open-access article which was selected by an in-house editor and fully peer-reviewed by external reviewers. It is distributed in accordance with the Creative Commons Attribution Non Commercial (CC BY-NC 4.0) license, which permits others to distribute, remix, adapt, build upon this work non-commercially, and license their derivative works on different terms, provided the original work is properly cited and the use is non-commercial. See: <http://creativecommons.org/licenses/by-nc/4.0/>

Manuscript source: Unsolicited manuscript

Correspondence to: Michele Di Martino, PhD, MD, Department

of Radiological Sciences, Oncology and Anatomical Pathology, "Sapienza" University of Rome, V.le Regina Elena 324, 00161 Rome, Italy. micdimartino@hotmail.it
Telephone: +39-6-49974511
Fax: +39-6-490243

Received: May 4, 2016

Peer-review started: May 5, 2016

First decision: May 27, 2016

Revised: June 30, 2016

Accepted: July 31, 2016

Article in press: July 31, 2016

Published online: October 21, 2016

Abstract

AIM

To establish a threshold value for liver fat content between healthy children and those with non-alcoholic fatty liver disease (NAFLD) by using magnetic resonance imaging (MRI), with liver biopsy serving as a reference standard.

METHODS

The study was approved by the local ethics committee, and written informed consent was obtained from all participants and their legal guardians before the study began. Twenty-seven children with NAFLD underwent liver biopsy to assess the presence of nonalcoholic steatohepatitis. The assessment of liver fat fraction was performed using MRI, with a high field magnet and 2D gradient-echo and multiple-echo T1-weighted sequence with low flip angle and single-voxel point-resolved ¹H MR-Spectroscopy (¹H-MRS), corrected for T1 and T2* decays. Receiver operating characteristic curve analysis was used to determine the best cut-off value. Lin coefficient test was used to evaluate the

correlation between histology, MRS and MRI-PDFF. A Mann-Whitney *U*-test and multivariate analysis were performed to analyze the continuous variables.

RESULTS

According to MRS, the threshold value between healthy children and those with NAFLD is 6%; using MRI-PDFF, a cut-off value of 3.5% is suggested. The Lin analysis revealed a good fit between the histology and MRS as well as MRI-PDFF.

CONCLUSION

MRS is an accurate and precise method for detecting NAFLD in children.

Key words: Magnetic resonance spectroscopy; Magnetic resonance imaging-PDFF; Obesity; Non-alcoholic fatty liver disease; Children

© The Author(s) 2016. Published by Baishideng Publishing Group Inc. All rights reserved.

Core tip: Differentiating normal from pathologic liver fat storage in children could depend on technical measurements. Using MR-spectroscopy, a cut-off value of 6% demonstrates the best diagnostic performance, otherwise magnetic resonance imaging (MRI)-PDFF cut-off value of 3.5% better discriminates normal weight from obese children. It is confirmed that MRS is an accurate and precise method for detecting non-alcoholic fatty liver disease in children. However, MRI-PDFF- is a feasible alternative to MRS for quantifying liver steatosis.

Di Martino M, Pacifico L, Bezzi M, Di Miscio R, Sacconi B, Chiesa C, Catalano C. Comparison of magnetic resonance spectroscopy, proton density fat fraction and histological analysis in the quantification of liver steatosis in children and adolescents. *World J Gastroenterol* 2016; 22(39): 8812-8819 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v22/i39/8812.htm> DOI: <http://dx.doi.org/10.3748/wjg.v22.i39.8812>

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is emerging as a leading cause of chronic liver disease in children and adolescents, with a prevalence in the general population ranging from 3%-10% in normal weight children and 80% in obese children^[1-2]. Children with NAFLD are usually asymptomatic and garner clinical attention because of elevated liver enzymes or fatty liver being observed during an ultrasound examination. The measurement of liver enzymes alone is not sufficient for accurate fatty liver screening in overweight children because enzymatic abnormalities correlates poorly or not at all with early steatosis^[3,4]. At present, liver biopsy represents the reference standard for diagnosing liver steatosis, although the

drawbacks of this invasive technique are well known. It is associated with morbidity and mortality, it has sampling errors, and it is not appropriate for screening, longitudinal monitoring, or evaluating treatment response^[4]. Among the currently available imaging modalities, magnetic resonance imaging (MRI) and ¹H MR spectroscopy (MRS) are the most reproducible, safe, and accurate imaging techniques that can be used in clinical trials and epidemiologic studies^[5]. However, MRS has limited clinical applicability or availability because it requires sophisticated post-processing methods, and not every MRI is routinely equipped with MRS capabilities. Recent improvements in MRI can provide the magnetic resonance imaging-estimated proton density fat fraction (MRI-PDFF), which is a novel biomarker that has demonstrated robust correlation and equivalency with MRS^[6-10]. In addition, MRI-PDFF allows fat mapping of the entire liver, and it can be used with any clinical MRI platform, whereas MRS measures fat biochemically in small regions of interest (ROIs). To the best of our knowledge, few studies have used MRS and histology to investigate liver fat content in adolescents^[11-14]. A recently published paper suggested similar fat-storage between overweight children and adults, and the study proposed the same cut-off value for normal and pathologic storage^[15]. The aim of our study was to validate a cut-off value for a pediatric population and to correlate the data with laboratory/chemistry results and subcutaneous and visceral adipose tissue (SAT and VAT) findings.

MATERIALS AND METHODS

The study has been approved by the ethics committee, and written informed consent was obtained from all participants and their legal guardians before the study began. From October 2013 to December 2014, 93 Caucasian obese children and adolescents [body mass index (BMI) above the 95th percentile for age and gender] were referred to the Hepatology Outpatient Unit of the Department of Pediatrics to confirm or rule out the presence of NALFD. All enrolled subjects underwent the following measurements: fast blood samples [glucose, cholesterol, triglycerides, alanine aminotransferase (ALT), aspartate aminotransferase (AST), insulin and γ -glutamyl transferase (γ -GT)] and BMI. They also underwent MRI to quantify liver steatosis and evaluate SAT and VAT. Five children were excluded because their data imaging was not suitable for post-processing due to several motion artifacts. The study population included 27 patients (16 males and 11 females; mean age, 13 years; range, 9-18 years) who were scheduled for liver biopsy to assess the presence of nonalcoholic steatohepatitis (NASH) or other liver diseases. Liver biopsy was performed within two weeks of the MR examination to avoid any bias, such as diet modification. An age- and sex-matched control group of 27 healthy Caucasian children, who

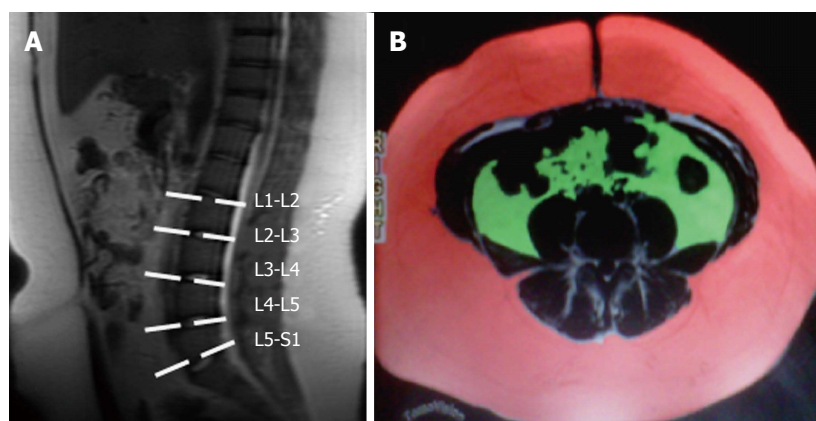


Figure 1 Subcutaneous and visceral fat measurement technique. A: Sagittal T1-weighted localizer MR image used to select levels for analysis (L1-L2, L2-L3, L3-L4, L4-L5, L5-S1); B: Axial T1-weighted MR image with water suppression at the L4-L5 level. Contrast was manually adjusted to select signal from subcutaneous (red) and visceral (green) adipose tissue. Area of this fat was calculated by the workstation and summarized with that of other levels.

also had blood chemistry results available, were recruited (11 males and 16 females; mean age, 12 years; range 8-18 years).

MR imaging

MRI exams were using a 3T magnet system (GE Discovery 750; General Electric Healthcare, Milwaukee, WI, United States), with a peak gradient amplitude of 50 mT/m and a 200 μ sec time to peak. An eight-element body torso-array coil system was used. Before the spectroscopy acquisition, a T2-weighted image in the coronal plane (TR 1300, TE 125, FA 90°, slice thickness 6 mm, matrix 288 \times 192) and a T1-weighted axial image (TR 4 ms/TE 1 ms, FA 60°, slice thickness 6 mm, matrix 288 \times 192) were acquired. To quantify the hepatic fat fraction (HFF), an axial breath-hold low-flip angle, T1-weighted, 2D multiple-echo, spoiled gradient-echo (GRE) sequence (TR5.1/TE from 0.8 to 3.8, flip angle 5°, field of view 33 cm; section thickness, 10 mm; intersection gap, 0, matrix 128 \times 128, acquisition time 2 \times 17 s) was used. MRS was performed using one 20 mm \times 20 mm \times 20 mm voxels placed in segment VI-VII (as close as possible to the site of liver biopsy) and avoiding the artifact, major blood vessels and biliary ducts. All spectra were obtained in the stimulated echo acquisition mode (STEAM, TR 4000 ms), using a breath hold sequence with an acquisition time of approximately 24 s. Field homogeneity was automatically adjusted for each voxel. The T2 relaxation times of both metabolites were determined from their peak amplitudes at each echo time using an exponential least-squares fitting algorithm, and saturation bands were used^[8]. For the quantification of subcutaneous and visceral adipose tissue (VAT and SAT), a 3D GRE T1-weighted sequence on an axial plane (TR4.1, TE 1.1, flip angle 15°, matrix 320 \times 192, section thickness 6 mm reconstructed 3 mm, intersection gap 0) was acquired using the IDEAL imaging and Dixon method, which enabled the

separation between water and fat components using the chemical shift MR technique.

Quantitative analysis

Images from multiple-echo GRE sequencing were subsequently analyzed with software provided by the manufacturer (Functool, GE Healthcare, Milwaukee, WI, United States); this procedure has been previously published^[16]. A ROI measuring 2-3 cm in diameter was drawn at the same site as the voxel used for 1H-MRS to avoid extrahepatic fat and large vessels. Magnetic resonance spectra were reconstructed on a dedicated workstation using the SAGE Dev2 0017.1 software (General Electric Healthcare, Milwaukee, WI, United States). Raw data were zero-filled once, and no filter was used. Spectra were referenced to residual water and the dominant methylene lipid (-CH₃ and -CH₂) peak at δ 4.8-5.2 and δ 0.9-1.1 and 1.3-1.6 and 2.1-2.3, respectively. The fat fraction percentage (FF) was defined as follows: $FF = FA / (FA + WA) \times 100$, where FA is the area under the fat peak and WA is the area under the water peak. The fat-only dataset from the T1-weighted sequence was transferred to a personal computer and analyzed using commercially available software (Slice-O-Matic; Tomovision Inc, Montreal Canada), the procedure for which has been described elsewhere^[17,18]. Briefly, SAT and VAT were calculated from 5 images extending from 5 cm below L4-L5 to 15 cm above L4-L5. A free-form ROI and manual thresholding were used to select tissue of fat within the subcutaneous and visceral adipose tissue (Figure 1).

Histopathologic analysis

With ultrasound guidance and within two weeks of the MR examination, a percutaneous needle liver biopsy was performed using an 18-gauge needle, with the patients under local anesthesia. To obtain an adequate sample, biopsy specimens were obtained twice from all patients at two different sites in the right hepatic

Table 1 Clinical analysis and anthropometry of the study population and the control group

	Patients with NAFLD			Control group		
	Median	SD	95%CI	Median	SD	95%CI
BMI	28.7 ¹	4.07	26.2-29.58	23.75	3.5	21.86-25.84
ALP	185	104.54	122.21-244.81	219.0	103.00	136.13-303.8
ALT	31	52.21	30-59.5	16.5	50.7	15-20.45
AST	25	25.78	21.14-30.85	22	25.21	20-26
Bilirubin	0.53	0.4074	0.38-0.68	0.59	0.21	0.37-0.67
γ-GT	21	12.24	17.14-28.97	11	9.98	10-12
Insuline	17.6 ¹	11.46	12.12-24.14	11.6	3.4	8.17-12.34
Cholesterol	154	38.56	130.44-168.55	142.5	28.8	136-167
Blood glucose	85	7.08	83.14-89.70	83	3.6	81-84
Triglycerides	77	68.86	68.68-114.65	60.5	24.64	50-84.45
VAT	368.35 ¹	258.23	334.9-501.7	275.9	76.02	252.4-299.9
SAT	1949.22 ¹	1184.9	1743.6-2886.0	1352.9	746.95	840.3-1539.4

¹Significantly higher values of BMI (0.0002), VAT (< 0.0001), SAT (0.0001), ALT (< 0.0001) and Insulin (0.0008) were reported. γ-GT: γ-glutamyl transferase; BMI: Body mass index; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase.

lobe (VI and VII segments). Liver specimens that were at least 1.5 cm in length and contained at least 10-11 complete portal tracts were considered to be adequate for histological assessment. Liver steatosis was determined by estimating the percentage of fat-containing hepatocytes on hematoxylin-eosin-stained specimens. The grading system for liver steatosis was based on the NASH Clinical Research Network criteria^[19]: grade 0, less than 5% steatosis; grade 1, 6%-33% steatosis; grade 2, 34%-66% steatosis; and grade 3, greater than 66% steatosis. The grading system incorporates the accepted normal value for histopathologic liver fat, which is less than 5%, and it is the standard applied in the clinical assessment of severity of liver steatosis by hepatologists and gastroenterologists.

Statistical analysis

To estimate the proper sample size in the correlation between the MR imaging and liver biopsy, a power analysis was conducted, considering a coefficient correlation of 0.6, α error = 0.05 and power $1 - \beta = 0.90$; the number of subjects was 21. Receiver operating characteristic (ROC) curve analysis was used to determine the best cut-off values for MRS and MRI-PDFF between the control group and children with NAFLD. The Pearson correlation coefficient was calculated among histology, MRS and MRI-PDFF. We also estimated agreement by using the 95% limit-of-agreement method developed by Bland and Altman^[20]. An analysis of the study population and control groups was performed to determine the median \pm SD, and comparisons were made using the Mann-Whitney *U* test. Multivariate analysis of the continuous variables was performed to evaluate which variables were useful for predicting liver steatosis. A *P* value less than 0.05 was considered to indicate significant difference. Statistical analysis was performed using the MedCalc Software (V.13.1.2, Acaciaaan 22 m 8400, Onsted, Belgium), except for the multivariate analysis, which was calculated using the JMP software (JMP.11, SAS

Institute Inc., Cary, NC, United States).

RESULTS

The clinical characteristics of the study population and control group are presented in Table 1.

Of the 27 patients who underwent liver biopsy, 11 (40.7%) children demonstrated grade 1 steatosis, 9 (33.3%) children demonstrated grade 2 and 7 (26%) demonstrated grade 3 (mean, 43.3% \pm 26; range, 10%-90%). The mean lipid content for MRS was 30% \pm 18 (median, 30%; range, 5%-66%), whereas the MRI-PDFF mean fat fraction was 11% \pm 7 (median, 10%; range, 1%-33%) (Figure 2). The following HFF values were recorded in the control group: MRS (mean, 4.4% \pm 2.5; median, 4%; range, 0.8%-13%) and MRI-PDFF (mean, 2.5 \pm 2; median, 1.9%; range, 0.7%-11%).

The ROC curve analysis suggested a cut-off value of 6% for MRS to discriminate between patients with and without steatosis (sensitivity, 92.6%; specificity, 95.7%; 6 false positive calls); conversely, MRI-PDFF suggested a cut-off value of 3.5% (sensitivity, 89%; specificity, 88%; 4 false positive calls) (Figure 3).

For MR spectroscopy, compared with the histology results, the Pearson test revealed a correlation of 0.68, (*P* = 0.0001) MRI-PDFF showed slightly lower values of 0.63, 0 (*P* = 0.0005).

Excellent correlation was reported between the MR techniques (*p* 0.81, *P* < 0.0001). Bland Altman plot reveals all points within were within the 95% limit of agreement, a possible bias encountered in the evaluation of MR-PDFF at medium and high level of hepatic steatosis (Figure 4).

Compared to the control group, significantly higher BMI (0.0002), VAT (< 0.0001), SAT (0.0001), ALT (< 0.0001) and insulin (0.0008) values were reported in the study population. Multivariate analysis of the quantitative variables demonstrated good correlation among VAT, SAT, BMI and insulin in terms of predicting liver steatosis (0.80, 0.51, 0.50 and 0.52, respectively).

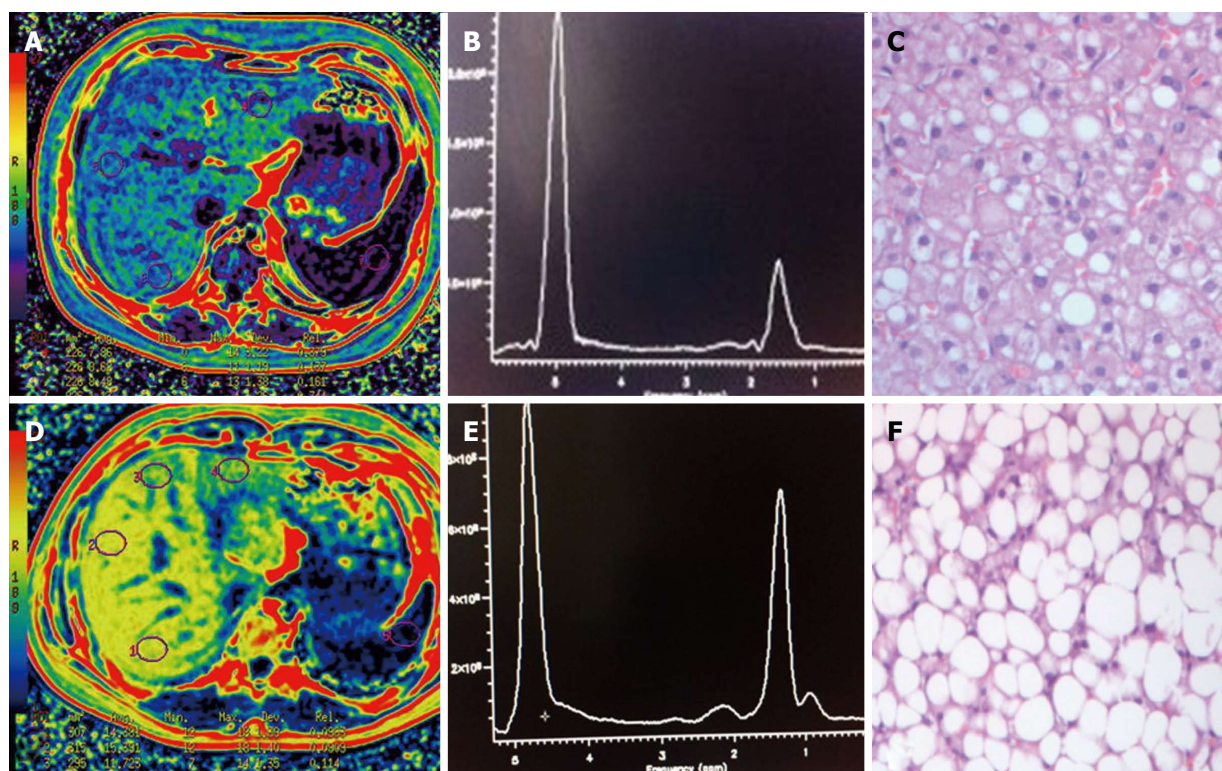


Figure 2 Mean lipid content for MR-Spectroscopy. A-C: MRI-PDFF reveals a hepatic fat fraction of 7%, MRS quantified 6.5% of liver steatosis and histological analysis 30% of liver steatosis; D-F: Severe hepatic steatosis in a 8-year-old girl: MRI-PDFF 25%, MRS 50% and histologic analysis 90% of liver steatosis respectively.

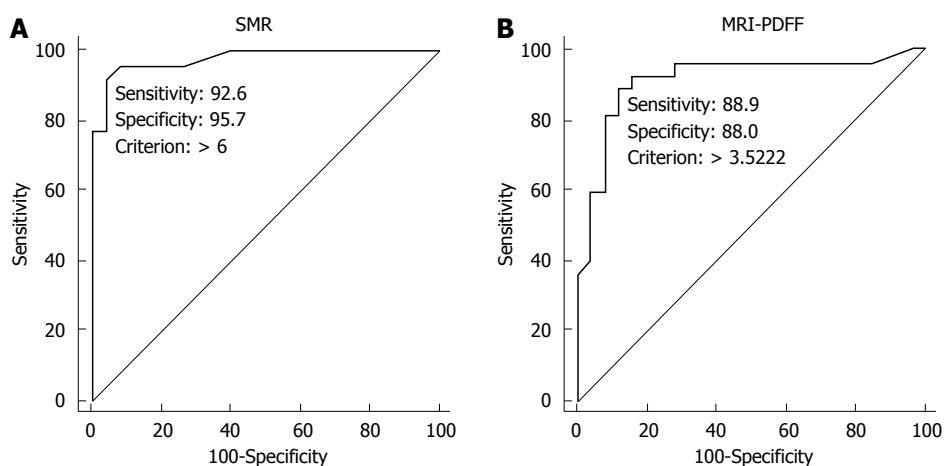


Figure 3 Receiver operating characteristic curve analysis of MR Spectroscopy (A) and MR imaging-PDFF (B) for discrimination of healthy from non-alcoholic fatty liver disease children.

DISCUSSION

Our results suggest that MRS is an accurate, non-invasive diagnostic technique for quantifying liver steatosis in a pediatric population and that MRI-PDFF is a feasible alternative technique. For MRS, the same cut-off value of 5% can be used to diagnosis liver steatosis in adolescents and adults; in contrast, when MRI-PDFF is used to quantify liver steatosis, a cut-off value of 3.5% is more appropriate. For clinical and morphologic analyses, the BMI, VAT, SAT and insulin

levels should be jointly considered promising tools for predicting liver steatosis.

In a pediatric population, precise measurement of the degree of liver steatosis is of clinical interest because it has demonstrated an association with subclinical signs of atherosclerosis and changes in myocardial functions^[21-24]. At present, no laboratory test can be used for the non-invasive quantification of liver steatosis, and ALT values may be elevated only in severe cases^[25]. Although our analysis reported significantly higher levels of ALT and insulin in pre-

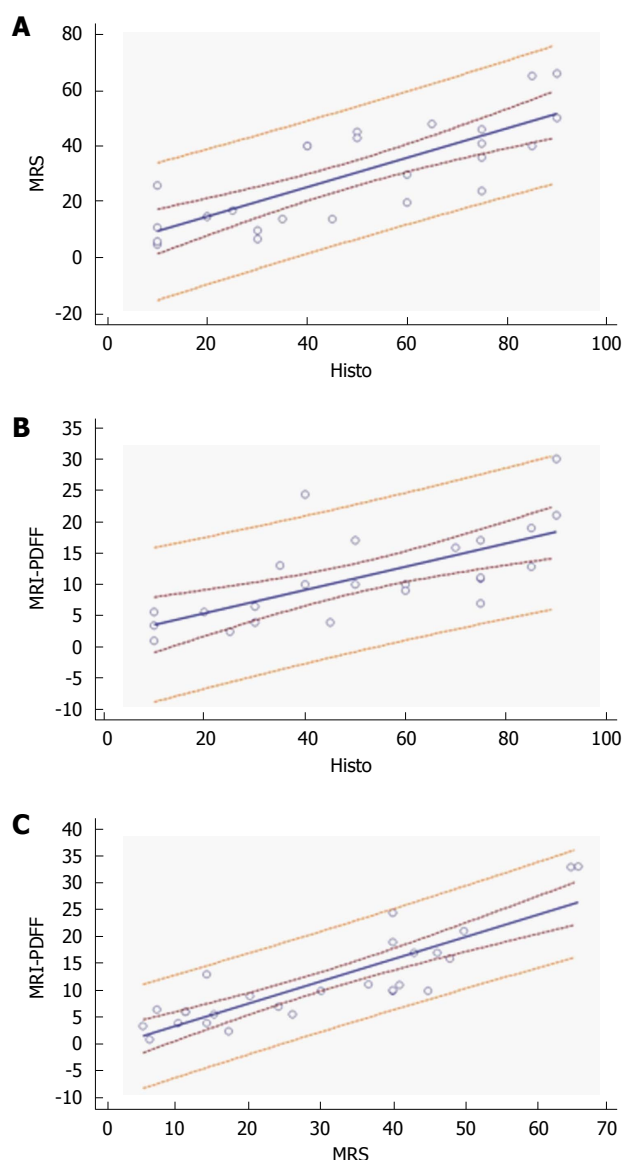


Figure 4 Evaluation of magnetic resonance imaging-estimated-proton density fat fraction at medium and high level of hepatic steatosis. A, B: Differences between liver fat fraction estimated by using ^1H MR spectroscopy and triple-echo sequence compared with histology were plotted against means, with 95% confidence intervals (Bland-Altman plot); C: All data points were within limits of agreement (dotted lines), corresponding to 1.96 SDs from mean. MRS: MR-Spectroscopy.

dicting liver steatosis, only in severe cases were the results beyond normal ranges. Anthropometry data revealed significantly higher BMI, SAT and VAT values in children with NAFLD, as previously described in the literature^[26,27]; unfortunately, multivariate analysis demonstrated only moderate to good values in predicting liver steatosis (range, 0.80 - 0.50).

Among the imaging modalities, MRS can provide a reliable estimation of the weight fraction of liver fat content, and it is now considered to be the most accurate non-invasive method; for these reasons, it is used extensively in published reports^[28-32]. In addition, in our experience, MRS demonstrated excellent correlation with liver biopsy, with good

concordance. However, MRS is an expensive and primarily research-based tool that is not always available^[33,34]. MRI-PDFF has been proposed as a feasible and simple alternative method for quantifying liver steatosis^[10,11,35-37]. Our data demonstrated good correlation between histology. However a probable error should develop in the differentiation between moderate and high grade of steatosis: this limit of chemical shift technique is attributed to the presence of both water and fat (fat-water signal dominance ambiguity) in the single voxel and it is not of clinical relevance for patients treatment and management^[38,39]. MR-PDFF suggests a lower threshold (3.5%) to define hepatic steatosis in children, in agreement with a recent publication by Rehm *et al.*^[40]. Our multiple-echo sequence has several advantages over MRS. First, the acquisition time is short, easily fitting within a breath hold. Second, the fat content can be measured throughout the liver instead of in only one voxel or a few voxels; this point is of major importance because fat distribution is often heterogeneous. Third, no spatial miss-registration errors occur because the OP and IP images are acquired simultaneously. Fourth, post-processing is considerably easier and faster than with MRS.

Some study limitations should be noted. First, the site-to-site reproducibility of our technique was evaluated only in segment VII. Second, our control group did not have histologically confirmed healthy livers because it would have been unethical to subject normal individuals to liver biopsy assessments. Thirdly, the three parameters (MRS, MR-PDFF and histology) tested in our study assess different aspects of steatosis. Histology reveals the percentage of hepatocytes that contain vesicles of fat, MR-PDFF determines the proportion of mobile protons contained within fat molecules and MRS shows the peaks of methyl and methylene ($-\text{CH}_2$ and CH_3) protons in the triglyceride molecule.

Finally, Bland-Altman plot analysis demonstrates a difficulty of MR-PDFF in the differentiation between moderate to severe steatosis; however this drawback has not clinical relevance because treatment and management of patients is quite similar.

In conclusion, MRS was confirmed as the most accurate non-invasive method for quantifying liver steatosis in obese children.

Multiple-echo gradient-echo MR sequence may also be an easily and rapidly performed technique used to quantify liver steatosis in a pediatric population; this technique demonstrated excellent agreement with MRS and should be considered as reference standard in longitudinal studies or clinical trials. Together, BMI, VAT, SAT and insulin measurements provide the most powerful test for predicting liver steatosis. Further studies should be conducted to better match data from histological and MRI analyses and to establish MRI thresholds for different grades of steatosis.

COMMENTS

Background

Non-alcoholic fatty liver disease (NAFLD) is emerging as a leading cause of chronic liver disease in children and adolescents, with a prevalence in the general population ranging from 3%-10% in normal weight children and 80% in obese children. Children with NAFLD are usually asymptomatic and garner clinical attention because of elevated liver enzymes or fatty liver being observed during an ultrasound examination.

Research frontiers

A recently published paper suggested similar fat-storage between overweight children and adults, and the study proposed the same cut-off value for normal and pathologic storage.

Innovations and breakthroughs

Body mass index, subcutaneous and visceral adipose tissue and insulin measurements provide the most powerful test for predicting liver steatosis.

Applications

This study should be conducted to better match data from histological and MRI analyses and to establish MRI thresholds for different grades of steatosis.

Peer-review

Interesting study, reasonable N. Needs more description of methods, results; I think with a careful revision, these authors can justify their conclusions with the data presented.

REFERENCES

- 1 Giorgio V, Prono F, Graziano F, Nobili V. Pediatric non alcoholic fatty liver disease: old and new concepts on development, progression, metabolic insight and potential treatment targets. *BMC Pediatr* 2013; **13**: 40 [PMID: 23530957 DOI: 10.1186/1471-2431-13-40]
- 2 Widhalm K, Ghods E. Nonalcoholic fatty liver disease: a challenge for pediatricians. *Int J Obes (Lond)* 2010; **34**: 1451-1467 [PMID: 20838401 DOI: 10.1038/ijo.2010.185]
- 3 Feldstein AE, Charatcharoenwithaya P, Treeprasertsuk S, Benson JT, Enders FB, Angulo P. The natural history of non-alcoholic fatty liver disease in children: a follow-up study for up to 20 years. *Gut* 2009; **58**: 1538-1544 [PMID: 19625277 DOI: 10.1136/gut.2008.171280]
- 4 Loomba R, Sirlin CB, Schwimmer JB, Lavine JE. Advances in pediatric nonalcoholic fatty liver disease. *Hepatology* 2009; **50**: 1282-1293 [PMID: 19637286 DOI: 10.1002/hep.23119]
- 5 Kotronen A, Yki-Järvinen H. Fatty liver: a novel component of the metabolic syndrome. *Arterioscler Thromb Vasc Biol* 2008; **28**: 27-38 [PMID: 17690317 DOI: 10.1161/ATVBAHA.107.147538]
- 6 Noureddin M, Lam J, Peterson MR, Middleton M, Hamilton G, Le TA, Bettencourt R, Changchien C, Brenner DA, Sirlin C, Loomba R. Utility of magnetic resonance imaging versus histology for quantifying changes in liver fat in nonalcoholic fatty liver disease trials. *Hepatology* 2013; **58**: 1930-1940 [PMID: 23696515 DOI: 10.1002/hep.26455]
- 7 Reeder SB, Cruite I, Hamilton G, Sirlin CB. Quantitative assessment of liver fat with magnetic resonance imaging and spectroscopy. *J Magn Reson Imaging* 2011; **34**: speone
- 8 Yokoo T, Shieh-morteza M, Hamilton G, Wolfson T, Schroeder ME, Middleton MS, Bydder M, Gamst AC, Kono Y, Kuo A, Patton HM, Horgan S, Lavine JE, Schwimmer JB, Sirlin CB. Estimation of hepatic proton-density fat fraction by using MR imaging at 3.0 T. *Radiology* 2011; **258**: 749-759 [PMID: 21212366 DOI: 10.1148/radiol.10100659]
- 9 Meisamy S, Hines CD, Hamilton G, Sirlin CB, McKenzie CA, Yu H, Brittain JH, Reeder SB. Quantification of hepatic steatosis with T1-independent, T2-corrected MR imaging with spectral modeling of fat: blinded comparison with MR spectroscopy. *Radiology* 2011; **258**: 767-775 [PMID: 21248233 DOI: 10.1148/radiol.10100708]
- 10 Hines CD, Frydrychowicz A, Hamilton G, Tudorascu DL, Vigen KK, Yu H, McKenzie CA, Sirlin CB, Brittain JH, Reeder SB. T(1) independent, T(2) (*) corrected chemical shift based fat-water separation with multi-peak fat spectral modeling is an accurate and precise measure of hepatic steatosis. *J Magn Reson Imaging* 2011; **33**: 873-881 [PMID: 21448952 DOI: 10.1002/jmri.22514]
- 11 Hamilton G, Yokoo T, Bydder M, Cruite I, Schroeder ME, Sirlin CB, Middleton MS. In vivo characterization of the liver fat ¹H MR spectrum. *NMR Biomed* 2011; **24**: 784-790 [PMID: 21834002 DOI: 10.1002/nbm.1622]
- 12 Cali AM, De Oliveira AM, Kim H, Chen S, Reyes-Mugica M, Escalera S, Dziura J, Taksali SE, Kursawe R, Shaw M, Savoye M, Pierpont B, Constable RT, Caprio S. Glucose dysregulation and hepatic steatosis in obese adolescents: is there a link? *Hepatology* 2009; **49**: 1896-1903 [PMID: 19434725 DOI: 10.1002/hep.22858]
- 13 Chabanova E, Bille DS, Thisted E, Holm JC, Thomsen HS. (1)H MRS assessment of hepatic steatosis in overweight children and adolescents: comparison between 3T and open 1T MR-systems. *Abdom Imaging* 2013; **38**: 315-319 [PMID: 22736224 DOI: 10.1007/s00261-012-9930-2]
- 14 Tang A, Tan J, Sun M, Hamilton G, Bydder M, Wolfson T, Gamst AC, Middleton M, Brunt EM, Loomba R, Lavine JE, Schwimmer JB, Sirlin CB. Nonalcoholic fatty liver disease: MR imaging of liver proton density fat fraction to assess hepatic steatosis. *Radiology* 2013; **267**: 422-431 [PMID: 23382291 DOI: 10.1148/radiol.12120896]
- 15 Larson-Meyer DE, Newcomer BR, VanVrancken-Tompkins CL, Sothorn M. Feasibility of assessing liver lipid by proton magnetic resonance spectroscopy in healthy normal and overweight prepubertal children. *Diabetes Technol Ther* 2010; **12**: 207-212 [PMID: 20151771 DOI: 10.1089/dia.2009.0069]
- 16 Idilman IS, Aniktar H, Idilman R, Kabacam G, Savas B, Elhan A, Celik A, Bahar K, Karcaaltincaba M. Hepatic steatosis: quantification by proton density fat fraction with MR imaging versus liver biopsy. *Radiology* 2013; **267**: 767-775 [PMID: 23382293 DOI: 10.1148/radiol.13121360]
- 17 Demerath EW, Ritter KJ, Couch WA, Rogers NL, Moreno GM, Choh A, Lee M, Remsberg K, Czerwinski SA, Chumlea WC, Siervogel RM, Towne B. Validity of a new automated software program for visceral adipose tissue estimation. *Int J Obes (Lond)* 2007; **31**: 285-291 [PMID: 16770332 DOI: 10.1038/sj.ijo.0803409]
- 18 Ross R, Freeman J, Hudson R, Janssen I. Abdominal obesity, muscle composition, and insulin resistance in premenopausal women. *J Clin Endocrinol Metab* 2002; **87**: 5044-5051 [PMID: 12414870 DOI: 10.1210/jc.2002-020570]
- 19 Brunt EM. Nonalcoholic steatohepatitis: definition and pathology. *Semin Liver Dis* 2001; **21**: 3-16 [PMID: 11296695 DOI: 10.1055/s-2001-12925]
- 20 Guu B, Petit JM, Loffroy R, Ben Salem D, Aho S, Masson D, Hillon P, Krause D, Cercueil JP. Quantification of liver fat content: comparison of triple-echo chemical shift gradient-echo imaging and in vivo proton MR spectroscopy. *Radiology* 2009; **250**: 95-102 [PMID: 19092092 DOI: 10.1148/radiol.2493080217]
- 21 Villanova N, Moscaticello S, Ramilli S, Bugianesi E, Magalotti D, Vanni E, Zoli M, Marchesini G. Endothelial dysfunction and cardiovascular risk profile in nonalcoholic fatty liver disease. *Hepatology* 2005; **42**: 473-480 [PMID: 15981216 DOI: 10.1002/hep.20781]
- 22 Targher G, Day CP, Bonora E. Risk of cardiovascular disease in patients with nonalcoholic fatty liver disease. *N Engl J Med* 2010; **363**: 1341-1350 [PMID: 20879883 DOI: 10.1056/NEJMr0912063]
- 23 Pacifico L, Di Martino M, De Merulis A, Bezzi M, Osborn JF, Catalano C, Chiesa C. Left ventricular dysfunction in obese children and adolescents with nonalcoholic fatty liver disease. *Hepatology* 2014; **59**: 461-470 [PMID: 23843206 DOI: 10.1002/hep.26610]
- 24 Santoro N, Caprio S. Nonalcoholic fatty liver disease/nonalcoholic steatohepatitis in obese adolescents: a looming marker of cardiac

- dysfunction. *Hepatology* 2014; **59**: 372-374 [PMID: 23913480 DOI: 10.1002/hep.26663]
- 25 **Sagi R**, Reif S, Neuman G, Webb M, Phillip M, Shalitin S. Nonalcoholic fatty liver disease in overweight children and adolescents. *Acta Paediatr* 2007; **96**: 1209-1213 [PMID: 17655622 DOI: 10.1111/j.1651-2227.2007.00399.x]
 - 26 **Taksali SE**, Caprio S, Dziura J, Dufour S, Calí AM, Goodman TR, Papademetris X, Burgert TS, Pierpont BM, Savoye M, Shaw M, Seyal AA, Weiss R. High visceral and low abdominal subcutaneous fat stores in the obese adolescent: a determinant of an adverse metabolic phenotype. *Diabetes* 2008; **57**: 367-371 [PMID: 17977954 DOI: 10.2337/db07-0932]
 - 27 **Maffeis C**, Manfredi R, Trombetta M, Sordelli S, Storti M, Benuzzi T, Bonadonna RC. Insulin sensitivity is correlated with subcutaneous but not visceral body fat in overweight and obese prepubertal children. *J Clin Endocrinol Metab* 2008; **93**: 2122-2128 [PMID: 18397988 DOI: 10.1210/jc.2007-2089]
 - 28 **Fonvig CE**, Bille DS, Chabanova E, Nielsen TR, Thomsen HS, Holm JC. Muscle fat content and abdominal adipose tissue distribution investigated by magnetic resonance spectroscopy and imaging in obese children and youths. *Pediatr Rep* 2012; **4**: e11
 - 29 **Kottronen A**, Peltonen M, Hakkarainen A, Sevastianova K, Bergholm R, Johansson LM, Lundbom N, Rissanen A, Ridderstråle M, Groop L, Orho-Melander M, Yki-Järvinen H. Prediction of non-alcoholic fatty liver disease and liver fat using metabolic and genetic factors. *Gastroenterology* 2009; **137**: 865-872 [PMID: 19524579 DOI: 10.1053/j.gastro.2009.06.005]
 - 30 **Longo R**, Pollesello P, Ricci C, Masutti F, Kvam BJ, Bercich L, Crocè LS, Grigolato P, Paoletti S, de Bernard B. Proton MR spectroscopy in quantitative in vivo determination of fat content in human liver steatosis. *J Magn Reson Imaging* 1995; **5**: 281-285 [PMID: 7633104 DOI: 10.1002/jmri.1880050311]
 - 31 **Szczepaniak LS**, Babcock EE, Schick F, Dobbins RL, Garg A, Burns DK, McGarry JD, Stein DT. Measurement of intracellular triglyceride stores by H spectroscopy: validation in vivo. *Am J Physiol* 1999; **276**: E977-E989 [PMID: 10329993]
 - 32 **van Werven JR**, Marsman HA, Nederveen AJ, Smits NJ, ten Kate FJ, van Gulik TM, Stoker J. Assessment of hepatic steatosis in patients undergoing liver resection: comparison of US, CT, T1-weighted dual-echo MR imaging, and point-resolved 1H MR spectroscopy. *Radiology* 2010; **256**: 159-168 [PMID: 20574093 DOI: 10.1148/radiol.10091790]
 - 33 **Chalasani N**. Nonalcoholic fatty liver disease liver fat score and fat equation to predict and quantitate hepatic steatosis: promising but not prime time! *Gastroenterology* 2009; **137**: 772-775 [PMID: 19638269 DOI: 10.1053/j.gastro.2009.07.032]
 - 34 **Springer F**, Machann J, Claussen CD, Schick F, Schwenzer NF. Liver fat content determined by magnetic resonance imaging and spectroscopy. *World J Gastroenterol* 2010; **16**: 1560-1566 [DOI: 10.3748/wjg.v16.i13.1560]
 - 35 **Reeder SB**, Robson PM, Yu H, Shimakawa A, Hines CD, McKenzie CA, Brittain JH. Quantification of hepatic steatosis with MRI: the effects of accurate fat spectral modeling. *J Magn Reson Imaging* 2009; **29**: 1332-1339 [PMID: 19472390 DOI: 10.1002/jmri.21751]
 - 36 **Machann J**, Thamer C, Schnoedt B, Stefan N, Haring HU, Claussen CD, Fritsche A, Schick F. Hepatic lipid accumulation in healthy subjects: a comparative study using spectral fat-selective MRI and volume-localized 1H-MR spectroscopy. *Magn Reson Med* 2006; **55**: 913-917 [PMID: 16506186 DOI: 10.1002/mrm.20825]
 - 37 **Cowin GJ**, Jonsson JR, Bauer JD, Ash S, Ali A, Osland EJ, Purdie DM, Clouston AD, Powell EE, Galloway GJ. Magnetic resonance imaging and spectroscopy for monitoring liver steatosis. *J Magn Reson Imaging* 2008; **28**: 937-945 [PMID: 18821619 DOI: 10.1002/jmri.21542]
 - 38 **Yokoo T**, Bydder M, Hamilton G, Middleton MS, Gamst AC, Wolfson T, Hassanein T, Patton HM, Lavine JE, Schwimmer JB, Sirlin CB. Nonalcoholic fatty liver disease: diagnostic and fat-grading accuracy of low-flip-angle multiecho gradient-recalled-echo MR imaging at 1.5 T. *Radiology* 2009; **251**: 67-76 [PMID: 19221054 DOI: 10.1148/radiol.2511080666]
 - 39 **Africa JA**, Newton KP, Schwimmer JB. Lifestyle Interventions Including Nutrition, Exercise, and Supplements for Nonalcoholic Fatty Liver Disease in Children. *Dig Dis Sci* 2016; **61**: 1375-1386 [PMID: 27041377 DOI: 10.1007/s10620-016-4126-1]
 - 40 **Rehm JL**, Wolfgram PM, Hernando D, Eickhoff JC, Allen DB, Reeder SB. Proton density fat-fraction is an accurate biomarker of hepatic steatosis in adolescent girls and young women. *Eur Radiol* 2015; **25**: 2921-2930 [PMID: 25916386 DOI: 10.1007/s00330-015-3724-1]

P- Reviewer: Shah RV, Wang CY S- Editor: Qi Y L- Editor: A
E- Editor: Wang CH



Randomized Clinical Trial

22-gauge core vs 22-gauge aspiration needle for endoscopic ultrasound-guided sampling of abdominal masses

William Sterlacci, Athanasios D Sioulas, Lothar Veits, Pervin Gönüllü, Guido Schachschal, Stefan Groth, Mario Anders, Christos K Kontos, Theodoros Topalidis, Andrea Hinsch, Michael Vieth, Thomas Rösch, Ulrike W Denzer

William Sterlacci, Lothar Veits, Michael Vieth, Institute of Pathology, Clinic of Bayreuth, 95445 Bayreuth, Germany

Athanasios D Sioulas, Pervin Gönüllü, Guido Schachschal, Stefan Groth, Mario Anders, Thomas Rösch, Ulrike W Denzer, Department of Interdisciplinary Endoscopy, University Hospital Hamburg-Eppendorf, 20246 Hamburg, Germany

Christos K Kontos, Department of Biochemistry and Molecular Biology, University of Athens, 15701 Athens, Greece

Theodoros Topalidis, Institute of Pathology, 30539 Hannover, Germany

Andrea Hinsch, Institute of Pathology, University Hospital Hamburg-Eppendorf, 20246 Hamburg, Germany

Author contributions: Denzer UW conceived the idea and designed the study, performed the endoscopies, collected the data, reviewed the draft, and approved the final manuscript; Sioulas AD performed the literature search and drafted and approved the final manuscript; Sterlacci W, Veits L, Topalidis T, Hinsch A and Vieth M performed the cytohistological analyses of the specimens, reviewed the draft, and approved the final manuscript; Kontos CK analyzed the data, reviewed the draft, and approved the final manuscript; Schachschal G, Groth S and Anders M performed the endoscopies, reviewed the draft, and approved the final manuscript; Rösch T critically reviewed the draft, and approved the final manuscript; all the authors contributed to this manuscript.

Institutional review board statement: This study was reviewed and approved by the Institutional Clinical Research Ethics Committee of the University Hospital Hamburg-Eppendorf (study number: PV 3835).

Clinical trial registration statement: The study has been registered at Clinicaltrials.gov (ID: NCT02181140).

Informed consent statement: All of the individuals who participated in the study provided their written informed consent prior to study enrollment.

Conflict-of-interest statement: None to declare.

Data sharing statement: No additional data are available.

Open-Access: This article is an open-access article which was selected by an in-house editor and fully peer-reviewed by external reviewers. It is distributed in accordance with the Creative Commons Attribution Non Commercial (CC BY-NC 4.0) license, which permits others to distribute, remix, adapt, build upon this work non-commercially, and license their derivative works on different terms, provided the original work is properly cited and the use is non-commercial. See: <http://creativecommons.org/licenses/by-nc/4.0/>

Manuscript source: Invited manuscript

Correspondence to: Athanasios D Sioulas, MD, PhD, Department of Interdisciplinary Endoscopy, University Hospital Hamburg-Eppendorf, Martinistraße 52, 20246 Hamburg, Germany. athsioulas@yahoo.gr
Telephone: +49-0-407410
Fax: +49-0-407410-44420

Received: June 25, 2016

Peer-review started: June 27, 2016

First decision: August 8, 2016

Revised: August 21, 2016

Accepted: September 14, 2016

Article in press: September 14, 2016

Published online: October 21, 2016

Abstract

AIM

To compare the aspiration needle (AN) and core biopsy needle (PC) in endoscopic ultrasound-guided fine needle aspiration (EUS-FNA) of abdominal masses.

METHODS

Consecutive patients referred for EUS-FNA were included in this prospective single-center trial. Each patient underwent a puncture of the lesion with both standard 22-gauge (G) AN (Echo Tip Ultra; Cook Medical, Bloomington, Indiana, United States) and the novel 22G PC (EchoTip ProCore; Cook Medical, Bloomington, Indiana, United States) in a randomized fashion; histology was attempted in the PC group only. The main study endpoint was the overall diagnostic accuracy, including the contribution of histology to the final diagnosis. Secondary outcome measures included material adequacy, number of needle passes, and complications.

RESULTS

Fifty six consecutive patients (29 men; mean age 68 years) with pancreatic lesions ($n = 38$), lymphadenopathy ($n = 13$), submucosal tumors ($n = 4$), or others lesions ($n = 1$) underwent EUS-FNA using both of the needles in a randomized order. AN and PC reached similar overall results for diagnostic accuracy (AN: 88.9 vs PC: 96.1, $P = 0.25$), specimen adequacy (AN: 96.4% vs PC: 91.1%, $P = 0.38$), mean number of passes (AN: 1.5 vs PC: 1.7, $P = 0.14$), mean cellularity score (AN: 1.7 vs PC: 1.1, $P = 0.058$), and complications (none). A diagnosis on the basis of histology was achieved in the PC group in 36 (64.3%) patients, and in 2 of those as the sole modality. In patients with available histology the mean cellularity score was higher for AN (AN: 1.7 vs PC: 1.0, $P = 0.034$); no other differences were of statistical significance.

CONCLUSION

Both needles achieved high overall diagnostic yields and similar performance characteristics for cytological diagnosis; histological analysis was only possible in 2/3 of cases with the new needle.

Key words: Endoscopic ultrasound; Cytology; Fine needle aspiration; Abdominal tumors; Core biopsy needle

© The Author(s) 2016. Published by Baishideng Publishing Group Inc. All rights reserved.

Core tip: Endoscopic ultrasound-guided fine needle aspiration and cytological analysis of the obtained material represents an established modality for diagnosis of intra- and paramural lesions. Recently developed fenestrated needles enable specimen acquisition for histological analysis aiming to improve diagnostic accuracy. We prospectively compared the 22 gauge standard aspiration needle with the same-diameter novel core biopsy needle in sampling of abdominal masses. Both needles yielded similar overall diagnostic accuracy, while no significant differences were evident regarding sample adequacy for the analysis, quality, and cellularity of specimens, number of needle passes, feasibility, and complications. The diagnostic contribution of histology with the novel needle was limited.

Sterlacci W, Sioulas AD, Veits L, Gönüllü P, Schachschal G, Groth S, Anders M, Kontos CK, Topalidis T, Hinsch A, Vieth M, Rösch T, Denzer UW. 22-gauge core vs 22-gauge aspiration needle for endoscopic ultrasound-guided sampling of abdominal masses. *World J Gastroenterol* 2016; 22(39): 8820-8830 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v22/i39/8820.htm> DOI: <http://dx.doi.org/10.3748/wjg.v22.i39.8820>

INTRODUCTION

Endoscopic ultrasound (EUS) has become widely-used for diagnostic purposes regarding lesions arising from the pancreas, upper gastrointestinal tract, as well as adjacent structures, such as the liver and lymph nodes. In this setting, EUS-guided fine needle aspiration (EUS-FNA) is currently considered a technique with an excellent safety profile with reported sensitivity of 60% to 95% and overall diagnostic accuracy ranging from 60% to 90%^[1-3].

However, EUS-FNA performance is dependent on numerous factors, including those related to target lesion (location, size, characteristics), technical details (type of needle used, aspiration/biopsy method, number of passes, material processing), and involved personnel (endoscopist expertise, presence of on-site experienced cytopathologist)^[4-7]. Usually, cytology is the basis of EUS-FNA based tissue diagnosis. However, cytological specimens obtained by means of standard EUS-FNA are of limited value in diagnosing entities like gastrointestinal stromal tumors, lymphomas, and autoimmune pancreatitis that mostly require additional immunocytochemistry and/or flow cytometry (e.g., lymphomas) or tissue processing and immunohistochemical evaluation for an accurate classification^[8-10]. Furthermore, individualized tumor therapy may mandate a more detailed immunohistological analysis in the future that may not be sufficiently covered by cytology.

To overcome these limitations of cytology and to reliably retrieve samples suitable for histopathological analysis, several new needles have been developed in recent years, with variable success^[10-12]; most recently, a novel needle (EchoTip ProCore; Cook Medical, Bloomington, Indiana, United States) has been developed that features a hollowed-out reverse bevel. This side fenestration promises to enable the acquisition of core biopsy specimens with preserved architecture and thus improve diagnostic yield. Fulfilling those expectations, recent published results on the performance of ProCore needles demonstrate high diagnostic accuracy rates from 82% to 96%^[13-17].

We hypothesized that this novel needle is superior in diagnostic performance, given its advanced technical characteristics. Therefore, we conducted this randomized study in order to prospectively compare the standard 22-gauge (G) aspiration needle (AN) with the 22G EchoTip ProCore needle (PC) in terms of diagnostic accuracy, adequacy of the obtained material

for evaluation, histocytological quality of the samples, technical feasibility, and related complications.

MATERIALS AND METHODS

This single-center prospective study was conducted at the Department of Interdisciplinary Endoscopy of the University Hospital Hamburg-Eppendorf in Germany from August 2011 to November 2013. The study protocol was approved by the Institutional Clinical Research Ethics Committee (study number: PV 3835) and was registered at Clinicaltrials.gov (ID: NCT02181140). All of the enrolled patients provided written informed consent for the procedure and study participation.

Patients

A total of 56 patients, between 18 and 85 years old, were enrolled in the study. All of the patients with an indication for EUS-FNA for the assessment of pancreatic lesions, paramural mass lesions, or subepithelial tumors (SET) were included. Exclusion criteria were: (1) very difficult or impossible access to the target site (*i.e.*, post-operative anatomic alterations, interpositioned vessels); (2) cystic lesions without solid tissue; (3) coagulopathy (Quick time < 40% or platelets-PLTs < 40000/mm³) or ongoing anticoagulant medications except ASS; and (4) poor performance status (Eastern Cooperative Oncology Group-ECOG IV).

Data collection

For each eligible patient, the following data were recorded: basic characteristics [age, gender, body mass index (BMI)], symptoms (pain, jaundice, weight loss), lesion location, laboratory data [complete blood count-CBC, international normalized ratio (INR)], and available imaging studies prior to EUS.

During the study, the following parameters were recorded: location, size, and echogenicity of the lesions, dose of the propofol administered for sedation, number of passes for each needle, specimen adequacy for evaluation, cellularity and cytological/histological quality of the material, cytohistological analysis result, and complications within 24 h after the intervention.

Follow-up was performed by telephone interviews, hospital visits, and chart reviews until the patient's death or termination of the study. Inquiries included symptoms, laboratory and/or imaging tests, and subsequent interventions, namely repeated tissue acquisition and surgery.

Procedures

All of the procedures were carried out by 4 experienced endoscopists, assisted by one endoscopy nurse. A linear array echoendoscope (GF-UCT 180, Olympus Europa, Hamburg, Germany) connected to a processor featuring a color Doppler function (Aloka Alpha 7,

Hitachi Medical Corporation, Tokyo, Japan) was used in all of the examinations. The patients remained in the left lateral position under conscious sedation by means of intravenous propofol administration. Oxygen *via* the nasal cannula and recording of the vital signs were continuously provided. No on-site cytopathologist was available.

Following careful scope manipulations, the endoscopist obtained visualization of the target lesion and, using the color Doppler function, excluded vessel interposition along the puncture route. The lesion parameters, including exact location, size, and echogenicity, were assessed and recorded. Subsequently, the puncture of the mass by both the standard 22G aspiration needle (AN; Echo Tip Ultra; Cook Medical, Bloomington, Indiana, United States) and the novel 22G core biopsy needle (PC; EchoTip ProCore; Cook Medical, Bloomington, Indiana, United States) was performed in a randomized order as determined by a computer-generated randomization assignment that took place prior to the procedure. The PC has a 5.2F shaft, a core trap sized 2 mm, and a reverse-bevel length of 5.9 mm. The same sampling technique was used for both needles to avoid technical biases. In detail, after the needle had successfully entered the lesion, its stylet was removed and suction was applied using a 10 mL syringe. During each puncture, the needle was moved back and forth between 10 and 20 times and, at the end, it was withdrawn from the mass after suction was released. The number of passes depended on the examiner's estimation of the yielded material with a maximum 3 passes being attempted with each device according to the specifications of the ethics committee.

Following each pass, the further processing of the acquired material was performed by the endosonographer, given the lack of an on-site cytopathologist. This was done, in detail, as following:

In the case of the standard AN, the specimens were smeared onto glass slides by stylet's reintroduction or by air flushing into the needle. Cytological evaluation was subsequently undertaken after air dried fixation and staining with the Giemsa method.

Regarding the PC, the material was completely flushed out with saline solution. When small-core biopsy cylinders (defined as whitish pieces of tissue with apparent bulk, which did not consist of blood and, therefore, dissolved in saline solution) were identified, they were retrieved by syringe suction and subsequently placed into formalin for histological analysis. The remaining material was used for the preparation of cytological smears. Core biopsy cylinders were subsequently cut to obtain hematoxylin-eosin stained sections. Cytological smears were fixed in ethanol and stained with Papanikolaou method.

The biopsy material was evaluated due to diagnostic and quality parameters by a study cytopathologist who was blinded to the type of the needle used.

Table 1 Patient demographics and baseline characteristics

Parameter	Value
No. of patients	56
Age (yr), mean (SD)	68 (12)
Sex, <i>n</i> (%)	
Male	29 (51.8)
Female	27 (48.2)
BMI (kg.m ⁻²), mean (SD)	25.6 (3.6)
Presenting symptom(s) (%)	
Pain	22.4
Weight loss	28.3
Jaundice	19.6

BMI: Body mass index.

Classification of results

The cytological and histological findings by EUS-FNA were classified as positive for malignancy if an unequivocal diagnosis of malignancy was made. It is noteworthy that gastrointestinal stromal tumors, neuroendocrine tumors (NET), and intraductal papillary mucinous neoplasia cases were included in the group of malignancies for the analysis of diagnostic parameters. The acquired material was regarded as adequate for cytological/histological analysis using the cytopathology quality scoring as described below.

Study end points

The main outcome parameters used for comparisons between the standard aspiration and ProCore needles were: (1) the rate of correct diagnosis of the obtained material and related diagnostic discrimination values (sensitivity, specificity, diagnostic accuracy). The gold standard criteria for diagnosis were considered as one or more of the following: definite EUS-FNA (see above), surgical resection, or clinical follow-up exceeding 12 mo. In the PC group, the cytology and histology results were considered together for the overall diagnosis; in the AN group, only cytology was used for diagnosis; and (2) the percentage of cases in which the collected specimen was regarded by the cytopathologist as adequate for cytological/histological examination defined as a cytology/histology quality score 1-3 (s. below).

Secondary endpoints included comparisons of several performance parameters, such as the following: (1) the number of needle passes needed to achieve a gain of a macroscopically optimal sample; (2) the quality of the cytological/histological specimens was rated with scores from 0 to 3 [0, non-representative; 1, representation questionable (poorly preserved, crush artifacts, overlapping cell groups); 2, representation limited (scant amount of diagnostic cells); and 3, representative], as modified from Payne *et al.*^[18]; (3) the cellularity of specimens was expressed by a score in a scale from 0 to 2 (0, poorly preserved (cellularity not reliably assessable); 1, low cellularity; 2, high

cellularity); and (4) the rate of procedure-related complications. These included bleeding, perforation, acute pancreatitis, hemobilia, or death; patients were specifically followed for 24 h after the intervention.

Sample size assumption and power calculations

Sample size was based on an inferiority design comparing both needles in diagnostic accuracy. Assuming the PC to be superior (sensitivity: 75% vs 60%), 53 evaluable patients were required (power 80%, alpha 0.05) to show superiority of the core needle.

Statistical analysis

Descriptive statistics for continuous variables are presented as means or medians with standard deviations (SD) or range, respectively. Categorical variables are reported as absolute values and percentages. Sensitivity, specificity, and diagnostic accuracy are calculated according to their definitions. Of note, the analyses only considered cases with adequate material.

Differences between the performance of AN and PC regarding the sample adequacy for evaluation as well as the rates of correct diagnosis were assessed using the McNemar's test. Comparisons between the number of needle passes, cellularity score, quality of cytological/histological material score and the complication rates were assessed using the non-parametric Wilcoxon rank sum test. The level of statistical significance for all the tests was defined at a probability value of less than 0.05 ($P < 0.05$). Datasets were compiled by using Microsoft Excel and all the statistical analyses were performed with IBM SPSS Statistics V22.0 software (SPSS Inc., Chicago, IL, United States).

RESULTS

Patients' demographics and baseline characteristics

A total of 56 patients were finally included in the study (Figure 1); patient demographics and baseline characteristics are presented in detail in Table 1. About 2/3 of the lesions were in the pancreas; lesion parameters are shown in Table 2. Confirmation of the final diagnoses was performed on the basis of surgery ($n = 26$), definite EUS-FNA result ($n = 16$), clinical follow-up for more than 12 mo ($n = 6$), or by more than one means ($n = 8$). The mean propofol dose administered for sedation was 482 mg.

Technical and diagnostic performance of the aspiration needle (cytology)

Technically successful advancement of the AN into the target lesion and sample collection was achieved in all of the cases. Inadequate for cytological analysis material was obtained in 2 cases, including pancreatic NET ($n = 1$) and gastric antrum SET ($n = 1$). A

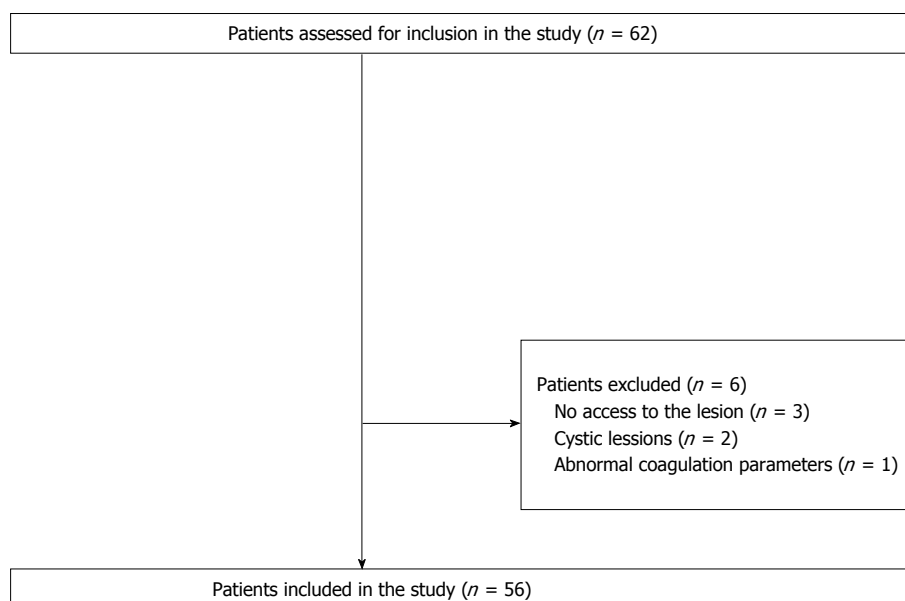


Figure 1 Study flowchart showing numbers of included and excluded patients.

Table 2 Lesion characteristics

Parameter	n (%)
Location	
Pancreas	38 (67.9)
Lymph nodes	13 (23.2)
SMT	4 (7.1)
Other	1 (1.8)
Diameter (mm), mean (SD)	33 (12)
Echogenicity on EUS ¹	
Hyper-/hypo-/iso-echoic	7 (12.7)/44 (80)/2 (3.6)
Non-homogeneous	2 (3.6)
Final diagnosis	
Pancreatic adenocarcinoma	25 (44.6)
Pancreatic NET	7 (12.5)
Lymph node metastasis	6 (10.7)
Inflammatory lymph node	5 (8.9)
GIST	3 (5.4)
Chronic pancreatitis	2 (3.6)
Pancreatic metastasis ²	2 (3.6)
Cholangiocarcinoma	1 (1.8)
Pancreatic lymphoma	1 (1.8)
Lymphoma	1 (1.8)
Leiomyoma	1 (1.8)
IPMN	1 (1.8)
Lymphoma renal infiltration	1 (1.8)
Gold standard method	
Surgery	26 (46.4)
Definite EUS-FNA	16 (28.6)
Clinical follow-up (> 12 mo)	6 (10.7)
Combination	8 (14.3)

¹Data available in 55/56 patients; ²Non-small-cell lung cancer ($n = 1$), malignant melanoma ($n = 1$). EUS-FNA: Endoscopic ultrasound-guided fine needle aspiration; NET: Neuroendocrine tumor; SMT: Submucosal tumor; IPMN: Intraductal papillary mucinous neoplasm.

wrong diagnosis was reached in 6 patients. Missed cases comprised pancreatic adenocarcinoma ($n = 2$), inflammatory lymph nodes ($n = 2$), IPMN ($n = 1$), and pancreatic lymphoma ($n = 1$). No procedure-related

complications were captured. Detailed performance characteristics of AN are shown in Table 3.

Technical and diagnostic performance of the ProCore needle (cytology or histology)

Technical success of EUS-FNA using the PC was universal (100%) and yielded adequate specimens (36 histological and 15 cytological) in all but 5 (pancreatic adenocarcinoma; $n = 4$, gastric antrum SET; $n = 1$) cases. A correct diagnosis, compared to the gold standard, was achieved in 49 evaluable patients. Missed cases included pancreatic adenocarcinoma ($n = 1$) and pancreatic lymphoma ($n = 1$). No adverse events were encountered (Table 3).

Comparisons of the aspiration needle and ProCore needle (in all patients)

Adequate for interpretation material was obtained by the usage of at least one needle in 55/56 cases (98.2%). The gastric antrum SET case ($n = 1$) allowed no sufficient specimen collection in general. At least one device correctly diagnosed 53 of the 55 eligible for the assessment individuals (96.4%) with the exception of pancreatic adenocarcinoma ($n = 1$) and pancreatic lymphoma ($n = 1$). The collection of adequate material by both needles was achieved in 50/56 cases (89.3%). In 47 of them (94%), the cytological/histological analysis results were in agreement, while in only 2 of these patients (4.4%) both specimens yielded an incorrect diagnosis (missed cases; pancreatic adenocarcinoma, $n = 1$ and pancreatic lymphoma, $n = 1$).

As shown in Table 3, there was no statistically significant difference between the AN and the PC in terms of the adequacy of specimens for the evaluation, mean number of passes, mean cellularity score, median specimen cytological or histological quality

Table 3 Technical characteristics and outcomes of endoscopic ultrasound-guided fine needle aspiration with the 2 needles in all patients ($n = 56$) and in patients with an available histological specimen ($n = 36$)

Characteristic	Type of needle (all cases/histology cases)		
	AN ($n = 56/36$)	PC ($n = 56/36$)	<i>P</i> value
Needle passes, mean (SD)	1.5 (0.6)/1.5 (0.7)	1.7 (0.6)/1.7 (0.6)	0.14/0.16
Cellularity, mean (SD)	1.7 (0.6)/1.7 (0.6)	1.1 (0.3)/1 (0)	0.058/0.034 ²
Cytologic/histologic quality, median (range)	2.6 (0-3)/ 3 (0-3)	2.4 (0-3)/3 (0-3)	0.083/0.49
Adequacy for diagnosis, n (%)	54 (96.4)/35 (97.2)	51 (91.1)/36 (100)	0.38/0.99
Correct diagnosis ¹ , n (%)	48/54 (88.9)/30/35 (85.7)	49/51 (96.1)/34/36 (94.4)	0.25/0.25

¹When adequate for analysis material was obtained; ²Statistically significant difference ($P < 0.05$). A difference is considered as statistically significant if $P < 0.05$. AN: Aspiration needle; PC: ProCore needle.

Table 4 Sensitivity, specificity and diagnostic accuracy of the 2 needles for the diagnosis of malignancy

	Sensitivity	Specificity	Diagnostic accuracy
All lesions ¹			
AN	91.5%	71.4%	88.9%
PC	95.4%	85.7%	94.1%
Pancreatic mass ²			
AN	88.5%	100.0%	89.2%
PC	93.8%	100.0%	94.1%
Lymph nodes ³			
AN	100.0%	60.0%	84.6%
PC	100.0%	80.0%	92.3%

¹Evaluable cases; AN: 54/56, PC: 51/56; ²Evaluable cases; AN: 37/38, PC: 34/38; ³Evaluable cases; both needles: 13/13. AN: Aspiration needle; PC: ProCore needle.

score, rates of correct diagnosis, technical success (100% in both) or complications (none in both).

Comparison between the aspiration needle and ProCore needle (in patients with available histology)

In the 36 patients in whom histological material was obtained with PC, AN achieved better mean cellularity score ($P = 0.034$). Differences regarding the adequacy of specimens for evaluation, mean number of passes, median specimen quality score, rates of correct diagnosis, technical success, and complications were of no statistical importance (Table 3).

Table 4 summarizes the results of the two needles and provides separate data on pancreatic mass and lymph node subgroups.

Figures 2 and 3 present various lesions as shown during EUS, including their appearance in the cytological/histological analysis.

DISCUSSION

EUS-FNA has been established as a safe and efficient technique for the diagnosis of solid lesions accessible from the upper gastrointestinal tract. Many prospective and retrospective series have reported reliable sensitivity, specificity, and overall diagnostic accuracy, mostly depending on the methodological factors, including on-site cytopathological evaluation, type

of needle used, and number of needle passes^[19,20]. In almost all of these studies, the diagnosis rested on cytological analysis. This appears to be well established; however, cytological analysis suffers from several principal limitations such as subjective interpretation with the lack of a standardized second-opinion process as is usual in histopathology, different categories with regards to the certainty of diagnosis ("suspicious" results are mostly counted positive in the respective studies), and further limitations concerning tissue characterization. In this setting, the complementary to routine cytology use of one or more immunocytochemical markers as well as flow cytometry (e.g., in lymphomas) seems to improve diagnostic accuracy. Moreover, molecular genetic analysis (e.g., assay for K-ras or p53 gene mutations by RT-PCR), although currently not a routine component of specimen analysis, increases EUS-FNA sensitivity, especially in patients with small tumors. These techniques may also serve the need for individualized tumor therapy in the future.

Given the limitations of cytology and the additional time and cost for the presence of a cytopathologist, efforts for the acquisition of core tissues suitable for histological analysis have been made leading to a variety of technical modifications beyond increasing needle size. Accordingly, an 18G FNA needle, a 19G FNA needle with a modified suction technique, as well as the Trucut device have been developed; however, studies indicate a somewhat lower efficacy when a transduodenal approach has to be used^[21-25]. To overcome this disadvantage, the core tissues collected by EUS-FNA using standard 22G needles have undergone histological assessment^[26-30]. The results of the respective studies varied, but showed that adequate specimens for histology were recovered in more than 80% of patients, histology demonstrated good accuracy rates, and combined cytological and histological analysis might be superior to either method alone. With the exception of procedural times and costs, no other significant difference between these two material processing methods was described.

Aiming to procure larger tissue specimens that enable histologic and immunophenotypic characterization, a novel needle device with side fenestration

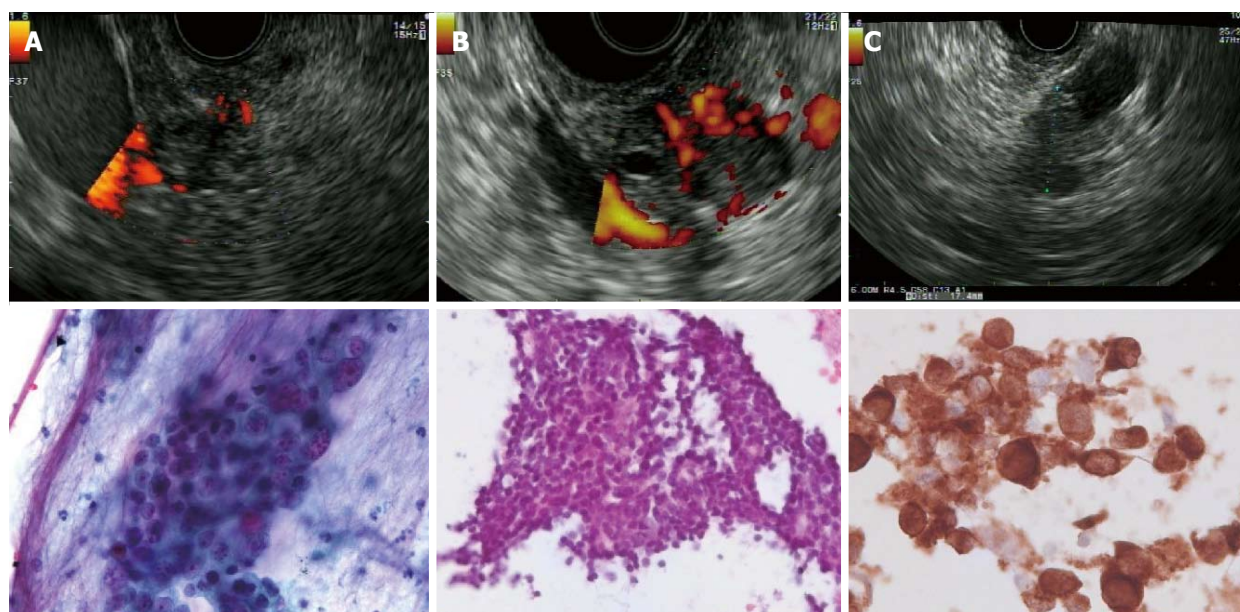


Figure 2 Endoscopic ultrasound and cytology images from various lesions. A: Adenocarcinoma of the pancreatic head. Cytology shows a cluster of carcinoma cells with crowding and nuclear pleomorphism with anisokaryosis and prominent nucleoli (Pap test); B: Neuroendocrine tumors (insulinoma) of the pancreas. Cytology shows a group of small monomorphic cells with uniform round nuclei and fine chromatin; C: Pancreatic metastasis of malignant melanoma: Cytology shows pleomorphic tumor cells with anisokaryosis and a high nuclear-to-cytoplasmic ratio. Positive staining for melanoma antigen recognized by T-cells-1.

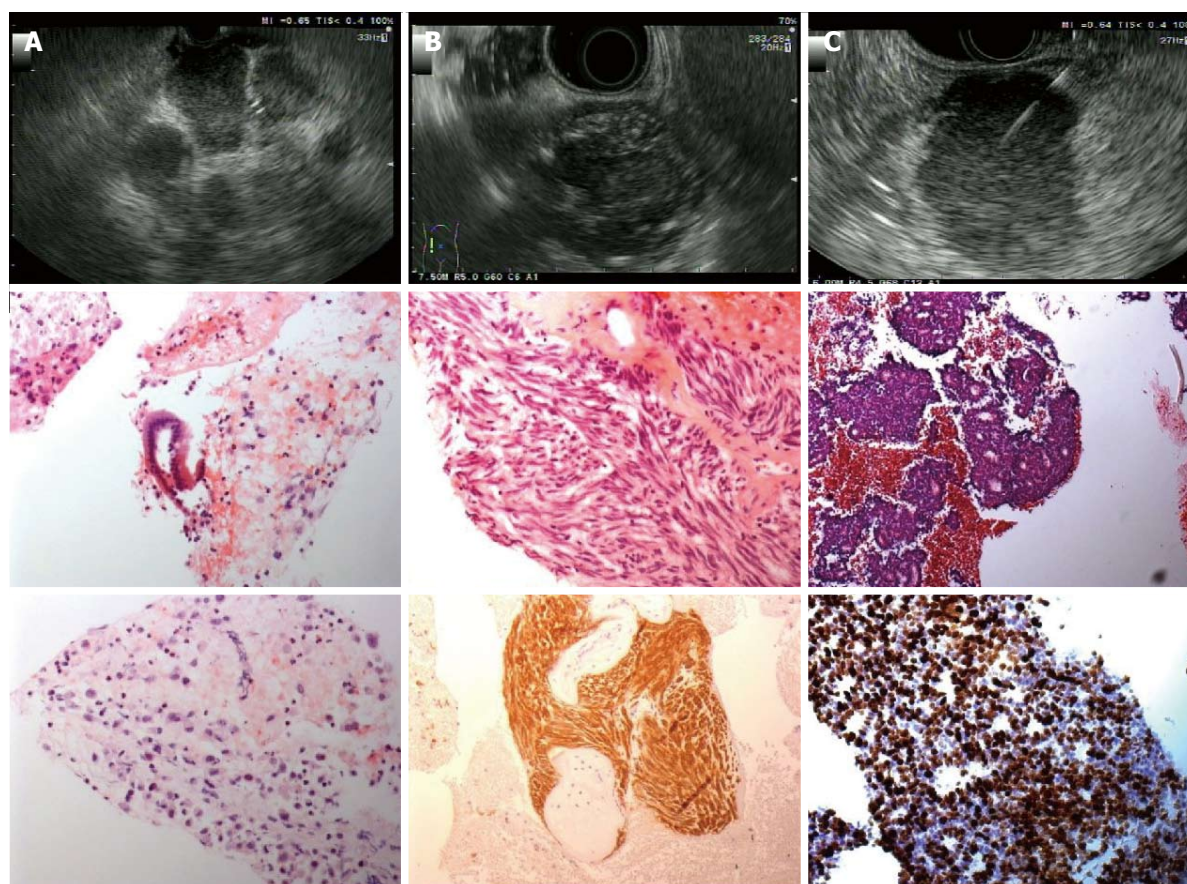


Figure 3 Endoscopic ultrasound and histology images from various lesions. A: Lymph node metastases of a pancreatic adenocarcinoma. Histology shows pleomorphic carcinoma cells with anisokaryosis, high nuclear-to-cytoplasmic ratio and prominent nucleoli [hematoxylin-eosin (HE) staining]; B: Gastric gastrointestinal stromal tumors originated from the muscle layer. Histology shows interlacing fascicles of uniform spindle cells with cigar-shaped nuclei (HE). Diffuse immunohistochemical positivity for CD117; C: Neuroendocrine tumors located in the pancreatic body. Histology shows sheets with rosettes of small to medium-sized, monomorphic cells with round, uniform nuclei and fine chromatin (HE staining). Immunohistochemical MIB1-proliferation rate: 80%.

Table 5 Published comparative trials regarding EchoTip ProCore needle performance

Ref.	Design	No. of lesions	Target	Needles	Diagnostic yield	Sample adequacy	Comments
Witt <i>et al</i> ^[32]	Retrospective	18 per needle type	Diverse	PC 22G vs AN 22G	Equivalent	Equivalent	PC: fewer passes needed
Strand <i>et al</i> ^[33]	RCT	32 punctured by both needles	Pancreas	PC 22G vs AN 22G	AN > PC	Equivalent	Only 2 passes with PC vs 5 with AN, PC technical failure in 16 cases
Bang <i>et al</i> ^[34]	RCT	28 per needle type	Pancreas	PC 22G vs AN 22G	Equivalent	Equivalent	On-site cytopathologist, needles of different manufactures
Lee <i>et al</i> ^[35]	RCT	58 per needle type	Pancreas	PC 22/25G vs AN 22/25G	Equivalent	N/A	On-site cytopathologist, PC: fewer passes needed
Hucl <i>et al</i> ^[36]	RCT	145 punctured by both needles	Diverse	PC 22G vs AN 22G	Equivalent	Equivalent	Only histology, PC: fewer passes needed
Mavrogenis <i>et al</i> ^[37]	RCT	28 punctured by both needles	Pancreas + LNs	PC 25G vs AN 22G	Equivalent	Equivalent	Different needle gauges, "slow pull" sampling technique
Vanbiervliet <i>et al</i> ^[39]	RCT	80 punctured by both needles	Pancreas	PC 22G vs AN 22G	Equivalent	Cytology: equivalent Histology: PC > AN	Only 1 pass with PC vs 2 with AN
Kim <i>et al</i> ^[40]	RCT	10 with AN, 12 with PC	SET	PC 22G vs AN 22G	PC > AN	PC > AN	Only histology, PC: fewer passes needed
Alatawi <i>et al</i> ^[41]	RCT	50 per needle type	Pancreas	PC 22G vs AN 22G	Equivalent	Equivalent, cellularity: PC > AN	Equivalent results after 2 passes with PC vs 3 with AN

PC: ProCore needle; AN: Aspiration needle; RCT: Randomized controlled trial; SET: Subepithelial tumors; LNs: Lymph nodes.

has been recently introduced (EchoTip ProCore). Initially, a 19G version became commercially available, followed by 22G and most recently 25 G. With respect to the 22G PC, the published case series report rates of adequate material for histologic analysis between 52.9% and 88.5% and overall diagnostic accuracy from 82% to 89%^[13,15,16]. To enhance its role, a recently published study equals two passes using this reverse-beveled needle with the current gold-standard of FNA with on-site cytopathology assessment^[31].

In our randomized study, we directly compared the performance of PC against standard AN (both 22G) on the basis of adequacy of obtained material for subsequent analysis (either cytological or histological) and the ability to provide a correct diagnosis. Although the utilization of both needles appeared technically feasible in all cases, we failed to demonstrate any statistically significant difference between the PC and AN in terms of the adequacy of tissue for the evaluation and rates of correct diagnosis. Moreover, no significant differences were encountered regarding the number of needle passes, specimen cellularity and quality scores and complication rates.

Our results are in line with those of most studies comparing the 22G version of the two needles (Table 5)^[32-37] and a recently published meta-analysis of nine comparative studies^[38]. On the contrary, Vanbiervliet *et al*^[39] reported in 2014 the significant superiority of the aspiration needle in both overall (cytology and histology) and solely histology adequacy in pancreatic lesions. However, this study is biased in favor of AN since two passes with this needle are compared with only one pass with the PC. In contrast, the PC achieved better adequacy when sampling subepithelial tumors,

according to Kim *et al*^[40].

The mean number of needle passes needed to achieve a satisfactory specimen did not differ in the present study. This finding opposes that of most similar studies that favor the PC device for fewer passes to achieve an adequate specimen^[32,35,36,39,41]. Interestingly, our number of needle passes was much lower than that recommended in several EUS-FNA studies, although it did not lead to substantially lower accuracies^[6,42].

The present study has several strengths, including its prospective randomized design, uniform sampling method for both needles, use of needles from the same manufacturer, and head-to-head comparisons that allow for improved statistical validity. Nevertheless, it also bears some limitations. Firstly, there was only one participating center. Secondly, the endoscopists could not be blinded for the needle due to obvious reasons. Thirdly, we enrolled patients with diverse lesion locations. Fourthly, the number of participant is rather small. Fifthly, the lack of an on-site cytopathologist, such as in most of the non-american series and daily routine procedures, may have prevented the optimal initial assessment and processing of the obtained material. Finally, the material obtained with either needle was subject to different preparation procedures - histology can only be attempted with the PC needle and, therefore, possible technical biases may have occurred. On the other hand, the aim of the PC needle is to add histology to the diagnostic armamentarium of EUS-FNA. We could show that this contributed very little to the overall diagnostic accuracy.

Our results may be of limited generalizability given the above mentioned methodological factors. However,

they definitely add to the existing body of available literature showing that the two needles do not differ significantly concerning overall technical and diagnostic performance. Keeping that in mind, endoscopists could possibly base the choice of needle type for EUS-FNA on other parameters (*i.e.*, availability, cost, and procedural times).

In conclusion, our study pointed out that, when compared to the standard 22G FNA needle, the reverse-beveled PC of the same gauge yielded a similar overall diagnostic accuracy and performed equally in terms of the sample adequacy for cytological analysis, quality and cellularity of the obtained specimens. This is in line with the majority of published comparative studies. No significant differences regarding the needed number of passes, technical feasibility, and complications were addressed. The overall contribution of histology to the diagnosis with the PC needle was limited.

ACKNOWLEDGMENTS

Athanasios D Sioulas is a scholar of the Hellenic Society of Gastroenterology.

COMMENTS

Background

Endoscopic ultrasound-guided fine needle aspiration (EUS-FNA) is an established modality for tissue sampling of intra- and paramural lesions, while cytological analysis represents the diagnostic standard of care. The recent development of fenestrated needles facilitates the acquisition of specimens that are also suitable for histological analysis thereby promising the improvement of diagnostic accuracy. The authors aimed to compare the standard aspiration needle (AN) with the novel core biopsy needle (PC) of an identical diameter (22G) in terms of various performance characteristics.

Research frontiers

According to these results, both needles performed equally in terms of overall diagnostic accuracy, sample adequacy for analysis, quality and cellularity of specimens, number of needle passes, feasibility, and complications. Interestingly, the histological analysis of the specimens obtained with the PC needle showed no additive contribution to the diagnosis.

Innovations and breakthroughs

The current study adds to the growing body of evidence supporting that both AN and PC demonstrate similar technical and performance characteristics when used for tissue sampling with EUS-FNA.

Applications

Keeping the results of the prospective clinical trial in mind, endoscopists could base their choice of needle type for EUS-FNA on other parameters (*i.e.*, availability, cost, and procedural times).

Terminology

EUS-FNA refers to the acquisition of abnormal tissue samples with specialized needles under endoscopic ultrasound guidance. The diagnostic accuracy of a method represents the association between its result and the disease status of the study participant. The cellularity of a specimen reflects the number of its constituent cells.

Peer-review

This study is well conducted and its conclusions will certainly contribute to the

future use of EUS-FNA as a safe and established method of tissue acquisition, as well as make the needle type choice an easier decision.

REFERENCES

- 1 **Wiersema MJ**, Vilmann P, Giovannini M, Chang KJ, Wiersema LM. Endosonography-guided fine-needle aspiration biopsy: diagnostic accuracy and complication assessment. *Gastroenterology* 1997; **112**: 1087-1095 [PMID: 9097990]
- 2 **Eloubeidi MA**, Chen VK, Eltoun IA, Jhala D, Chhieng DC, Jhala N, Vickers SM, Wilcox CM. Endoscopic ultrasound-guided fine needle aspiration biopsy of patients with suspected pancreatic cancer: diagnostic accuracy and acute and 30-day complications. *Am J Gastroenterol* 2003; **98**: 2663-2668 [PMID: 14687813 DOI: 10.1111/j.1572-0241.2003.08666.x]
- 3 **O'Toole D**, Palazzo L, Arotçarena R, Dancour A, Aubert A, Hammel P, Amaris J, Ruszniewski P. Assessment of complications of EUS-guided fine-needle aspiration. *Gastrointest Endosc* 2001; **53**: 470-474 [PMID: 11275888 DOI: 10.1067/mge.2001.112839]
- 4 **Siddiqui AA**, Brown LJ, Hong SK, Draganova-Tacheva RA, Korenblit J, Loren DE, Kowalski TE, Solomides C. Relationship of pancreatic mass size and diagnostic yield of endoscopic ultrasound-guided fine needle aspiration. *Dig Dis Sci* 2011; **56**: 3370-3375 [PMID: 21688127 DOI: 10.1007/s10620-011-1782-z]
- 5 **Hawes RH**. The evolution of endoscopic ultrasound: improved imaging, higher accuracy for fine needle aspiration and the reality of endoscopic ultrasound-guided interventions. *Curr Opin Gastroenterol* 2010; **26**: 436-444 [PMID: 20703111 DOI: 10.1097/MOG.0b013e32833d1799]
- 6 **Erickson RA**, Sayage-Rabie L, Beissner RS. Factors predicting the number of EUS-guided fine-needle passes for diagnosis of pancreatic malignancies. *Gastrointest Endosc* 2000; **51**: 184-190 [PMID: 10650262]
- 7 **Logroño R**, Waxman I. Interactive role of the cytopathologist in EUS-guided fine needle aspiration: an efficient approach. *Gastrointest Endosc* 2001; **54**: 485-490 [PMID: 11577312]
- 8 **Kopelman Y**, Marmor S, Ashkenazi I, Fireman Z. Value of EUS-FNA cytological preparations compared with cell block sections in the diagnosis of pancreatic solid tumours. *Cytopathology* 2011; **22**: 174-178 [PMID: 20482717 DOI: 10.1111/j.1365-2303.2010.00766.x]
- 9 **Ribeiro A**, Vazquez-Sequeiros E, Wiersema LM, Wang KK, Clain JE, Wiersema MJ. EUS-guided fine-needle aspiration combined with flow cytometry and immunocytochemistry in the diagnosis of lymphoma. *Gastrointest Endosc* 2001; **53**: 485-491 [PMID: 11275890 DOI: 10.1067/mge.2001.112841]
- 10 **Mizuno N**, Bhatia V, Hosoda W, Sawaki A, Hoki N, Hara K, Takagi T, Ko SB, Yatabe Y, Goto H, Yamao K. Histological diagnosis of autoimmune pancreatitis using EUS-guided trucut biopsy: a comparison study with EUS-FNA. *J Gastroenterol* 2009; **44**: 742-750 [PMID: 19434362 DOI: 10.1007/s00535-009-0062-6]
- 11 **Thomas T**, Kaye PV, Ragunath K, Aithal G. Efficacy, safety, and predictive factors for a positive yield of EUS-guided Trucut biopsy: a large tertiary referral center experience. *Am J Gastroenterol* 2009; **104**: 584-591 [PMID: 19262518 DOI: 10.1038/ajg.2008.97]
- 12 **Larghi A**, Verna EC, Stavropoulos SN, Rotterdam H, Lightdale CJ, Stevens PD. EUS-guided trucut needle biopsies in patients with solid pancreatic masses: a prospective study. *Gastrointest Endosc* 2004; **59**: 185-190 [PMID: 14745390]
- 13 **Fabbri C**, Luigiano C, Maimone A, Tarantino I, Baccarini P, Fornelli A, Liotta R, Polifemo A, Barresi L, Traina M, Virgilio C, Cennamo V. Endoscopic ultrasound-guided fine-needle biopsy of small solid pancreatic lesions using a 22-gauge needle with side fenestration. *Surg Endosc* 2015; **29**: 1586-1590 [PMID: 25303907 DOI: 10.1007/s00464-014-3846-6]
- 14 **Iwashita T**, Nakai Y, Samarasena JB, Park DH, Zhang Z, Gu M, Lee JG, Chang KJ. High single-pass diagnostic yield of a new 25-gauge core biopsy needle for EUS-guided FNA biopsy in solid pancreatic lesions. *Gastrointest Endosc* 2013; **77**: 909-915 [PMID: 23039077 DOI: 10.1016/j.gie.2012.11.041]

- 23433596 DOI: 10.1016/j.gie.2013.01.001]
- 15 **Larghi A**, Iglesias-Garcia J, Poley JW, Monges G, Petrone MC, Rindi G, Abdulkader I, Arcidiacono PG, Costamagna G, Biermann K, Bories E, Doglioni C, Dominguez-Muñoz JE, Hassan C, Bruno M, Giovannini M. Feasibility and yield of a novel 22-gauge histology EUS needle in patients with pancreatic masses: a multicenter prospective cohort study. *Surg Endosc* 2013; **27**: 3733-3738 [PMID: 23644834 DOI: 10.1007/s00464-013-2957-9]
 - 16 **Paik WH**, Park Y, Park DH, Hong SM, Lee BU, Choi JH, Lee SS, Seo DW, Lee SK, Kim MH. Prospective evaluation of new 22 gauge endoscopic ultrasound core needle using capillary sampling with stylet slow-pull technique for intra-abdominal solid masses. *J Clin Gastroenterol* 2015; **49**: 199-205 [PMID: 24921417 DOI: 10.1097/MCG.0000000000000084]
 - 17 **Iglesias-Garcia J**, Poley JW, Larghi A, Giovannini M, Petrone MC, Abdulkader I, Monges G, Costamagna G, Arcidiacono P, Biermann K, Rindi G, Bories E, Doglioni C, Bruno M, Dominguez-Muñoz JE. Feasibility and yield of a new EUS histology needle: results from a multicenter, pooled, cohort study. *Gastrointest Endosc* 2011; **73**: 1189-1196 [PMID: 21420083 DOI: 10.1016/j.gie.2011.01.053]
 - 18 **Payne M**, Staerckel G, Gong Y. Indeterminate diagnosis in fine-needle aspiration of the pancreas: reasons and clinical implications. *Diagn Cytopathol* 2009; **37**: 21-29 [PMID: 18973122 DOI: 10.1002/dc.20949]
 - 19 **Iglesias-Garcia J**, Dominguez-Munoz JE, Abdulkader I, Larino-Noia J, Eugenyeva E, Lozano-Leon A, Forteza-Vila J. Influence of on-site cytopathology evaluation on the diagnostic accuracy of endoscopic ultrasound-guided fine needle aspiration (EUS-FNA) of solid pancreatic masses. *Am J Gastroenterol* 2011; **106**: 1705-1710 [PMID: 21483464 DOI: 10.1038/ajg.2011.119]
 - 20 **Turner BG**, Cizginer S, Agarwal D, Yang J, Pitman MB, Brugge WR. Diagnosis of pancreatic neoplasia with EUS and FNA: a report of accuracy. *Gastrointest Endosc* 2010; **71**: 91-98 [PMID: 19846087 DOI: 10.1016/j.gie.2009.06.017]
 - 21 **Larghi A**, Verna EC, Ricci R, Seerden TC, Galasso D, Carnuccio A, Uchida N, Rindi G, Costamagna G. EUS-guided fine-needle tissue acquisition by using a 19-gauge needle in a selected patient population: a prospective study. *Gastrointest Endosc* 2011; **74**: 504-510 [PMID: 21872709 DOI: 10.1016/j.gie.2011.05.014]
 - 22 **Wittmann J**, Kocjan G, Sgouras SN, Deheragoda M, Pereira SP. Endoscopic ultrasound-guided tissue sampling by combined fine needle aspiration and trucut needle biopsy: a prospective study. *Cytopathology* 2006; **17**: 27-33 [PMID: 16417562 DOI: 10.1111/j.1365-2303.2006.00313.x]
 - 23 **Levy MJ**. Endoscopic ultrasound-guided trucut biopsy of the pancreas: prospects and problems. *Pancreatol* 2007; **7**: 163-166 [PMID: 17592229 DOI: 10.1159/000104240]
 - 24 **Varadarajulu S**, Fraig M, Schmulewitz N, Roberts S, Wildi S, Hawes RH, Hoffman BJ, Wallace MB. Comparison of EUS-guided 19-gauge Trucut needle biopsy with EUS-guided fine-needle aspiration. *Endoscopy* 2004; **36**: 397-401 [PMID: 15100946 DOI: 10.1055/s-2004-814316]
 - 25 **Binmoeller KF**, Thul R, Rathod V, Henke P, Brand B, Jabusch HC, Soehendra N. Endoscopic ultrasound-guided, 18-gauge, fine needle aspiration biopsy of the pancreas using a 2.8 mm channel convex array echoendoscope. *Gastrointest Endosc* 1998; **47**: 121-127 [PMID: 9512275]
 - 26 **Möller K**, Papanikolaou IS, Toerner T, Delicha EM, Sarbia M, Schenck U, Koch M, Al-Abadi H, Meining A, Schmidt H, Schulz HJ, Wiedenmann B, Rösch T. EUS-guided FNA of solid pancreatic masses: high yield of 2 passes with combined histologic-cytologic analysis. *Gastrointest Endosc* 2009; **70**: 60-69 [PMID: 19394012 DOI: 10.1016/j.gie.2008.10.008]
 - 27 **Iglesias-Garcia J**, Dominguez-Munoz E, Lozano-Leon A, Abdulkader I, Larino-Noia J, Antunez J, Forteza J. Impact of endoscopic ultrasound-guided fine needle biopsy for diagnosis of pancreatic masses. *World J Gastroenterol* 2007; **13**: 289-293 [PMID: 17226911 DOI: 10.3748/wjg.v13.i2.289]
 - 28 **Voss M**, Hammel P, Molas G, Palazzo L, Dancour A, O'Toole D, Terris B, Degott C, Bernades P, Ruszniewski P. Value of endoscopic ultrasound guided fine needle aspiration biopsy in the diagnosis of solid pancreatic masses. *Gut* 2000; **46**: 244-249 [PMID: 10644320]
 - 29 **Papanikolaou IS**, Adler A, Wegener K, Al-Abadi H, Dürr A, Koch M, Pohl H, Abou-Rebyeh H, Veltzke-Schlieker W, Wiedenmann B, Rösch T. Prospective pilot evaluation of a new needle prototype for endoscopic ultrasonography-guided fine-needle aspiration: comparison of cytology and histology yield. *Eur J Gastroenterol Hepatol* 2008; **20**: 342-348 [PMID: 18334879 DOI: 10.1097/MEG.0b013e3282f2a5cf]
 - 30 **Brais RJ**, Davies SE, O'Donovan M, Simpson BW, Cook N, Darbonne WC, Chilcott S, Lolkema MP, Neesse A, Lockley M, Corrie PG, Jodrell DI, Praseedom RK, Huguet EL, Jah A, Jamieson NV, de Sauvage FJ, Tuveson DA, Carroll NR. Direct histological processing of EUS biopsies enables rapid molecular biomarker analysis for interventional pancreatic cancer trials. *Pancreatol* 2012; **12**: 8-15 [PMID: 22487467 DOI: 10.1016/j.pan.2011.12.009]
 - 31 **Lin M**, Hair CD, Green LK, Vela SA, Patel KK, Qureshi WA, Shaib YH. Endoscopic ultrasound-guided fine-needle aspiration with on-site cytopathology versus core biopsy: a comparison of both techniques performed at the same endoscopic session. *Endosc Int Open* 2014; **2**: E220-E223 [PMID: 26135096 DOI: 10.1055/s-0034-1377611]
 - 32 **Witt BL**, Adler DG, Hilden K, Layfield LJ. A comparative needle study: EUS-FNA procedures using the HD ProCoreTM and EchoTip[®] 22-gauge needle types. *Diagn Cytopathol* 2013; **41**: 1069-1074 [PMID: 23513000 DOI: 10.1002/dc.22971]
 - 33 **Strand DS**, Jeffus SK, Sauer BG, Wang AY, Stelow EB, Shami VM. EUS-guided 22-gauge fine-needle aspiration versus core biopsy needle in the evaluation of solid pancreatic neoplasms. *Diagn Cytopathol* 2014; **42**: 751-758 [PMID: 24550162 DOI: 10.1002/dc.23116]
 - 34 **Bang JY**, Hebert-Magee S, Trevino J, Ramesh J, Varadarajulu S. Randomized trial comparing the 22-gauge aspiration and 22-gauge biopsy needles for EUS-guided sampling of solid pancreatic mass lesions. *Gastrointest Endosc* 2012; **76**: 321-327 [PMID: 22658389 DOI: 10.1016/j.gie.2012.03.1392]
 - 35 **Lee YN**, Moon JH, Kim HK, Choi HJ, Choi MH, Kim DC, Lee TH, Cha SW, Cho YD, Park SH. Core biopsy needle versus standard aspiration needle for endoscopic ultrasound-guided sampling of solid pancreatic masses: a randomized parallel-group study. *Endoscopy* 2014; **46**: 1056-1062 [PMID: 25098611 DOI: 10.1055/s-0034-1377558]
 - 36 **Hucl T**, Wee E, Anuradha S, Gupta R, Ramchandani M, Rakesh K, Shrestha R, Reddy DN, Lakhtakia S. Feasibility and efficiency of a new 22G core needle: a prospective comparison study. *Endoscopy* 2013; **45**: 792-798 [PMID: 24068588 DOI: 10.1055/s-0033-1344217]
 - 37 **Mavrogenis G**, Weynand B, Sibille A, Hassaini H, Deprez P, Gillain C, Warzée P. 25-gauge histology needle versus 22-gauge cytology needle in endoscopic ultrasonography-guided sampling of pancreatic lesions and lymphadenopathy. *Endosc Int Open* 2015; **3**: E63-E68 [PMID: 26134775 DOI: 10.1055/s-0034-1390889]
 - 38 **Bang JY**, Hawes R, Varadarajulu S. A meta-analysis comparing ProCore and standard fine-needle aspiration needles for endoscopic ultrasound-guided tissue acquisition. *Endoscopy* 2016; **48**: 339-349 [PMID: 26561917 DOI: 10.1055/s-0034-1393354]
 - 39 **Vanbiervliet G**, Napoléon B, Saint Paul MC, Sakarovitch C, Wangermez M, Bichard P, Subtil C, Koch S, Grandval P, Gincul R, Karsenti D, Heyries L, Duchmann JC, Bourgaux JF, Levy M, Calament G, Fumex F, Pujol B, Lefort C, Poincloux L, Pagenault M, Bonin EA, Fabre M, Barthet M. Core needle versus standard needle for endoscopic ultrasound-guided biopsy of solid pancreatic masses: a randomized crossover study. *Endoscopy* 2014; **46**: 1063-1070 [PMID: 25098612 DOI: 10.1055/s-0034-1377559]
 - 40 **Kim GH**, Cho YK, Kim EY, Kim HK, Cho JW, Lee TH, Moon JS. Comparison of 22-gauge aspiration needle with 22-gauge biopsy needle in endoscopic ultrasonography-guided subepithelial

- tumor sampling. *Scand J Gastroenterol* 2014; **49**: 347-354 [PMID: 24325591 DOI: 10.3109/00365521.2013.867361]
- 41 **Alatawi A**, Beuvon F, Grabar S, Leblanc S, Chaussade S, Terris B, Barret M, Prat F. Comparison of 22G reverse-beveled versus standard needle for endoscopic ultrasound-guided sampling of solid pancreatic lesions. *United European Gastroenterol J* 2015; **3**: 343-352 [PMID: 26279842 DOI: 10.1177/2050640615577533]
- 42 **LeBlanc JK**, Ciaccia D, Al-Assi MT, McGrath K, Imperiale T, Tao LC, Vallery S, DeWitt J, Sherman S, Collins E. Optimal number of EUS-guided fine needle passes needed to obtain a correct diagnosis. *Gastrointest Endosc* 2004; **59**: 475-481 [PMID: 15044881]

P- Reviewer: Beg M, Stoos-Veic T, Yusuf MA **S- Editor:** Yu J

L- Editor: A **E- Editor:** Wang CH



Progression from low-grade dysplasia to malignancy in patients with Barrett's esophagus diagnosed by two or more pathologists

Harsha Moole, Jaymon Patel, Zohair Ahmed, Abhiram Duvvuri, Sreekar Vennelaganti, Vishnu Moole, Sowmya Dharmapuri, Raghuveer Boddireddy, Pratyusha Yedama, Naveen Bondalapati, Achuta Uppu, Prashanth Vennelaganti, Srinivas Puli

Harsha Moole, Jaymon Patel, Zohair Ahmed, Department of Medicine, University of Illinois College of Medicine at Peoria, Peoria, IL 61637, United States

Harsha Moole, Department of Medicine, Apogee Medical Group, Davenport, IA 52804, United States

Abhiram Duvvuri, Sreekar Vennelaganti, Division of Gastroenterology and Hepatology, Kansas City Veteran Affairs Medical Center, Kansas City, MO 64128, United States

Vishnu Moole, Sowmya Dharmapuri, Raghuveer Boddireddy, Pratyusha Yedama, Department of Medicine, NTR University of Health Sciences, Andhra Pradesh 520008, India

Naveen Bondalapati, Department of Medicine, Barnes Jewish Christian Medical Group, Christian Hospital, St. Louis, MO 63136, United States

Achuta Uppu, Department of Medicine, Albert Einstein College of Medicine, Bronx, NY 10461, United States

Prashanth Vennelaganti, Division of Gastroenterology and Hepatology, University of Kansas Medical Center, Kansas City, MO 66160, United States

Srinivas Puli, Division of Gastroenterology and Hepatology, University of Illinois College of Medicine at Peoria, IL 61637, United States

Author contributions: Moole H conception and design of the study, acquisition of data, analysis and interpretation of data, drafting the article, critical revision, and final approval; Patel J acquisition of data, analysis and interpretation of data, drafting the article, final approval; Ahmed Z acquisition of data, analysis and interpretation of data, drafting the article, final approval; Duvvuri A interpretation of data, revising the article, final approval; Vennelaganti S interpretation of data, revising the article, final approval; Moole V acquisition of data, interpretation of data, drafting the article, revising the article,

final approval; Dharmapuri S interpretation of data, revising the article, final approval; Boddireddy R acquisition of data, analysis and interpretation of data, drafting the article, final approval; Yedama P acquisition of data, analysis and interpretation of data, drafting the article, final approval; Bondalapati N acquisition of data, analysis and interpretation of data, drafting the article, final approval; Uppu A acquisition of data, analysis and interpretation of data, drafting the article, final approval; Vennelaganti P interpretation of data, revising the article, final approval; Puli S conception and design of the study, critical revision, final approval.

Conflict-of-interest statement: The authors deny any conflict of interest.

Data sharing statement: No additional data are available.

Open-Access: This article is an open-access article which was selected by an in-house editor and fully peer-reviewed by external reviewers. It is distributed in accordance with the Creative Commons Attribution Non Commercial (CC BY-NC 4.0) license, which permits others to distribute, remix, adapt, build upon this work non-commercially, and license their derivative works on different terms, provided the original work is properly cited and the use is non-commercial. See: <http://creativecommons.org/licenses/by-nc/4.0/>

Manuscript source: Invited manuscript

Correspondence to: Harsha Moole, MD, Clinical Associate, Department of Medicine, University of Illinois College of Medicine at Peoria, 1 Illini Dr, Peoria, IL 61637, United States. harsha1778@yahoo.co.in
Telephone: +1-309-6557257
Fax: +1-844-8936705

Received: July 11, 2016
Peer-review started: July 12, 2016
First decision: August 19, 2016
Revised: September 4, 2016

Accepted: September 28, 2016

Article in press: September 28, 2016

Published online: October 21, 2016

Abstract

AIM

To evaluate annual incidence of low grade dysplasia (LGD) progression to high grade dysplasia (HGD) and/or esophageal adenocarcinoma (EAC) when diagnosis was made by two or more expert pathologists.

METHODS

Studies evaluating the progression of LGD to HGD or EAC were included. The diagnosis of LGD must be made by consensus of two or more expert gastrointestinal pathologists. Articles were searched in Medline, Pubmed, and Embase. Pooled proportions were calculated using fixed and random effects model. Heterogeneity among studies was assessed using the I^2 statistic.

RESULTS

Initial search identified 721 reference articles, of which 53 were selected and reviewed. Twelve studies ($n = 971$) that met the inclusion criteria were included in this analysis. Among the total original LGD diagnoses in the included studies, only 37.49% reached the consensus LGD diagnosis after review by two or more expert pathologists. Total follow up period was 1532 patient-years. In the pooled consensus LGD patients, the annual incidence rate (AIR) of progression to HGD and/or EAC was 10.35% (95%CI: 7.56-13.13) and progression to EAC was 5.18% (95%CI: 3.43-6.92). Among the patients down staged from original LGD diagnosis to No-dysplasia Barrett's esophagus, the AIR of progression to HGD and EAC was 0.65% (95%CI: 0.49-0.80). Among the patients down staged to Indefinite for dysplasia, the AIR of progression to HGD and EAC was 1.42% (95%CI: 1.19-1.65). In patients with consensus HGD diagnosis, the AIR of progression to EAC was 28.63% (95%CI: 13.98-43.27).

CONCLUSION

When LGD is diagnosed by consensus agreement of two or more expert pathologists, its progression towards malignancy seems to be at least three times the current estimates, however it could be up to 20 times the current estimates. Biopsies of all Barrett's esophagus patients with LGD should be reviewed by two expert gastroenterology pathologists. Follow-up strict surveillance programs should be in place for these patients.

Key words: Barrett's esophagus; Low grade dysplasia; High grade dysplasia; Esophageal adenocarcinoma; Annual incidence of progression; Systematic review; Meta-analysis

© The Author(s) 2016. Published by Baishideng Publishing Group Inc. All rights reserved.

Core tip: Current estimates suggest that annual incidence of progression from low grade dysplasia (LGD) to high grade dysplasia and/or esophageal adenocarcinoma is 0.5% to 4% per year. Current estimates are based on diagnosis made by one pathologist. Recent studies indicate that when the diagnosis of LGD is made by two or more expert pathologists, LGD progression is grossly underestimated. When LGD is diagnosed by consensus agreement of two or more expert pathologists, its progression towards malignancy seems to be at least three times the current estimates, however it could be up to 20 times the current estimates. Biopsies of all Barrett's esophagus patients with LGD should be reviewed by two expert gastroenterology pathologists. Follow-up strict surveillance programs should be in place for these patients.

Moole H, Patel J, Ahmed Z, Duvvuri A, Vennelaganti S, Moole V, Dharmapuri S, Boddireddy R, Yedama P, Bondalapati N, Uppu A, Vennelaganti P, Puli S. Progression from low-grade dysplasia to malignancy in patients with Barrett's esophagus diagnosed by two or more pathologists. *World J Gastroenterol* 2016; 22(39): 8831-8843 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v22/i39/8831.htm> DOI: <http://dx.doi.org/10.3748/wjg.v22.i39.8831>

INTRODUCTION

Barrett's esophagus (BE), first described in 1950, is a condition in which the normal esophageal squamous epithelium is replaced by columnar epithelium in a process known as metaplasia^[1]. The development of esophageal adenocarcinoma (EAC) occurs when BE progresses through dysplasia. Dysplasia in BE is classified as either low grade dysplasia (LGD) or high grade dysplasia (HGD). The populations at risk for developing BE include individuals with chronic gastroesophageal reflux disease (GERD), obesity, male gender, have a smoking history, and are Caucasian^[2-4].

The epidemiology of BE varies greatly owing to advancements in knowledge, diagnostic approach, and surveillance strategies. BE is prevalent in 10%-20% of patients with GERD, 2%-7% of general population, and incidence is between 23.1 and 32.7 per 100000^[5-11]. The risk of progression to malignancy has been found to be lower in Barrett's esophagus without dysplasia^[12-14]. Indefinite for dysplasia (IDBE), indicating no clear evidence of dysplasia, poses an additional diagnostic challenge. Increasing dysplasia levels were associated with increased detection of malignancy in a greater number of patients in shorter time intervals^[8].

Further epidemiological analysis has been performed in BE patients assessing the incidence of progression from LGD to HGD and/or progression to EAC. The rate of progression is also variable and

has been reported to be between 0.5% to 4% per year^[10,11]. This wide range of progression incidence is primarily attributed to inter-observer variability in the assessment amongst the reading pathologists. Although LGD has been clearly defined by its histological features, there is a substantial subjective component involved in making the diagnosis. Recent studies indicate the progression of LGD is grossly underestimated. This may be attributed to the expertise of the reading pathologist, in addition to the number of pathologists assessing the biopsies. Given the concern for the risk of progression, accurate assessment of dysplasia is vital for appropriate risk stratification and surveillance strategies. Large statistical analyses have been conducted on the progression of LGD diagnosed by single pathologists. However, there is no statistical analyses in the form of meta-analysis evaluating LGD progression diagnosed by two or more expert pathologists.

The recognition of BE and prevention of progression to esophageal adenocarcinoma is quickly becoming a national public health concern. EAC incidence has more than quadrupled over the last few decades, and is alarmingly becoming a leader for cancer mortality. The aim of this systematic review and meta-analysis was to evaluate annual incidence of LGD progression (to HGD and/or EAC) when the diagnosis was made by two or more expert pathologists. Primary outcomes are to evaluate the annual incidence rate (AIR) of HGD and EAC in patients with LGD, diagnosed by consensus agreement of two or more expert gastroenterology pathologists. Secondary outcomes are to evaluate the AIR of HGD and EAC in patients down staged to No dysplasia in Barrett's esophagus (NDBE) and Indefinite for dysplasia in Barrett's esophagus (IDBE) from the original LGD diagnosis. AIR of EAC from HGD was also evaluated. A sub-group meta-analysis was performed on prospective studies only, evaluating the same variables.

MATERIALS AND METHODS

Study selection criteria

Studies that evaluated the progression of LGD to HGD or EAC were included in this analysis. The diagnosis of LGD must be made by consensus agreement of two or more expert gastroenterology pathologists. Studies that evaluated patients with an original diagnosis of EAC were excluded. Patients with prior surgery or procedural interventions for EAC/LGD/HGD management were also excluded. We included both prospective and retrospective studies. Studies without original data, perspective articles, review articles, and expert opinions were excluded from this meta-analysis. Only full text articles, peer reviewed and published in international journals were included in this analysis. If there were duplicate studies, the most complete and latest study was included in this meta-analysis.

Data collection and extraction

The study design was written in accordance to PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) statement. Articles were systematically searched in Medline, PubMed, Ovid journals, EMABSE, Cumulative Index for Nursing and Allied Health Literature, ACP journal club, DARE, International Pharmaceutical Abstracts, old Medline, Medline nonindexed citations, OVID Healthstar, and Cochrane Central Register of Controlled Trials. The search was performed for the years 1966 to June 2016. Abstracts were manually searched in the major gastroenterology journals for the past 3 years. Study authors for the abstracts included in this analysis were contacted when the required data for the outcome measures could not be determined from the publications. The MeSH search headings used were "barrett's esophagus", "barrett's oesophagus", "low grade dysplasia", "high grade dysplasia", "esophageal adenocarcinoma", "oesophageal adenocarcinoma". The reference lists of the included studies were manually searched for any relevant publications. Two authors (HM and VM) independently searched and extracted the data into an abstraction form. Any differences were resolved by mutual agreement. If the disagreement persisted, the final decision was made by a third author (SP) after reviewing the relevant information. The agreement between reviewers for the collected data was quantified using the Cohen's κ ^[15]. Data was extracted from the selected studies and entered into a standardized data collection form. The following variables were recorded: name and year of study; country where study was performed; type of study; age in years-median; male/female distribution in percentage; total number of patients included; source of patient registry; diagnosis criteria for LGD; esophageal biopsy information (endoscopic details regarding the site of biopsies and pathology details regarding the methods of fixing the biopsy slide); number of patients with original LGD diagnosis (made by a single pathologist); follow-up period in months (median); follow up period in patient years; number of patients with a consensus diagnosis of LGD; number of patients with a consensus diagnosis of HGD at the beginning of study; AIR (expressed as percent) for HGD and EAC in consensus LGD patients; AIR for EAC only in consensus LGD patients; AIR for HGD and EAC in patients down staged to NDBE; AIR for HGD and EAC in patients down staged to IDBE.

Definitions

For the purpose of this meta-analysis, we have used definitions that are most widely accepted by various gastroenterology organizations and that were used in most of the studies included in this analysis. Barrett's esophagus was defined as any red colored epithelium visible on endoscopy, above the

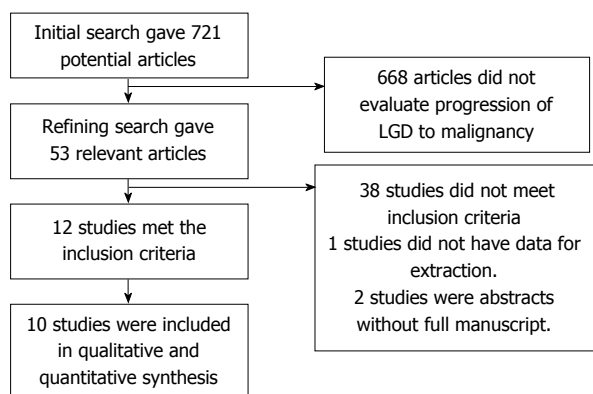


Figure 1 Flow diagram: Search results.

lower esophageal sphincter/proximal end of gastric folds, biopsies of which reveal intestinal metaplasia. The progression of BE towards malignancy was classified into "no dysplasia", "indefinite for dysplasia", "low grade dysplasia", "high grade dysplasia" and "adenocarcinoma". This classification is in accordance with Vienna classification of gastrointestinal epithelial neoplasia^[16]. Original LGD/HGD diagnosis refers to the patients labelled as having LGD/HGD diagnosed by a single pathologist. Consensus or Confirmed LGD/HGD diagnosis refers to the patients actually having LGD/HGD after two or more expert pathologists reviewed the biopsies and agreed upon the diagnosis.

Quality of studies

Clinical trials designed with a control and treatment arms can be assessed for quality of the study. A number of criteria have been used to assess this quality of a study (e.g., randomization, selection bias of the arms in the study, concealment of allocation, and blinding of outcome). Jadad score was used to evaluate the quality of randomized studies. Cochrane Collaborations and the Quality of Reporting of Meta-analysis guidelines were followed to assess the quality of studies^[17,18]. Quality of nonrandomized studies included in this meta-analysis was assessed using Newcastle-Ottawa Scale^[19].

Statistical methods

This meta-analysis was performed by calculating pooled proportions. First the individual study proportion of annual incidence rates of HGD and EAC, consensus LGD diagnosis, etc., was transformed into a quantity using Freeman-Tukey variant of the arcsine square root transformed proportion. The pooled proportion is calculated as the back-transform of the weighted mean of the transformed proportions, using inverse arcsine variance weights for the fixed effects model and DerSimonian-Laird weights for the random effects model^[20,21]. Forest plots were drawn to show the point estimates in each study in relation to the summary pooled estimate. The width of the point estimates in the Forest plots indicates the assigned

weight to that study. The heterogeneity among studies was tested using I² statistic and Cochran's Q test based upon inverse variance weights^[22]. I² of 0–39% was considered as non-significant heterogeneity, 40%–75% as moderate heterogeneity, and 76%–100% as considerable heterogeneity. If *P* value is > 0.10, it rejects the null hypothesis that the studies are heterogeneous. The effect of publication and selection bias on the summary estimates was tested by both Harbord-Egger bias indicator^[23] and Begg-Mazumdar bias indicator^[24]. Also, funnel plots were constructed to evaluate potential publication bias^[25,26]. Microsoft Excel 2013 software was used to perform statistics for this meta-analysis. Subgroup analysis was performed on prospective studies evaluating the AIR of HGD and EAC in confirmed LGD patients.

RESULTS

Study selection

Initial search identified 721 reference articles, in which 53 articles were selected and reviewed. Data was extracted from 12 studies^[10,27-37] (*n* = 971) that evaluated the progression of LGD towards malignancy. All the studies are published as full text articles. Figure 1 shows the flow diagram of search results. All the pooled estimates given are estimates calculated either by fixed or random effects model. Fixed effect model was preferred when heterogeneity was low and random effect model was preferred when heterogeneity was high. Among the 12 studies included in this analysis, seven studies^[10,28,30,32-34,36] initially selected patients with LGD diagnosis made by single pathologist, however was reviewed later by two or more expert pathologists to make confirmation diagnosis after a consensus. Remainder of the five studies directly included patient with confirmed diagnosis of LGD/HGD. Eight studies out of the 12 studies were retrospective studies^[27-34], the others were prospective studies. Subgroup analysis was performed on all prospective trials^[10,35-37]. The included studies were distributed all over the world. Most patient registries of the included studies were specialist center based, except three studies^[29,32,36] that included patients from community based cohorts. One study^[29] included patients from both specialist center and community based registries. Patients in individual studies were on optimal proton pump inhibitor therapy (PPI) – once or twice daily regimen.

The total number of patients included in this meta-analysis is 971. Nine hundred and seventy-one represents total number of original and confirmed LGD cases. However, the total number of confirmed LGD cases after consensus by two or more expert pathologists is 418, with a predominantly male population (72%). Among the total original LGD diagnoses in the included studies, only 37.49% (95%CI: 25.72-50.1) [*I*² (inconsistency) = 89.7% (95%CI: 81.2%-93.3%), Egger: bias = 4.66 (95%CI:

Table 1 Basic characteristics of the included studies

Ref.	Country	Type	Age: years - Median	Sex: Male%	N LGD - prior to panel review	Follow up - median - months	Follow up - Patient years	N LGD (after panel review) consensus diagnosis	N HGD (after panel review) consensus diagnosis
Picardo <i>et al</i> ^[27] 2015	Ireland	R	59	67%	NA	50	354.17	85	60
Duits <i>et al</i> ^[28] 2014	Netherlands	R	63	76%	293	39	256.75	79	NA
von Rahden <i>et al</i> ^[29] 2008	Germany	R	59	72%	NA	24	114.00	57	NA
Lim <i>et al</i> ^[30] 2007	United Kingdom	R	67	80%	34	96	112.00	14	1
Vieth <i>et al</i> ^[31] 2006	Germany	R	61	68%	NA	54	85.50	19	10
Basu <i>et al</i> ^[32] 2004	United Kingdom	R	74	74%	16	60	50.00	10	3
Montgomery <i>et al</i> ^[33] 2001	United States	R	65	72%	26	24	30.00	15	15
Skacel <i>et al</i> ^[34] 2000	United States	R	67	84%	25	26	36.83	17	NA
Younes <i>et al</i> ^[35] 2011	United States	P	NA	NA	NA	35	81.67	28	NA
Wani <i>et al</i> ^[10] 2011	United States	P	61	85%	210	74	252.83	41	NA
Curvers <i>et al</i> ^[36] 2010	Netherlands	P	59	67%	147	51	93.50	22	NA
Srivastava <i>et al</i> ^[37] 2007	United States	P	64	91%	NA	25	64.58	31	46

R: Retrospective; P: Prospective; NA: Not available.

1.40-7.91), $P = 0.01$] reached the consensus LGD diagnosis after review by two or more expert pathologists. Median age of the patients was 65 years. Table 1 shows the baseline characteristics of the studies. Table 2 shows other key characteristics of the individual studies. In summary, the methods used to diagnose confirmed LGD cases in all the studies were mostly similar. Two or more pathologists had to review the biopsies and come to an agreement regarding the diagnosis of LGD. Biopsies were obtained from four quadrants at at-least 2 cm intervals all along the length of Barrett's esophagus. Hematoxylin Eosin staining of paraffin/formalin/Hollande's embedded biopsy specimens was used. The P for χ^2 heterogeneity for all the pooled accuracy estimates was > 0.10 . The agreement between reviewers for the collected data gave a Cohen's κ value of 1.0. The follow up period in individual studies ranged from 24-96 mo. Median follow up period was 50 mo. Cumulative follow up period in all the included studies was 1532 patient years.

Annual incidence rate of malignant transformation in confirmed LGD

Malignant transformation of LGD includes transformation into HGD and or EAC. In the pooled consensus LGD patients, the AIR of progression to HGD and EAC was 10.35% (95%CI: 7.56-13.13). Bias indicators for this variable were: Begg-Mazumdar: Kendall's tau $b = 0.27$, $P = 0.25$; Egger: bias = 10.22 (95%CI: 5.42-15.03), $P = 0.0008$. Heterogeneity for this variable was assessed using I^2 (inconsistency) = 98.4% (95%CI: 98.1%-98.6%). Figure 2 is a forest plot representing the pooled and individual AIR for malignant (HGD and or EAC) transformation in confirmed LGD patients. Figure 3 is a funnel plot assessing the publication bias for same variable. Due to significant heterogeneity among the studies, we have attempted to exclude two studies that were extreme outliers (Wani *et al*^[10] with an AIR 0.84%

and Montgomery *et al*^[33] with an AIR 26.7%). AIR for HGD and EAC, calculated after exclusion of these two studies was 10.17 (95%CI: 7.59-12.75). I^2 (inconsistency) = 96%, Egger: bias = 7.80 (95%CI: 1.67-13.95), $P = 0.02$.

In pooled patient population, progression of confirmed LGD to EAC only was 5.18% (95%CI: 3.43-6.92). Date for this variable was available only in eight studies^[10,27,30-34,37]. Bias indicators for this variable were: Begg-Mazumdar: Kendall's tau $b = 0.79$, $P = 0.0055$; Egger: bias = 7.54 (95%CI: 3.03-12.05) $P = 0.0064$. I^2 (inconsistency) = 96.9% (95%CI: 95.9%-97.6%).

Annual incidence rate of EAC transformation in confirmed HGD

Four studies enrolled confirmed HGD cases into their patient population^[30,32,33,37]. These patients were followed up to assess the rate of transformation into EAC. Total patients included in this analysis was 65, with a predominantly male population (74%). Median age of the patients was 65 years. In patients with consensus HGD diagnosis, the AIR of progression to EAC was 28.63 (95%CI: 13.98-43.27). I^2 (inconsistency) = 95.1%. Egger: bias = 7.65 (95%CI: -20.61-35.92), $P = 0.36$. Figure 4 is a forest plot representing the pooled and individual AIR for EAC transformation in confirmed HGD patients.

Annual incidence rate of malignant transformation in down staged LGD

Original diagnosis of LGD, after review by two or more expert pathologists was either confirmed as LGD after consensus or was down staged to one of the two: NDBE or IDBE. In patients down staged to NDBE, data was available from nine studies^[27-31,33-35,36]. AIR of malignant transformation (HGD and or EAC) in confirmed NDBE patients after down staging was 0.65% (95%CI: 0.49-0.80). I^2 (inconsistency) = 38.4%. Egger: bias = -1.94 (95%CI: -4.69-0.83),

Table 2 Key characteristics of individual studies

Study	Patient registry	Diagnosis method of LGD	Biopsy details
Picardo <i>et al</i> ^[27] 2015	Specialist center based registry	Expert pathologist panel: 2 pathologists required to make diagnosis	Four-quadrant biopsies every 1 cm of Barrett's esophagus
Duits <i>et al</i> ^[28] 2014	Specialist center based registry	Expert pathologists panel: At-least 2 pathologists required to make diagnosis	H&E stained slides of paraffin embedded biopsy specimens
von Rahden <i>et al</i> ^[29] 2008	Specialist center and Community population based registry	Expert pathologists panel: 3 pathologists required to make diagnosis	Multiple biopsies at different levels of Barrett's esophagus
Lim <i>et al</i> ^[30] 2007	Specialist center based registry	Expert pathologists panel: 5 pathologists required to make diagnosis	Four to ten (sometimes more) biopsies taken from Barrett's area. Hematoxylin and eosin staining
Vieth <i>et al</i> ^[31] 2006	Specialist center based registry	Biopsies assessed twice by two pathologists in a blinded fashion	Four biopsies every 2 cm in relation to the Barrett's esophagus length
Basu <i>et al</i> ^[32] 2004	Community based cohort	Experienced gastrointestinal pathologist assessed histological sections, with confirmation by a colleague if high-grade dysplasia or worse was suspected. All cases of low-grade dysplasia were reviewed at a regular gastrointestinal histopathology meeting	2-cm interval quadrantic biopsies in the entire length of Barrett's esophagus
Montgomery <i>et al</i> ^[33] 2001	Specialist center based registry	Expert pathologists panel: 12 pathologists required to make diagnosis - reviewed blindly twice by each pathologist	Multiple biopsies at different levels of Barrett's esophagus. Submitted biopsy specimen had to show the worst lesion that the patient was known to have at the time of the initial known endoscopy
Skacel <i>et al</i> ^[34] 2000	Specialist center based registry	Expert pathologists panel: LGD cases were randomized and blindly reviewed by three gastrointestinal pathologists	Four-quadrant biopsies taken using jumbo forceps at intervals of < 2 cm throughout the length of the Barrett's segment, with additional biopsies of any endoscopic lesions. All biopsy specimens had been fixed in formalin or Hollande's solution
Younes <i>et al</i> ^[45] 2011	Specialist center based registry	Expert pathologist panel: 2 pathologists required to make diagnosis	Biopsies from two or more levels in barrett's esophagus. Hematoxylin-eosin-stained sections of formalin-fixed and paraffin-embedded tissue
Wani <i>et al</i> ^[10] 2011	Specialist center based registry	Consensus diagnosis among two or more pathologists: defined as agreement between the local GI pathologist and expert central pathologists	At least 4 quadrant biopsies every 2 cm with either a standard or jumbo biopsy forceps. Hematoxylin Eosin stained slides of paraffin-embedded biopsy specimens
Curvers <i>et al</i> ^[36] 2010	Community based cohort	Expert pathologist panel: 2 pathologists required to make diagnosis	All visible abnormalities were sampled, followed by random sampling of the Barrett segment in four quadrants every 2 cm. Hematoxylin and eosin stained slides of paraffin-embedded biopsy specimens
Srivastava <i>et al</i> ^[37] 2007	Specialist center based registry	Expert pathologists panel: 3 pathologists required to make diagnosis	Four-quadrant endoscopic esophageal mucosal biopsies were obtained at every 1-2 cm. All four-quadrant Hollande's or formalin fixed biopsies were embedded into one paraffin block and serial 4 µm thick tissue sections were cut and stained with hematoxylin and eosin

$P = 0.14$. Figure 5 is a forest plot representing the pooled and individual AIR for malignant transformation in confirmed NDBE patients. Figure 6 is a funnel plot assessing the publication bias for same variable.

IDBE data was available in four studies^[27,28,35,36]. Among the patients down staged to IDBE, the AIR of progression to HGD and or EAC was 1.42% (95%CI: 1.18-1.65). I^2 (inconsistency) = 97.9%. Egger: bias = 6.48 (95%CI: -59.45-72.41), $P = 0.71$. Figure 7 is a forest plot representing the pooled and individual AIR for malignant transformation in confirmed IDBE patients.

Subgroup analysis of prospective studies

Subgroup analysis was performed on all prospective trials^[10,35-37]. Four studies were included in this analysis. The total number of patients included in this subgroup was 122, with a predominantly male

population (67%). Median age of the patients was 61 years. In the subgroup analysis of prospective studies, AIR of malignant (HGD and or EAC) transformation in confirmed LGD patients was 7.98 (95%CI: 3.64-12.31). I^2 (inconsistency) = 98.4%. Egger: bias = 11.49 (95%CI: 7.46-15.53), $P = 0.006$. Figure 8 is a forest plot representing the pooled and individual AIR for malignant (HGD and or EAC) transformation in confirmed LGD patients.

DISCUSSION

There is a wide variation in the incidence of adenocarcinoma stemming from Barrett's esophagus in the current literature. Existing endoscopic surveillance studies show anywhere from 1 in 52 to 1 in 441 patient-years of surveillance. The lack of large prospective studies makes it difficult to determine

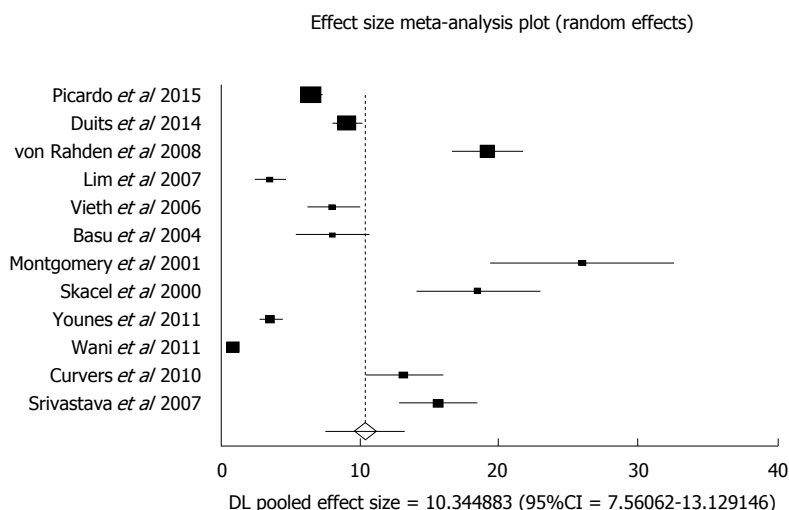


Figure 2 Forest plot representing the pooled and individual annual incidence rate for malignant (high grade dysplasia and/or esophageal adenocarcinoma) transformation in confirmed low grade dysplasia patients.

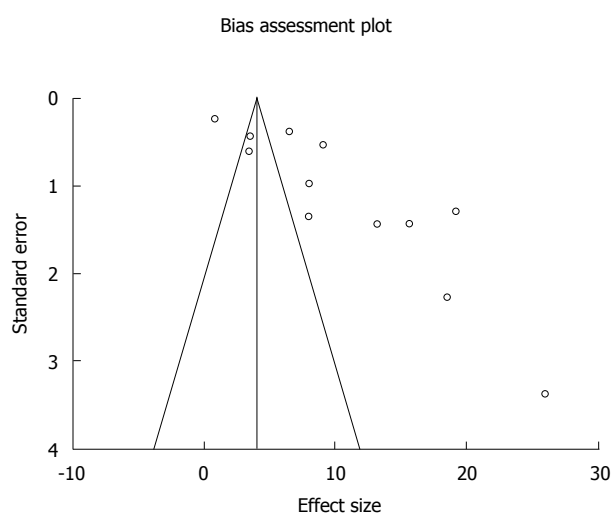


Figure 3 Funnel plot assessing for publication bias (annual incidence rate for malignant transformation in confirmed low grade dysplasia patients).

the efficacy and cost-effectiveness of widespread surveillance programs, and thus there is a large discrepancy between surveillance programs^[38-41]. Basu *et al*^[32] conducted a retrospective analysis of the Barrett's epithelium surveillance database to assess the utility of a surveillance program and to determine the natural incidence of dysplasia in this population. The overall prevalence of LGD in the 5-year period was 7.2%, with an annual incidence of 1.4%. None of these patients progressed to HGD. This study disagreed with the previous study by van Sandick *et al*^[42], who concluded an increased progression to HGD from the initial low grade population. The disagreement may be due to study limitations including small sample size and short duration of follow up in the study by Basu *et al*^[32]. Overall, this study concluded that annual endoscopic surveillance showed little reward, but there are variations among regions^[43] and surveillance

intervals should also be based on the cancer incidence of that specific region.

Another factor affecting surveillance intervals and the true rate of progression to adenocarcinoma is interobserver variability in grading dysplasia. Outcomes in a study by Montgomery *et al*^[33], support that IDBE and LGD should have identical surveillance and follow up as they had similar frequencies of development of adenocarcinoma, but IDBE was slower to progress than LGD by at least 42 mo. Increasing dysplasia was also associated with increased frequency of ulcers found on exam. The study found that approximately 1 of 50 patients with Barrett's esophagus without dysplasia progressed to HGD at approximately 6 years. Conversely, those with any grade of dysplasia or evidence of ulceration should be followed up in shorter intervals. They further concluded that criteria for the diagnosis of dysplasia are reliable. Patients with Barrett's esophagus without dysplasia may be followed with endoscopy and biopsies every few years while those with IDBE, any grade of dysplasia, or evidence of ulceration require shorter follow up intervals.

Dysplasia is diagnosed when epithelial atypia also involves the surface epithelium^[44]. In cases where the surface epithelium is denuded, uninvolved, or unable to be evaluated, the grading is changed to indeterminate dysplasia or indefinite for dysplasia (IDBE)^[45]. Younes *et al*^[35] demonstrated with statistical significance that patients with an initial biopsy diagnosis of either IDBE-Multifocal, LGD, or LGD-Multifocal were more likely to progress to HGD than those with an initial diagnosis of NDBE or IDBE. Unfortunately, there exists a large breadth of interobserver variability, especially in IDBE, where there are no clear guidelines for diagnosis.

Interobserver variability may result in under-diagnosis, such as in the study by Montgomery *et al*^[33] or in over-diagnosis as demonstrated in the study by Curvers *et al*^[36]. In Curvers *et al*^[36] over 75% of cases

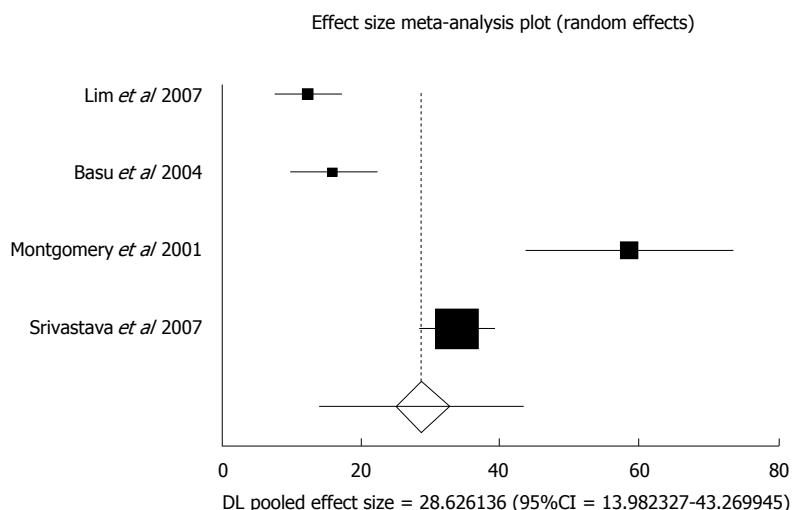


Figure 4 Forest plot representing the pooled and individual annual incidence rate for esophageal adenocarcinoma transformation in confirmed high grade dysplasia patients.

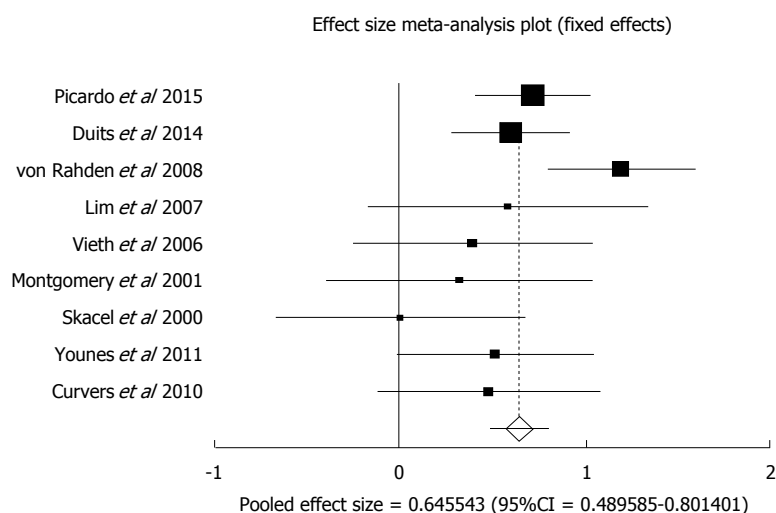


Figure 5 Forest plot representing the pooled and individual annual incidence rate for malignant (high grade dysplasia and/or esophageal adenocarcinoma) transformation in confirmed No dysplasia in Barrett's esophagus patients.

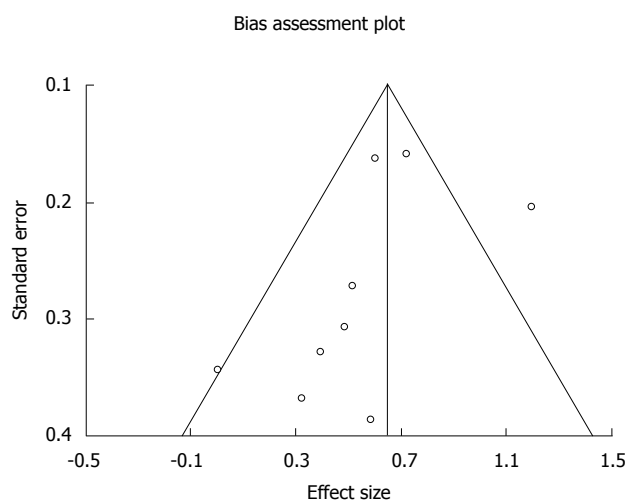


Figure 6 Funnel plot assessing for publication bias (annual incidence rate for malignant transformation in confirmed No dysplasia in Barrett's esophagus patients).

with previously diagnosed low grade dysplasia were downgraded to NDBE, which were shown to have an incidence rate of 0.49% per patient year. This is in contrast to the study by Montgomery *et al*^[33] in which pathologists rated IDBE despite marked atypia to avoid over-diagnosis. Also, a true diagnosis of low grade dysplasia, which is defined here as one diagnosed by an expert panel of pathologists, carries a 13.4% incidence of progression per patient year. Though low grade dysplasia may be over-diagnosed, those cases which are confirmed by a consensus diagnosis with a panel of expert pathologists should not be underestimated and warrant close follow-up.

Duits *et al*^[28] conducted a large, retrospective cohort which studied the incidence rates of HGD and EAC in patients diagnosed with LGD by an expert panel of pathologists. Seventy-three percent of the initial community cohort of patients were downgraded to a diagnosis of IDBE or NDBD. When the diagnosis

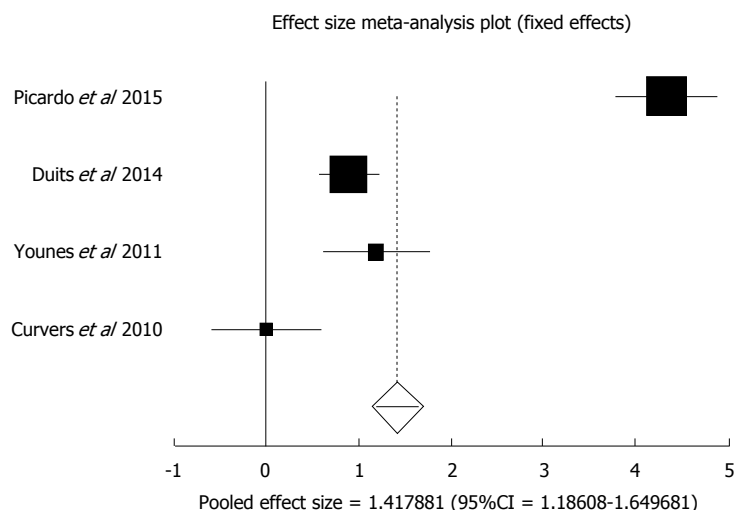


Figure 7 Forest plot representing the pooled and individual annual incidence rate for malignant (high grade dysplasia and/or esophageal adenocarcinoma) transformation in confirmed Indefinite for dysplasia in Barrett's esophagus patients.

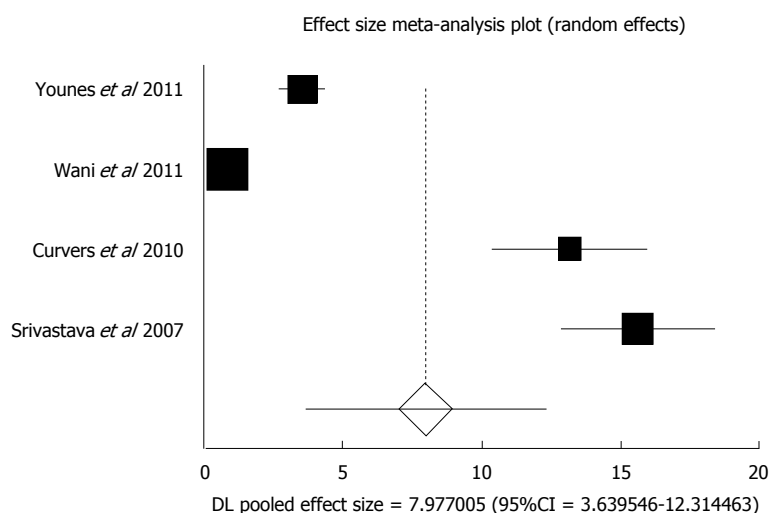


Figure 8 Forest plot representing the pooled and individual annual incidence rate for malignant (high grade dysplasia and/or esophageal adenocarcinoma) transformation in confirmed low grade dysplasia patients - Only prospective studies.

of low grade dysplasia was confirmed by an expert panel, the risk of progression to HGD or EAC was 9.1% annually and had a five-year cumulative progression risk of 33.3%, which was much higher than expected. This study again highlights the initial over-diagnosis of low grade dysplasia. Unfortunately, this leads to an underestimation of the risk to malignant progression in cases of low grade dysplasia which have been confirmed by an expert panel.

Skacel *et al*^[34] further evaluated the degree of interobserver variability in the histological diagnosis of LGD and impact on reported risk of progression to HGD or EAC. Biopsy specimens were reviewed individually by three GI pathologists, blind to the previous diagnosis, and found the individual GI pathologists' diagnosis did not correlate with disease progression. When at least 2 of the pathologists agreed on the histological diagnosis, there was a

statistically significant progression (7 out of 17, $P = 0.04$) When all 3 pathologists agreed, 4 out of the 5 patients progressed ($P = 0.012$). Of the 8 patients where there was no agreement between pathologists, 0 of these patients progressed

A study by Weston *et al*^[46] showed 5 out of 62 patients initially diagnosed with LGD progressed, but it is unclear if the initial diagnosis was confirmed by an expert panel^[46]. In a study by Reid *et al*^[47], it was noted that a consensus diagnosis of low grade dysplasia suggests an increased risk of progression to high grade dysplasia as compared to identifications where pathologists disagree on the diagnosis.

There has been little evaluation of the extent of LGD as a risk factor for the development of EAC, and studies on HGD as a risk factor were conflicting. Previous studies evaluated the extent of HGD as a risk factor for developing adenocarcinoma in Barrett's esophagus,

but they did not evaluate LGD^[48,49]. Srivastava *et al.*^[37] conducted a study assessing the total numbers of both LGD and HGD crypts, the extent of dysplasia, and total dysplasia correlated with EAC development. In those who progressed to EAC, the number of dysplastic crypts per patient (115.2) including HGD and LGD, were significantly higher than in nonprogressors (56.2, $P = 0.01$). When the crypts were stratified by dysplasia grade, the patients who developed EAC had a marginally greater mean number of LGD crypts (93.9) as compared with patients that did not progress (41.2, $P = 0.07$). Per patient, the mean proportion of LGD crypts were also found to be greater in progressors (46.4% vs 26%, $P = 0.037$), but more significantly than the mean number of crypts. Interestingly, neither the mean number ($P = 0.14$) nor the mean proportion ($P = 0.20$) of HGD crypts per patient was significantly associated with development of EAC. The study concluded that the extent of LGD is a significant risk factor for developing esophageal adenocarcinoma, while the extent of HGD was not found to be an independent risk factor. However, the presence of HGD is associated with a greater relative risk for developing adenocarcinoma.

A registry of patients with Barrett's esophagus has been found to be beneficial with respect to patient management as well as for identifying populations at greater risk in need of alternate surveillance intervals. The utility of a registry is further increased where surveillance is not cost-effective for all^[50]. Esophageal adenocarcinoma has increased drastically in Europe, and is predicted to increase even higher in the near future^[51-53]. Picardo *et al.*^[27] established a Barrett's registry in 2008 consisting of 1093 patients from the Republic of Ireland. Of the 73 patients with a diagnosis of LGD with endoscopic follow up beyond 1 year, 46 (65%) had histological regression, 8 progressed to HGD, and 6 to EAC. This study showed that the absolute risk of EAC was higher than reported in whole population studies. Incidence was higher in this study as compared to a Danish population study and another study conducted from the Northern Ireland Barrett's Register^[12,54].

The strengths of this meta-analysis include the high quality methodology of statistical analysis, high quality methodology used in individual studies, relatively greater number of studies that met the inclusion criteria, and large total number of patients included in this analysis ($n = 971$). This is the first meta-analysis to pool the evidence for AIR of malignancy in confirmed LGD patients.

The limitations of this study include: There was a significant level of heterogeneity among studies included in this analysis. Random effects model was used to calculate pooled effects for most of the variables when the heterogeneity was high. We also performed a pooled subgroup analysis after excluding the outliers, with the intention of reducing the

heterogeneity. We were unable to perform financial impact analysis due to the lack of data from the individual studies. The local expertise of pathologists/expert pathologists/gastrointestinal pathologists plays a key role in the outcomes. The variability of their expertise is one of the most significant reasons for heterogeneity among studies. A few other important reasons for heterogeneity among studies could be exclusion of prevalent cases of malignancies, and design of individual studies. There were retrospective studies included in this meta-analysis. In order to mitigate this issue, we have performed a sub-group analysis on prospective studies only. Due to the paucity of data available from individual studies, we were not able to analyze the relation between the length of Barrett's, extent of dysplasia and progression to malignancy.

Since the incidence of esophageal adenocarcinoma is rising, it is vital to focus on all possible preventive measures to halt or slow the progression from BE to EAC. Based on the results of this analysis, the incidence of malignancy in confirmed LGD patients is much higher than the current estimates. For these reasons, it is of paramount importance to confirm the diagnosis of patients labelled as LGD. Once a diagnosis of LGD has been confirmed, these patients would benefit from closer follow-up and surveillance. In patients with LGD, most gastroenterology societies recommend surveillance endoscopy with biopsies every 12 mo. However, with more recent evidence suggesting the true rate of transformation is higher, there may be a role for more frequent endoscopic surveillance or sooner procedural intervention. These questions should be answered with further large prospective trials.

Studies with statistically significant positive results tend to be published and cited. Additionally, smaller studies may show larger treatment effects compared to larger studies. This publication and selection bias may affect the summary estimates. The bias can be estimated using Egger bias indicators and the construction of funnel plots, whose shape can be affected by bias. In the present meta-analysis and systematic review, bias calculations both Egger^[23] and Begg-Mazumdar^[24] bias indicators showed no statistically significant bias. Furthermore, analysis using funnel plots showed no significant publication bias among the studies included in the present analysis.

Overall, when LGD is diagnosed by consensus agreement of two or more expert pathologists, its progression towards malignancy appears to be at least three times the current estimates, and may be up to 20 times the current estimates. Biopsies of all Barrett's esophagus patients with LGD should be reviewed by two expert gastroenterology pathologists. Follow-up strict surveillance programs should be in place for these patients. Large prospective studies are required to evaluate if confirmed LGD patients should have follow

up surveillance more frequently than every year.

COMMENTS

Background

Esophageal adenocarcinoma incidence has more than quadrupled over the last few decades, and is alarmingly becoming a leader for cancer mortality. The recognition of Barrett's esophagus and prevention of progression to esophageal adenocarcinoma is quickly becoming a national public health concern.

Research frontiers

Recent studies indicate the progression from Barrett's esophagus to Low grade dysplasia is grossly underestimated. This may be attributed to the expertise of the reading pathologist, in addition to the number of pathologists assessing the biopsies. Given the concern for the risk of progression, accurate assessment of dysplasia is vital for appropriate risk stratification and surveillance strategies.

Innovations and breakthroughs

Based on the results of this study, when Low grade dysplasia is diagnosed by consensus agreement of two or more expert pathologists, its progression towards malignancy appears to be at least three times the current estimates, and may be up to 20 times the current estimates.

Applications

Biopsies of all Barrett's esophagus patients with low grade dysplasia (LGD) should be reviewed by two expert gastroenterology pathologists. Follow-up strict surveillance programs should be in place for these patients. Large prospective studies are required to evaluate if confirmed LGD patients should have follow up surveillance more frequently than every year.

Terminology

Barrett's Esophagus is a condition in which the normal esophageal squamous epithelium is replaced by columnar epithelium in a process known as metaplasia.

Peer-review

In their manuscript the authors performed is a systematic review and meta-analysis to evaluate annual incidence of LGD progression to high grade dysplasia and/or esophageal adenocarcinoma when diagnosis was made by two or more expert pathologists.

REFERENCES

- 1 **Barrett NR.** Chronic peptic ulcer of the oesophagus and 'oesophagitis'. *Br J Surg* 1950; **38**: 175-182 [PMID: 14791960 DOI: 10.1002/bjs.18003815005]
- 2 **Corley DA,** Kubo A, Levin TR, Block G, Habel L, Rumore G, Quesenberry C, Buffler P. Race, ethnicity, sex and temporal differences in Barrett's oesophagus diagnosis: a large community-based study, 1994-2006. *Gut* 2009; **58**: 182-188 [PMID: 18978173 DOI: 10.1136/gut.2008.163360]
- 3 **Shiota S,** Singh S, Anshasi A, El-Serag HB. Prevalence of Barrett's Esophagus in Asian Countries: A Systematic Review and Meta-analysis. *Clin Gastroenterol Hepatol* 2015; **13**: 1907-1918 [PMID: 26260107 DOI: 10.1016/j.cgh.2015.07.050]
- 4 **Kamat P,** Wen S, Morris J, Anandasabapathy S. Exploring the association between elevated body mass index and Barrett's esophagus: a systematic review and meta-analysis. *Ann Thorac Surg* 2009; **87**: 655-662 [PMID: 19161814 DOI: 10.1016/j.athorac.2008.08.003]
- 5 **Ronkainen J,** Aro P, Storskrubb T, Johansson SE, Lind T, Bolling-Sternevald E, Vieth M, Stolte M, Talley NJ, Agr  us L. Prevalence of Barrett's esophagus in the general population: an endoscopic study. *Gastroenterology* 2005; **129**: 1825-1831 [PMID: 16344051 DOI: 10.1053/j.gastro.2005.08.053]
- 6 **Rex DK,** Cummings OW, Shaw M, Cumings MD, Wong RK, Vasudeva RS, Dunne D, Rahmani EY, Helper DJ. Screening for Barrett's esophagus in colonoscopy patients with and without heartburn. *Gastroenterology* 2003; **125**: 1670-1677 [PMID: 14724819 DOI: 10.1053/j.gastro.2003.09.030]
- 7 **Hayeck TJ,** Kong CY, Spechler SJ, Gazelle GS, Hur C. The prevalence of Barrett's esophagus in the US: estimates from a simulation model confirmed by SEER data. *Dis Esophagus* 2010; **23**: 451-457 [PMID: 20353441 DOI: 10.1111/j.1442-2050.2010.01054.x]
- 8 **van Soest EM,** Dieleman JP, Siersema PD, Sturkenboom MC, Kuipers EJ. Increasing incidence of Barrett's oesophagus in the general population. *Gut* 2005; **54**: 1062-1066 [PMID: 15857935 DOI: 10.1136/gut.2004.063685]
- 9 **Ford AC,** Forman D, Reynolds PD, Cooper BT, Moayyedi P. Ethnicity, gender, and socioeconomic status as risk factors for esophagitis and Barrett's esophagus. *Am J Epidemiol* 2005; **162**: 454-460 [PMID: 16076833 DOI: 10.1093/aje/kwi218]
- 10 **Wani S,** Falk GW, Post J, Yeran L, Hall M, Wang A, Gupta N, Gaddam S, Singh M, Singh V, Chuang KY, Boolchand V, Gavini H, Kuczynski J, Sud P, Bansal A, Rastogi A, Mathur SC, Young P, Cash B, Goldblum J, Lieberman DA, Sampliner RE, Sharma P. Risk factors for progression of low-grade dysplasia in patients with Barrett's esophagus. *Gastroenterology* 2011; **141**: 1179-1186, 1186.e1 [PMID: 21723218 DOI: 10.1053/j.gastro.2011.06.055]
- 11 **Sinh P,** Anaparthi R, Young PE, Gaddam S, Thota P, Balasubramanian G, Singh M, Higbee AD, Wani S, Gupta N, Rastogi A, Mathur SC, Bansal A, Horwhat JD, Cash BD, Falk GW, Lieberman DA, Vargo JJ, Sampliner RE, Sharma P. Clinical outcomes in patients with a diagnosis of "indefinite for dysplasia" in Barrett's esophagus: a multicenter cohort study. *Endoscopy* 2015; **47**: 669-674 [PMID: 25910065 DOI: 10.1055/s-0034-1391966]
- 12 **Bhat S,** Coleman HG, Yousef F, Johnston BT, McManus DT, Gavin AT, Murray LJ. Risk of malignant progression in Barrett's esophagus patients: results from a large population-based study. *J Natl Cancer Inst* 2011; **103**: 1049-1057 [PMID: 21680910 DOI: 10.1093/jnci/djr203]
- 13 **Chandrasoma P,** Wickramasinghe K, Ma Y, DeMeester T. Is intestinal metaplasia a necessary precursor lesion for adenocarcinomas of the distal esophagus, gastroesophageal junction and gastric cardia? *Dis Esophagus* 2007; **20**: 36-41 [PMID: 17227308 DOI: 10.1111/j.1442-2050.2007.00638.x]
- 14 **Fitzgerald RC,** di Pietro M, Raganath K, Ang Y, Kang JY, Watson P, Trudgill N, Patel P, Kaye PV, Sanders S, O'Donovan M, Bird-Lieberman E, Bhandari P, Jankowski JA, Attwood S, Parsons SL, Loft D, Lagergren J, Moayyedi P, Lyraatzopoulos G, de Caestecker J. British Society of Gastroenterology guidelines on the diagnosis and management of Barrett's oesophagus. *Gut* 2014; **63**: 7-42 [PMID: 24165758 DOI: 10.1136/gutjnl-2013-305372]
- 15 **Brennan P,** Silman A. Statistical methods for assessing observer variability in clinical measures. *BMJ* 1992; **304**: 1491-1494 [PMID: 1611375]
- 16 **Schlemper RJ,** Riddell RH, Kato Y, Borchard F, Cooper HS, Dawsey SM, Dixon MF, Fenoglio-Preiser CM, Fl  jou JF, Geboes K, Hattori T, Hirota T, Itabashi M, Iwafuchi M, Iwashita A, Kim YI, Kirchner T, Klimpfinger M, Koike K, Lauwers GY, Lewin KJ, Oberhuber G, Offner F, Price AB, Rubio CA, Shimizu M, Shimoda T, Sipponen P, Solcia E, Stolte M, Watanabe H, Yamabe H. The Vienna classification of gastrointestinal epithelial neoplasia. *Gut* 2000; **47**: 251-255 [PMID: 10896917 DOI: 10.1136/gut.47.2.251]
- 17 **Jadad AR,** Moore RA, Carroll D, Jenkinson C, Reynolds DJ, Gavaghan DJ, McQuay HJ. Assessing the quality of reports of randomized clinical trials: is blinding necessary? *Control Clin Trials* 1996; **17**: 1-12 [PMID: 8721797 DOI: 10.1016/0197-2456(95)00134-4]
- 18 **Stroup DF,** Berlin JA, Morton SC, Olkin I, Williamson GD, Rennie D, Moher D, Becker BJ, Sipe TA, Thacker SB. Meta-analysis of observational studies in epidemiology: a proposal for reporting. Meta-analysis Of Observational Studies in Epidemiology (MOOSE) group. *JAMA* 2000; **283**: 2008-2012 [PMID: 10789670]

- DOI: 10.1001/jama.283.15.2008]
- 19 **Wells GA**, Shea B, O'Connell D, Peterson J, Welch V, Losos M, Tugwell P. The Newcastle-Ottawa Scale (NOS) for assessing the quality of nonrandomized studies in meta-analyses; 2014. Available from: URL: http://www.ohri.ca/programs/clinical_epidemiology/oxford.asp
 - 20 **Stuart A**, Ord JK. Kendall's Advanced Theory of Statistics (6th edition). London: Edward Arnold, 1994
 - 21 **DerSimonian R**, Laird N. Meta-analysis in clinical trials. *Control Clin Trials* 1986; **7**: 177-188 [PMID: 3802833 DOI: 10.1016/0197-2456(86)90046-2]
 - 22 **Deeks JJ**. Systematic reviews of evaluations of diagnostic and screening tests. In: Egger M, Smith GD, Altman DG, editors. Systematic Reviews in Health Care. Meta-analysis in context. London: BMJ Books, 2001
 - 23 **Harbord RM**, Egger M, Sterne JA. A modified test for small-study effects in meta-analyses of controlled trials with binary endpoints. *Stat Med* 2006; **25**: 3443-3457 [PMID: 16345038 DOI: 10.1002/sim.2380]
 - 24 **Begg CB**, Mazumdar M. Operating characteristics of a rank correlation test for publication bias. *Biometrics* 1994; **50**: 1088-1101 [PMID: 7786990 DOI: 10.2307/2533446]
 - 25 **Sterne JA**, Egger M, Smith GD. Systematic reviews in health care: Investigating and dealing with publication and other biases in meta-analysis. *BMJ* 2001; **323**: 101-105 [PMID: 11451790]
 - 26 **Sterne JA**, Egger M. Funnel plots for detecting bias in meta-analysis: guidelines on choice of axis. *J Clin Epidemiol* 2001; **54**: 1046-1055 [PMID: 11576817 DOI: 10.1016/S0895-4356(01)00377-8]
 - 27 **Picardo SL**, O'Brien MP, Feighery R, O'Toole D, Ravi N, O'Farrell NJ, O'Sullivan JN, Reynolds JV. A Barrett's esophagus registry of over 1000 patients from a specialist center highlights greater risk of progression than population-based registries and high risk of low grade dysplasia. *Dis Esophagus* 2015; **28**: 121-126 [PMID: 24428806 DOI: 10.1111/dote.12166]
 - 28 **Duits LC**, Phoa KN, Curvers WL, Ten Kate FJ, Meijer GA, Seldenrijk CA, Offerhaus GJ, Visser M, Meijer SL, Krishnadath KK, Tijssen JG, Mallant-Hent RC, Bergman JJ. Barrett's oesophagus patients with low-grade dysplasia can be accurately risk-stratified after histological review by an expert pathology panel. *Gut* 2015; **64**: 700-706 [PMID: 25034523 DOI: 10.1136/gutjnl-2014-307278]
 - 29 **von Rahden BH**, Stein HJ, Weber A, Vieth M, Stolte M, Rösch T, Schmid RM, Sarbia M, Meining A. Critical reappraisal of current surveillance strategies for Barrett's esophagus: analysis of a large German Barrett's database. *Dis Esophagus* 2008; **21**: 685-689 [PMID: 18847456 DOI: 10.1111/j.1442-2050.2008.00857.x]
 - 30 **Lim CH**, Treanor D, Dixon MF, Axon AT. Low-grade dysplasia in Barrett's esophagus has a high risk of progression. *Endoscopy* 2007; **39**: 581-587 [PMID: 17611911 DOI: 10.1055/s-2007-966592]
 - 31 **Vieth M**, Schubert B, Lang-Schwarz K, Stolte M. Frequency of Barrett's neoplasia after initial negative endoscopy with biopsy: a long-term histopathological follow-up study. *Endoscopy* 2006; **38**: 1201-1205 [PMID: 17163319 DOI: 10.1055/s-2006-944993]
 - 32 **Basu KK**, Pick B, de Caestecker JS. Audit of a Barrett's epithelium surveillance database. *Eur J Gastroenterol Hepatol* 2004; **16**: 171-175 [PMID: 15075990 DOI: 10.1097/01.meg.0000085551.45167.d2]
 - 33 **Montgomery E**, Goldblum JR, Greenston JK, Haber MM, Lamps LW, Lauwers GY, Lazenby AJ, Lewin DN, Robert ME, Washington K, Zahurak ML, Hart J. Dysplasia as a predictive marker for invasive carcinoma in Barrett esophagus: a follow-up study based on 138 cases from a diagnostic variability study. *Hum Pathol* 2001; **32**: 379-388 [PMID: 11331954 DOI: 10.1053/hupa.2001.23511]
 - 34 **Skacel M**, Petras RE, Gramlich TL, Sigel JE, Richter JE, Goldblum JR. The diagnosis of low-grade dysplasia in Barrett's esophagus and its implications for disease progression. *Am J Gastroenterol* 2000; **95**: 3383-3387 [PMID: 11151865 DOI: 10.1111/j.1572-0241.2000.03348.x]
 - 35 **Younes M**, Lauwers GY, Ertan A, Ergun G, Verm R, Bridges M, Woods K, Meriano F, Schmulen C, Johnson C, Barroso A, Schwartz J, McKechnie J, Lechago J. The significance of "indefinite for dysplasia" grading in Barrett metaplasia. *Arch Pathol Lab Med* 2011; **135**: 430-432 [PMID: 21466357 DOI: 10.1043/2010-0097-OA.1]
 - 36 **Curvers WL**, ten Kate FJ, Krishnadath KK, Visser M, Elzer B, Baak LC, Bohmer C, Mallant-Hent RC, van Oijen A, Naber AH, Scholten P, Busch OR, Blaauwgeers HG, Meijer GA, Bergman JJ. Low-grade dysplasia in Barrett's esophagus: overdiagnosed and underestimated. *Am J Gastroenterol* 2010; **105**: 1523-1530 [PMID: 20461069 DOI: 10.1038/ajg.2010.171]
 - 37 **Srivastava A**, Hornick JL, Li X, Blount PL, Sanchez CA, Cowan DS, Ayub K, Maley CC, Reid BJ, Odze RD. Extent of low-grade dysplasia is a risk factor for the development of esophageal adenocarcinoma in Barrett's esophagus. *Am J Gastroenterol* 2007; **102**: 483-493; quiz 694 [PMID: 17338734 DOI: 10.1111/j.1572-0241.2007.01073.x]
 - 38 **Hameeteman W**, Tytgat GN, Houthoff HJ, van den Tweel JG. Barrett's esophagus: development of dysplasia and adenocarcinoma. *Gastroenterology* 1989; **96**: 1249-1256 [PMID: 2703113]
 - 39 **Van der Veen AH**, Dees J, Blankensteijn JD, Van Blankenstein M. Adenocarcinoma in Barrett's oesophagus: an overrated risk. *Gut* 1989; **30**: 14-18 [PMID: 2920919]
 - 40 **Williamson WA**, Ellis FH, Gibb SP, Shahian DM, Aretz HT, Heatley GJ, Watkins E. Barrett's esophagus. Prevalence and incidence of adenocarcinoma. *Arch Intern Med* 1991; **151**: 2212-2216 [PMID: 1953225]
 - 41 **Drewitz DJ**, Sampliner RE, Garewal HS. The incidence of adenocarcinoma in Barrett's esophagus: a prospective study of 170 patients followed 4.8 years. *Am J Gastroenterol* 1997; **92**: 212-215 [PMID: 9040193]
 - 42 **van Sandick JW**, van Lanschot JJ, Kuiken BW, Tytgat GN, Offerhaus GJ, Obertop H. Impact of endoscopic biopsy surveillance of Barrett's oesophagus on pathological stage and clinical outcome of Barrett's carcinoma. *Gut* 1998; **43**: 216-222 [PMID: 10189847]
 - 43 **Jankowski JA**, Provenzale D, Moayyedi P. Esophageal adenocarcinoma arising from Barrett's metaplasia has regional variations in the west. *Gastroenterology* 2002; **122**: 588-590 [PMID: 11845805]
 - 44 **Sampliner RE**. Updated guidelines for the diagnosis, surveillance, and therapy of Barrett's esophagus. *Am J Gastroenterol* 2002; **97**: 1888-1895 [PMID: 12190150 DOI: 10.1111/j.1572-0241.2002.05910.x]
 - 45 **Younes M**, Lechago J, Chakraborty S, Ostrowski M, Bridges M, Meriano F, Solcher D, Barroso A, Whitman D, Schwartz J, Johnson C, Schmulen AC, Verm R, Balsaver A, Carlson N, Ertan A. Relationship between dysplasia, p53 protein accumulation, DNA ploidy, and Glut1 overexpression in Barrett metaplasia. *Scand J Gastroenterol* 2000; **35**: 131-137 [PMID: 10720109 DOI: 10.1080/003655200750024281]
 - 46 **Weston A**, Sharma P, Topalowski M. Low-grade dysplasia in Barrett's esophagus: Variable fate during long-term prospective follow-up. *Gastroenterology* 1999; **116**: 1349A
 - 47 **Reid BJ**, Haggitt RC, Rubin CE, Roth G, Surawicz CM, Van Belle G, Lewin K, Weinstein WM, Antonioli DA, Goldman H. Observer variation in the diagnosis of dysplasia in Barrett's esophagus. *Hum Pathol* 1988; **19**: 166-178 [PMID: 3343032]
 - 48 **Buttar NS**, Wang KK, Sebo TJ, Riehle DM, Krishnadath KK, Lutzke LS, Anderson MA, Petterson TM, Burgart LJ. Extent of high-grade dysplasia in Barrett's esophagus correlates with risk of adenocarcinoma. *Gastroenterology* 2001; **120**: 1630-1639 [PMID: 11375945 DOI: 10.1053/gast.2001.25111]
 - 49 **Dar MS**, Goldblum JR, Rice TW, Falk GW. Can extent of high grade dysplasia in Barrett's oesophagus predict the presence of adenocarcinoma at oesophagectomy? *Gut* 2003; **52**: 486-489 [PMID: 12631655]
 - 50 **Provenzale D**, Schmitt C, Wong JB. Barrett's esophagus: a new look at surveillance based on emerging estimates of cancer risk.

- Am J Gastroenterol* 1999; **94**: 2043-2053 [PMID: 10445526 DOI: 10.1111/j.1572-0241.1999.01276.x]
- 51 **Cook MB**, Chow WH, Devesa SS. Oesophageal cancer incidence in the United States by race, sex, and histologic type, 1977-2005. *Br J Cancer* 2009; **101**: 855-859 [PMID: 19672254 DOI: 10.1038/sj.bjc.6605246]
- 52 **Steevens J**, Botterweck AA, Dirx MJ, van den Brandt PA, Schouten LJ. Trends in incidence of oesophageal and stomach cancer subtypes in Europe. *Eur J Gastroenterol Hepatol* 2010; **22**: 669-678 [PMID: 19474750 DOI: 10.1097/MEG.0b013e32832ca091]
- 53 **Post PN**, Siersema PD, Van Dekken H. Rising incidence of clinically evident Barrett's oesophagus in The Netherlands: a nationwide registry of pathology reports. *Scand J Gastroenterol* 2007; **42**: 17-22 [PMID: 17190757 DOI: 10.1080/00365520600815654]
- 54 **Hvid-Jensen F**, Pedersen L, Drewes AM, Sørensen HT, Funch-Jensen P. Incidence of adenocarcinoma among patients with Barrett's esophagus. *N Engl J Med* 2011; **365**: 1375-1383 [PMID: 21995385 DOI: 10.1056/NEJMoa1103042]

P- Reviewer: Deng B, Francisco G, Gao C, Tang Y, Tarnawski AS

S- Editor: Qi Y **L- Editor:** A **E- Editor:** Wang CH



Cyclophosphamide-associated enteritis: A rare association with severe enteritis

Linda S Yang, Karla Cameron, Tim Papaluca, Chamara Basnayake, Louise Jactett, Penelope McKelvie, David Goodman, Barbara Demediuk, Sally J Bell, Alexander J Thompson

Linda S Yang, Karla Cameron, Tim Papaluca, Chamara Basnayake, Barbara Demediuk, Sally J Bell, Alexander J Thompson, Department of Gastroenterology, St. Vincent's Hospital, Fitzroy, Victoria 3065, Australia

Louise Jactett, Penelope McKelvie, Department of Pathology, St. Vincent's Hospital, Fitzroy, Victoria 3065, Australia

David Goodman, Department of Nephrology, St. Vincent's Hospital, Fitzroy, Victoria 3065, Australia

Author contributions: Yang LS wrote the paper; Yang LS and Cameron K collected the clinical data; Papaluca T, Basnayake C, Goodman D, Demediuk B, Bell SJ and Thompson AJ were the treating team and supervised the manuscript writing; Jactett L and McKelvie P provided the figures.

Conflict-of-interest statement: The authors have no conflicts of interest or funding from any organisation to disclose.

Institutional review board statement: This case report was exempt from the Institutional Review Board standards at St. Vincent's Hospital, Melbourne in Australia.

Informed consent statement: A written informed consent was waived by the Institutional Review Board given this case report presents no more than minimal risk to the patient involved.

Open-Access: This article is an open-access article which was selected by an in-house editor and fully peer-reviewed by external reviewers. It is distributed in accordance with the Creative Commons Attribution Non Commercial (CC BY-NC 4.0) license, which permits others to distribute, remix, adapt, build upon this work non-commercially, and license their derivative works on different terms, provided the original work is properly cited and the use is non-commercial. See: <http://creativecommons.org/licenses/by-nc/4.0/>

Manuscript source: Unsolicited manuscript

Correspondence to: Linda S Yang, MBBS, Department of Gastroenterology, St. Vincent's Hospital, 41 Victoria Pde, Fitzroy, Victoria 3065, Australia. lindaviva0912@gmail.com
Telephone: +61-3-425726761

Fax: +61-3-92313590

Received: November 3, 2015

Peer-review started: November 4, 2015

First decision: December 11, 2015

Revised: January 5, 2016

Accepted: March 18, 2016

Article in press: March 18, 2016

Published online: October 21, 2016

Abstract

Cyclophosphamide is a potent cytotoxic agent used in many clinical settings. The main risks of cyclophosphamide therapy include hematological disorders, infertility, hemorrhagic cystitis and malignancies. Gastrointestinal side effects reported to date are often non-specific and not severe. We present the first case of a fatal small bowel enteritis and pan-colitis which appears to be associated with cyclophosphamide. We aim to raise the readers' awareness of this significant adverse event to facilitate clinical suspicion and early recognition in potential future cases.

Key words: Enteritis; Colitis; Cyclophosphamide; Small bowel; Pan-colitis

© **The Author(s) 2016.** Published by Baishideng Publishing Group Inc. All rights reserved.

Core tip: The well-known adverse effects associated with cyclophosphamide include bone marrow suppression, hemorrhagic cystitis and malignancies. This case report describes the first case of fatal and irreversible small bowel enteritis and pan-colitis associated with cyclophosphamide.

Yang LS, Cameron K, Papaluca T, Basnayake C, Jactett L,

McKelvie P, Goodman D, Demediuk B, Bell SJ, Thompson AJ. Cyclophosphamide-associated enteritis: A rare association with severe enteritis. *World J Gastroenterol* 2016; 22(39): 8844-8848 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v22/i39/8844.htm> DOI: <http://dx.doi.org/10.3748/wjg.v22.i39.8844>

INTRODUCTION

Cyclophosphamide is an alkylating agent with cytotoxic and immunosuppressive properties. It is frequently used to treat a variety of inflammatory and malignant conditions, either as a sole agent or in combination with chemotherapeutic drugs or glucocorticoids. Toxicities associated with cyclophosphamide are well-described. Significant adverse effects include dose-related leukopenia, hemorrhagic cystitis, infertility, secondary malignancies and pulmonary toxicity^[1]. Reported gastrointestinal side effects include dose-related nausea, stomatitis and a single case of hemorrhagic colitis. In this report, we describe the case of a male patient with fatal small bowel enteritis and pan-colitis which was associated with four weeks of cyclophosphamide therapy.

CASE REPORT

A 60-year-old male was diagnosed with histologically confirmed glomerulonephritis secondary to anti-neutrophil cytoplasmic antibody -positive microscopic polyangiitis, following investigations for elevated creatinine on routine blood test. He was treated in hospital with intravenous methylprednisolone (500 mg daily) and oral cyclophosphamide (100 mg daily) for three days. He was discharged home on a weaning course of prednisolone and cyclophosphamide, with normalisation of his renal function.

Two weeks following this admission, the patient was admitted to a regional hospital with a two day history of nausea, vomiting and diarrhea with intolerance of oral intake. His wife had had similar symptoms recently. The patient developed large volume watery diarrhea, up to eight liters per day. He required transfer to a tertiary hospital intensive care unit where he received hemofiltration for hypovolemic acute kidney injury. Cyclophosphamide was initially reduced to 50 mg daily and then ceased in setting of potential infectious pathology. The patient had received approximately one month of cyclophosphamide, up to 2.1 g of total dose. Empiric antimicrobial therapy was commenced including tazobactam and piperacillin, intravenous metronidazole and ganciclovir. His stool specimen showed secretory diarrhoea with no infective agents identified. Vasoactive intestinal polypeptide and chromogranin levels were also non-diagnostic.

Serial computed tomographs of his abdomen revealed diffuse mural thickening of the small and

large bowel. Upper and lower endoscopic evaluations demonstrated denuded and erythematous mucosa in the duodenum, as well as from sigmoid colon to terminal ileum with no significant interval change in macroscopic appearance (Figure 1). The rectum was relatively spared. Histopathology showed full thickness mucosal ulceration and inflammation throughout the terminal ileum and large bowel (Figure 2). There were no features of inflammatory bowel disease, vasculitis or viral inclusions.

His diarrhea persisted up to six liters daily despite empirical treatment with maximal doses of anti-diarrheals, octreotide and cholestyramine. Repeat imaging, stool specimens, endoscopic evaluation and histopathology failed to reveal an infectious, neuroendocrine, inflammatory or neoplastic etiology. Repeat colonic biopsies showed regenerative mucosal changes. In particular, viral polymerase chain reaction (PCR) and bacterial and fungal cultures were negative on repeated testing. He required continuous intravenous therapy, electrolyte replacements and total parenteral nutrition for severe hypoalbuminemia. Infliximab was administered as empirical therapy without significant clinical or endoscopic improvement.

The patient subsequently developed septic complications with *Enterobacter* and *Candida glabrata* bacteremia. He returned to intensive care and subsequently died from severe acute respiratory distress syndrome.

Post-mortem examination showed multiple areas of hemorrhagic ulceration in the small bowel. Histology of the ulcerated areas in small bowel showed minimal residual mucosa. Similar changes, but less severe were seen in sections of ascending and transverse colon. A small amount of retained mucosa was seen in the descending colon with relative sparing of the rectum (Figure 3). No evidence of vasculitis or thromboemboli was seen in multiple sections of the bowel wall and mesentery. No definite infectious etiology was identified in histological sections (no bacteria or fungal organisms seen on PAS or Gram stains, immunohistochemistry for cytomegalovirus, herpes simplex virus-1 and -2 were negative). Viral PCR from the small and large bowel tissue detected herpes simplex virus-1 DNA, but the clinical significance of this was uncertain in the absence of consistent immunohistochemistry and previously negative PCR.

Sections from all lobes of the lungs revealed changes of diffuse alveolar damage (shock lung) and metastatic pulmonary calcification. Culture detected *Enterobacter faecium*, *Candida krusei* and *Pneumocystis jiroveci*. *Candida krusei* was also cultured in small and large bowel.

DISCUSSION

We present a rare case of severe multifocal ulcerative enterocolitis affecting much of the small bowel and colon, which occurred in close temporal relation to

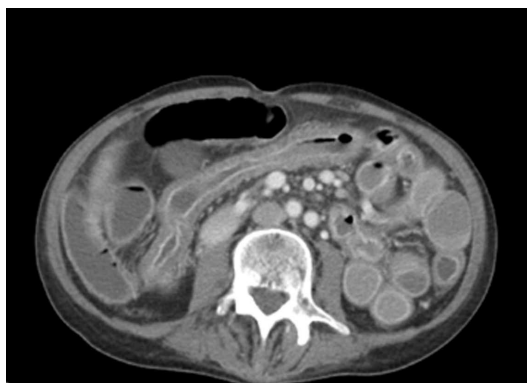


Figure 1 Initial computed tomography of abdomen showing diffuse mural thickening of the small and large bowel. Approximately 2 wk following cyclophosphamide treatment.

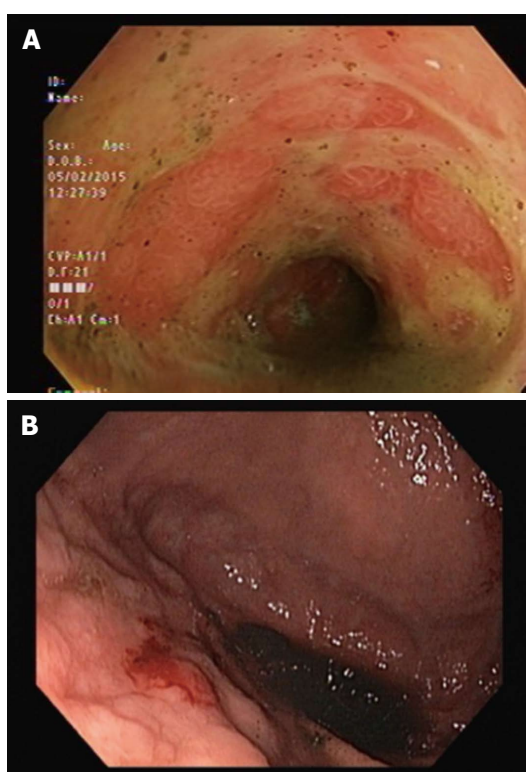


Figure 2 Denuded small and large bowel mucosa on endoscopic evaluation. Approximately 4 wk following cessation of cyclophosphamide. A: Duodenum; B: Descending colon.

cyclophosphamide. To our knowledge, this is the first case report of cyclophosphamide-associated enterocolitis in literature. It is a rare but serious adverse event that clinicians should consider in setting of cyclophosphamide.

Severe gastrointestinal toxicity is not commonly associated with cyclophosphamide and data on the association between cyclophosphamide and enteropathy are scarce. There is a single case report of hemorrhagic colitis following cyclophosphamide treatment of polymyositis^[2]. The findings of this case report were similar to our case, but confined

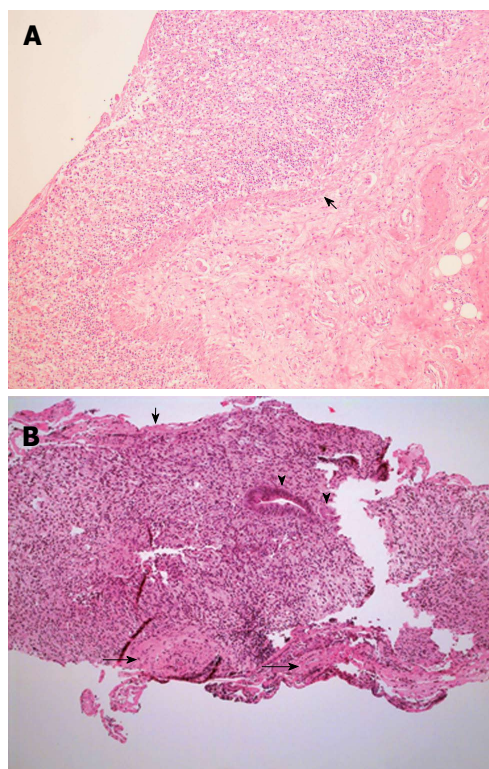


Figure 3 Post-mortem histopathology. A: Small bowel. The high power view of small bowel shows focal full thickness mucosal ulceration with inflamed granulation tissue and preservation of the muscularis mucosae (arrow); B: Colon. Endoscopic biopsy of the left colon approximately one month after commencing cyclophosphamide showed surface fibrin exudate (short arrow), severe mucosal ulceration and granulation tissue with a few residual glands (arrowheads). There were no viral inclusions or granulomas. Preserved muscularis mucosae is seen (long arrows). Hematoxylin-eosin staining, magnification $\times 100$.

to the colon. In that report, endoscopic and histologic examination revealed nonspecific colitis with ulcerations, which resolved after cessation of cyclophosphamide and hydrocortisone rectal enemas. Other available literature mainly describes the therapeutic use of pulse cyclophosphamide in gastrointestinal vasculitis and refractory inflammatory bowel disease. Although not commonly utilized, monthly intravenous cyclophosphamide has been shown to be safe and efficacious in induction and maintenance of remission in severe steroid-refractory inflammatory bowel disease^[3,4]. Gastrointestinal vasculitis, as seen in polyarteritis nodosa, also responds to cyclophosphamide^[5].

This case was a diagnostic and therapeutic challenge due to the unusual presentation and scarcity of similar case reports in literature. We considered the diagnosis of a severe neutropenic typhilitis secondary to cyclophosphamide. Cyclophosphamide has been associated with neutropenic enterocolitis, which usually occurs within two weeks of chemotherapy^[6]. However, it is associated with profound neutropenia on presentation and course of disease. Our patient did not demonstrate significant neutropenia even at

nadir. Treatment for an auto-immune or vasculitic cause of enteritis with IV steroid and infliximab was unsuccessful. Tissue PCR and cultures for infective causes were negative ante-mortem but prolonged immunosuppression is associated with high risks of secondary infection. Whether this contributed to failure of mucosal regeneration in this case is unclear.

The pathophysiology of cyclophosphamide-associated enteritis is difficult to ascertain in this case. If the hemorrhagic enterocolitis was related to the cyclophosphamide, one postulated etiology would be extensive mucosal necrosis with such profound loss of the stem cell niches at the base of the crypts that no repopulation of the mucosal epithelium was possible. This does not seem likely as there was evidence of mucosal regeneration on repeat biopsies and at postmortem. This would be the first case of this scenario occurring with cyclophosphamide, which is used widely for treatment of inflammatory bowel disease including patients with extensive ulceration and for patients with vasculitis of the gastrointestinal tract. It is not clear why this patient would have suffered such a severe insult with a total dose of only 2 grams. His course was complicated by acute hypovolaemic renal failure, metabolic acidosis and sepsis with *Enterobacter* and *Candida* septicemia.

Chemotherapy-related diarrhea is common and is often attributed to alimentary mucositis, which has a complex pathophysiology including alterations in the intestinal microbiome. Abrupt changes in the microbiome may result in excessive generation of reactive oxygen species in the epithelium, thereby upregulating pro-inflammatory cytokines^[7]. Changes in the microbial flora in this patient may have contributed to the ongoing enterocolitis even after cyclophosphamide was ceased.

The mechanisms of hemorrhagic cystitis due to cyclophosphamide have been shown to be specific to the bladder and attributed to urotoxicity of acrolein, a metabolite of the drug, and causing damage *via* multiple factors including tumor necrosis factor- α , interleukin-1- β , cyclooxygenase-2, reactive oxygen and nitric oxide species, and PARP activation. This complication was not seen in our patient.

Large volume fluid and electrolyte replacement and parenteral nutrition is required but volumes above three to four litres a day can only be sustained in inpatients, and intestinal transplantation may need consideration. Intestinal transplant was contraindicated in this case due to sepsis and malnutrition. Lastly, empiric treatment of viral and fungal infections seen in immunocompromised patients may be considered.

In conclusion, with this case report, we aim to highlight a potentially fatal and irreversible enteropathy associated with cyclophosphamide. The etiology, risk factors and treatment of this condition are not yet understood. However, early suspicion of this adverse event may facilitate timely cessation of the offending

drug and provision of early supportive care and prophylactic antimicrobial therapy to prevent significant morbidity or mortality.

COMMENTS

Case characteristics

A 60-year-old man with glomerulonephritis presented with a 2-d history of severe watery diarrhoea and worsening renal function, 1 mo after oral cyclophosphamide therapy.

Clinical diagnosis

Severe enteritis and rectum-sparing pan-colitis, leading to acute renal injury, malabsorption and severe electrolyte disturbances, and ultimately fatal septic complications with *Enterobacter* and *Candida glabrata* bacteremia.

Differential diagnosis

Infective colitis, inflammatory bowel disease, vasculitis, neutropenic typhilitis, carcinoid syndrome, ischemic bowel.

Laboratory diagnosis

Acute renal injury resolved with intravenous hydration and a short course of hemofiltration. Persistent hypokalemia, hypomagnesemia, hypophosphatemia and hypoalbuminemia. Raised inflammatory markers, non-diagnostic vasoactive intestinal polypeptide and chromogranin levels. His stool specimen showed secretory diarrhoea with no infective agents cultured. On post-mortem biopsy, *Enterobacter faecium*, *Candida krusei* and *Pneumocystis jiroveci* were isolated in lungs and colon.

Imaging diagnosis

Computed tomography of the abdomen showed diffuse mural thickening of the small and large bowel.

Pathological diagnosis

Upper and lower endoscopy demonstrated denuded and erythematous mucosa in the duodenum, as well as from sigmoid colon to terminal ileum. Endoscopic biopsy of the small bowel and colon showed full thickness ulceration and granulation tissue with a few residual glands. There were no viral inclusions or granulomas.

Treatment

Prophylactic antibiotics, antivirals and antifungals, anti-diarrhoeals, intravenous fluids and electrolytes, total parenteral nutrition, glucocorticoids and infliximab.

Related reports

There is a single case report of hemorrhagic colitis following cyclophosphamide treatment of polymyositis. The findings of this case report were similar to our case, but confined to the colon. In that report, endoscopic and histologic examination revealed nonspecific colitis with ulcerations, which resolved after cessation of cyclophosphamide and hydrocortisone rectal enemas.

Term explanation

Cyclophosphamide is an alkylating cytotoxic agent used in a variety of clinical settings as chemotherapeutic agent or immunosuppressant. Its main adverse effects include marrow suppression, infertility, hemorrhagic cystitis and malignancies. Gastrointestinal side effects rare and often non-specific and non-fatal.

Experiences and lessons

This is a rare complication in association with cyclophosphamide therapy. Due to the unusual presentation and scarcity of similar case reports in literature, this case can be a diagnostic and therapeutic challenge. Early suspicion and recognition of this adverse event may help prevent significant morbidity or mortality in potential future cases.

Peer-review

The report is well written. In case of any suspected case of colitis after administering cyclophosphamide, timely cessation of the offending drug should be advised to facilitate early, aggressive supportive care for the affected patient. Moreover, prophylactic antimicrobial therapy should be considered to prevent significant morbidity or mortality. This important message is to be disseminated to protect future adverse events during cyclophosphamide treatment.

REFERENCES

- 1 **Fraiser LH**, Kanekal S, Kehrer JP. Cyclophosphamide toxicity. Characterising and avoiding the problem. *Drugs* 1991; **42**: 781-795 [PMID: 1723374]
- 2 **Bujakowska O**, Kur-Zalewska J, Tlustochowicz W. Hemorrhagic colitis as a complication of treatment with cyclophosphamide. *Reumatologica* 2011; **49**: 275-278
- 3 **Barta Z**, Tóth L, Zeher M. Pulse cyclophosphamide therapy for inflammatory bowel disease. *World J Gastroenterol* 2006; **12**: 1278-1280 [PMID: 16534885 DOI: 10.3748/wjg.v12.i8.1278]
- 4 **Rall JM**, Mach SA, Dash PK. Intrahippocampal infusion of a cyclooxygenase-2 inhibitor attenuates memory acquisition in rats. *Brain Res* 2003; **968**: 273-276 [PMID: 12663097]
- 5 **Fauci AS**, Katz P, Haynes BF, Wolff SM. Cyclophosphamide therapy of severe systemic necrotizing vasculitis. *N Engl J Med* 1979; **301**: 235-238 [PMID: 36563 DOI: 10.1056/NEJM197908023010503]
- 6 **Moran H**, Yaniv I, Ashkenazi S, Schwartz M, Fisher S, Levy I. Risk factors for typhlitis in pediatric patients with cancer. *J Pediatr Hematol Oncol* 2009; **31**: 630-634 [PMID: 19644402 DOI: 10.1097/MPH.0b013e3181b1ee28]
- 7 **Stringer AM**, Al-Dasooqi N, Bowen JM, Tan TH, Radzuan M, Logan RM, Mayo B, Keefe DM, Gibson RJ. Biomarkers of chemotherapy-induced diarrhoea: a clinical study of intestinal microbiome alterations, inflammation and circulating matrix metalloproteinases. *Support Care Cancer* 2013; **21**: 1843-1852 [PMID: 23397098 DOI: 10.1007/s00520-013-1741-7]

P- Reviewer: Ingle SB, Krishnan T, Rostami K **S- Editor:** Yu J
L- Editor: A **E- Editor:** Zhang FF



Crowned dens syndrome developed after an endoscopic retrograde cholangiopancreatography procedure

Hiroyasu Nakano, Kazunari Nakahara, Yosuke Michikawa, Keigo Suetani, Ryo Morita, Nobuyuki Matsumoto, Fumio Itoh

Hiroyasu Nakano, Kazunari Nakahara, Yosuke Michikawa, Keigo Suetani, Ryo Morita, Nobuyuki Matsumoto, Fumio Itoh, Division of Gastroenterology and Hepatology, Department of Internal Medicine, St. Marianna University School of Medicine, Kanagawa 216-8511, Japan

Author contributions: Nakano H, Nakahara K and Matsumoto N designed the report; Nakano H, Nakahara K, Michikawa Y, Suetani K and Morita R were attending doctors for the patient; Itoh F organized the report; and Nakano H wrote the paper.

Informed consent statement: Written informed consent for endoscopic intervention was obtained from the patient and patient's families.

Conflict-of-interest statement: Authors have no conflict of interest.

Open-Access: This article is an open-access article which was selected by an in-house editor and fully peer-reviewed by external reviewers. It is distributed in accordance with the Creative Commons Attribution Non Commercial (CC BY-NC 4.0) license, which permits others to distribute, remix, adapt, build upon this work non-commercially, and license their derivative works on different terms, provided the original work is properly cited and the use is non-commercial. See: <http://creativecommons.org/licenses/by-nc/4.0/>

Manuscript source: Unsolicited manuscript

Correspondence to: Hiroyasu Nakano, MD, Division of Gastroenterology and Hepatology, Department of Internal Medicine, St. Marianna University School of Medicine, 2-16-1, Sugao, Miyamae-ku, Kawasaki City, Kanagawa 216-8511, Japan. bluesky0314@gmail.com
Telephone: +81-44-9778111
Fax: +81-44-9765805

Received: April 4, 2016
Peer-review started: April 4, 2016
First decision: May 12, 2016
Revised: May 26, 2016
Accepted: July 20, 2016
Article in press: July 20, 2016
Published online: October 21, 2016

Abstract

We present a unique case of crowned dens syndrome (CDS) that developed after endoscopic retrograde cholangiopancreatography (ERCP) in a patient who presented with fever and neck pain. Administration of non-steroidal anti-inflammatory drugs was extremely effective for relieving fever and neck pain, and in the improvement of inflammatory markers. To the best of our knowledge, this is the first case report of CDS caused by an ERCP procedure. In a patient with fever and neck pain after an ERCP procedure, CDS should be considered in the differential diagnosis.

Key words: Acute cholangitis; Pseudogout; Endoscopic retrograde cholangiopancreatography; Crowned dens syndrome

© The Author(s) 2016. Published by Baishideng Publishing Group Inc. All rights reserved.

Core tip: There are numerous differential diagnoses for patients with fever after an endoscopic retrograde cholangiopancreatography (ERCP). A careful evaluation of medical history and a physical examination provide clues to making a definitive diagnosis. Retrograde cholangitis is a well-known complication of ERCP; additionally, pseudogout is known to develop after severe illnesses or surgery. This case report describes a patient with pseudogout of the neck, which is also known as crowned dens syndrome (CDS). CDS should be considered in a patient with fever and neck pain after an ERCP procedure.

Nakano H, Nakahara K, Michikawa Y, Suetani K, Morita R, Matsumoto N, Itoh F. Crowned dens syndrome developed after an endoscopic retrograde cholangiopancreatography procedure. *World J Gastroenterol* 2016; 22(39): 8849-8852 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v22/i39/8849.htm>
DOI: <http://dx.doi.org/10.3748/wjg.v22.i39.8849>

INTRODUCTION

endoscopic retrograde cholangiopancreatography (ERCP) is an established technique for the removal of common bile duct stones in patients with cholangitis. It is well known that there are several complications that arise after ERCP, such as hemorrhage, gastrointestinal perforation, pancreatitis, and ascending cholangitis. Additionally, fever and neck pain are frequently encountered in clinical settings.

Crowned dens syndrome (CDS) is characterized by neck pain and fever, computed tomography (CT) findings of periodontoid calcification, and a clinical entity of pseudogout. Pseudogout is characterized by the involvement of large joints that most commonly affects knees and wrists. Radiographs demonstrate chondrocalcinosis within articular joints. Aspiration of joint fluid shows calcium pyrophosphate crystals in the joint space. The etiology of pseudogout still remains unclear; however, an excessive mechanical load on cervical motion may be a factor that triggers CDS. Here, we present a case of CDS in a patient who developed stiff neck pain after an ERCP procedure. We propose that CDS might be caused by the patient lying in the prone position in ERCP, as the patient is restricted of cervical rotation movements.

CASE REPORT

An 87-year-old woman with a past medical history of diabetes mellitus and hypertension presented to our hospital complaining of frequent vomiting and jaundice. She took anti-hypertensives and oral glycemic control medications. On arrival, she was alert and had stable vital signs, except for a low grade fever of 37.2 °C. Physical examination showed jaundice without epigastric tenderness, and Murphy's sign. Laboratory tests showed a marked elevation of inflammatory markers and hepatobiliary enzymes, which showed the following lab values: WBC 11100/ μ L, CRP 14.27 mg/dL, procalcitonin 1.56 μ g/L, AST 356 IU/L, ALT 174 IU/L, ALP 836 IU/L, γ -GTP 240IU/L, LDH 327 IU/L, and a total bilirubin of 5.7 mg/dL. Uric acid of 3.3 g/dL was within normal limits. Abdominal computed tomography (CT) revealed multiple stones in the bile duct (Figure 1). The patient was diagnosed with choledocholithiasis and acute cholangitis with moderate severity^[1], and emergency endoscopic stone removal with endoscopic sphincterotomy (EST) was performed on the day of admission (Figure 2), after which her symptoms resolved. We perform ERCP following sedation with hydroxyzine pamoate and pentazocine as premedication, and midazolam is administered intravenously. Additional midazolam is given in incremental doses during the procedure as needed.

On day 2 after the ERCP procedure, her temperature rose to 38 °C without any elevation of serum biliary enzymes. As we suspected retrograde cholangitis, we continued to administer antibiotics; however, her



Figure 1 Abdominal computed tomography scan on admission. There are multiple small computed tomography-high density stones on lower common bile duct (white arrow).

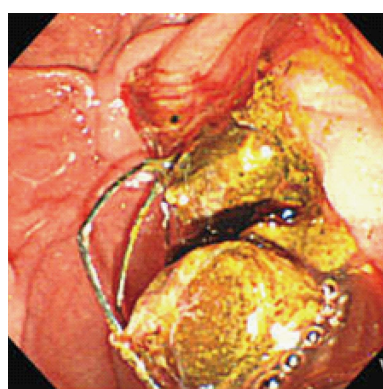


Figure 2 Emergent endoscopic retrograde cholangiopancreatography. With endoscopic sphincterotomy and mechanical lithotripsy, all stones could be removed successfully without any complications of either hemorrhage or gastrointestinal perforation.

temperature remained at 37–38 °C. On day 4, she complained of neck pain, right wrist pain, and left knee pain. The patient did not exhibit confusion or convulsion. In particular, neck pain was of moderate to severe intensity and was aggravated by the rotation of the head. Both neck stiffness and jolt accentuation of headaches was positive. Although serum biliary enzymes decreased after the ERCP procedure, laboratory tests showed a marked elevation of inflammatory markers. Based on her physical examination, the differential diagnosis was meningitis, retropharyngeal abscess, infective spondylitis, polymyalgia rheumatica, Lemierre's syndrome, and CDS. Two sets of blood cultures were obtained to rule out bacteremia, and the results were later shown to be negative. A head CT revealed no space-occupying lesions; however, there was a sharply defined periodontoid calcification on the patient's cervical CT (Figure 3A and B), which confirmed a diagnosis of CDS. In addition, radiographs of the wrist and knee showed crystal deposits in the joint spaces, which are consistent with a diagnosis of pseudogout (Figure 3C and D). She has never experienced nuchal pain or other joint pain before. Based on this diagnosis, treatment with the non-steroidal anti-inflammatory

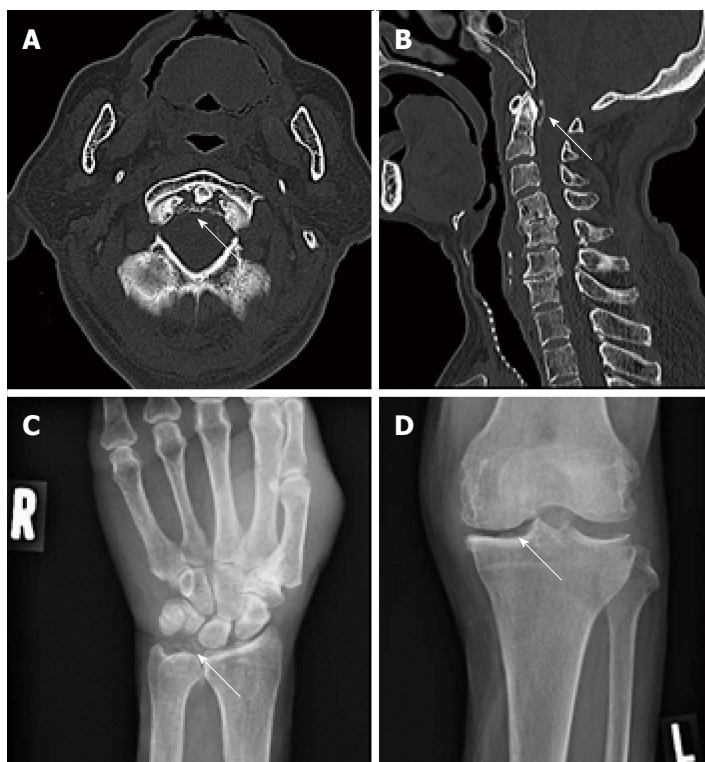


Figure 3 Cervical computed tomography and radiograph of the right wrist and left knee on day 4 after endoscopic retrograde cholangiopancreatography. A: A sharply defined calcification of periodontoid articular ligaments is noted (white arrow); B: The same as (A) on sagittal view; C: Crystal deposit in the right wrist joint space (white arrow); D: Crystal deposit in left knee joint space (white arrow).

drug loxoprofen (180 mg per day) was started, and her fever and neck, wrist, and knee pain dramatically improved. Inflammatory markers on laboratory tests showed a marked improvement after administration of loxoprofen. She was discharged on the 14th day after admission.

DISCUSSION

CDS is a clinico-radiographic entity characterized by an acute onset of neck pain, fever, nuchal rigidity, jolt accentuation of headaches, general signs of inflammation, and a periodontoid calcification on cervical CT images^[2]. In the present case, despite the removal of the bile duct stones, the patient presented with persisting fever, neck pain, a significant restriction of neck rotation and elevation of serum inflammatory markers. Crystal deposits on CT are mainly composed of calcium pyrophosphate dehydrate crystals. Because of its rarity and lack of awareness, CDS is sometimes misdiagnosed as meningitis, spondylitis, bone metastasis, or polymyalgia rheumatica^[3]. As CDS is recognized to be a subtype of pseudogout, plain radiographs of other joints, such as the knees and wrists, even though there are no specific symptoms, might be helpful in detecting calcification in the articular cartilage. Laboratory tests are helpful to diagnose CDS, as elevation of CRP and the erythrocyte sedimentation rate caused by an inflammatory reac-

tion is common.

The pathogenesis of CDS involves the phagocytosis of crystals by inflammatory cells, which initiates a sequence of inflammatory responses in articular joints^[4]. However, what triggers inflammation is still unclear. The development of CDS after the onset of myocardial or cerebral ischemia has been reported^[5], which indicates that severe illnesses may trigger an inflammatory response. No previous reports have indicated that ERCP may be a trigger for CDS. We hypothesize that there are two causative factors for the onset of CDS: systemic inflammation caused by cholangitis or head rotation with the patient in the prone position during ERCP. These factors could trigger CDS in elderly adults with periodontoid calcification, inducing a local inflammatory response in joint spaces.

We have seen three similar cases of CDS that developed after an ERCP procedure. The precise clinical manifestations of three CDS cases are showed on Table 1. All patients were febrile and over 80 years old with acute cholangitis. Interestingly, the onset of CDS after ERCP and total procedure time of ERCP differed in each case. We need to accumulate more cases whether these factors are associated with the onset of CDS.

According to our patients diagnosed with CDS, all of them have never experienced nuchal pain nor diagnosed with pseudogout. Whether long session

Table 1 Precise clinical manifestations of three crowned dens syndrome cases

No.	Age	Sex	Day	Fever	Site	Time(min)	WBC(μ L)	CRP(mg/dL)	Treatment
1	82	Male	day 16	39.2 °C	Neck	65	4800	0.10	NSAIDs
2	85	Female	day 1	38.6 °C	Neck, Ankle	30	11500	4.93	NSAIDs
3	83	Male	day 3	38.0 °C	Neck	22	7800	2.12	NSAIDs

Day: Onset of crowned dens syndrome after endoscopic retrograde cholangiopancreatography (ERCP) procedure; Site: Painful site of complaint; Time: Time requiring for ERCP procedure.

of ERCP with restricted head movement attributes to the onset of CDS is still unknown. In order to warn for endoscopist about the possibility of CDS, endoscopist should be cautious for the patient's position during ERCP if they have frequent episode of neck pain or have history of cervical spondylitis before.

In summary, CDS should be considered in the differential diagnosis when elderly patients are placed in the prone position with the head rotated during an ERCP and subsequently complain of neck pain and fever after.

COMMENTS

Case characteristics

A 87-year-old female presented with fever and neck pain, who was diagnosed with Crowned dens syndrome (CDS) after an endoscopic retrograde cholangiopancreatography (ERCP) that was detected with a careful physical examination and radiographic findings.

Clinical diagnosis

The neck computed tomography (CT) scan performed after the onset of fever and neck pain revealed a sharply defined periodontoid calcification, which was compatible with neck pseudogout, namely CDS.

Differential diagnosis

The authors performed complete physical examination (PE) when she complained of fever and neck pain. Based on PE, the differential diagnosis was meningitis, retropharyngeal abscess, infective spondylitis, polymyalgia rheumatica, Lemierre's syndrome and CDS.

Laboratory diagnosis

Inflammatory marker including white blood cells and C-reactive protein was gradually elevated, though hepato-biliary enzyme is decreasing after ERCP.

Imaging diagnosis

Head to neck CT was a key diagnostic tool to make a definitive diagnosis. Neck CT revealed a sharply periodontoid calcification.

Treatment

NSAID with Loxoprofen 180 mg per day was extremely effective to relieve her symptoms, namely fever and neck pain.

Term explanation

CDS is characterized by neck pain and fever, CT findings of periodontoid calcification, and a clinical entity of pseudogout which is largely affected by the large joints such as knees and wrists.

Experiences and lessons

Clinicians need to know that CDS is not rare disease entity, but is easily to be missed when not in clinician's mind. If elderly patients, who are under an endoscopic procedure with head rotating, are complaining of fever and neck pain, CDS is one of the differential diagnosis. Focusing on patient's physical examination on knees and wrists whether there are inflammatory findings is extremely important. If you suspect this disease, cervical CT scan or affected joint X-ray will help you to make a definitive diagnosis.

Peer-review

Arthrocentesis is a diagnostic intervention of pseudogout. The authors regard patient's clinical symptoms based on history, physical examination and radiographic findings as pseudogout. Another important differential diagnosis of joint pain, especially presenting with mono-arthritis, is a septic arthritis. If you judge patient's complaint as not-typical symptoms, arthrocentesis is needed to be performed in order to differentiate septic arthritis or pseudogout.

REFERENCES

- 1 **Miura F**, Takada T, Strasberg SM, Solomkin JS, Pitt HA, Gouma DJ, Garden OJ, Büchler MW, Yoshida M, Mayumi T, Okamoto K, Gomi H, Kusachi S, Kiriya S, Yokoe M, Kimura Y, Higuchi R, Yamashita Y, Windsor JA, Tsuyuguchi T, Gabata T, Itoi T, Hata J, Liao KH. TG13 flowchart for the management of acute cholangitis and cholecystitis. *J Hepatobiliary Pancreat Sci* 2013; **20**: 47-54 [PMID: 23307003 DOI: 10.1007/s00534-012-0563-1]
- 2 **Bouvet JP**, le Parc JM, Michalski B, Benlahrache C, Auquier L. Acute neck pain due to calcifications surrounding the odontoid process: the crowned dens syndrome. *Arthritis Rheum* 1985; **28**: 1417-1420 [PMID: 4084331 DOI: 10.1002/art.1780281215]
- 3 **Aouba A**, Vuillemin-Bodaghi V, Mutschler C, De Bandt M. Crowned dens syndrome misdiagnosed as polymyalgia rheumatica, giant cell arteritis, meningitis or spondylitis: an analysis of eight cases. *Rheumatology (Oxford)* 2004; **43**: 1508-1512 [PMID: 15316123 DOI: 10.1093/rheumatology/keh370]
- 4 **Constantin A**, Marin F, Bon E, Fedele M, Lagarrigue B, Bouteiller G. Calcification of the transverse ligament of the atlas in chondrocalcinosis: computed tomography study. *Ann Rheum Dis* 1996; **55**: 137-139 [PMID: 8712865 DOI: 10.1136/ard.55.2.137]
- 5 **Takakuni M**, Nakamura M, Suenaga T. Pseudogout as a complication of stroke. *Clin Neurol* 2008; **48**: 563-567

P-Reviewer: Lai KH, Kayaalp C, Tantau A, Yan SL **S-Editor:** Qi Y
L-Editor: A **E-Editor:** Wang CH





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 1007-9327



9 771007 932045