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

# World Journal of *Gastroenterology*

*World J Gastroenterol* 2021 November 14; 27(42): 7210-7401

Published by Baishideng Publishing Group Inc

World Journal of Gastroenterology

Contents

Weekly Volume 27 Number 42 November 14, 2021

## FRONTIER



## OPINION REVIEW



## REVIEW



## MINIREVIEWS



## ORIGINAL ARTICLE

### Basic Study

## ABOUT COVER

Editorial Board Member of *World Journal of Gastroenterology*, Fabio Grizzi, PhD, Head, Histology Core, Department of Immunology and Inflammation, IRCCS Humanitas Research Hospital, Via Manzoni 56, Rozzano 20089, Milan, Italy. fabio.grizzi@humanitasresearch.it

## AIMS AND SCOPE

The primary aim of *World Journal of Gastroenterology* (*WJG*, *World J Gastroenterol*) is to provide scholars and readers from various fields of gastroenterology and hepatology with a platform to publish high-quality basic and clinical research articles and communicate their research findings online. *WJG* mainly publishes articles reporting research results and findings obtained in the field of gastroenterology and hepatology and covering a wide range of topics including gastroenterology, hepatology, gastrointestinal endoscopy, gastrointestinal surgery, gastrointestinal oncology, and pediatric gastroenterology.

## INDEXING/ABSTRACTING

The *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, and Scopus. The 2021 edition of Journal Citation Report® cites the 2020 impact factor (IF) for *WJG* as 5.742; Journal Citation Indicator: 0.79; IF without journal self cites: 5.590; 5-year IF: 5.044; Ranking: 28 among 92 journals in gastroenterology and hepatology; and Quartile category: Q2. The *WJG*'s CiteScore for 2020 is 6.9 and Scopus CiteScore rank 2020: Gastroenterology is 19/136.

## RESPONSIBLE EDITORS FOR THIS ISSUE

**Production Editor:** *Ji-Hong Liu*; **Production Department Director:** *Yu-Jie Ma*; **Editorial Office Director:** *Ze-Mao Gong*.

**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**
Andrzej S Tarnawski, Subrata Ghosh

**EDITORIAL BOARD MEMBERS**
http://www.wjgnet.com/1007-9327/editorialboard.htm

**PUBLICATION DATE**
November 14, 2021



**INSTRUCTIONS TO AUTHORS**
https://www.wjgnet.com/bpg/gerinfo/204

**GUIDELINES FOR ETHICS DOCUMENTS**
https://www.wjgnet.com/bpg/GerInfo/287

**GUIDELINES FOR NON-NATIVE SPEAKERS OF ENGLISH**
https://www.wjgnet.com/bpg/gerinfo/240

**PUBLICATION ETHICS**
https://www.wjgnet.com/bpg/GerInfo/288

**PUBLICATION MISCONDUCT**
https://www.wjgnet.com/bpg/gerinfo/208

**ARTICLE PROCESSING CHARGE**
https://www.wjgnet.com/bpg/gerinfo/242

**STEPS FOR SUBMITTING MANUSCRIPTS**
https://www.wjgnet.com/bpg/GerInfo/239

**ONLINE SUBMISSION**
https://www.f6publishing.com

World Journal of Gastroenterology

Submit a Manuscript: https://www.f6publishing.com

*World J Gastroenterol* 2021 November 14; 27(42): 7210-7232

DOI: 10.3748/wjg.v27.i42.7210

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

FRONTIER

# Frontiers in antibiotic alternatives for *Clostridioides difficile* infection

Matthew Phanchana, Phurt Harnvoravongchai, Supapit Wongkuna, Tanaporn Phetruen, Wichuda Phothichaisri, Supakan Panturat, Methinee Pipatthana, Sitthivut Charoensutthivarakul, Surang Chankhamhaengdecha, Tavan Janvilisri

**ORCID number:** Matthew Phanchana 0000-0002-7736-7500; Phurt Harnvoravongchai 0000-0001-6730-5712; Supapit Wongkuna 0000-0003-3766-5391; Tanaporn Phetruen 0000-0003-0636-1803; Wichuda Phothichaisri 0000-0003-4056-9923; Supakan Panturat 0000-0003-1012-4499; Methinee Pipatthana 0000-0002-5296-7278; Sitthivut Charoensutthivarakul 0000-0002-4447-3438; Surang Chankhamhaengdecha 0000-0002-5414-4072; Tavan Janvilisri 0000-0002-6376-5575.

**Author contributions:** Phanchana M and Janvilisri T conceptualized and conceived the idea; all authors have been involved equally and have read and approved the final manuscript; Janvilisri T supervised the project.

**Supported by** Research Cluster (Multi-generation Researchers) Grant, Mahidol University, No. MRC-MGR 02/2563.

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



**Matthew Phanchana,** Department of Molecular Tropical Medicine and Genetics, Faculty of Tropical Medicine, Mahidol University, Bangkok 10400, Thailand

**Phurt Harnvoravongchai, Surang Chankhamhaengdecha,** Department of Biology, Faculty of Science, Mahidol University, Bangkok 10400, Thailand

**Supapit Wongkuna, Tanaporn Phetruen, Wichuda Phothichaisri, Supakan Panturat, Methinee Pipatthana, Tavan Janvilisri,** Department of Biochemistry, Faculty of Science, Mahidol University, Bangkok 10400, Thailand

**Sitthivut Charoensutthivarakul,** School of Bioinnovation and Bio-based Product Intelligence, Faculty of Science, Mahidol University, Bangkok 10400, Thailand

**Corresponding author:** Tavan Janvilisri, PhD, Professor, Department of Biochemistry, Faculty of Science, Mahidol University, 272 Rama VI Road, Thung Phayathai, Ratchathewi, Bangkok 10400, Thailand. tavan.jan@mahidol.ac.th

## Abstract

*Clostridioides difficile* (*C. difficile*) is a gram-positive, anaerobic spore-forming bacterium and a major cause of antibiotic-associated diarrhea. Humans are naturally resistant to *C. difficile* infection (CDI) owing to the protection provided by healthy gut microbiota. When the gut microbiota is disturbed, *C. difficile* can colonize, produce toxins, and manifest clinical symptoms, ranging from asymptomatic diarrhea and colitis to death. Despite the steady-if not rising-prevalence of CDI, it will certainly become more problematic in a world of antibiotic overuse and the post-antibiotic era. *C. difficile* is naturally resistant to most of the currently used antibiotics as it uses multiple resistance mechanisms. Therefore, current CDI treatment regimens are extremely limited to only a few antibiotics, which include vancomycin, fidaxomicin, and metronidazole. Therefore, one of the main challenges experienced by the scientific community is the development of alternative approaches to control and treat CDI. In this Frontier article, we collectively summarize recent advances in alternative treatment approaches for CDI. Over the past few years, several studies have reported on natural product-derived compounds, drug repurposing, high-throughput library screening, phage therapy, and fecal microbiota transplantation. We also include an update on vaccine development, pre- and probiotics for CDI, and toxin antidote approaches. These measures tackle CDI at every stage of disease pathology *via* multiple mechanisms. We also discuss the

**Specialty type:** Microbiology

**Country/Territory of origin:** Thailand

**Peer-review report's scientific quality classification**

Grade A (Excellent): A, A
Grade B (Very good): 0
Grade C (Good): 0
Grade D (Fair): 0
Grade E (Poor): 0

**Received:** March 21, 2021
**Peer-review started:** March 21, 2021
**First decision:** April 30, 2021
**Revised:** May 12, 2021
**Accepted:** October 25, 2021
**Article in press:** October 25, 2021
**Published online:** November 14, 2021

**P-Reviewer:** Ong LT, Sales-Campos H
**S-Editor:** Ma YJ
**L-Editor:** A
**P-Editor:** Ma YJ

gaps and concerns in these developments. The next epidemic of CDI is not a matter of if but a matter of when. Therefore, being well-equipped with a collection of alternative therapeutics is necessary and should be prioritized.

**Key Words:** Bacteriophage; Pharmaceutical; *Clostridioides difficile*; Alternative therapy; Drug resistance; Fecal microbiota



**Core Tip:** *Clostridioides difficile* is considered a threat to public health owing to increases in treatment failure over the past few years. Current antibiotic treatment options are highly limited. Therefore, alternative strategies are critical. Herein, we review recent advances in alternative therapeutics, including the development of new chemical entities, fecal microbiota transplantation, pre- and pro-biotic, antitoxin antibodies, use of bacteriophages, and vaccines. We also highlight the concerns, limitations, and directions for each of these developments.

**Citation:** Phanchana M, Harnvoravongchai P, Wongkuna S, Phetruen T, Phothichaisri W, Panturat S, Pipatthana M, Charoensutthivarakul S, Chankhamhaengdecha S, Janvilisri T. Frontiers in antibiotic alternatives for *Clostridioides difficile* infection. *World J Gastroenterol* 2021; 27(42): 7210-7232
**URL:** https://www.wjgnet.com/1007-9327/full/v27/i42/7210.htm
**DOI:** https://dx.doi.org/10.3748/wjg.v27.i42.7210

## INTRODUCTION

*Clostridioides difficile* (*C. difficile*) is a gram-positive, spore-forming, toxin-producing, rod-shaped anaerobic bacterium. *C. difficile* infection (CDI) is a leading cause of nosocomial infections in several countries. The source of *C. difficile* still remains debatable. Studies have proposed that *C. difficile* was part of the human gut commensal bacterial community, as the bacterium could often be isolated from neonates[1,2]. The pathogenicity of *C. difficile* was believed to be attributed to its toxin-production properties; however, recent developments have suggested that outgrowth and colonization are also a pivotal feature of pathogenicity. Under normal conditions, when the gut microbiota is in balance, in a stage called "eubiosis", *C. difficile* can neither multiply nor colonize the gut, thus preventing it from causing disease. On the other hand, when the composition of the gut microbiota is altered from its normal state, so-called "dysbiosis" occurs, which allows *C. difficile* to multiply and colonize[3,4]. Once colonized, *C. difficile* can produce up to 3 toxins, *i.e.*, toxin A (TcdA), toxin B (TcdB), and binary toxin (CDT). The first two are prominent virulent factors, whilst the latter is controversial[5-7]. The binary toxin is believed to enhance the toxicity and pathogenicity of the primary toxins; however, only a few reports have shown that PCR-negative, but CDT-positive, *TcdA* and *TcdB* can cause CDI[8,9]. Details of the pathogenesis of *C. difficile* can be found in reviews elsewhere[5,7,10,11].

CDI is one of the most prominent causes of healthcare-associated infections (HAI). In the United States and European countries, *C. difficile* has been ranked among the top 10 causes of HAI[12]. The challenges of CDI control and prevention are intrinsic drug resistance and environmental resistant spore of *C. difficile*. To date, CDI treatment with most antibiotics has generally resulted in failure. Use of certain antibiotics, such as clindamycin, cephalosporins, quinolones, and penicillins reportedly increased the risk of CDI[13]. Since these antibiotics are broad-spectrum and deplete other gut microbiota that inhibit *C. difficile* multiplication[13], the use of such antibiotics increases CDI risk. To the best of our knowledge, some classes of antibiotics do not increase the risk of CDI, including tetracyclines and aminoglycosides[14]. The antibiotics currently used to treat CDI are vancomycin and fidaxomicin, whereas metronidazole is the antibiotic of choice only when the first two are not available[15]. Along with treatment, prevention is a good control strategy for CDI in most settings. Multiple strategies are being implemented to reduce the rate of CDI, *e.g.*, case management, infrastructure, and antibiotic stewardship, in hospitals[16]. To make all

these control measures meaningful, monitoring of *C. difficile* situations is necessary to ensure that *C. difficile* does not develop resistance to current antibiotics, which would threaten the control and treatment of CDI. There has been an increase in the number of fatal cases of CDI in the past decade[17]. In most countries, a national surveillance system for CDI is still lacking, resulting in under-represented cases of CDI.

Current antibiotic options seem to have reduced efficacy. Recent CDI treatment guidelines have moved away from metronidazole because its efficacy has reduced from 95% pre-2000 to 75% since[15]. A similar reduction has been observed for vancomycin, from 98% to 85%[15]. The increases in recurrent episodes of CDI have reflected the failure of current antibiotics to sustain positive treatment outcomes[18]. Therefore, there is a critical need to develop alternative treatment approaches for CDI (Figure 1). In fact, several alternative approaches are under development, and some are more advanced than others. The development of new antibiotics is the most active research field. Fecal microbiota transplantation (FMT), including pre/probiotic approaches, has also gained extensive research interest in the past few years, as evidenced by several clinical trial registrations. Phage therapy, antitoxin antibody, and vaccines are trying to catch up with others in the league. Notably, there is one antitoxin antibody, bezlotoxumab, approved for preventing the recurrence of *C. difficile* by the United States Food and Drug Administration (FDA), in 2016.

We explored the developments that are currently in the pipeline for alternative therapies by examining data from the ClinicalTrials website. Several trials are investigating current antibiotics for CDI but in various aspects, most commonly in conjunction with FMT or pre/probiotic or antitoxin antibodies. Several molecular entities, such as nitazoxanide, are under investigation. Based on this insight, we extrapolated that the developing treatment options aim to treat/prevent recurrent CDI rather than the initial episode of CDI. Interestingly, currently no trials have been reported on bacteriophage therapy, despite this technology having been active since the early 1990s[19], and its therapeutic application having been demonstrated in other pathogenic bacteria[20]. This indicates that some complexities underline the technology.

## SMALL MOLECULES TAKE THE LEAD IN CDI TREATMENT DEVELOPMENT

In the realm of drug discovery and development, small molecules dominate the pharmacy shelf, irrespective of the sources of the small molecules, *e.g.*, natural product-derived/inspired or (semi-)synthetics. According to the United States FDA database, small molecules account for most approved drugs. Regarding the nature and pathogenesis of *C. difficile*, the drugs used to treat CDI have a specific set of requirements, although no target product profiles have been proposed. We believe that the community consensus for the expected properties of the CDI drugs is that they could effectively kill vegetative and spore stages with low systemic absorption (high colonic concentration)[21,22]. Currently in clinical trials, several candidate compounds are being investigated for the development of CDI drugs. Petrosillo *et al*[23] have published a comprehensive review on the development of small molecules for CDI drugs.

Cadazolid is a synthetic oxazolidinone, a derivative of linezolid, which was developed for CDI and also exhibited potency against other enterococci[24]. Cadazolid exhibits good clearance of *C. difficile* with sub-mM MIC and limited effects on other gut microorganisms[25]. Its mechanism of action is protein synthesis inhibition by binding to peptidyl transferase center (PTC). Although cadazolid shares similar mechanism to linezolid, cadazolid is active against linezolid-resistant *C. difficile*[24]. The results from phase 3 clinical trials of cadazolid demonstrated a comparable clinical cure to vancomycin, good safety profile and well tolerated. However, it did not show non-inferiority to vancomycin, which subsequently resulted in the discontinuation of the development by the developer.

Nitazoxanide is an FDA-approved drug with an indication for treatments of cryptosporidiosis and giardiasis. At the millimolar range, this drug inhibited multiple gram-negative and gram-positive anaerobic bacteria, *e.g.*, *Bacteroides* spp., *Prevotella* spp., *C. difficile*, *C. perfringens* and *Mycobacterium tuberculosis*[26]. The mechanism of action of nitazoxanide has been suggested to involve the inhibition of pyruvate ferredoxin oxidoreductase (PFOR) in anaerobic energy metabolism[27] and the disruption of membrane potential in aerobic bacteria[28]. In addition, studies have shown that nitazoxanide is effective against various RNA and DNA viruses including coronavirus

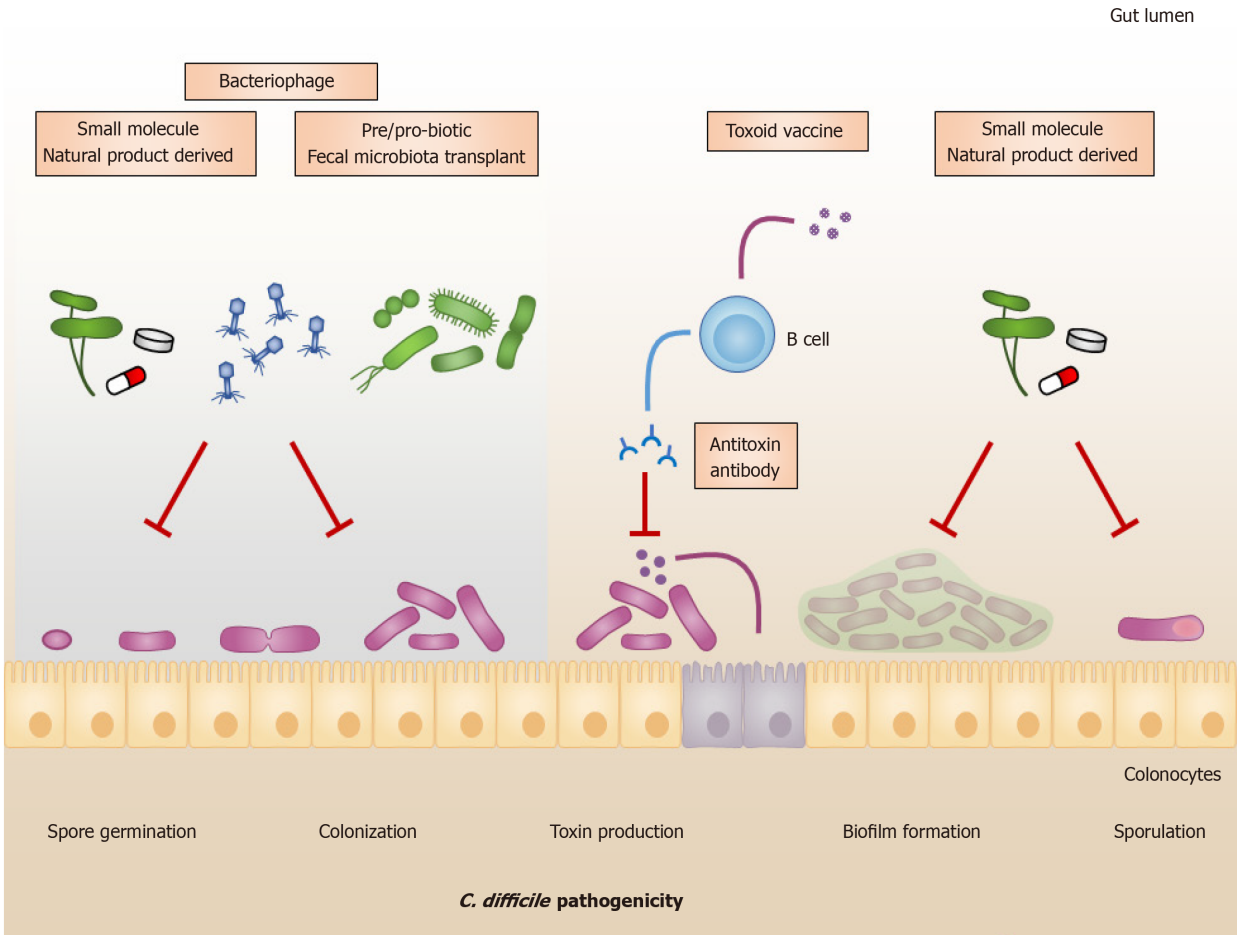


**Figure 1 Alternative approaches for *Clostridioides difficile* infection treatment under development.** Different approaches are aiming at the different pathological stages of *Clostridioides difficile* (*C. difficile*) infection. Small molecule and natural product derived have broad range activity from vegetative cell inhibition to biofilm and spore effects. Bacteriophage therapy affects mostly the vegetative stage of *C. difficile*. Fecal microbiota transplantation and pre- and pro-biotics aim to restore the balance of the gut microbiota mitigating the chance of *C. difficile* population and production of toxins. Vaccine and antitoxin antibody are targeting toxin neutralization.

[29] and SARS-CoV-2[30]. The result of an early phase-3 trial have suggested that the effectiveness of nitazoxanide is comparable to that of vancomycin, although noninferiority to vancomycin is inconclusive owing to small sample size and early termination[31]. Hence, the mechanism of action of nitazoxanide compared with current CDI treatments is anticipated from other ongoing trials because of the high expectations for nitazoxanide in the treatment of recurrent CDI[32].

Ridinilazole (SMT19969) is an antibiotic developed specifically for CDI. It exhibits good anticlostridial activity, with different magnitude, against multiple *Clostridium* spp. and also *C. difficile* with minimal effect to other gut microbiome[33,34] and bile acid profiles[35]. The mechanism of action of ridinilazole is believed to be unique from that of other drugs. It does not interfere with cell division by inhibiting cell wall synthesis; it does so by decreasing septum formation[36]. Ridinilazole has a low systemic absorption and, therefore, has a high colonic concentration, making it a good agent for CDI treatment[34]. The results obtained from a phase-2 clinical trial demonstrated sustained clinical response with ridinilazole treatment, noninferiority over vancomycin, and good tolerance[37]. These results altogether support further development of ridinilazole for clinical use. Phase-3 clinical trials are ongoing.

Advancements in technology and compound libraries has helped considerably in screening thousands of compounds, and has been made possible within a much shorter time. In the past few decades, several FDA-approved drugs were initially screened from large compound libraries through phenotypic screening[38]. A recent analysis showed that more than 16 million compounds can be purchased from various chemical suppliers[39]. This extends our opportunity to discover even more hits for drug discovery and development.

Apart from a rationally designed drug approach or large library screening, one recent popular drug development approach is drug repurposing or repositioning. The approach is regarded as a shortcut for a lengthy conventional drug development pipeline, as safety and pharmacokinetic data are readily available, which allows the compounds to proceed directly into clinical trials. Many works have demonstrated approved drugs can be repurposed for *C. difficile*[40,41]. The screening of both random compound libraries and approved drugs has become a very intriguing approach for drug development in the past years. Several studies conducted using this approach have been published[30,40,41]. However, if the route of administration is different from the approved indication, preclinical stages are necessary to evaluate the safety profile[42].

We examined the molecular similarity among the FDA-approved drugs from screening campaigns mentioned in this paper[40,43]. Molecular clustering of these drugs will help us predict a key biomolecular pathway or target the hits interact with or act upon. Molecular analysis was performed using the KNIME Analytics Platform, free and open-source software, aiming to solve large-data analysis problems[44]. KNIME offers an extensive toolset for data pre-processing and transformation as well as visualization and it can be equipped with extensions and nodes that are suitable for pharmaceutical research. We employed KNIME 4.1.2 equipped with various extensions, including RDKit KNIME integration, KNIME Distance Matrix Extension, and KNIME-CDK to create our workflow[45,46]. The workflow of the molecular analysis of the hits is shown in Scheme 1 of the supplement file.

The results shown in Table 1 and Supplementary material revealed that those hits can be categorized structurally into two large clusters and six smaller clusters based on molecular clustering analysis (> 3 hits per cluster). The largest group of clusters belongs to the β-lactam antibiotics (clusters 88–97, Supplementary material in SI) and the second largest is the tetracycline and its derivatives (clusters 98–99, Supplementary material in SI). Although these drugs are well-known antibiotics and are expected to exert some biological activity against *C. difficile*, the clinical use of these materials might not be of best interest since they are broad-spectrum antibiotics that could cause concern in gut microbiota dysbiosis and may drive an undesired side-effect of antibiotic resistance elsewhere in the body[47]. Other known antibiotics, such as aminoglycosides (cluster 123) and benzalkoniums cationic surfactant (cluster 61) which could be used topically as antiseptic were also identified.

Interestingly, metronidazole, one of the current treatments for *C. difficile*, and its derivatives (cluster 42) also show up as one cluster. Further exploration in this chemical moiety seems a way forward to avoid cross-resistance with current antibiotics as the drugs within this class exert their biological activity through the formation of nitroso radical. The mechanism that causes microbial DNA damage is not related to a certain enzyme and, therefore, makes it more challenging for the bacteria to develop resistance. Antifungal imidazoles were identified in another interesting cluster (cluster 21). These drugs act as an inhibitor of lanosterol 14α-demethylase, which catalyzes the formation of ergosterol, an important sterol found in eukaryotic cell membranes[48]. Although the mechanism of this class of drugs in *C. difficile* is not known, the result may prompt scientists to investigate the potential of these materials further, since these azoles are not currently in use as antibiotics, and it could avoid unwanted antibiotic cross-resistance with other current drugs[40,43]. Furthermore, most of the drugs identified from the FDA-approved panels resulted in drugs with anthelmintic (parasitic) indication[40]. It is possible that these parasites share a similar anaerobic/microaerophilic environment, and to some extent anaerobic metabolism, to that of *C. difficile*, thereby allowing those drugs to be active against *C. difficile*. It would be very interesting to observe whether this hypothesis remains valid when more screenings are performed. Although several drugs and lead compounds have been identified in the literature, only a few have been put forward into the development pipeline. This is largely due to the lack of academic and industrial partnerships. We have seen what the scientific community has achieved, in an unprecedented time scale, with the development of COVID-19 vaccines with the leading role of big pharmaceutical companies and their academic partners. This emphasizes the importance of academic-industrial partnerships in drug and vaccine developments, as this field of research is resource-intensive and requires considerable funding and workforce. Nevertheless, non-profit organizations can play an important role in drug discovery and development as well evidenced by the approval of anti-tubercular pretomanid, which was led by the TB Alliance[49].

**Table 1 Representatives of anti-*Clostridioides difficile* chemical clusters from reported literature**

| Cluster No. | Compound class |
|---|---|
| 21 | Antifungal imidazoles |
| 42 | Metronidazole and its derivatives |
| 61 | Benzalkonium cationic surfactants |
| 88–97 | β-lactams |
| 98–99 | Tetracycline and its derivatives |
| 123 | Aminoglycosides |

## NATURAL PRODUCTS SERVE AS A GOOD SOURCE OF ANTI-CLOSTRIDIAL ACTIVITIES

Humans have been using natural products as traditional medicine for treating several illnesses for centuries. It has been estimated that approximately 500000 plant species exist on the planet, but only 1% of them have been explored for bioactive compounds [50]. A detailed analysis of FDA-approved drugs from 1931 to 2013 revealed that natural products and their derivatives represented over 30% of new drugs[51]. Due to the increasing prevalence of antibiotic resistance in several pathogenic bacteria, the prescribed antibiotics may no longer be effective. Therefore, exploration of plant-derived compounds could provide us a tremendous opportunity to discover novel bioactive agents.

Several plant extracts and plant-derived compounds possess antibacterial activity against *C. difficile* and their action has been investigated previously. Roshan *et al*[52] reported the anti-*C. difficile* activities of natural products that are commercially available as both processed and unprocessed products. Some processed products such as aloe vera gel, peppermint oil, artichoke capsules, and garlic tablets demonstrated antimicrobial activity against *C. difficile* with MICs of 16% (v/v), 8% (v/v), 75–150 mg/mL, and 37.5–75 mg/mL, respectively[52]. Regarding the unprocessed products, allicin (derived from garlic) and cinnamon powder demonstrated antimicrobial activity against *C. difficile* with MICs of 2.3–4.7 and 75 mg/mL, respectively. Zingerone (derived from ginger) and menthol (derived from peppermint) inhibited *C. difficile* at the same inhibition concentration of 9.4 mg/mL, whereas trans-cinnamaldehyde, an active constituent found in cinnamon bark, exhibited strong inhibitory activity with an MIC of 0.2 mg/mL[52]. δ-3-Carene, a monoterpene derived from the root of *Asarum heterotropoides*, exhibited anticlostridial activity with an MIC of 0.7 mg/mL with a less potential of suppressing beneficial intestinal bacteria[53]. Moreover, an essential oil extract that contains 16.5% of δ-3-carene exhibited antimicrobial activity against *C. difficile* at a concentration of 0.25% (v/v)[54]. Xanthohumol, derived from *Humulus lupulus* L., exhibited anti-*C. difficile* activity with MICs of 0.032–0.107 mg/mL against 28 different *C. difficile* isolates[55]. Crude methanol extract of the bark of *Mammea africana* demonstrated anti-*C. difficile* activity with an MIC of 2 μg/mL. The identified active flavonic compound in *M. africana*, mammeisin or mammea A/AA, exhibited strong inhibitory activity with an MIC of 0.25 μg/mL[56]. Curcumin (phenol), an active agent derived from *Curcuma longa*, is reportedly active against 27 *C. difficile* strains, with MICs ranging between 16 and 32 μg/mL. The inhibition was specific to *C. difficile* as it did not affect beneficial gut microbiota[57]. Curcumin was also able to reduce sporulation and toxin production in *C. difficile*[57].

Several studies have reported that the anticlostridial activity of crude plant extracts contain unidentified active compounds. For instance, pomegranate extract was shown to exhibit specific inhibitory activity against 23 tested isolates of *C. difficile* with MICs of 12.5–25 μg/mL, but no inhibitory effect was observed on the tested normal intestinal bacteria (MIC > 400 μg/mL)[58]. Investigation of a commercial animal supplement, BIOCITRO, a citrus fruit extract, revealed an inhibitory effect on *C. difficile* with MICs of 16–32 μg/mL with a possible mode of action of disrupting polysaccharides and carbohydrates of the cell wall[59]. In addition, methanolic extract of the leaf and rhizomes of *Aristolochia paucinervis* Pomel demonstrated an inhibitory effect on *C. difficile*, with concentrations ranging from 8 to 64 μg/mL[60].

Several compounds are known to kill *C. difficile* through changes in cell permeability. The ability of membrane disruption was suggested for zingerone, menthol, and trans-cinnamaldehyde[61]. Asiatic acid, an active triterpenoid derived

from *Centella asiatica*, exhibited significant antimicrobial activity against *C. difficile* strains isolated from different sources by disrupting the cytoplasmic membrane with MICs of 10–20 μg/mL[62]. Lauric acid, an active component found in virgin coconut oil, exhibited anticlostridial activity at a 250-μmol/L concentration through the membrane disruption mechanism[63]. Cannabidiol, an active ingredient derived from cannabis, could inhibit *C. difficile* at concentrations of 2–4 μg/mL, possibly by disrupting the bacterial membrane[64]. Another study of cannabidiol in caco-2 cells infected with *C. difficile* reported its potential to inhibit toxin A-induced cytotoxicity [65]. Interestingly, data analysis of hospitalization from the Healthcare Cost and Utilization Project in 2014 suggested that patients associated with cannabis usage had potentially lower risk for CDI by 28%[66].

The dormant spores of *C. difficile* contribute to transmission and link with the pathogenesis of CDI. *C. difficile* spores can persist in harsh conditions. They can then translocate to the intestinal tract, germinate in response to specific bile salts, and initiate infection. Most antibiotics are inactive against *C. difficile* spores because of the spores' intrinsic durability. In addition to vegetative cell activity, natural products are active against several spore stages of *C. difficile*. Peppermint oil and trans-cinnamaldehyde have been shown to exhibit sporicidal activity, which reduces the number of spores by up to 200 times when spores were exposed for 7 d[67]. Several compounds such as allicin and carvacrol, essential oils found in oregano, and fresh onion bulb extracts could inhibit spore outgrowth[67,68]. As *C. difficile* spores play an essential role in CDI, the inhibition of sporulation is an attractive strategy to reduce infection. A study conducted by Roshan *et al*[67] showed that the subinhibitory concentration of coconut oil, fresh onion bulb, and fresh ginger can reduce the number of spores production by 90%. Another study conducted on Manuka honey or Leptospermum honey also reported the inhibition of sporulation in *C. difficile*[69]. Baicalin, a flavonoid derivative present in the root of *Scutellaria baicalensis*, could inhibit both sporulation and outgrowth of *C. difficile* at a concentration of 1.6 mmol/L. It also reduced toxin production by downregulating *tcdA* and *tcdB* gene expression[70]. Toxin A and toxin B produced by *C. difficile* are cytotoxic and cause colitis. Leptospermum honey, fresh onion bulb, and trans-cinnamaldehyde were able to reduce the cytotoxicity of *C. difficile* toxins in Vero cells by 70% and toxin production by 40%[71].

In an *in vivo* mouse model, it was observed that berberine, a compound derived from the genus *Berberis*, has the potential to prevent recurrent CDI and restore gut community, either alone or in combination with vancomycin[72]. It is noteworthy that successful therapy by endoscopic lavage with Manuka honey was reported in the patient with vancomycin treatment failure[73].

## ANTITOXIN ANTIBODIES WITH ANTIBIOTICS IMPROVE TREATMENT SUSTAINABILITY

Toxin production has long been associated with CDI pathogenicity. Classical antibiotic treatments effectively eliminate the pathogen, although they inevitably affect the normal gut microbiota to some extent. Antitoxin antibody treatment aims to neutralize the toxicity of the toxin, rather than interfere directly with the bacterium. Therefore, it can reduce clinical severity, in conjunction with antibiotic treatment, and reduce recurrent CDI. To date, the only FDA-approved antitoxin antibody for CDI treatment is bezlotoxumab, a human monoclonal antibody against toxin B. A detailed study demonstrated that bezlotoxumab binds to carbohydrate-binding pockets on toxin B, which directly prevents the interaction between the toxin and host cells[74]. This hypothesis was supported by a mutant antibody that does not bind to Fc receptors of host immune cells, which provides similar toxin neutralization and protection effects as wild-type antibody[75]. Its toxin A counterpart, actoxumab, did not demonstrate improvement in clinical efficacy, and therefore it was discontinued during the phase 3 trial[76]. These results support the hypothesis that TcdB is a more prominent virulent factor than other toxins and that the interplay between these toxins is a complex process.

IM-01 is an experimental polyclonal antibody for inflammatory bowel disease, including Crohn's disease, and CDI. IM-01 is produced using a chicken egg technique by immunizing hens with toxin A, toxin B, and *C. difficile* spores. Currently, detailed information on this intervention is lacking, but its phase 2 clinical trial is being recruited. The results from patent filing showed good antibody production, toxin neutralization, reduction of spore burden, and inhibition of vegetative cell growth[77]. These results indicate that IM-01 is a promising development for CDI treatment;

however, it is too early to state whether it would work effectively independently or in combination with antibiotics.

Colostrum is a fluid produced by the mammary glands immediately after the birth of a newborn. It has been demonstrated that cows persistently injected with antigens will produce antibodies in the colostrum, which are collectively referred to as hyperimmune bovine colostrum (HBC). A preclinical piglet model study showed that HBC can reduce the severity of CDI[78]. A limitation of using colostrum is the scale of production, which is limited to a few hours after birth. Therefore, some studies have examined the use of milk or whey protein isolate (WPI) instead of colostrum for CDI treatment. WPI exhibited superior and sustained treatment outcome compared with vancomycin in a hamster model[79]. Considering its clinical use, we found only one terminated phase 2 trial, which was completed, but there were no results, of the whey protein concentrate MucoMilk.

Altogether, antitoxin antibodies in any form appear to be a significant candidate for alternative treatment of CDI, since preclinical studies have demonstrated good inhibition of vegetative cells and spores and neutralization effect of toxins in initial and recurrent episodes of CDI. Nonetheless, it will take some time to observe this group of treatments for clinical use, as production is more complicated and potentially expensive.

## FECAL MICROBIOTA TRANSPLANT IS A VERY ATTRACTIVE APPROACH FOR CDI

Although antibiotic administration is the first-line treatment for CDI, severe outbreaks and recurrent episodes remain excessive due to a significant increase in resistant strains[80]. Treatment options for CDI, especially recurrent CDI, become limited, due to which the development of alternative therapeutics is highly required. In particular, CDI develops when the indigenous microbiota is dysbiotic, typically *via* exposure to antibiotics, thereby allowing *C. difficile* to acquire nutrient niches in the gut and cause disease[13,81]. Based on this concept, restoration of the healthy microbial community, eubiosis, can help eliminate and prevent this growing problem. To date, researchers have expressed interest in biotherapeutic strategies that confer curative effects on recurrences. A burgeoning alternative treatment approach for recurrent CDI is FMT, which refers to the administration of feces collected from a healthy individual to the intestinal tract of a patient[82,83]. In general, FMT is applied to a patient with refractory CDI who had failed standard antibiotic treatments in an initial episode of CDI[84]. This approach emerged due to its unique feature, restoring the balance of the gut microbiota, which is unlikely to be achieved by other approaches. FMT provides multiple mechanisms of colonization resistance, such as competition for nutrients, production of antimicrobial peptides, and production of short-chain fatty acids (SCFAs) to inhibit vegetative growth and spore germination of *C. difficile*[85,86].

The first clinical trial of FMT, whose efficacy was significantly greater than the use of antibiotics for recurrent CDI treatment, was performed by van Nood *et al*[87] and published in 2013. Among the 16 patients, 13 with FMT recovered from CDI after the first infusion and the symptoms of 2 patients resolved after a second infusion, whereas only 3 of 13 patients resolved with vancomycin treatment alone. Other clinical studies have demonstrated that FMT provides better benefits than standard antibiotics for treating recurrent CDI by replenishing the balance of the gut microbiota. Currently, clinical studies are ongoing with the recruitment of patients with relapsing CDI to evaluate the safety and efficacy of FMT (Table 2). The upper and lower route of administration has been used in delivering the transplant. Several methods, including nasoduodenal, enema, upper endoscopy, colonoscopy, and oral capsules, are commonly used for administering FMT, with diverse outcomes[88]. A study using colonoscopy demonstrated the resolution of CDI in 18 of 20 patients treated with FMT compared with 5 of 19 patients treated with vancomycin[89]. Conversely, FMT administered by enema was found to have a low success rate in treating acute episodes of recurrent CDI[90]. To simplify administration, oral capsules of fecal filtrates have been developed and widely used. Patients with relapsing CDI had a decent response to FMT administered by oral capsules, wherein the resolution rate of recurrent CDI was similar to that observed with colonoscopy[91]. These findings demonstrated no definite preference of the administration route for FMT.

Another factor questioned in FMT is the preparation of fecal material. Initially, fresh stools from donors were used for infusion. As the process of collecting fresh stools is difficult, frozen and lyophilized stools were then considered for transplant delivery.

**Table 2 Recent clinical trials examining potential fecal microbiota transplantation for treatment of *Clostridioides difficile* infection**

| Current phase | Title | ClinicalTrials.gov Identifier | First posted |
|---|---|---|---|
| NA | Fecal microbiota transplantation for *C. difficile* infection | NCT01905709 | July 23, 2013 |
| | Immune response to FMT for *C. difficile* | NCT02797288 | June 13, 2016 |
| | Outcomes and data collection for fecal microbiota transplantation for the treatment of recurrent *C. difficile* | NCT03562741 | June 19, 2018 |
| | Fecal microbiota transplantation (FMT) for *C. difficile* (CEFTA) | NCT03712722 | October 1, 2018 |
| | Rescue fecal microbiota transplantation for national refractory intestinal infection | NCT03895593 | March 29, 2019 |
| | Safety and efficacy of fecal microbiota transplantation | NCT04014413 | July 10, 2019 |
| 1 | Fecal transplant for pediatric patients who have recurrent *C. difficile* infection (FMT) | NCT02134392 | May 9, 2014 |
| 2 | Stool transplants to treat refractory *C. difficile* | NCT02127398 | April 30, 2014 |
| | FMT versus antimicrobials for initial treatment of recurrent CDI | NCT02255305 | October 2, 2014 |
| | Fecal microbiota therapy for recurrent *C. difficile* infection | NCT02686645 | February 19, 2016 |
| | Phase II trial of fecal microbiota transplant (FMT) for VRE and CRE patients | NCT03643887 | August 23, 2018 |
| | Fecal microbiota transplantation (FMT) plus fidaxomicin for severe of fulminant *C. difficile* | NCT03760484 | November 30, 2018 |
| | Multicentre blinded comparison of lyophilized sterile fecal filtrate to lyophilized fecal microbiota transplant in recurrent *C. difficile* infection | NCT03806803 | January 16, 2019 |
| | FMT and bezltoxumab compared to FMT and placebo for patients with IBD and CDI (ICON-2) | NCT03829475 | February 4, 2019 |
| | PMT for severe-CDI | NCT03970200 | May 31, 2019 |
| | Penn microbiome therapy (PMT) for recurrent *C. difficile* infection | NCT03973697 | June 4, 2019 |
| 3 | Fecal transplantation for primary *C. difficile* infection (COLONIZE) | NCT03796650 | January 8, 2019 |
| | Microbiota restoration therapy for recurrent *C. difficile* infection (PUNCH CD3-OLS) (CD3-OLS) | NCT03931941 | April 30, 2019 |
| | Fecal microbiota transplantation for primary *C. difficile* diarrhea | NCT02801656 | June 16, 2016 |

FMT: Fecal microbiota transplantation; *C. difficile*: *Clostridioides difficile*; PMT: Penn microbiome therapy.

Several clinical trials claimed that frozen stool was comparable to fresh stool with no loss of effectiveness[92,93]. In contrast, lyophilization reduced the efficacy of the transplant material compared with fresh and frozen materials[94]. In particular, frozen fecal material is becoming available for commercial purchase. In addition to its use for treating multiple episodes of recurrent CDI, FMT has been proposed as a promising treatment approach for severe and complicated CDI. Evidence obtained from clinical trials has shown that FMT improved survival in severe cases, including immunocompromised patients[95,96].

Although FMT is a favorable therapeutic option for recurrent CDI, a major concern is that there is no universal or industrial standard such as a defined bacterial formula; moreover, there is no information on the mechanism associated with FMT-screening methods for adverse transmission events and the period of treatment[97,98]. Such limitations of FMT led to the discovery of beneficial microorganisms in fecal materials that activate colonization resistance and the determination of their roles in inhibiting *C. difficile*. Correspondingly, the refined stool-derived microbial suspension RBX2660 has been successfully used to treat relapsing CDI, and is currently under a phase 2b study[99]; furthermore, SER-109 and SER-262, defined microbial preparations containing spore-forming bacteria purified from human feces and formulated as a capsule, are currently being investigated in a phase 3 and 1b trial[100,101]. These data conclude that FMT continues to be a recommended therapy for recurrent CDI, and in the meantime, a number of studies continue identifying essential bacteria species from the feces of healthy donors to formulate standard microbial preparations approved by the FDA.

Another point of concern for FMT is the unintentional transfer of drug-resistant bacteria, which could possibly lead to complicated infection and death. There have been multiple bacteremia incidents in patients who received FMT[102], some of which were severe and life-threatening. This indicates the need for a standardized protocol for stool preparation, to minimize potential drug-resistant bacterial infection from FMT, including, but not limited to, extended-spectrum beta-lactamase (EBSL)-producing *E. coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *etc.*[103]. Recently, a caution has been issued regarding the transmission of SARS-CoV-2 in FMT[104].

## PROBIOTICS AND PROBIOTIC PROMOTE GUT BALANCING

Probiotics are live microorganisms that confer health benefits on the host when administered in adequate amounts[105,106]. They can improve host immunity by producing beneficial metabolites, such as SCFAs, as well as preventing enteric infections *via* colonization resistance mechanisms, such as competition for nutrients, inhibition of bile acid conversion, and production of antimicrobial peptides[107,108]. Numerous probiotic strains and probiotic mixtures have been evaluated to combat CDI in *in vitro* studies; however, there are limitations due to the lack of evidence from human clinical trials.

*Lactobacillus rhamnosus* GG and *Saccharomyces boulardii*, the most common probiotics, have provided promising applications against CDI and have been widely investigated in clinical trials. The efficacy of *S. boulardii* in CDI management was first examined by McFarland *et al*[109], who reported a significant reduction in recurrence rates after the administration of *S. boulardii* twice a day for 4 wk during and after antibiotic treatment. Their study demonstrated a significant reduction in the CDI recurrence rate in the *S. boulardii* treatment group compared with the placebo group. Moreover, patients with relapsing CDI had a statistically significant response to *S. boulardii* compared with placebo. The probiotic effect of *L. rhamnosus* GG in a clinical study was first verified by Gorbach *et al*[110], in 1987. They successfully used the organism to treat 5 patients with multiple recurrent CDI episodes. In addition to its beneficial effects in treating refractory CDI, *L. rhamnosus* GG displayed a protective potential in healthy individuals[111]. Further meta-analytical studies have been performed to confirm the usefulness of *S. boulardii* and *L. rhamnosus* GG in the prevention of CDI [112,113]. In addition to single-probiotic strains, probiotic mixtures have been developed for quite some time. The probiotic mixture BioK+ containing *L. acidophilus* CL1285, *L. rhamnosus* CL2, and *L. casei* LBC80R was able successfully to reduce the CDI rate from 18.0 to 2.3 cases per 10000 patients. Furthermore, new mixtures have been developed and are in the process of clinical trials[114]. The probiotic mixture of *L. casei* DN-114 001, *S. thermophilus*, and *L. bulgaricus* was randomly administered to hospital inpatients twice-daily during, and for 1 wk after, antibiotic treatment[115]. This probiotic mixture showed a positive result, wherein no individual in the probiotic group developed CDI compared with 9 of 53 individuals who contracted CDI in the placebo group. Another study evaluated the efficacy of the probiotic mixture of *L. acidophilus* and *L. casei*[116]. The probiotic doses were varied and administered within 1.5 d of initial antibiotic therapy and then continued for 5 d after the final antibiotic dose. Both higher dose and lower dose probiotic groups showed significantly reduced CDI incidence rates, at 1.2% and 9.4%, respectively, compared with 23.8% in the placebo group. The most recent clinical trial of a multi-strain probiotic consisting of *L. acidophilus* NCFM, ATCC700396, *L. paracasei* Lcp-37, ATCC SD5275, *Bifidobacterium lactis* Bi-07, ATCC SC5220, and *B. lactis* B1-04, ATCC SD5219 has been conducted[117]. A phase 2 study evaluated the potential benefits of this probiotic mixture by administration daily for 4 wk. The results revealed a shorter duration of diarrhea in patients with an initial episode of mild-to-moderate CDI compared with the placebo group. Based on the available evidence, probiotics are strongly advocated as an alternative for preventing and treating CDI. However, the high heterogeneity in existing studies indicates that the beneficial effects of probiotics are rather subjective[118]. Therefore, discovering novel probiotics and understanding their functions and interactions have been continuing to improve the probiotic effects in a clinical setting.

It is known that diet has a key influence on the composition and functions of the gut microbiota[119]. Several types of fiber, particularly, galacto-oligosaccharides (GOS) and fructo-oligosaccharides (FOS), have been found to increase the abundance of common probiotic bacteria, such as *Bifidobacterium* and *Lactobacillus* species[120]. Hence, plant-based foods containing dietary fiber are generally accepted as favorable for gut health. Certain fiber types, including FOS, GOS, and inulin, are considered to

be prebiotics, which are defined as substrates that are selectively used by host microorganisms conferring a health benefit[121]. The combination of probiotics and prebiotics has been proposed as an alternative, known as synbiotics, to prevent and treat refractory CDI. A recent study investigated the effects of a synbiotic, *L. plantarum* DSM 21379 and xylitol, on the germination of *C. difficile* spores[122]. *In vitro* experiments demonstrated that the synbiotic completely inhibited the germination of *C. difficile* spores. Moreover, the administration of this synbiotic for 5–6 d before ampicillin and *C. difficile* challenge in mice reduced the CDI incidence from 44% to 22% mortality. Another *in vitro* study examined the inhibitory capability of four different *Bifidobacterium* sp. strains combined with various prebiotics against *C. difficile* growth. Using oligo-fructosaccharides as a carbon source, it was observed that *B. longum* and *B. breve* rescued the survival of a cell line exposed to *C. difficile* cell-free supernatant. These findings indicate that a probiotic strain requires a specific prebiotic substrate. To produce more effective synbiotics to control CDI, it is necessary to determine the optimal prebiotic for each probiotic.

## BACTERIOPHAGE AND ITS PRODUCTS SPECIFICALLY ELIMINATE *C. DIFFICILE*

As bacteria-infecting viruses, bacteriophages or phages have received attention for potential use as an alternative treatment for several bacterial infections. The high specificity to their bacterial hosts and the self-replicating mechanism of phages are claimed to have advantages over other approaches. Although *C. difficile* phages have been discovered and investigated since 1983[123], the use of phages for human infection is restricted due to some limitations. The key bacteriophage studies of *C. difficile* are summarized in Table 3.

*C. difficile* phages were first described for bacterial typing purposes[123]. Morphological analysis showed that most of them are either *Myoviridae* or *Siphoviridae*, belonging to the order *Caudovirales*. They possess dsDNA as their genetic materials [124,125]. Currently, all 26 complete genomes of *C. difficile* phages are characterized as temperate; they can alternate their life cycles between lytic and lysogenic cycles[126, 127]. This is a major constraint, as most therapeutic phage applications require a virulent phage, *i.e.*, a phage with a strictly lytic life cycle. Therefore, current research has been focusing on the use of temperate phage and phage-derived proteins to combat CDI[128-131]. At the very beginning, phage therapy was performed as a single-phage treatment to ensure its capacity. It has been demonstrated that phiCD27 significantly reduced the growth of *C. difficile* cells, as well as the production of toxin A and toxin B in an *in vitro* batch fermentation and artificial gut model. Furthermore, phiCD27 treatment did not affect commensal microbiota in both models, suggesting high specificity on the bacterial target[132]. However, using only one phage has some limitations due to narrow host range and lysogenic capacity. Therefore, a combination of different phages or phage cocktails has become more intriguing[133-135].

A phage cocktail was successfully developed both *in vitro* and *in vivo*, as observed in the study conducted by Nale *et al*[134]. Optimized cocktails of phiCDHM1 to phiCDHM6 and phiCDHS1 were tested against *C. difficile* ribotypes 076, 014/020, and 027 strains. The best combination included phiCDHM 1, 2, 4, and 6, which could completely kill *C. difficile* without regrowth. The CDI hamster model showed a significantly lower number of spores in the cecum and colon with the combination of phiCDHM 1, 2, 5, and 6. The same phage mixture was used in the wax moth larva *Galleria mellonella*. The efficiency of using phage combined with antibiotics, including vancomycin and clindamycin, was evaluated. The results suggested that phage could be used as a supplement to antibiotic treatment and prevent the onset of CDI. Prophylaxis was the most effective therapy with 100% protection, and efficiency was reduced when used as a remedial treatment. Moreover, the authors found that the phage could penetrate and prevent biofilm formation in the *C. difficile* ribotype 014/020[136]. Phage treatment in an artificial gut model was further investigated using these sets of phage combinations. A six-log reduction in *C. difficile* growth was observed after 5 h in the prophylaxis group, and the vegetative cells were completely removed within 24 h[136]. Furthermore, metagenomic analysis was conducted using fecal samples of volunteers to observe the impact of phage therapy on the total gut microbiome. Another *in vitro* study conducted on the human colonic cell line HT-29, which is the CDI site, reported that phiCDHS1 preferentially adsorbed onto HT-29 cells, thereby promoting the interaction between the phage and bacterial cells[137]. It has also been shown that either bacterial lysis by phage or the phage itself was

**Table 3 Key experiments in bacteriophage for *Clostridioides difficile* infection treatment**

| Phage | Experiment | Finding | Ref. |
|---|---|---|---|
| phiCD140 | A single dose of phage treatment for *C. difficile* infection in hamsters | Surviving of phage treated hamster | [135] |
| phiCD27 | Phage treatment of CDI in an *in vitro* batch fermentation and human colon model | (1) Reduction of both vegetative cell and toxin A and toxin B productions from *C. difficile*; and (2) No impact on others gut microbes | [135] |
| phiCDHM1 to phiCDHM6, and phiCDHS1 | (1) Investigation for an effective phage combination; and (2) Phage delivered orally in hamster model every 8 h after *C. difficile* challenge | (1) Discovery of phage-resistant colonies after a single phage treatment; and (2) Reduction of *C. difficile* amount and colonization using phage combination *in vivo* | [124] |
| phiCDHM1, 2, 5, and 6 | (1) Phage treatment before and after the biofilm formation; (2) First time using *Galleria mellonella* (wax moth) model for *C. difficile* phage; and (3) Using phage in combination with antibiotics (vancomycin) | (1) Reduction and prevention of the biofilm establishment *in vitro*; and (2) Disease prevention in the prophylaxis group and increasing the wax moth survival rates | [130] |
| phiCDHM1, 2, 5, and 6 | (1) Optimized temperate phage cocktail to treat in batch fermentation model; and (2) First metagenomic analysis of phage treatment on gut microbiome | (1) *C. difficile* elimination after 24 h in prophylactic condition while maintain other microbiota components; and (2) No significant impact on other bacterial groups in human gut | [127] |
| phiCDHS1 | Measurement of planktonic and adhered *C. difficile* cells and free phage to human colon tumorigenic cell line HT-29 | (1) Reduction of planktonic and adhered *C. difficile*; and (2) No cytotoxicity to human cells | [129] |
| phiCD24-2 | (1) Using engineered phage delivered Type 1-B CRISPR system as antimicrobial agent *in vitro* and *in vivo*; and (2) Mutation of phage lysogenic gene by the cI repressor and integrase gene deletion | (1) *C. difficile* eradication effectively in engineered phage comparing with wild-type phage; and (2) Detection of lysogen due to potentially functional complements from *C. difficile* prophage | [125] |

*C. difficile*: *Clostridioides difficile*.

nontoxic to the colonic cells.

Recently, phiCD24-2 has been engineered to contain a genome targeting CRISPR-Cas3, which is commonly found in the genome of *C. difficile*[135]. The major characteristics of the engineered phage or CRISPR-enhanced phage (crPhage) have been investigated. There was no difference in phage morphology or host range compared with the wild-type phage (wtPhage). The efficiency of phage treatment for *C. difficile* was determined both *in vitro* and *in vivo*. crPhage demonstrated a higher efficiency to reduce the growth of vegetative cells than wtPhage in both models. However, the bacterial number rebounded by 24 h, suggesting lysogination into the host genome rather than bacterial lysis. The mouse model exhibited a significant difference in the number of *C. difficile* cells recovered from mouse feces between wtPhage and crPhage treatments, indicating the superiority of crPhage treatment *in vivo*. The CRISPR approach enhances phage efficiency during the lytic cycle as bacterial lysis can occur *via* two independent mechanisms, including genome damage and phage lytic activity *via* endolysin and holin expression. The challenge of engineered phages remains due to lysogenic conversion. Therefore, the removal of cI repressor and integrase genes was performed to generate lysogenic phage mutants[135]. Although lysogen from *in vitro* culture was not detected, it was detectable in mouse feces. These findings suggested the functional complement in the *C. difficile* genome for those removed genes.

An alternative to using temperate phages for therapeutic purposes is to utilize their products. Endolysin, a peptidoglycan hydrolase enzyme, is encoded by the phage genome. Endolysin is required to disrupt the bacterial cell wall to release phage progeny at the final step of viral infection. The endolysin CD27L is derived from the phage CD27, which is the first phage endolysin characterized in *C. difficile*. The specificity test demonstrated that CD27L was active against a panel of 30 *C. difficile* strains, including a hypervirulent ribotype 027[125]. The N-terminal truncated CD27L, $CD27L_{1–179}$, improved the lytic activity when tested against *C. difficile*. $CD27L_{1–179}$ exhibited a slightly broader lytic range than the full-length phage. It was also active against *Listeria* spp. However, both CD27L and $CD27L_{1–179}$ did not harm the selected gut commensal bacteria[130]. Another endolysin retrieved from *C. difficile* 630 prophage has been described. The lytic activity of full-length PlyCD, and that of a truncated N-terminal containing the catalytic domain $PlyCD_{1–174}$, were evaluated. Similar to CD27L, the truncated $PlyCD_{1–174}$ exhibited greater lytic activity than its full-length counterpart and also displayed a broader activity range against *C. difficile* strains than the full-length PlyCD. The bactericidal assay demonstrated that $PlyCD_{1–174}$

reduced more than 4-log growth of the *C. difficile* hypervirulent MLST2 strains 217B, 615H, and UK1 (027). This result highlighted the potency of $PlyCD_{1-174}$ against the crucial clinical strains. Interestingly, $PlyCD_{1-174}$ exhibited a synergistic effect with vancomycin pretreatment. The combination treatment between vancomycin and $PlyCD_{1-174}$ *in vivo* demonstrated significant inhibition of *C. difficile* growth (> 2-log). Unfortunately, the *in vivo* study of $PlyCD_{1-174}$ was unsuccessful due to inconsistent results. *Ex vivo* experiments were conducted using infected mouse cecum and anus as an infecting area. $PlyCD_{1-174}$ exhibited 2-log reduction of *C. difficile* cell growth, indicating the activity of $PlyCD_{1-174}$ in the gastrointestinal environment[131]. The mechanism by which truncated endolysins exhibit higher efficiency than the wild-type counterpart remains to be explored.

Phage-derived proteins with high specificity to *C. difficile* strains could be developed for targeted therapy. Diffiocin, a contractile R-type phage tail-like bacteriocin, was originally derived from the *C. difficile* strain CD4 (Diffiocin-4). This protein exhibits a higher efficiency when fused with the receptor-binding protein (RBP) of prophage phi027 in the genome of strain R20291 (Av-CD291.1 and Av-CD291.2). Interestingly, avidocin is stably active throughout the mouse gastrointestinal tract when supplied in drinking water containing 4% sucrose and 1% sodium bicarbonate. The specificity of avidocin-CDs was evaluated across *C. difficile* strains, based on which the relationship between RBPs in avidocin-CDs construct and *slpA* allele was concluded[129]. Despite the advances in the knowledge of bacteriophage biology, the use of phage for therapeutic purposes remains much of a challenge. The first challenge is lysogenic conversion, which results in ineffective treatment outcomes. Therefore, the remaining challenge is to identify or engineer the phage to obtain a strictly lytic phage. The subsequent concern is the battle between the host and the phage that occurs during phage infection. Some bacteria naturally mutate and become resistant to phage infection using different mechanisms, *e.g.*, extracellular modification or intracellular modification, as mentioned in a review elsewhere[138]. Still, there is room for lytic phage hunters and phage modification, which are desperately needed. However, to apply this technology effectively in clinical practice, further research is warranted to overcome these limitations.

## ILEOSTOMY AND COLONIC LAVAGE ARE RESERVED FOR FULMINANT CDI

At the other end of the spectrum, one alternative treatment is the removal of part of the gut, *i.e.*, colectomy, ileostomy, loop ileostomy, and colonic lavage. This treatment approach is largely reserved for severe and complicated (fulminant) CDI as it is the most invasive treatment. Earlier, colectomy was opted, but it did not improve the clinical outcome, resulting in some mortality[139]. Diverting loop ileostomy and colonic lavage were developed at the Pittsburgh School of Medicine and therefore referred to as the Pittsburgh protocol. This protocol exhibited benefits over conventional colectomy and end ileostomy, which decreased the mortality rate and preserved the colon[140]. We found 2 clinical trials that were terminated due to slow recruitment of participants; hence, there is limited insight into how this method confers benefit to patients with CDI. This indicates to some degree that the current treatment regimen is sufficient to save patients' lives without involving this invasive intervention.

## VACCINES ARE THE HOPE FOR CDI CONTROL AND PREVENTION

Current treatment approaches for CDI concentrate primarily on antibiotics. Although antibiotic administration commonly serves as the first-line therapy, prolonged disruption of the normal microbiota can result in recurrent CDI, with incidences of up to 45% after antibiotic therapy[141]. The incidence of healthcare-associated CDI has reduced over time, although there have been recent reports on the growing number of cases of community-associated CDI[142]. Therefore, there exists an urgent need to control and prevent CDI transmission. To date, several preventive remedies for recurrent CDI have been proposed, including antibiotics and FMT. Although their efficacy has been proven, none are recommended by the Infectious Disease Society of America (IDSA), and the protracted efficacy is still questionable. Therefore, vaccines could be a valuable option to serve as long-term prophylaxis for CDI due to their memory function.

Several vaccine candidates have been developed and launched to animal and clinical trials. Currently, a total of 21 clinical trials on *C. difficile* vaccines have been reported, of which 3 were terminated. Most of the vaccine studies have been developed based on 3 formulations, *viz.*, toxoid, recombinant peptide, and surface-associated antigen. In this section, we shall briefly describe each vaccine formulation regarding its development and efficacy in preventing CDI.

Because toxin A and toxin B are key virulence determinants of CDI and associated with the severity of colon damage, they become a remarkable target for vaccine development. The alteration of toxin structure by chemical treatment, thereby inactivating toxicity while preserving its immunogenicity—termed as toxoid—has been developed for over 3 decades. Toxoid-based vaccine use in animal models has demonstrated a satisfactory production level of serum antibody responses against both toxins and a preventive efficacy against a lethal outcome of CDI[143]. The first clinical trial on toxoid-based vaccine in healthy individuals was introduced 20 years ago. It was observed that the majority of study subjects developed a massive level of specific antibodies for both toxins. Although some adverse reactions were documented, the vaccine was demonstrated to be safe and immunogenic in healthy adults[144]. This information paves the way for further development of the next generation of formulated vaccine candidates that are currently under investigation.

The use of a recombinant protein-based design is another approach for vaccine development to avoid residual toxins, which are not completely inactivated by chemical, and also maximizes the protective effect using only the toxin domain responsible for immunogenicity. Furthermore, this genetic modification allows uncomplicated manufacture of vaccine preparations compared with toxoid production. Three functional domains of both toxins, including glucosyl-transferase (GT) and cysteine protease (CP), central translocation (T), and C-terminal receptor-binding domain (RBD), were tested for immunogenic validation. The first study conducted by Lyerly *et al*[145] on the use of the RBD of toxin A reported partial protection against CDI and death in a hamster model. The genetic modifications of toxin A RBD were widely investigated by several research groups. These modifications included the use of RBD subdomain, recombinant fusion with other immunogenic proteins, and combination with a mucosal adjuvant[146]. These constructions exhibited strong action by evoking a complete seroconversion for toxin A and preventing fluid secretion and histological changes. Although seroconversion against toxin A was found to be greater than that against toxin B, an optimal vaccine needs to include both toxin A and toxin B fragments to achieve maximum protective efficacy. Therefore, later developments focused on the combination of recombinant peptides with/without immune adjuvants. The fusion peptide, containing fragments of toxin A and toxin B, generates an immune response to both toxins. Complete protection against toxin A was observed at all doses, whereas less immunogenicity toward toxin B was noticed[147]. Co-administration of the combined recombinant protein with the adjuvant also demonstrated satisfactory positive protection, hence this vaccine formulation is currently in phase 1 clinical trial.

To overcome the limitations of previous vaccine candidates related to the prevention of CDI, surface protein antigens have emerged as an alternative target for vaccine development. Several studies have confirmed the induction of immune response during infection against surface layer protein (SLP), including flagella components, adhesin, fibronectin-binding protein, and cysteine protease[148,149]. However, none of the vaccinations provided significant protection in animal models. Furthermore, glycans, the polysaccharide coat on the surface of the bacterium, serve as another target for eliciting specific antibodies. It has been documented that all 3 glycan structures, PSI, PSII and PSIII, conjugated with other immune inducers stimulated specific antibodies, including IgA and IgG, in animal models[150,151]. It was also shown that immune cells can recognize both native and synthesized glycans, supporting the importance of this molecule to be developed as a vaccine and/or a vaccine additive.

There have been several attempts to develop vaccines for CDI. Unfortunately, there is still no approved vaccine to prevent initial and/or recurrent CDI. Nevertheless, there are 2 potential vaccine candidates reaching the final stage of development[152-154]. VLA84, a chimeric protein containing truncated toxin A and toxin B, developed by Valneva Austria GmbH, demonstrated good efficacy in a phase 1 trial[155,156]. It is currently under phase 2 clinical trial (NCT02316470). This clinical phase is a randomized placebo-controlled study of a total of 500 healthy subjects aged > 50 years. The subjects were separated into 4 groups, which individually received different vaccine doses, including VLA84 75 μg without alum, VLA84 200 μg with and without alum, and phosphate-buffered saline as the placebo group. All subjects received 3

doses of vaccination on days 0, 7, and 28. The immunogenicity and safety of VLA84 were evaluated after the last vaccination for up to 6 mo. Based on the primary outcome, it was reported that seroconversion for IgG was ≥ 4-fold increase for toxin A and toxin B on day 56. However, efficacy and data analysis are yet to be reported.

Pfizer's vaccine has been developed using genetically modified full-length toxin A and toxin B, using a novel detoxification process that preserves the critical epitopes responsible for immunogenicity for maximizing the production of neutralizing antibodies[157,158]. This vaccine entered clinical trials in 2012 and received Fast Track designation from the United States FDA in 2014. The phase 2 clinical trial was completed on patients aged 50–85 years (NCT02117570). The vaccine induced robust immune responses and exerted protective effects in preclinical models. The Pfizer vaccine is currently undergoing phase 3 clinical trials with ≥ 17000 subjects in 23 countries. Subjects aged ≥ 50 years will receive 3-dose vaccinations at months 0, 1, and 6. Volunteers will be followed up for 3 years after the last vaccination. As the trial is ongoing, the results of data analysis from this vaccine are expected in the near future.

Although several vaccines have been developed for decades, aiming to serve as a prophylaxis for CDI, there are concerns and limitations for rapid, long-lasting, and protective immunity. Further efforts are still required to identify optimal dose, dosing schedule, and vaccine formulation, and also to determine potential application for high-risk healthy populations and immunocompromised individuals. Regarding the promising efficacy of a CDI vaccine, if approved, it will provide the primary prevention and reduction of CDI cases worldwide.

## CONCLUSION

CDI is a serious healthcare concern as most countries move towards aging societies [159]; *C. difficile* can have maximum impact on this age group. CDI treatment is also threatened by treatment failures, especially recurrent CDI. Therefore, an urgent need exists to develop alternative treatment approaches. Small molecules and natural products have been subjected to the most advanced progress compared with other approaches, followed by an erupting trend of FMT. Both single and combinational therapies appear to be the way forward, such as antibiotic/FMT, antibiotic/antitoxin antibody, and antibiotic/synbiotic. Vaccine development is highly anticipated as the results are extremely promising and would provide a significant tool for CDI prevention and control in community and healthcare settings. Bacteriophage therapy has to overcome the grand challenge before it can be used in clinical practice. These developments are the future of CDI treatment; they require a huge amount of effort and capital; meanwhile, the management of antibiotic use, hygiene precautions and education, and monitoring systems, must be implemented to reduce the incidence of CDI.

## ACKNOWLEDGEMENTS

Authors would like to thank Paul Adams for kindly proofreading the final manuscript and the Faculty of Tropical Medicine for subsidizing the English editing service on the first manuscript.

## REFERENCES

1 **Kuiper GA**, van Prehn J, Ang W, Kneepkens F, van der Schoor S, de Meij T. *Clostridium difficile* infections in young infants: Case presentations and literature review. *IDCases* 2017; **10**: 7-11 [PMID: 28791215 DOI: 10.1016/j.idcr.2017.07.005]

2 **Tullus K**, Aronsson B, Marcus S, Möllby R. Intestinal colonization with Clostridium difficile in infants up to 18 months of age. *Eur J Clin Microbiol Infect Dis* 1989; **8**: 390-393 [PMID: 2502403 DOI: 10.1007/BF01964052]

3 **Hooks KB**, O'Malley MA. Dysbiosis and Its Discontents. *mBio* 2017; **8** [PMID: 29018121 DOI: 10.1128/mBio.01492-17]

4 **Seekatz AM**, Young VB. Clostridium difficile and the microbiota. *J Clin Invest* 2014; **124**: 4182-4189 [PMID: 25036699 DOI: 10.1172/JCI72336]

5 **Chandrasekaran R**, Lacy DB. The role of toxins in Clostridium difficile infection. *FEMS Microbiol Rev* 2017; **41**: 723-750 [PMID: 29048477 DOI: 10.1093/femsre/fux048]

6 **Kuehne SA**, Collery MM, Kelly ML, Cartman ST, Cockayne A, Minton NP. Importance of toxin A,

toxin B, and CDT in virulence of an epidemic Clostridium difficile strain. *J Infect Dis* 2014; **209**: 83-86 [PMID: 23935202 DOI: 10.1093/infdis/jit426]

7 **Smits WK**, Lyras D, Lacy DB, Wilcox MH, Kuijper EJ. Clostridium difficile infection. *Nat Rev Dis Primers* 2016; **2**: 16020 [PMID: 27158839 DOI: 10.1038/nrdp.2016.20]

8 **Androga GO**, Hart J, Foster NF, Charles A, Forbes D, Riley TV. Infection with Toxin A-Negative, Toxin B-Negative, Binary Toxin-Positive Clostridium difficile in a Young Patient with Ulcerative Colitis. *J Clin Microbiol* 2015; **53**: 3702-3704 [PMID: 26354812 DOI: 10.1128/JCM.01810-15]

9 **Eckert C**, Emirian A, Le Monnier A, Cathala L, De Montclos H, Goret J, Berger P, Petit A, De Chevigny A, Jean-Pierre H, Nebbad B, Camiade S, Meckenstock R, Lalande V, Marchandin H, Barbut F. Prevalence and pathogenicity of binary toxin-positive Clostridium difficile strains that do not produce toxins A and B. *New Microbes New Infect* 2015; **3**: 12-17 [PMID: 25755885 DOI: 10.1016/j.nmni.2014.10.003]

10 **Abt MC**, McKenney PT, Pamer EG. Clostridium difficile colitis: pathogenesis and host defence. *Nat Rev Microbiol* 2016; **14**: 609-620 [PMID: 27573580 DOI: 10.1038/nrmicro.2016.108]

11 **Poxton IR**, McCoubrey J, Blair G. The pathogenicity of Clostridium difficile. *Clin Microbiol Infect* 2001; **7**: 421-427 [PMID: 11591205 DOI: 10.1046/j.1198-743x.2001.00287.x]

12 **Suetens C**, Latour K, Kärki T, Ricchizzi E, Kinross P, Moro ML, Jans B, Hopkins S, Hansen S, Lyytikäinen O, Reilly J, Deptula A, Zingg W, Plachouras D, Monnet DL; The Healthcare-Associated Infections Prevalence Study Group. Prevalence of healthcare-associated infections, estimated incidence and composite antimicrobial resistance index in acute care hospitals and long-term care facilities: results from two European point prevalence surveys, 2016 to 2017. *Euro Surveill* 2018; **23** [PMID: 30458912 DOI: 10.2807/1560-7917.ES.2018.23.46.1800516]

13 **Vardakas KZ**, Trigkidis KK, Boukouvala E, Falagas ME. Clostridium difficile infection following systemic antibiotic administration in randomised controlled trials: a systematic review and meta-analysis. *Int J Antimicrob Agents* 2016; **48**: 1-10 [PMID: 27216385 DOI: 10.1016/j.ijantimicag.2016.03.008]

14 **O'Grady K**, Knight DR, Riley TV. Antimicrobial resistance in Clostridioides difficile. *Eur J Clin Microbiol Infect Dis* 2021 [PMID: 34427801 DOI: 10.1007/s10096-021-04311-5]

15 **McDonald LC**, Gerding DN, Johnson S, Bakken JS, Carroll KC, Coffin SE, Dubberke ER, Garey KW, Gould CV, Kelly C, Loo V, Shaklee Sammons J, Sandora TJ, Wilcox MH. Clinical Practice Guidelines for Clostridium difficile Infection in Adults and Children: 2017 Update by the Infectious Diseases Society of America (IDSA) and Society for Healthcare Epidemiology of America (SHEA). *Clin Infect Dis* 2018; **66**: e1-e48 [PMID: 29462280 DOI: 10.1093/cid/cix1085]

16 **Baur D**, Gladstone BP, Burkert F, Carrara E, Foschi F, Döbele S, Tacconelli E. Effect of antibiotic stewardship on the incidence of infection and colonisation with antibiotic-resistant bacteria and Clostridium difficile infection: a systematic review and meta-analysis. *Lancet Infect Dis* 2017; **17**: 990-1001 [PMID: 28629876 DOI: 10.1016/S1473-3099(17)30325-0]

17 **Negrut N**, Nistor-Cseppento DC, Khan SA, Pantis C, Maghiar TA, Maghiar O, Aleya S, Rus M, Tit DM, Aleya L, Rahdar A, Bungau S. Clostridium difficile infection epidemiology over a period of 8 years—a single centre study. *Sustainability* 2020; **12**: 4439 [DOI: 10.3390/su12114439]

18 **Marra AR**, Perencevich EN, Nelson RE, Samore M, Khader K, Chiang HY, Chorazy ML, Herwaldt LA, Diekema DJ, Kuxhausen MF, Blevins A, Ward MA, McDanel JS, Nair R, Balkenende E, Schweizer ML. Incidence and Outcomes Associated With Clostridium difficile Infections: A Systematic Review and Meta-analysis. *JAMA Netw Open* 2020; **3**: e1917597 [PMID: 31913488 DOI: 10.1001/jamanetworkopen.2019.17597]

19 **Principi N**, Silvestri E, Esposito S. Advantages and Limitations of Bacteriophages for the Treatment of Bacterial Infections. *Front Pharmacol* 2019; **10**: 513 [PMID: 31139086 DOI: 10.3389/fphar.2019.00513]

20 **Dedrick RM**, Guerrero-Bustamante CA, Garlena RA, Russell DA, Ford K, Harris K, Gilmour KC, Soothill J, Jacobs-Sera D, Schooley RT, Hatfull GF, Spencer H. Engineered bacteriophages for treatment of a patient with a disseminated drug-resistant Mycobacterium abscessus. *Nat Med* 2019; **25**: 730-733 [PMID: 31068712 DOI: 10.1038/s41591-019-0437-z]

21 **Amidon S**, Brown JE, Dave VS. Colon-targeted oral drug delivery systems: design trends and approaches. *AAPS PharmSciTech* 2015; **16**: 731-741 [PMID: 26070545 DOI: 10.1208/s12249-015-0350-9]

22 **Jarrad AM**, Karoli T, Blaskovich MA, Lyras D, Cooper MA. Clostridium difficile drug pipeline: challenges in discovery and development of new agents. *J Med Chem* 2015; **58**: 5164-5185 [PMID: 25760275 DOI: 10.1021/jm5016846]

23 **Petrosillo N**, Granata G, Cataldo MA. Novel Antimicrobials for the Treatment of *Clostridium difficile* Infection. *Front Med (Lausanne)* 2018; **5**: 96 [PMID: 29713630 DOI: 10.3389/fmed.2018.00096]

24 **Mendes RE**, Rhomberg PR, Locher HH, Jones RN. Activity of cadazolid against Gram-positive clinical isolates, including linezolid-resistant subsets with defined resistance mechanisms. ICACC; 2013; North Liberty, IA, USA. Available from: https://www.jmilabs.com/data/posters/ICAAC2013/E-144.PDF

25 **Chilton CH**, Crowther GS, Baines SD, Todhunter SL, Freeman J, Locher HH, Athanasiou A, Wilcox MH. In vitro activity of cadazolid against clinically relevant Clostridium difficile isolates and in an in vitro gut model of C. difficile infection. *J Antimicrob Chemother* 2014; **69**: 697-705 [PMID: 24128668 DOI: 10.1093/jac/dkt411]

26 **Odingo J**, Bailey MA, Files M, Early JV, Alling T, Dennison D, Bowman J, Dalai S, Kumar N, Cramer J, Masquelin T, Hipskind PA, Parish T. In Vitro Evaluation of Novel Nitazoxanide Derivatives against *Mycobacterium tuberculosis*. *ACS Omega* 2017; **2**: 5873-5890 [PMID: 30023755 DOI: 10.1021/acsomega.7b00892]

27 **Hoffman PS**, Sisson G, Croxen MA, Welch K, Harman WD, Cremades N, Morash MG. Antiparasitic drug nitazoxanide inhibits the pyruvate oxidoreductases of Helicobacter pylori, selected anaerobic bacteria and parasites, and Campylobacter jejuni. *Antimicrob Agents Chemother* 2007; **51**: 868-876 [PMID: 17158936 DOI: 10.1128/AAC.01159-06]

28 **de Carvalho LP**, Darby CM, Rhee KY, Nathan C. Nitazoxanide Disrupts Membrane Potential and Intrabacterial pH Homeostasis of Mycobacterium tuberculosis. *ACS Med Chem Lett* 2011; **2**: 849-854 [PMID: 22096616 DOI: 10.1021/ml200157f]

29 **Rossignol JF**. Nitazoxanide: a first-in-class broad-spectrum antiviral agent. *Antiviral Res* 2014; **110**: 94-103 [PMID: 25108173 DOI: 10.1016/j.antiviral.2014.07.014]

30 **Stachulski AV**, Taujanskas J, Pate SL, Rajoli RKR, Aljayyoussi G, Pennington SH, Ward SA, Hong WD, Biagini GA, Owen A, Nixon GL, Leung SC, O'Neill PM. Therapeutic Potential of Nitazoxanide: An Appropriate Choice for Repurposing versus SARS-CoV-2? *ACS Infect Dis* 2021; **7**: 1317-1331 [PMID: 33352056 DOI: 10.1021/acsinfecdis.0c00478]

31 **Musher DM**, Logan N, Bressler AM, Johnson DP, Rossignol JF. Nitazoxanide versus vancomycin in Clostridium difficile infection: a randomized, double-blind study. *Clin Infect Dis* 2009; **48**: e41-e46 [PMID: 19133801 DOI: 10.1086/596552]

32 **Rafiullah F**, Kanwal S, Majeed UM, Korsten MA, Cheema FH, Luthra M, Sohail MR. Successful use of nitazoxanide in the treatment of recurrent Clostridium difficile infection. *BMJ Case Rep* 2011; **2011** [PMID: 22674696 DOI: 10.1136/bcr.04.2011.4123]

33 **Brook I**, Wexler HM, Goldstein EJ. Antianaerobic antimicrobials: spectrum and susceptibility testing. *Clin Microbiol Rev* 2013; **26**: 526-546 [PMID: 23824372 DOI: 10.1128/CMR.00086-12]

34 **Vickers R**, Robinson N, Best E, Echols R, Tillotson G, Wilcox M. A randomised phase 1 study to investigate safety, pharmacokinetics and impact on gut microbiota following single and multiple oral doses in healthy male subjects of SMT19969, a novel agent for Clostridium difficile infections. *BMC Infect Dis* 2015; **15**: 91 [PMID: 25880933 DOI: 10.1186/s12879-015-0759-5]

35 **Qian X**, Yanagi K, Kane AV, Alden N, Lei M, Snydman DR, Vickers RJ, Lee K, Thorpe CM. Ridinilazole, a narrow spectrum antibiotic for treatment of *Clostridioides difficile* infection, enhances preservation of microbiota-dependent bile acids. *Am J Physiol Gastrointest Liver Physiol* 2020; **319**: G227-G237 [PMID: 32597706 DOI: 10.1152/ajpgi.00046.2020]

36 **Bassères E**, Endres BT, Khaleduzzaman M, Miraftabi F, Alam MJ, Vickers RJ, Garey KW. Impact on toxin production and cell morphology in Clostridium difficile by ridinilazole (SMT19969), a novel treatment for C. difficile infection. *J Antimicrob Chemother* 2016; **71**: 1245-1251 [PMID: 26895772 DOI: 10.1093/jac/dkv498]

37 **Vickers RJ**, Tillotson GS, Nathan R, Hazan S, Pullman J, Lucasti C, Deck K, Yacyshyn B, Maliakkal B, Pesant Y, Tejura B, Roblin D, Gerding DN, Wilcox MH; CoDIFy study group. Efficacy and safety of ridinilazole compared with vancomycin for the treatment of Clostridium difficile infection: a phase 2, randomised, double-blind, active-controlled, non-inferiority study. *Lancet Infect Dis* 2017; **17**: 735-744 [PMID: 28461207 DOI: 10.1016/S1473-3099(17)30235-9]

38 **Swinney DC**, Anthony J. How were new medicines discovered? *Nat Rev Drug Discov* 2011; **10**: 507-519 [PMID: 21701501 DOI: 10.1038/nrd3480]

39 **Volochnyuk DM**, Ryabukhin SV, Moroz YS, Savych O, Chuprina A, Horvath D, Zabolotna Y, Varnek A, Judd DB. Evolution of commercially available compounds for HTS. *Drug Discov Today* 2019; **24**: 390-402 [PMID: 30399443 DOI: 10.1016/j.drudis.2018.10.016]

40 **AbdelKhalek A**, Mohammad H, Mayhoub AS, Seleem MN. Screening for potent and selective anticlostridial leads among FDA-approved drugs. *J Antibiot (Tokyo)* 2020; **73**: 392-409 [PMID: 32132676 DOI: 10.1038/s41429-020-0288-3]

41 **AbdelKhalek A**, Seleem MN. Repurposing the Veterinary Antiprotozoal Drug Ronidazole for the Treatment of Clostridioides difficile Infection. *Int J Antimicrob Agents* 2020; **56**: 106188 [PMID: 33045352 DOI: 10.1016/j.ijantimicag.2020.106188]

42 **Pushpakom S**, Iorio F, Eyers PA, Escott KJ, Hopper S, Wells A, Doig A, Guilliams T, Latimer J, McNamee C, Norris A, Sanseau P, Cavalla D, Pirmohamed M. Drug repurposing: progress, challenges and recommendations. *Nat Rev Drug Discov* 2019; **18**: 41-58 [PMID: 30310233 DOI: 10.1038/nrd.2018.168]

43 **Pal R**, Seleem MN. Screening of Natural Products and Approved Oncology Drug Libraries for Activity against Clostridioides difficile. *Sci Rep* 2020; **10**: 5966 [PMID: 32249833 DOI: 10.1038/s41598-020-63029-0]

44 **Fillbrunn A**, Dietz C, Pfeuffer J, Rahn R, Landrum GA, Berthold MR. KNIME for reproducible cross-domain analysis of life science data. *J Biotechnol* 2017; **261**: 149-156 [PMID: 28757290 DOI: 10.1016/j.jbiotec.2017.07.028]

45 **Beisken S**, Meinl T, Wiswedel B, de Figueiredo LF, Berthold M, Steinbeck C. KNIME-CDK: Workflow-driven cheminformatics. *BMC Bioinformatics* 2013; **14**: 257 [PMID: 24103053 DOI: 10.1186/1471-2105-14-257]

46 **Mazanetz MP**, Marmon RJ, Reisser CB, Morao I. Drug discovery applications for KNIME: an open source data mining platform. *Curr Top Med Chem* 2012; **12**: 1965-1979 [PMID: 23110532 DOI: 10.2174/156802612804910331]

47 **Kong KF**, Schneper L, Mathee K. Beta-lactam antibiotics: from antibiosis to resistance and bacteriology. *APMIS* 2010; **118**: 1-36 [PMID: 20041868 DOI: 10.1111/j.1600-0463.2009.02563.x]

48 **Sheehan DJ**, Hitchcock CA, Sibley CM. Current and emerging azole antifungal agents. *Clin Microbiol Rev* 1999; **12**: 40-79 [PMID: 9880474 DOI: 10.1128/CMR.12.1.40]

49 **de la Torre BG**, Albericio F. The Pharmaceutical Industry in 2019. An Analysis of FDA Drug Approvals from the Perspective of Molecules. *Molecules* 2020; **25** [PMID: 32050446 DOI: 10.3390/molecules25030745]

50 **Palombo EA**. Phytochemicals from traditional medicinal plants used in the treatment of diarrhoea: modes of action and effects on intestinal function. *Phytother Res* 2006; **20**: 717-724 [PMID: 16619336 DOI: 10.1002/ptr.1907]

51 **Patridge E**, Gareiss P, Kinch MS, Hoyer D. An analysis of FDA-approved drugs: natural products and their derivatives. *Drug Discov Today* 2016; **21**: 204-207 [PMID: 25617672 DOI: 10.1016/j.drudis.2015.01.009]

52 **Roshan N**, Riley TV, Hammer KA. Antimicrobial activity of natural products against Clostridium difficile in vitro. *J Appl Microbiol* 2017; **123**: 92-103 [PMID: 28489336 DOI: 10.1111/jam.13486]

53 **Perumalsamy H**, Jung MY, Hong SM, Ahn YJ. Growth-Inhibiting and morphostructural effects of constituents identified in Asarum heterotropoides root on human intestinal bacteria. *BMC Complement Altern Med* 2013; **13**: 245 [PMID: 24083511 DOI: 10.1186/1472-6882-13-245]

54 **Fraternale D**, Flamini G, Ricci D. Essential oil composition and antimicrobial activity of Angelica archangelica L. (Apiaceae) roots. *J Med Food* 2014; **17**: 1043-1047 [PMID: 24788027 DOI: 10.1089/jmf.2013.0012]

55 **Cermak P**, Olsovska J, Mikyska A, Dusek M, Kadleckova Z, Vanicek J, Nyc O, Sigler K, Bostikova V, Bostik P. Strong antimicrobial activity of xanthohumol and other derivatives from hops (Humulus lupulus L.) on gut anaerobic bacteria. *APMIS* 2017; **125**: 1033-1038 [PMID: 28960474 DOI: 10.1111/apm.12747]

56 **Canning C**, Sun S, Ji X, Gupta S, Zhou K. Antibacterial and cytotoxic activity of isoprenylated coumarin mammea A/AA isolated from Mammea africana. *J Ethnopharmacol* 2013; **147**: 259-262 [PMID: 23466248 DOI: 10.1016/j.jep.2013.02.026]

57 **Mody D**, Athamneh AIM, Seleem MN. Curcumin: A natural derivative with antibacterial activity against Clostridium difficile. *J Glob Antimicrob Resist* 2020; **21**: 154-161 [PMID: 31622683 DOI: 10.1016/j.jgar.2019.10.005]

58 **Finegold SM**, Summanen PH, Corbett K, Downes J, Henning SM, Li Z. Pomegranate extract exhibits in vitro activity against Clostridium difficile. *Nutrition* 2014; **30**: 1210-1212 [PMID: 24976424 DOI: 10.1016/j.nut.2014.02.029]

59 **de Nova PJG**, Carvajal A, Prieto M, Rubio P. *In vitro* Susceptibility and Evaluation of Techniques for Understanding the Mode of Action of a Promising Non-antibiotic Citrus Fruit Extract Against Several Pathogens. *Front Microbiol* 2019; **10**: 884 [PMID: 31105673 DOI: 10.3389/fmicb.2019.00884]

60 **Gadhi CA**, Weber M, Mory F, Benharref A, Lion C, Jana M, Lozniewski A. Antibacterial activity of Aristolochia paucinervis Pomel. *J Ethnopharmacol* 1999; **67**: 87-92 [PMID: 10616964 DOI: 10.1016/s0378-8741(98)00212-8]

61 **Roshan N**, Riley TV, Knight DR, Steer JH, Hammer KA. Natural products show diverse mechanisms of action against Clostridium difficile. *J Appl Microbiol* 2019; **126**: 468-479 [PMID: 30412324 DOI: 10.1111/jam.14152]

62 **Harnvoravongchai P**, Chankhamhaengdecha S, Ounjai P, Singhakaew S, Boonthaworn K, Janvilisri T. Antimicrobial Effect of Asiatic Acid Against *Clostridium difficile* Is Associated With Disruption of Membrane Permeability. *Front Microbiol* 2018; **9**: 2125 [PMID: 30245677 DOI: 10.3389/fmicb.2018.02125]

63 **Shilling M**, Matt L, Rubin E, Visitacion MP, Haller NA, Grey SF, Woolverton CJ. Antimicrobial effects of virgin coconut oil and its medium-chain fatty acids on Clostridium difficile. *J Med Food* 2013; **16**: 1079-1085 [PMID: 24328700 DOI: 10.1089/jmf.2012.0303]

64 **Blaskovich MAT**, Kavanagh AM, Elliott AG, Zhang B, Ramu S, Amado M, Lowe GJ, Hinton AO, Pham DMT, Zuegg J, Beare N, Quach D, Sharp MD, Pogliano J, Rogers AP, Lyras D, Tan L, West NP, Crawford DW, Peterson ML, Callahan M, Thurn M. The antimicrobial potential of cannabidiol. *Commun Biol* 2021; **4**: 7 [PMID: 33469147 DOI: 10.1038/s42003-020-01530-y]

65 **Gigli S**, Seguella L, Pesce M, Bruzzese E, D'Alessandro A, Cuomo R, Steardo L, Sarnelli G, Esposito G. Cannabidiol restores intestinal barrier dysfunction and inhibits the apoptotic process induced by *Clostridium difficile* toxin A in Caco-2 cells. *United European Gastroenterol J* 2017; **5**: 1108-1115 [PMID: 29238589 DOI: 10.1177/2050640617698622]

66 **Adejumo AC**, Bukong TN. Cannabis use and risk of Clostridioides difficile infection: Analysis of 59,824 hospitalizations. *Anaerobe* 2020; **61**: 102095 [PMID: 31493498 DOI: 10.1016/j.anaerobe.2019.102095]

67 **Roshan N**, Riley TV, Hammer KA. Effects of natural products on several stages of the spore cycle of Clostridium difficile in vitro. *J Appl Microbiol* 2018; **125**: 710-723 [PMID: 29675852 DOI: 10.1111/jam.13889]

68 **Mooyottu S**, Flock G, Venkitanarayanan K. Carvacrol reduces Clostridium difficile sporulation and spore outgrowth in vitro. *J Med Microbiol* 2017; **66**: 1229-1234 [PMID: 28786786 DOI: 10.1099/jmm.0.000515]

69 **Yu L**, Palafox-Rosas R, Luna B, She RC. The Bactericidal Activity and Spore Inhibition Effect of

Manuka Honey against *Clostridioides Difficile*. *Antibiotics (Basel)* 2020; **9** [PMID: 33050172 DOI: 10.3390/antibiotics9100684]

70 **Pellissery AJ**, Vinayamohan PG, Venkitanarayanan K. *In vitro* antivirulence activity of baicalin against *Clostridioides difficile*. *J Med Microbiol* 2020; **69**: 631-639 [PMID: 32216868 DOI: 10.1099/jmm.0.001179]

71 **Roshan N**, Riley TV, Knight DR, Hammer KA. Effect of natural products on the production and activity of Clostridium difficile toxins in vitro. *Sci Rep* 2018; **8**: 15735 [PMID: 30356168 DOI: 10.1038/s41598-018-33954-2]

72 **Wultańska D**, Piotrowski M, Pituch H. The effect of berberine chloride and/or its combination with vancomycin on the growth, biofilm formation, and motility of Clostridioides difficile. *Eur J Clin Microbiol Infect Dis* 2020; **39**: 1391-1399 [PMID: 32140903 DOI: 10.1007/s10096-020-03857-0]

73 **Giles SL**, Laheij RJF. Successful treatment of persistent Clostridium difficile infection with manuka honey. *Int J Antimicrob Agents* 2017; **49**: 522-523 [PMID: 28257905 DOI: 10.1016/j.ijantimicag.2017.02.005]

74 **Orth P**, Xiao L, Hernandez LD, Reichert P, Sheth PR, Beaumont M, Yang X, Murgolo N, Ermakov G, DiNunzio E, Racine F, Karczewski J, Secore S, Ingram RN, Mayhood T, Strickland C, Therien AG. Mechanism of action and epitopes of Clostridium difficile toxin B-neutralizing antibody bezlotoxumab revealed by X-ray crystallography. *J Biol Chem* 2014; **289**: 18008-18021 [PMID: 24821719 DOI: 10.1074/jbc.M114.560748]

75 **Yang Z**, Ramsey J, Hamza T, Zhang Y, Li S, Yfantis HG, Lee D, Hernandez LD, Seghezzi W, Furneisen JM, Davis NM, Therien AG, Feng H. Mechanisms of protection against Clostridium difficile infection by the monoclonal antitoxin antibodies actoxumab and bezlotoxumab. *Infect Immun* 2015; **83**: 822-831 [PMID: 25486992 DOI: 10.1128/IAI.02897-14]

76 **Wilcox MH**, Gerding DN, Poxton IR, Kelly C, Nathan R, Birch T, Cornely OA, Rahav G, Bouza E, Lee C, Jenkin G, Jensen W, Kim YS, Yoshida J, Gabryelski L, Pedley A, Eves K, Tipping R, Guris D, Kartsonis N, Dorr MB; MODIFY I and MODIFY II Investigators. Bezlotoxumab for Prevention of Recurrent Clostridium difficile Infection. *N Engl J Med* 2017; **376**: 305-317 [PMID: 28121498 DOI: 10.1056/NEJMoa1602615]

77 **Maiti PK**, inventor Immunimed Inc. , assignee. Use of polyclonal antibodies against Clostridium Difficile for treatment of inflammatory bowel disease. US patent US20180362618A1. 2017. Available from: https://patents.google.com/patent/US10513552B2/en

78 **Sponseller JK**, Steele JA, Schmidt DJ, Kim HB, Beamer G, Sun X, Tzipori S. Hyperimmune bovine colostrum as a novel therapy to combat Clostridium difficile infection. *J Infect Dis* 2015; **211**: 1334-1341 [PMID: 25381448 DOI: 10.1093/infdis/jiu605]

79 **Heidebrecht HJ**, Weiss WJ, Pulse M, Lange A, Gisch K, Kliem H, Mann S, Pfaffl MW, Kulozik U, von Eichel-Streiber C. Treatment and Prevention of Recurrent Clostridium difficile Infection with Functionalized Bovine Antibody-Enriched Whey in a Hamster Primary Infection Model. *Toxins (Basel)* 2019; **11** [PMID: 30736358 DOI: 10.3390/toxins11020098]

80 **Sholeh M**, Krutova M, Forouzesh M, Mironov S, Sadeghifard N, Molaeipour L, Maleki A, Kouhsari E. Antimicrobial resistance in Clostridioides (Clostridium) difficile derived from humans: a systematic review and meta-analysis. *Antimicrob Resist Infect Control* 2020; **9**: 158 [PMID: 32977835 DOI: 10.1186/s13756-020-00815-5]

81 **Reeves AE**, Theriot CM, Bergin IL, Huffnagle GB, Schloss PD, Young VB. The interplay between microbiome dynamics and pathogen dynamics in a murine model of Clostridium difficile Infection. *Gut Microbes* 2011; **2**: 145-158 [PMID: 21804357 DOI: 10.4161/gmic.2.3.16333]

82 **Gupta S**, Allen-Vercoe E, Petrof EO. Fecal microbiota transplantation: in perspective. *Therap Adv Gastroenterol* 2016; **9**: 229-239 [PMID: 26929784 DOI: 10.1177/1756283X15607414]

83 **Sbahi H**, Di Palma JA. Faecal microbiota transplantation: applications and limitations in treating gastrointestinal disorders. *BMJ Open Gastroenterol* 2016; **3**: e000087 [PMID: 27239328 DOI: 10.1136/bmjgast-2016-000087]

84 **Mullish BH**, Quraishi MN, Segal JP, McCune VL, Baxter M, Marsden GL, Moore DJ, Colville A, Bhala N, Iqbal TH, Settle C, Kontkowski G, Hart AL, Hawkey PM, Goldenberg SD, Williams HRT. The use of faecal microbiota transplant as treatment for recurrent or refractory *Clostridium difficile* infection and other potential indications: joint British Society of Gastroenterology (BSG) and Healthcare Infection Society (HIS) guidelines. *Gut* 2018; **67**: 1920-1941 [PMID: 30154172 DOI: 10.1136/gutjnl-2018-316818]

85 **Khoruts A**, Staley C, Sadowsky MJ. Faecal microbiota transplantation for Clostridioides difficile: mechanisms and pharmacology. *Nat Rev Gastroenterol Hepatol* 2021; **18**: 67-80 [PMID: 32843743 DOI: 10.1038/s41575-020-0350-4]

86 **Segal JP**, Mullish BH, Quraishi MN, Iqbal T, Marchesi JR, Sokol H. Mechanisms underpinning the efficacy of faecal microbiota transplantation in treating gastrointestinal disease. *Therap Adv Gastroenterol* 2020; **13**: 1756284820946904 [PMID: 32952613 DOI: 10.1177/1756284820946904]

87 **van Nood E**, Vrieze A, Nieuwdorp M, Fuentes S, Zoetendal EG, de Vos WM, Visser CE, Kuijper EJ, Bartelsman JF, Tijssen JG, Speelman P, Dijkgraaf MG, Keller JJ. Duodenal infusion of donor feces for recurrent Clostridium difficile. *N Engl J Med* 2013; **368**: 407-415 [PMID: 23323867 DOI: 10.1056/NEJMoa1205037]

88 **Kim KO**, Gluck M. Fecal Microbiota Transplantation: An Update on Clinical Practice. *Clin Endosc* 2019; **52**: 137-143 [PMID: 30909689 DOI: 10.5946/ce.2019.009]

89 **Cammarota G**, Masucci L, Ianiro G, Bibbò S, Dinoi G, Costamagna G, Sanguinetti M, Gasbarrini

A. Randomised clinical trial: faecal microbiota transplantation by colonoscopy vs. vancomycin for the treatment of recurrent Clostridium difficile infection. *Aliment Pharmacol Ther* 2015; **41**: 835-843 [PMID: 25728808 DOI: 10.1111/apt.13144]

90 **Hui W**, Li T, Liu W, Zhou C, Gao F. Fecal microbiota transplantation for treatment of recurrent C. difficile infection: An updated randomized controlled trial meta-analysis. *PLoS One* 2019; **14**: e0210016 [PMID: 30673716 DOI: 10.1371/journal.pone.0210016]

91 **Kao D**, Roach B, Silva M, Beck P, Rioux K, Kaplan GG, Chang HJ, Coward S, Goodman KJ, Xu H, Madsen K, Mason A, Wong GK, Jovel J, Patterson J, Louie T. Effect of Oral Capsule- vs Colonoscopy-Delivered Fecal Microbiota Transplantation on Recurrent Clostridium difficile Infection: A Randomized Clinical Trial. *JAMA* 2017; **318**: 1985-1993 [PMID: 29183074 DOI: 10.1001/jama.2017.17077]

92 **Hamilton MJ**, Weingarden AR, Unno T, Khoruts A, Sadowsky MJ. High-throughput DNA sequence analysis reveals stable engraftment of gut microbiota following transplantation of previously frozen fecal bacteria. *Gut Microbes* 2013; **4**: 125-135 [PMID: 23333862 DOI: 10.4161/gmic.23571]

93 **Lee CH**, Steiner T, Petrof EO, Smieja M, Roscoe D, Nematallah A, Weese JS, Collins S, Moayyedi P, Crowther M, Ropeleski MJ, Jayaratne P, Higgins D, Li Y, Rau NV, Kim PT. Frozen vs Fresh Fecal Microbiota Transplantation and Clinical Resolution of Diarrhea in Patients With Recurrent Clostridium difficile Infection: A Randomized Clinical Trial. *JAMA* 2016; **315**: 142-149 [PMID: 26757463 DOI: 10.1001/jama.2015.18098]

94 **Jiang ZD**, Ajami NJ, Petrosino JF, Jun G, Hanis CL, Shah M, Hochman L, Ankoma-Sey V, DuPont AW, Wong MC, Alexander A, Ke S, DuPont HL. Randomised clinical trial: faecal microbiota transplantation for recurrent Clostridum difficile infection - fresh, or frozen, or lyophilised microbiota from a small pool of healthy donors delivered by colonoscopy. *Aliment Pharmacol Ther* 2017; **45**: 899-908 [PMID: 28220514 DOI: 10.1111/apt.13969]

95 **Agrawal M**, Aroniadis OC, Brandt LJ, Kelly C, Freeman S, Surawicz C, Broussard E, Stollman N, Giovanelli A, Smith B, Yen E, Trivedi A, Hubble L, Kao D, Borody T, Finlayson S, Ray A, Smith R. The Long-term Efficacy and Safety of Fecal Microbiota Transplant for Recurrent, Severe, and Complicated Clostridium difficile Infection in 146 Elderly Individuals. *J Clin Gastroenterol* 2016; **50**: 403-407 [PMID: 26352106 DOI: 10.1097/MCG.0000000000000410]

96 **Fischer M**, Sipe B, Cheng YW, Phelps E, Rogers N, Sagi S, Bohm M, Xu H, Kassam Z. Fecal microbiota transplant in severe and severe-complicated Clostridium difficile: A promising treatment approach. *Gut Microbes* 2017; **8**: 289-302 [PMID: 28001467 DOI: 10.1080/19490976.2016.1273998]

97 **DeFilipp Z**, Bloom PP, Torres Soto M, Mansour MK, Sater MRA, Huntley MH, Turbett S, Chung RT, Chen YB, Hohmann EL. Drug-Resistant *E. coli* Bacteremia Transmitted by Fecal Microbiota Transplant. *N Engl J Med* 2019; **381**: 2043-2050 [PMID: 31665575 DOI: 10.1056/NEJMoa1910437]

98 **Wilcox MH**, McGovern BH, Hecht GA. The Efficacy and Safety of Fecal Microbiota Transplant for Recurrent *Clostridium difficile* Infection: Current Understanding and Gap Analysis. *Open Forum Infect Dis* 2020; **7**: ofaa114 [PMID: 32405509 DOI: 10.1093/ofid/ofaa114]

99 **Kwak S**, Choi J, Hink T, Reske KA, Blount K, Jones C, Bost MH, Sun X, Burnham CD, Dubberke ER, Dantas G; CDC Prevention Epicenter Program. Impact of investigational microbiota therapeutic RBX2660 on the gut microbiome and resistome revealed by a placebo-controlled clinical trial. *Microbiome* 2020; **8**: 125 [PMID: 32862830 DOI: 10.1186/s40168-020-00907-9]

100 **Ford C**, Litcofsky K, McGovern B, Pardi D, Nathan R, Hansen V, Brennan R, Pullman J, Bernardo P, Tomlinson A, Horgan K, Bryant J, Walsh E, Rodriguez M, Rogalin H, Wang E, Henn M. 1503. Engraftment of investigational microbiome drug, SER-262, in subjects receiving vancomycin is associated with reduced rates of recurrence after primary Clostridium infection (CDI). *Open Forum Infect Dis* 2019; **6**: S547-S548 [DOI: 10.1093/ofid/ofz360.1367]

101 **McGovern BH**, Ford CB, Henn MR, Pardi DS, Khanna S, Hohmann EL, O'Brien EJ, Desjardins CA, Bernardo P, Wortman JR, Lombardo MJ, Litcofsky KD, Winkler JA, McChalicher CWJ, Li SS, Tomlinson AD, Nandakumar M, Cook DN, Pomerantz RJ, Auninš JG, Trucksis M. SER-109, an Investigational Microbiome Drug to Reduce Recurrence After Clostridioides difficile Infection: Lessons Learned From a Phase 2 Trial. *Clin Infect Dis* 2021; **72**: 2132-2140 [PMID: 32255488 DOI: 10.1093/cid/ciaa387]

102 **Quera R**, Espinoza R, Estay C, Rivera D. Bacteremia as an adverse event of fecal microbiota transplantation in a patient with Crohn's disease and recurrent Clostridium difficile infection. *J Crohns Colitis* 2014; **8**: 252-253 [PMID: 24184170 DOI: 10.1016/j.crohns.2013.10.002]

103 **Vendrik KEW**, Terveer EM, Kuijper EJ, Nooij S, Boeije-Koppenol E, Sanders IMJG, van Lingen E, Verspaget HW, Berssenbrugge EKL, Keller JJ, van Prehn J; Netherlands Donor Faeces Bank Study Group. Periodic screening of donor faeces with a quarantine period to prevent transmission of multidrug-resistant organisms during faecal microbiota transplantation: a retrospective cohort study. *Lancet Infect Dis* 2021; **21**: 711-721 [PMID: 33275940 DOI: 10.1016/S1473-3099(20)30473-4]

104 **US Food and Drug Administration**. Safety alert regarding use of fecal microbiota for transplantation and additional safety protections pertaining to SARS-CoV-2 and COVID-19. 2020. Available from: https://www.fda.gov/vaccines-blood-biologics/safety-availability-biologics/safety-alert-regarding-use-fecal-microbiota-transplantation-and-additional-safety-protections

105 **Joint FAO/WHO**. Guidelines for the evaluation of probiotics in food. 2002. Available from:

https://www.who.int/foodsafety/fs_management/en/probiotic_guidelines.pdf

106 **Hill C**, Guarner F, Reid G, Gibson GR, Merenstein DJ, Pot B, Morelli L, Canani RB, Flint HJ, Salminen S, Calder PC, Sanders ME. Expert consensus document. The International Scientific Association for Probiotics and Prebiotics consensus statement on the scope and appropriate use of the term probiotic. *Nat Rev Gastroenterol Hepatol* 2014; **11**: 506-514 [PMID: 24912386 DOI: 10.1038/nrgastro.2014.66]

107 **Hudson LE**, Anderson SE, Corbett AH, Lamb TJ. Gleaning Insights from Fecal Microbiota Transplantation and Probiotic Studies for the Rational Design of Combination Microbial Therapies. *Clin Microbiol Rev* 2017; **30**: 191-231 [PMID: 27856521 DOI: 10.1128/CMR.00049-16]

108 **Mills JP**, Rao K, Young VB. Probiotics for prevention of Clostridium difficile infection. *Curr Opin Gastroenterol* 2018; **34**: 3-10 [PMID: 29189354 DOI: 10.1097/MOG.0000000000000410]

109 **McFarland LV**, Surawicz CM, Greenberg RN, Fekety R, Elmer GW, Moyer KA, Melcher SA, Bowen KE, Cox JL, Noorani Z. A randomized placebo-controlled trial of Saccharomyces boulardii in combination with standard antibiotics for Clostridium difficile disease. *JAMA* 1994; **271**: 1913-1918 [PMID: 8201735]

110 **Gorbach SL**, Chang TW, Goldin B. Successful treatment of relapsing Clostridium difficile colitis with Lactobacillus GG. *Lancet* 1987; **2**: 1519 [PMID: 2892070 DOI: 10.1016/s0140-6736(87)92646-8]

111 **Pace F**, Pace M, Quartarone G. Probiotics in digestive diseases: focus on Lactobacillus GG. *Minerva Gastroenterol Dietol* 2015; **61**: 273-292 [PMID: 26657927]

112 **Cameron D**, Hock QS, Kadim M, Mohan N, Ryoo E, Sandhu B, Yamashiro Y, Jie C, Hoekstra H, Guarino A. Probiotics for gastrointestinal disorders: Proposed recommendations for children of the Asia-Pacific region. *World J Gastroenterol* 2017; **23**: 7952-7964 [PMID: 29259371 DOI: 10.3748/wjg.v23.i45.7952]

113 **Valdés-Varela L**, Gueimonde M, Ruas-Madiedo P. Probiotics for Prevention and Treatment of Clostridium difficile Infection. *Adv Exp Med Biol* 2018; **1050**: 161-176 [PMID: 29383669 DOI: 10.1007/978-3-319-72799-8_10]

114 **McFarland LV**, Ship N, Auclair J, Millette M. Primary prevention of Clostridium difficile infections with a specific probiotic combining Lactobacillus acidophilus, L. casei, and L. rhamnosus strains: assessing the evidence. *J Hosp Infect* 2018; **99**: 443-452 [PMID: 29702133 DOI: 10.1016/j.jhin.2018.04.017]

115 **Hickson M**, D'Souza AL, Muthu N, Rogers TR, Want S, Rajkumar C, Bulpitt CJ. Use of probiotic Lactobacillus preparation to prevent diarrhoea associated with antibiotics: randomised double blind placebo controlled trial. *BMJ* 2007; **335**: 80 [PMID: 17604300 DOI: 10.1136/bmj.39231.599815.55]

116 **Gao XW**, Mubasher M, Fang CY, Reifer C, Miller LE. Dose-response efficacy of a proprietary probiotic formula of Lactobacillus acidophilus CL1285 and Lactobacillus casei LBC80R for antibiotic-associated diarrhea and Clostridium difficile-associated diarrhea prophylaxis in adult patients. *Am J Gastroenterol* 2010; **105**: 1636-1641 [PMID: 20145608 DOI: 10.1038/ajg.2010.11]

117 **Barker AK**, Duster M, Valentine S, Hess T, Archbald-Pannone L, Guerrant R, Safdar N. A randomized controlled trial of probiotics for Clostridium difficile infection in adults (PICO). *J Antimicrob Chemother* 2017; **72**: 3177-3180 [PMID: 28961980 DOI: 10.1093/jac/dkx254]

118 **Zmora N**, Zilberman-Schapira G, Suez J, Mor U, Dori-Bachash M, Bashiardes S, Kotler E, Zur M, Regev-Lehavi D, Brik RB, Federici S, Cohen Y, Linevsky R, Rothschild D, Moor AE, Ben-Moshe S, Harmelin A, Itzkovitz S, Maharshak N, Shibolet O, Shapiro H, Pevsner-Fischer M, Sharon I, Halpern Z, Segal E, Elinav E. Personalized Gut Mucosal Colonization Resistance to Empiric Probiotics Is Associated with Unique Host and Microbiome Features. *Cell* 2018; **174**: 1388-1405.e21 [PMID: 30193112 DOI: 10.1016/j.cell.2018.08.041]

119 **Rothschild D**, Weissbrod O, Barkan E, Kurilshikov A, Korem T, Zeevi D, Costea PI, Godneva A, Kalka IN, Bar N, Shilo S, Lador D, Vila AV, Zmora N, Pevsner-Fischer M, Israeli D, Kosower N, Malka G, Wolf BC, Avnit-Sagi T, Lotan-Pompan M, Weinberger A, Halpern Z, Carmi S, Fu J, Wijmenga C, Zhernakova A, Elinav E, Segal E. Environment dominates over host genetics in shaping human gut microbiota. *Nature* 2018; **555**: 210-215 [PMID: 29489753 DOI: 10.1038/nature25973]

120 **So D**, Whelan K, Rossi M, Morrison M, Holtmann G, Kelly JT, Shanahan ER, Staudacher HM, Campbell KL. Dietary fiber intervention on gut microbiota composition in healthy adults: a systematic review and meta-analysis. *Am J Clin Nutr* 2018; **107**: 965-983 [PMID: 29757343 DOI: 10.1093/ajcn/nqy041]

121 **Gibson GR**, Hutkins R, Sanders ME, Prescott SL, Reimer RA, Salminen SJ, Scott K, Stanton C, Swanson KS, Cani PD, Verbeke K, Reid G. Expert consensus document: The International Scientific Association for Probiotics and Prebiotics (ISAPP) consensus statement on the definition and scope of prebiotics. *Nat Rev Gastroenterol Hepatol* 2017; **14**: 491-502 [PMID: 28611480 DOI: 10.1038/nrgastro.2017.75]

122 **Rätsep M**, Kõljalg S, Sepp E, Smidt I, Truusalu K, Songisepp E, Stsepetova J, Naaber P, Mikelsaar RH, Mikelsaar M. A combination of the probiotic and prebiotic product can prevent the germination of Clostridium difficile spores and infection. *Anaerobe* 2017; **47**: 94-103 [PMID: 28465256 DOI: 10.1016/j.anaerobe.2017.03.019]

123 **Sell TL**, Schaberg DR, Fekety FR. Bacteriophage and bacteriocin typing scheme for Clostridium difficile. *J Clin Microbiol* 1983; **17**: 1148-1152 [PMID: 6874905 DOI: 10.1128/JCM.17.6.1148-1152.1983]

124 **Fortier LC**, Moineau S. Morphological and genetic diversity of temperate phages in Clostridium difficile. *Appl Environ Microbiol* 2007; **73**: 7358-7366 [PMID: 17890338 DOI: 10.1128/AEM.00582-07]

125 **Mayer MJ**, Narbad A, Gasson MJ. Molecular characterization of a Clostridium difficile bacteriophage and its cloned biologically active endolysin. *J Bacteriol* 2008; **190**: 6734-6740 [PMID: 18708505 DOI: 10.1128/JB.00686-08]

126 **Fortier LC**. Bacteriophages Contribute to Shaping *Clostridioides (Clostridium) difficile* Species. *Front Microbiol* 2018; **9**: 2033 [PMID: 30233520 DOI: 10.3389/fmicb.2018.02033]

127 **Li T**, Zhang Y, Dong K, Kuo CJ, Li C, Zhu YQ, Qin J, Li QT, Chang YF, Guo X, Zhu Y. Isolation and Characterization of the Novel Phage JD032 and Global Transcriptomic Response during JD032 Infection of Clostridioides difficile Ribotype 078. *mSystems* 2020; **5** [PMID: 32371470 DOI: 10.1128/mSystems.00017-20]

128 **Drulis-Kawa Z**, Majkowska-Skrobek G, Maciejewska B. Bacteriophages and phage-derived proteins--application approaches. *Curr Med Chem* 2015; **22**: 1757-1773 [PMID: 25666799 DOI: 10.2174/0929867322666150209152851]

129 **Gebhart D**, Lok S, Clare S, Tomas M, Stares M, Scholl D, Donskey CJ, Lawley TD, Govoni GR. A modified R-type bacteriocin specifically targeting Clostridium difficile prevents colonization of mice without affecting gut microbiota diversity. *mBio* 2015; **6** [PMID: 25805733 DOI: 10.1128/mBio.02368-14]

130 **Mayer MJ**, Garefalaki V, Spoerl R, Narbad A, Meijers R. Structure-based modification of a Clostridium difficile-targeting endolysin affects activity and host range. *J Bacteriol* 2011; **193**: 5477-5486 [PMID: 21803993 DOI: 10.1128/JB.00439-11]

131 **Wang Q**, Euler CW, Delaune A, Fischetti VA. Using a Novel Lysin To Help Control Clostridium difficile Infections. *Antimicrob Agents Chemother* 2015; **59**: 7447-7457 [PMID: 26392484 DOI: 10.1128/AAC.01357-15]

132 **Meader E**, Mayer MJ, Steverding D, Carding SR, Narbad A. Evaluation of bacteriophage therapy to control Clostridium difficile and toxin production in an in vitro human colon model system. *Anaerobe* 2013; **22**: 25-30 [PMID: 23685029 DOI: 10.1016/j.anaerobe.2013.05.001]

133 **Loc-Carrillo C**, Abedon ST. Pros and cons of phage therapy. *Bacteriophage* 2011; **1**: 111-114 [PMID: 22334867 DOI: 10.4161/bact.1.2.14590]

134 **Nale JY**, Spencer J, Hargreaves KR, Buckley AM, Trzepiński P, Douce GR, Clokie MR. Bacteriophage Combinations Significantly Reduce Clostridium difficile Growth In Vitro and Proliferation In Vivo. *Antimicrob Agents Chemother* 2016; **60**: 968-981 [PMID: 26643348 DOI: 10.1128/AAC.01774-15]

135 **Selle K**, Fletcher JR, Tuson H, Schmitt DS, McMillan L, Vridhambal GS, Rivera AJ, Montgomery SA, Fortier LC, Barrangou R, Theriot CM, Ousterout DG. *In Vivo* Targeting of Clostridioides difficile Using Phage-Delivered CRISPR-Cas3 Antimicrobials. *mBio* 2020; **11** [PMID: 32156803 DOI: 10.1128/mBio.00019-20]

136 **Nale JY**, Redgwell TA, Millard A, Clokie MRJ. Efficacy of an Optimised Bacteriophage Cocktail to Clear Clostridium difficile in a Batch Fermentation Model. *Antibiotics (Basel)* 2018; **7** [PMID: 29438355 DOI: 10.3390/antibiotics7010013]

137 **Shan J**, Ramachandran A, Thanki AM, Vukusic FBI, Barylski J, Clokie MRJ. Bacteriophages are more virulent to bacteria with human cells than they are in bacterial culture; insights from HT-29 cells. *Sci Rep* 2018; **8**: 5091 [PMID: 29572482 DOI: 10.1038/s41598-018-23418-y]

138 **Caflisch KM**, Suh GA, Patel R. Biological challenges of phage therapy and proposed solutions: a literature review. *Expert Rev Anti Infect Ther* 2019; **17**: 1011-1041 [PMID: 31735090 DOI: 10.1080/14787210.2019.1694905]

139 **Hall JF**, Berger D. Outcome of colectomy for Clostridium difficile colitis: a plea for early surgical management. *Am J Surg* 2008; **196**: 384-388 [PMID: 18519126 DOI: 10.1016/j.amjsurg.2007.11.017]

140 **Neal MD**, Alverdy JC, Hall DE, Simmons RL, Zuckerbraun BS. Diverting loop ileostomy and colonic lavage: an alternative to total abdominal colectomy for the treatment of severe, complicated Clostridium difficile associated disease. *Ann Surg* 2011; **254**: 423-7; discussion 427 [PMID: 21865943 DOI: 10.1097/SLA.0b013e31822ade48]

141 **McFarland LV**, Elmer GW, Surawicz CM. Breaking the cycle: treatment strategies for 163 cases of recurrent Clostridium difficile disease. *Am J Gastroenterol* 2002; **97**: 1769-1775 [PMID: 12135033 DOI: 10.1111/j.1572-0241.2002.05839.x]

142 **Popovich KJ**, Calfee DP, Patel PK, Lassiter S, Rolle AJ, Hung L, Saint S, Chopra V. The Centers for Disease Control and Prevention STRIVE Initiative: Construction of a National Program to Reduce Health Care-Associated Infections at the Local Level. *Ann Intern Med* 2019; **171**: S2-S6 [PMID: 31569228 DOI: 10.7326/M18-3529]

143 **Anosova NG**, Brown AM, Li L, Liu N, Cole LE, Zhang J, Mehta H, Kleanthous H. Systemic antibody responses induced by a two-component Clostridium difficile toxoid vaccine protect against C. difficile-associated disease in hamsters. *J Med Microbiol* 2013; **62**: 1394-1404 [PMID: 23518659 DOI: 10.1099/jmm.0.056796-0]

144 **Sehgal K**, Khanna S. Immune response against *Clostridioides difficile* and translation to therapy. *Therap Adv Gastroenterol* 2021; **14**: 17562848211014817 [PMID: 33995585 DOI: 10.1177/17562848211014817]

145 **Lyerly DM**, Johnson JL, Frey SM, Wilkins TD. Vaccination against lethal Clostridium difficile

enterocolitis with a nontoxic recombinant peptide of toxin A. *Curr Microbiol* 1990; **21**: 29-32 [DOI: 10.1007/BF02090096]

146 **Ward SJ**, Douce G, Dougan G, Wren BW. Local and systemic neutralizing antibody responses induced by intranasal immunization with the nontoxic binding domain of toxin A from Clostridium difficile. *Infect Immun* 1999; **67**: 5124-5132 [PMID: 10496886 DOI: 10.1128/IAI.67.10.5124-5132.1999]

147 **Ghose C**, Verhagen JM, Chen X, Yu J, Huang Y, Chenesseau O, Kelly CP, Ho DD. Toll-like receptor 5-dependent immunogenicity and protective efficacy of a recombinant fusion protein vaccine containing the nontoxic domains of Clostridium difficile toxins A and B and Salmonella enterica serovar typhimurium flagellin in a mouse model of Clostridium difficile disease. *Infect Immun* 2013; **81**: 2190-2196 [PMID: 23545305 DOI: 10.1128/IAI.01074-12]

148 **Péchiné S**, Janoir C, Collignon A. Variability of Clostridium difficile surface proteins and specific serum antibody response in patients with Clostridium difficile-associated disease. *J Clin Microbiol* 2005; **43**: 5018-5025 [PMID: 16207956 DOI: 10.1128/JCM.43.10.5018-5025.2005]

149 **Wright A**, Drudy D, Kyne L, Brown K, Fairweather NF. Immunoreactive cell wall proteins of Clostridium difficile identified by human sera. *J Med Microbiol* 2008; **57**: 750-756 [PMID: 18480333 DOI: 10.1099/jmm.0.47532-0]

150 **Adamo R**, Romano MR, Berti F, Leuzzi R, Tontini M, Danieli E, Cappelletti E, Cakici OS, Swennen E, Pinto V, Brogioni B, Proietti D, Galeotti CL, Lay L, Monteiro MA, Scarselli M, Costantino P. Phosphorylation of the synthetic hexasaccharide repeating unit is essential for the induction of antibodies to Clostridium difficile PSII cell wall polysaccharide. *ACS Chem Biol* 2012; **7**: 1420-1428 [PMID: 22620974 DOI: 10.1021/cb300221f]

151 **Monteiro MA**, Ma Z, Bertolo L, Jiao Y, Arroyo L, Hodgins D, Mallozzi M, Vedantam G, Sagermann M, Sundsmo J, Chow H. Carbohydrate-based Clostridium difficile vaccines. *Expert Rev Vaccines* 2013; **12**: 421-431 [PMID: 23560922 DOI: 10.1586/erv.13.9]

152 **Legenza LM**, Barnett SG, Rose WE. Vaccines in development for the primary prevention of Clostridium difficile infection. *J Am Pharm Assoc (2003)* 2017; **57**: 547-549 [PMID: 28712463 DOI: 10.1016/j.japh.2017.06.007]

153 **Leuzzi R**, Adamo R, Scarselli M. Vaccines against Clostridium difficile. *Hum Vaccin Immunother* 2014; **10**: 1466-1477 [PMID: 24637887 DOI: 10.4161/hv.28428]

154 **Riley TV**, Lyras D, Douce GR. Status of vaccine research and development for Clostridium difficile. *Vaccine* 2019; **37**: 7300-7306 [PMID: 30902484 DOI: 10.1016/j.vaccine.2019.02.052]

155 **Bézay N**, Ayad A, Dubischar K, Firbas C, Hochreiter R, Kiermayr S, Kiss I, Pinl F, Jilma B, Westritschnig K. Safety, immunogenicity and dose response of VLA84, a new vaccine candidate against Clostridium difficile, in healthy volunteers. *Vaccine* 2016; **34**: 2585-2592 [PMID: 27079932 DOI: 10.1016/j.vaccine.2016.03.098]

156 **Tian JH**, Glenn G, Flyer D, Zhou B, Liu Y, Sullivan E, Wu H, Cummings JF, Elllingsworth L, Smith G. Clostridium difficile chimeric toxin receptor binding domain vaccine induced protection against different strains in active and passive challenge models. *Vaccine* 2017; **35**: 4079-4087 [PMID: 28669616 DOI: 10.1016/j.vaccine.2017.06.062]

157 **Donald RGK**, Flint M, Kalyan N, Johnson E, Witko SE, Kotash C, Zhao P, Megati S, Yurgelonis I, Lee PK, Matsuka YV, Severina E, Deatly A, Sidhu M, Jansen KU, Minton NP, Anderson AS. A novel approach to generate a recombinant toxoid vaccine against Clostridium difficile. *Microbiology (Reading)* 2013; **159**: 1254-1266 [PMID: 23629868 DOI: 10.1099/mic.0.066712-0]

158 **Vidunas E**, Mathews A, Weaver M, Cai P, Koh EH, Patel-Brown S, Yuan H, Zheng ZR, Carriere M, Johnson JE, Lotvin J, Moran J. Production and Characterization of Chemically Inactivated Genetically Engineered Clostridium difficile Toxoids. *J Pharm Sci* 2016; **105**: 2032-2041 [PMID: 27233688 DOI: 10.1016/j.xphs.2016.04.017]

159 **United Nations**, Department of Economic and Social Affairs, Population Division. World population aging 2019: Highlight. 2019. Available from: https://www.un.org/en/development/desa/population/publications/pdf/ageing/WorldPopulationAgeing2019-Report.pdf

World Journal of Gastroenterology

Submit a Manuscript: https://www.f6publishing.com

*World J Gastroenterol* 2021 November 14; 27(42): 7233-7239

DOI: 10.3748/wjg.v27.i42.7233

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

*OPINION REVIEW*

# Serologic diagnosis of celiac disease: May it be suitable for adults?

Giuseppe Losurdo, Milena Di Leo, Edoardo Santamato, Monica Arena, Maria Rendina, Carmelo Luigiano, Enzo Ierardi, Alfredo Di Leo

**ORCID number:** Giuseppe Losurdo 0000-0001-7038-3287; Milena Di Leo 0000-0002-5933-8474; Edoardo Santamato 0000-0002-1433-8729; Monica Arena 0000-0002-2958-3655; Maria Rendina 0000-0003-0077-6629; Carmelo Luigiano 0000-0001-5719-3948; Enzo Ierardi 0000-0001-7275-5080; Alfredo Di Leo 0000-0003-2026-1200.

**Author contributions:** Losurdo G, Luigiano C, Ierardi E, and Di Leo A planned the study; Di Leo M, Arena M, Rendina M, and Ierardi E searched for the data; Losurdo G, Di Leo M, Santamato E, and Ierardi E wrote the paper; Luigiano C and Di Leo A critically revised the paper; all authors read and approved the final version of the manuscript.

**Conflict-of-interest statement:** The authors declare no conflict of interest for this manuscript.



**Giuseppe Losurdo, Edoardo Santamato, Maria Rendina, Enzo Ierardi, Alfredo Di Leo,** Section of Gastroenterology, Department of Emergency and Organ Transplantation, University of Bari, Bari 70124, Italy

**Milena Di Leo, Monica Arena, Carmelo Luigiano,** Unit of Digestive Endoscopy, San Paolo Hospital, Milan 20090, Italy

**Corresponding author:** Giuseppe Losurdo, MD, Academic Fellow, Doctor, Section of Gastroenterology, Department of Emergency and Organ Transplantation, University of Bari, Piazza Giulio Cesare, Bari 70124, Italy. giuseppelos@alice.it

## Abstract

The diagnosis of coeliac disease (CD) in adult patients requires the simultaneous assessment of clinical presentation, serology, and typical histological picture of villous atrophy. However, several years ago, the European Society of Pediatric Gastroenterology, Hepatology, and Nutrition guidelines approved new criteria for the diagnosis in children: Biopsy could be avoided when anti-transglutaminase antibody (TGA) values exceed the cut-off of × 10 upper limit of normal (ULN) and anti-endomysium antibodies are positive, independently from value. This "no biopsy" approach is a decisive need for pediatric population, allowing to avoid stressful endoscopic procedures in children, if unnecessary. This approach relies on the correlation existing in children between TGA levels and assessment of mucosal atrophy according to Marsh's classification. Several lines of evidence have shown that patients with villous atrophy have markedly elevated TGA levels. Therefore, we aim to perform a narrative review on the topic in adults. Despite that some studies confirmed that the × 10 ULN threshold value has a very good diagnostic performance, several lines of evidence in adults suggest that TGA cut off should be different from that of pediatric population for reaching a good correlation with histological picture. In conclusion, the heterogeneity of study reports as well as some conditions, which may hamper the serological diagnosis of CD (such as seronegative CD and non-celiac villous atrophy) and are much more common in adults than in children, could represent a limitation for the "no biopsy" approach to CD diagnosis in patients outside the pediatric age.

**Key Words:** Celiac disease; Villous atrophy; Serology; Biopsy; Anti-transglutaminase antibody

**Specialty type:** Gastroenterology and hepatology

**Country/Territory of origin:** Italy

**Peer-review report's scientific quality classification**
Grade A (Excellent): 0
Grade B (Very good): B, B, B
Grade C (Good): 0
Grade D (Fair): 0
Grade E (Poor): 0

**Received:** March 21, 2021
**Peer-review started:** March 21, 2021
**First decision:** April 29, 2021
**Revised:** May 2, 2021
**Accepted:** October 25, 2021
**Article in press:** October 25, 2021
**Published online:** November 14, 2021

**P-Reviewer:** Balaban YH, Jin SY, Sabelnikova EA
**S-Editor:** Ma YJ
**L-Editor:** Wang TQ
**P-Editor:** Ma YJ



**Core Tip:** A "no biopsy" approach to celiac disease diagnosis, based only on anti-transglutaminase antibody titer, is a well-established strategy in children and an appealing matter of debate in adults. Indeed, the same strategy is recommended by pediatric guidelines, since it allows to avoid about one third of upper endoscopy procedures. In adults, literature on the topic is flourishing even if the topic is still under-investigated, results are heterogeneous, and some conditions may be relevant limiting factors.

**Citation:** Losurdo G, Di Leo M, Santamato E, Arena M, Rendina M, Luigiano C, Ierardi E, Di Leo A. Serologic diagnosis of celiac disease: May it be suitable for adults? *World J Gastroenterol* 2021; 27(42): 7233-7239
**URL:** https://www.wjgnet.com/1007-9327/full/v27/i42/7233.htm
**DOI:** https://dx.doi.org/10.3748/wjg.v27.i42.7233

## INTRODUCTION

Celiac disease (CD) is the most common immune-mediated enteropathy. It affects subjects with a genetic predisposition based on the presence of a human leukocyte antigen (HLA) DQ2/DQ8 haplotype and polymorphisms of several other inflammatory genes. The same genes are frequently involved in several other autoimmune conditions, and this explains why a high rate of coeliac patients suffer from at least another immune-mediated disease[1,2].

CD is the only autoimmune disease certainly triggered by an exogenous factor, *i.e.*, the ingestion of gluten. Gluten is a complex of alcohol soluble proteins such as gliadins, avenins, and secalins. These rich-in-proline and glutamine peptides are difficultly hydrolyzed by humans for the absence of an enzyme called prolyl-endopeptidase on the brush border of enterocytes[3].

The global prevalence of CD is about 1%[4]. However, considerable differences exist among various countries. Additionally, it is more frequent in females (2:1-3:1), like other autoimmune diseases. Diagnosis may occur in every moment of life. In the past, CD was considered a disease of the childhood[4], but nowadays the trend is changing because the 50% of new diagnoses occur in people over 50 years old. The most important difference between pediatric and adult patients concerns symptoms at onset: In children the intestinal signs are more frequent, while in adults the extra-intestinal manifestations are more typical[5].

Clinical manifestations may include both intestinal and extra-intestinal symptoms. Intestinal manifestations are diarrhea, dyspepsia, bloating, and abdominal pain. Extraintestinal findings are weight loss, iron deficiency anemia, microcytic or megaloblastic anemia, and osteopenia[6].

Malabsorption is the consequence of the mucosal injury caused by humoral and cell-mediated autoimmunity. In fact, tissue transglutaminase 2 (TTG2), an intestinal enzyme, makes gluten peptides toxic by reactions of transamidation and deamidation [7]. Plasma cells release IgA antibodies against both self-components of the mucosal layer and deamidated gluten peptides. IgA molecules pass into the bloodstream as antibodies against transglutaminase 2 (TGA), endomysium (EMA), and deamidated gliadin peptides (DGPs) and their detection is useful for the diagnosis of CD[4,8].

On the other side, an immune response mediated by CD3+ T cells takes place. These CD3+ T lymphocytes are called intraepithelial lymphocytes (IELs). IELs infiltrate the mucosal layer, thus damaging enterocytes. In CD, IELs are usually more than 25/100 enterocytes and lose their normal pattern of distribution in the villous area, which is called base-tip pattern and is characterized by a few number of IELs located at the base of the villi. Conversely, in CD, IELs are abnormally distributed in the whole surface of the villi[9].

The number of IELs is one of the two main histological criteria used for assessment of mucosal damage according to Marsh classification; the other one is the reduction of the villous-crypt ratio. In the normal duodenum, the villi are 3-fold longer than Lieberkhun crypt depth; in CD, the flattening of villi causes an inversion of the normal ratio from 3:1 to 1:1 until to 1:3.

**Table 1 Sensitivity and specificity of serologic tests**

| Antibody | Sensitivity (range) | Specificity (range) |
|---|---|---|
| IgA TGA | 98% (78%-100%) | 98% (90%-100%) |
| EMA | 95% (86%-100%) | 99% (97%-100%) |
| IgA DGP | 88% (74%-100%) | 95% (90%-99%) |
| IgG TGA | 70% (45%-95%) | 95% (94%-100%) |
| IgG DGP | 80% (63%-95%) | 98% (90%-99%) |

DGP: Deamidated gliadin antibodies; EMA: Anti-endomysium antibodies; TGA: Anti-transglutaminase antibodies.

These histological findings are assessed on biopsy samples taken from the duodenum. At least two samples from the bulb and four from the second part of the duodenum should be taken in order to obtain an adequate sample[10,11].

## DIAGNOSIS

Currently, a combination of clinical presentation, serology, and histology is required to diagnose CD in adults.

A patient with suggestive intestinal or extraintestinal symptoms/signs should undergo a serological analysis to assess the IgA levels: IgA-class TGA are the most sensitive and specific antibodies for CD even if they do not allow to diagnose CD alone. The IgA-class TGA test is performed by enzyme-linked immunosorbent assay. It is reliable and inexpensive, and represents the most sensitive test for CD (98%), with a very low percentage of false positive when the titer is more than 5-fold the upper limit of normal value[12]. The hypothesis of CD should be confirmed by IgA-class EMA positivity. Indeed, IgA-class EMA measurement is the most specific test (near to 100%) but the test is immunofluorescence–based, so it is operator dependent for its difficult interpretation[13].

In patients with an IgA deficiency (a frequent condition in celiac patients), IgG levels should be assessed[3]. A summary of diagnostic performance of serologic tests in CD is reported in Table 1[14].

Antibodies against DPGs are not very useful in diagnosis, except if the patient is less than 2 years old; they could be considered in the follow-up, because their variations are very rapid after the starting of a gluten free diet (GFD)[15,16].

In adult population, endoscopy with duodenal biopsy samples is considered the gold standard for CD diagnosis. Several endoscopic findings may suggest CD with a high sensitivity and specificity. However, more than 33% of CD patients have a normal endoscopic appearance, so biopsy samples should be collected in all patients with suspected CD irrespectively of endoscopic appearance. During upper GI endoscopy, at least 4-6 specimens should be collected, including samples from the duodenal bulb, in order to increase the diagnostic yield[17]. In each pass of biopsy forceps, the endoscopist should take only a single biopsy specimen[18]. However, at least 10% of specimens may not have an acceptable quality, due to insufficient size or lack of orientation and, sometimes, endoscopy should be repeated. Moreover, endoscopy is an invasive procedure with risk of complications and expensive, and the sedation is often required due to the duration of the procedure.

A level 3 in Marsh assessment corresponds to a complete villous atrophy and is required to diagnose CD.

However, the European Society of Pediatric Gastroenterology, Hepatology, and Nutrition (ESPGHAN) guidelines in these last years stated that a different diagnostic algorithm could be used for children.

Then, guidelines stated that, if clinical features are present, TGA level overcomes the threshold of 10 × UNL, and EMAs are positive, histology and genetics could not be carried out. This conclusion relies on the strong association between TGA and Marsh's grade of atrophy[19].

This approach, despite being not applicable to all children, has changed the clinical practice since, at least in children, upper endoscopy is not easily performed. It has been estimated that the cited cut-off may avoid endoscopy in 18% of celiac children, with a sensitivity of 96.3 and specificity of 98.6%[20]. In another study, 29% of children

could have avoided biopsy *as per* the 2020 ESPGHAN guidelines, and levels of TGA ≥ 60 U/mL or DGP ≥ 28 U/mL had a 100% specificity and 100% positive predictive value (PPV) for CD. HLA typing and EMA did not improve the PPV in patients with a TGA level ≥ 60 U/mL, but addition of DGP ≥ 28 U/mL improved the diagnostic sensitivity albeit maintaining the 100% specificity[21].

The promising data found in pediatric literature have, therefore, pushed researchers to investigate whether a pure serologic approach could be used in adults with suspicion of CD. Therefore, we aimed to perform a narrative review on the topic in adults.

## A NO-BIOPSY, SEROLOGY-BASED APPROACH IN ADULTS: CURRENT EVIDENCE

Several studies supported the "no-biopsy strategy" in adult population. Sugai *et al*[22], in a prospective study, evaluated the diagnostic accuracy of duodenal biopsy and serology for CD diagnosis (TGA and DGP), in two cohorts of subjects with different pre-test probabilities. In the high-risk group (161 enrolled patients), the prevalence of CD was 39.1%, while in the low-risk group (518 enrolled patients), the CD prevalence was 3.3%. Using assay combinations, it would be possible to confirm or rule out a diagnosis of CD without biopsy in 92% of cases in both pre-test populations. Salmi *et al*[23] compared histological examination to serum and intestinal celiac autoantibodies in untreated CD. They corroborated a high sensitivity and specificity of autoantibodies TGAs for detection of CD with villous atrophy. In 2008, Hill *et al*[24] found that an IgA-class TGA level of × 10 ULN could be used as a diagnostic cut-off with a positive predictive value of 100% for CD in adults. A similar cut-off of TGA antibody level (× 10) was suggested by Beltran *et al*[25] for CD diagnosis, with a 100% specificity. However, the authors emphasized the necessity of local validation for the cut-off value. The study of Penny *et al*[26] confirmed that an IgA-class TGA titer of × 10 ULN had a 100% specificity as a cut-off value for detection of Marsh type 3 lesions.

Other cut-off values of TGA levels have also been suggested. In a retrospective study, Holmes *et al*[27] enrolled 270 CD adults with IgA-TGA levels measured and small bowel biopsy samples. The authors found that a cut-off greater than 45 U/mL (> × 8 ULN + 2SDs) had a PPV of 100% for CD. The same value was suggested by Tortora *et al*[28]. In their study, a cut-off value of TGA of 45 U/mL had a sensitivity of 70% and specificity of 100% for predicting Marsh ≥ 2 lesions. Moreover, the authors found that the best cut-off for predicting villous atrophy was 62.4 U/mL (sensitivity 69%, specificity 100%). A lower cut-off value of TGA was found in the retrospective study of Zanini *et al*[29]: They demonstrated a 100% specificity for duodenal atrophy with a cut-off value of five times higher than the ULN. The application of this diagnostic approach could avoid upper GI endoscopy in one out of three patients. In a multicenter retrospective analysis enrolling both pediatric and adult patients who underwent small-bowel biopsy for suspicion of CD and positivity for both TGA and EMA, Alessio *et al*[30] demonstrated that a TGA level ≥ × 7 ULN was able to diagnose CD with a specificity and PPV close to 100%. On the other hand, Di Tola *et al*[31] determined that the best TGA serum level/cut-off ratio was > 3.6 with a sensitivity of 76.8 % and PPV of 97.2 %. The use of threshold value for CD diagnosis could avoid endoscopy with biopsy in 75% of the patients. The authors also found a strong correlation between TGA serum levels/cut-off ratio and the degree of duodenal lesions.

The combination of serology for IgA-TGA and IgA-EMA for CD diagnosis was retrospectively evaluated by Wakim-Fleming *et al*[32]. In their cohort, a value of serum IgA-class TGA greater than 118 U had only a 2% false-positive rate. While, if the value of serum IgA TGA was between 21 and 118, the value of EMA at least 1:60 had a PPV of 83% for CD. IgA-class TGA level less than 20 U, in combination with an EMA dilution titer less than 1:10, had a negative predictive value of 92% for CD.

Oyaert *et al*[33] evaluated the use of IgA-class TGA value associated with IgG-DGP antibody for CD diagnosis, in both pediatric and adult populations. Patients with double positivity and high antibody levels (> 3 times and > 10 times ULN) had a high probability of having CD (likelihood ratio ≥ 649 for > 3 times ULN and ∞ for > 10 times ULN). However, the sensitivity was significantly higher for all test combinations in the group aged younger than 16 years compared to the adult group.

The study by Efthymakis *et al*[34] found that the optimal cut-off anti-TGA value was ≥ × 16 ULN. In this study, 11 different assays were used for TGA titer determination. Analyzing the two more prevalent, the authors found different optimal cut-off values

(14.3 × ULN *vs* 3.7 × ULN), even after standardization (-0.14 *vs* -1.2).

## SEROLOGY AND PERSISTENT ATROPHY IN FOLLOW-UP

Key endpoints in the follow-up of CD patients are the absence of symptoms and the achievement of mucosal healing, *i.e.*, regression of atrophy. After 6-12 mo of adhering to a GFD, serology becomes negative in 80% of the patients and in 90% after 5 years.

Unfortunately, a normal TGA level at follow-up does not predict recovery of villous atrophy. Really, the lack of declining values and/or persistently positive serology 1 year after starting a GFD strongly suggest gluten contamination. Indeed, a recent meta-analysis demonstrated that IgA-class TGA and IgA-class EMA detected persistent villous atrophy with a high specificity (83%) but low sensitivity (50%).

Of interest, this study emphasized a presumable different CD diagnostic tool pattern between pediatric and adult ages. Indeed, the area under the curve for villous atrophy prediction was higher for children than for adults (0.879 *vs* 0.781)[16].

## CONCLUSION

Biopsy-free strategy is a promising approach for the diagnosis of CD in adult population, with a sensitivity and specificity close to 100%. However, it should be highlighted that in adults the diagnosis of CD may be more challenging than in children, since villous atrophy and increased IELs might be related not only to CD, but even to other pathologic conditions, including drug damage, infections, or functional gastrointestinal disorders[35-41].

On the other hand, seronegative CD is a rare condition that may be found in adults. It should be always kept into account when clinical symptoms are highly suggestive of the disorder despite the absence of serological markers and, in this case, histological examination is the mandatory diagnostic tool[42,43].

Moreover, the possibility of false positivity of TGA has been described, especially after viral respiratory infections[44].

In conclusion, despite that the results show that biopsy-free strategy may be promising in adults, some cautions should be taken into account before performing a fully serologic diagnosis of CD. Indeed, the topic is still under-investigated, the results of the studies are heterogeneous, and some conditions, such as seronegative CD or intestinal damage due to causes other than gluten, may be relevant limiting factors. Furthermore, since most of studies are retrospective, the real possibility of avoiding endoscopic examination for diagnosing CD in adults is still a matter of debate and requires further research. Therefore, further studies with a standardized approach are still required to evaluate this strategy and determine the best cut-off.

## REFERENCES


1 **Gutierrez-Achury J**, Coutinho de Almeida R, Wijmenga C. Shared genetics in coeliac disease and other immune-mediated diseases. *J Intern Med* 2011; **269**: 591-603 [PMID: 21401738 DOI: 10.1111/j.1365-2796.2011.02375.x]

2 **Kaukinen K**, Lindfors K, Collin P, Koskinen O, Mäki M. Coeliac disease--a diagnostic and therapeutic challenge. *Clin Chem Lab Med* 2010; **48**: 1205-1216 [PMID: 20578966 DOI: 10.1515/CCLM.2010.241]

3 **Biesiekierski JR**. What is gluten? *J Gastroenterol Hepatol* 2017; **32** Suppl 1: 78-81 [PMID: 28244676 DOI: 10.1111/jgh.13703]

4 **Rubin JE**, Crowe SE. Celiac Disease. *Ann Intern Med* 2020; **172**: ITC1-ITC16 [PMID: 31905394 DOI: 10.7326/AITC202001070]

5 **Singh P**, Arora A, Strand TA, Leffler DA, Catassi C, Green PH, Kelly CP, Ahuja V, Makharia GK. Global Prevalence of Celiac Disease: Systematic Review and Meta-analysis. *Clin Gastroenterol Hepatol* 2018; **16**: 823-836.e2 [PMID: 29551598 DOI: 10.1016/j.cgh.2017.06.037]

6 **Hujoel IA**, Reilly NR, Rubio-Tapia A. Celiac Disease: Clinical Features and Diagnosis. *Gastroenterol Clin North Am* 2019; **48**: 19-37 [PMID: 30711209 DOI: 10.1016/j.gtc.2018.09.001]

7 **Di Sabatino A**, Vanoli A, Giuffrida P, Luinetti O, Solcia E, Corazza GR. The function of tissue transglutaminase in celiac disease. *Autoimmun Rev* 2012; **11**: 746-753 [PMID: 22326684 DOI: 10.1016/j.autrev.2012.01.007]

8 **Tye-Din J**. Interpreting tests for coeliac disease: Tips, pitfalls and updates. *Aust J Gen Pract* 2018; **47**: 28-33 [PMID: 29429314 DOI: 10.31128/AFP-10-17-4357]

9 **Ensari A**. Gluten-sensitive enteropathy (celiac disease): controversies in diagnosis and classification. *Arch Pathol Lab Med* 2010; **134**: 826-836 [PMID: 20524861 DOI: 10.5858/134.6.826]

10 **Lagana SM**, Bhagat G. Biopsy Diagnosis of Celiac Disease: The Pathologist's Perspective in Light of Recent Advances. *Gastroenterol Clin North Am* 2019; **48**: 39-51 [PMID: 30711210 DOI: 10.1016/j.gtc.2018.09.003]

11 **Lebwohl B**, Kapel RC, Neugut AI, Green PH, Genta RM. Adherence to biopsy guidelines increases celiac disease diagnosis. *GastrointestEndosc* 2011; **74**: 103-109 [PMID: 21601201 DOI: 10.1016/j.gie.2011.03.1236]

12 **Li M**, Yu L, Tiberti C, Bonamico M, Taki I, Miao D, Murray JA, Rewers MJ, Hoffenberg EJ, Agardh D, Mueller P, Stern M, Bonifacio E, Liu E. A report on the International Transglutaminase Autoantibody Workshop for Celiac Disease. *Am J Gastroenterol* 2009; **104**: 154-163 [PMID: 19098864 DOI: 10.1038/ajg.2008.8]

13 **Lebwohl B**, Rubio-Tapia A, Assiri A, Newland C, Guandalini S. Diagnosis of celiac disease. *GastrointestEndosc Clin N Am* 2012; **22**: 661-677 [PMID: 23083985 DOI: 10.1016/j.giec.2012.07.004]

14 **Leffler DA**, Schuppan D. Update on serologic testing in celiac disease. *Am J Gastroenterol* 2010; **105**: 2520-2524 [PMID: 21131921 DOI: 10.1038/ajg.2010.276]

15 **Husby S**, Murray JA, Katzka DA. AGA Clinical Practice Update on Diagnosis and Monitoring of Celiac Disease-Changing Utility of Serology and Histologic Measures: Expert Review. *Gastroenterology* 2019; **156**: 885-889 [PMID: 30578783 DOI: 10.1053/j.gastro.2018.12.010]

16 **Silvester JA**, Kurada S, Szwajcer A, Kelly CP, Leffler DA, Duerksen DR. Tests for Serum Transglutaminase and Endomysial Antibodies Do Not Detect Most Patients With Celiac Disease and Persistent Villous Atrophy on Gluten-free Diets: a Meta-analysis. *Gastroenterology* 2017; **153**: 689-701.e1 [PMID: 28545781 DOI: 10.1053/j.gastro.2017.05.015]

17 **McCarty TR**, O'Brien CR, Gremida A, Ling C, Rustagi T. Efficacy of duodenal bulb biopsy for diagnosis of celiac disease: a systematic review and meta-analysis. *Endosc Int Open* 2018; **6**: E1369-E1378 [PMID: 30410959 DOI: 10.1055/a-0732-5060]

18 **Latorre M**, Lagana SM, Freedberg DE, Lewis SK, Lebwohl B, Bhagat G, Green PH. Endoscopic biopsy technique in the diagnosis of celiac disease: one bite or two? *GastrointestEndosc* 2015; **81**: 1228-1233 [PMID: 25638509 DOI: 10.1016/j.gie.2014.10.024]

19 **Husby S**, Koletzko S, Korponay-Szabó I, Kurppa K, Mearin ML, Ribes-Koninckx C, Shamir R, Troncone R, Auricchio R, Castillejo G, Christensen R, Dolinsek J, Gillett P, Hróbjartsson A, Koltai T, Maki M, Nielsen SM, Popp A, Størdal K, Werkstetter K, Wessels M. European Society Paediatric Gastroenterology, Hepatology and Nutrition Guidelines for Diagnosing Coeliac Disease 2020. *J Pediatr Gastroenterol Nutr* 2020; **70**: 141-156 [PMID: 31568151 DOI: 10.1097/MPG.0000000000002497]

20 **Ho SS**, Keenan JI, Day AS. Role of serological tests in the diagnosis of coeliac disease in children in New Zealand. *J Paediatr Child Health* 2020; **56**: 1906-1911 [PMID: 32946186 DOI: 10.1111/jpc.15076]

21 **Thompson G**, Grover Z, Loh R, Mews C, Ravikumara M, Jevon G, D'Orsogna L, McLean-Tooke A. Assessment of European Society of Paediatric Gastroenterology Hepatology and Nutrition (ESPGHAN) guidelines in an Australian paediatric population. *Pathology* 2020; **52**: 568-575 [PMID: 32586687 DOI: 10.1016/j.pathol.2020.05.002]

22 **Sugai E**, Moreno ML, Hwang HJ, Cabanne A, Crivelli A, Nachman F, Vázquez H, Niveloni S, Argonz J, Mazure R, La Motta G, Caniggia ME, Smecuol E, Chopita N, Gómez JC, Mauriño E, Bai JC. Celiac disease serology in patients with different pretest probabilities: is biopsy avoidable? *World J Gastroenterol* 2010; **16**: 3144-3152 [PMID: 20593499 DOI: 10.3748/wjg.v16.i25.3144]

23 **Salmi TT**, Collin P, Reunala T, Mäki M, Kaukinen K. Diagnostic methods beyond conventional histology in coeliac disease diagnosis. *Dig Liver Dis* 2010; **42**: 28-32 [PMID: 19473894 DOI: 10.1016/j.dld.2009.04.004]

24 **Hill PG**, Holmes GK. Coeliac disease: a biopsy is not always necessary for diagnosis. *Aliment PharmacolTher* 2008; **27**: 572-577 [PMID: 18194500 DOI: 10.1111/j.1365-2036.2008.03609.x]

25 **Beltran L**, Koenig M, Egner W, Howard M, Butt A, Austin MR, Patel D, Sanderson RR, Goubet S, Saleh F, Lavender J, Stainer E, Tarzi MD. High-titre circulating tissue transglutaminase-2 antibodies predict small bowel villous atrophy, but decision cut-off limits must be locally validated. *Clin Exp Immunol* 2014; **176**: 190-198 [PMID: 24325651 DOI: 10.1111/cei.12249]

26 **Penny HA**, Raju SA, Lau MS, Marks LJ, Baggus EM, Bai JC, Bassotti G, Bontkes HJ, Carroccio A, Danciu M, Derakhshan MH, Ensari A, Ganji A, Green PHR, Johnson MW, Ishaq S, Lebwohl B, Levene A, Maxim R, MohagheghShalmani H, Rostami-Nejad M, Rowlands D, Spiridon IA, Srivastava A, Volta U, Villanacci V, Wild G, Cross SS, Rostami K, Sanders DS. Accuracy of a no-biopsy approach for the diagnosis of coeliac disease across different adult cohorts. *Gut* 2021; **70**: 876-883 [PMID: 33139268 DOI: 10.1136/gutjnl-2020-320913]

27 **Holmes GKT**, Forsyth JM, Knowles S, Seddon H, Hill PG, Austin AS. Coeliac disease: further evidence that biopsy is not always necessary for diagnosis. *Eur J Gastroenterol Hepatol* 2017; **29**: 640-645 [PMID: 28441689 DOI: 10.1097/MEG.0000000000000841]

28 **Tortora R**, Imperatore N, Capone P, De Palma GD, De Stefano G, Gerbino N, Caporaso N, Rispo A. The presence of anti-endomysial antibodies and the level of anti-tissue transglutaminases can be used to diagnose adult coeliac disease without duodenal biopsy. *Aliment PharmacolTher* 2014; **40**: 1223-1229 [PMID: 25263177 DOI: 10.1111/apt.12970]

29 **Zanini B**, Magni A, Caselani F, Lanzarotto F, Carabellese N, Villanacci V, Ricci C, Lanzini A. High tissue-transglutaminase antibody level predicts small intestinal villous atrophy in adult patients at high risk of celiac disease. *Dig Liver Dis* 2012; **44**: 280-285 [PMID: 22119616 DOI: 10.1016/j.dld.2011.10.013]

30 **Alessio MG**, Tonutti E, Brusca I, Radice A, Licini L, Sonzogni A, Florena A, Schiaffino E, Marus W, Sulfaro S, Villalta D; Study Group on Autoimmune Diseases of Italian Society of Laboratory Medicine. Correlation between IgA tissue transglutaminase antibody ratio and histological finding in celiac disease. *J Pediatr Gastroenterol Nutr* 2012; **55**: 44-49 [PMID: 22197946 DOI: 10.1097/MPG.0b013e3182470249]

31 **Di Tola M**, Marino M, Goetze S, Casale R, Di Nardi S, Borghini R, Donato G, Tiberti A, Picarelli A. Identification of a serum transglutaminase threshold value for the noninvasive diagnosis of symptomatic adult celiac disease patients: a retrospective study. *J Gastroenterol* 2016; **51**: 1031-1039 [PMID: 26928588 DOI: 10.1007/s00535-016-1188-y]

32 **Wakim-Fleming J**, Pagadala MR, Lemyre MS, Lopez R, Kumaravel A, Carey WD, Zein NN. Diagnosis of celiac disease in adults based on serology test results, without small-bowel biopsy. *Clin Gastroenterol Hepatol* 2013; **11**: 511-516 [PMID: 23305824 DOI: 10.1016/j.cgh.2012.12.015]

33 **Oyaert M**, Vermeersch P, De Hertogh G, Hiele M, Vandeputte N, Hoffman I, Bossuyt X. Combining antibody tests and taking into account antibody levels improves serologic diagnosis of celiac disease. *Clin Chem Lab Med* 2015; **53**: 1537-1546 [PMID: 25719330 DOI: 10.1515/cclm-2013-1099]

34 **Efthymakis K**, Serio M, Milano A, Laterza F, Bonitatibus A, Di Nicola M, Neri M. Application of the Biopsy-Sparing ESPGHAN Guidelines for Celiac Disease Diagnosis in Adults: A Real-Life Study. *Dig Dis Sci* 2017; **62**: 2433-2439 [PMID: 28717844 DOI: 10.1007/s10620-017-4672-1]

35 **Lauwers GY**, Fasano A, Brown IS. Duodenal lymphocytosis with no or minimal enteropathy: much ado about nothing? *Mod Pathol* 2015; **28** Suppl 1: S22-S29 [PMID: 25560597 DOI: 10.1038/modpathol.2014.135]

36 **Losurdo G**, Giorgio F, Piscitelli D, Montenegro L, Covelli C, Fiore MG, Giangaspero A, Iannone A, Principi M, Amoruso A, Barone M, Di Leo A, Ierardi E. May the assessment of baseline mucosal molecular pattern predict the development of gluten related disorders among microscopic enteritis? *World J Gastroenterol* 2016; **22**: 8017-8025 [PMID: 27672296 DOI: 10.3748/wjg.v22.i35.8017]

37 **Ierardi E**, Losurdo G, Iannone A, Piscitelli D, Amoruso A, Barone M, Principi M, Pisani A, Di Leo A. Lymphocytic duodenitis or microscopic enteritis and gluten-related conditions: what needs to be explored? *Ann Gastroenterol* 2017; **30**: 380-392 [PMID: 28655974 DOI: 10.20524/aog.2017.0165]

38 **Malamut G**, Cerf-Bensussan N, Cellier C. Identification of new cases of severe enteropathy has recently increased the spectrum of intestinal non-celiac villous atrophy. *Expert Rev Gastroenterol Hepatol* 2015; **9**: 719-721 [PMID: 25990839 DOI: 10.1586/17474124.2015.1039990]

39 **Burbure N**, Lebwohl B, Arguelles-Grande C, Green PH, Bhagat G, Lagana S. Olmesartan-associated sprue-like enteropathy: a systematic review with emphasis on histopathology. *Hum Pathol* 2016; **50**: 127-134 [PMID: 26997446 DOI: 10.1016/j.humpath.2015.12.001]

40 **Losurdo G**, Piscitelli D, Ierardi E, Di Leo A. Intraepithelial lymphocytes: bystanders or causative factors in functional gastrointestinal disorders? *Cell Mol Immunol* 2021; **18**: 1620-1621 [PMID: 33479415 DOI: 10.1038/s41423-020-00614-3]

41 **Losurdo G**, Piscitelli D, Giangaspero A, Principi M, Buffelli F, Giorgio F, Montenegro L, Sorrentino C, Amoruso A, Ierardi E, Di Leo A. Evolution of nonspecific duodenal lymphocytosis over 2 years of follow-up. *World J Gastroenterol* 2015; **21**: 7545-7552 [PMID: 26140001 DOI: 10.3748/wjg.v21.i24.7545]

42 **Ierardi E**, Losurdo G, Piscitelli D, Giorgio F, Sorrentino C, Principi M, Montenegro L, Amoruso A, Di Leo A. Seronegative celiac disease: where is the specific setting? *Gastroenterol Hepatol Bed Bench* 2015; **8**: 110-116 [PMID: 25926935]

43 **Schiepatti A**, Sanders DS, Zuffada M, Luinetti O, Iraqi A, Biagi F. Overview in the clinical management of patients with seronegative villous atrophy. *Eur J Gastroenterol Hepatol* 2019; **31**: 409-417 [PMID: 30557227 DOI: 10.1097/MEG.0000000000001340]

44 **De Leo L**, Quaglia S, Ziberna F, Vatta S, Martelossi S, Maschio M, Not T. Serum anti-tissue transglutaminase antibodies detected during febrile illness may not be produced by the intestinal mucosa. *J Pediatr* 2015; **166**: 761-763 [PMID: 25722272 DOI: 10.1016/j.jpeds.2014.12.005]

World Journal of Gastroenterology

Submit a Manuscript: https://www.f6publishing.com

*World J Gastroenterol* 2021 November 14; 27(42): 7240-7246

DOI: 10.3748/wjg.v27.i42.7240

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

OPINION REVIEW

# Digital surgery for gastroenterological diseases

Niall Philip Hardy, Ronan Ambrose Cahill

**ORCID number:** Niall Philip Hardy 0000-0002-7036-3910; Ronan Ambrose Cahill 0000-0002-1270-4000.

**Author contributions:** Hardy NP and Cahill RA were involved in the ideation, collation and drafting of this work.

**Supported by** Disruptive Technologies and Innovation Fund, Enterprise Ireland, Ireland.

**Conflict-of-interest statement:** Cahill RA receives speaker fees from Stryker Corp, Johnson and Johnson/Ethicon and Olympus, consultancy fees from Touch Surgery and DistalMotion, and research funding from Intuitive Surgery. Cahill RA also holds research funding from EU Horizon 2020 with Palliare and the Irish Government in collaboration with IBM Research in Ireland and Deciphex. Hardy NP is employed as a researcher in this collaboration.



**Niall Philip Hardy, Ronan Ambrose Cahill,** UCD Centre for Precision Surgery, University College Dublin, Dublin D07 Y9AW, Ireland

**Corresponding author:** Ronan Ambrose Cahill, FRCS, MBChB, MD, Professor, UCD Centre for Precision Surgery, University College Dublin, Eccles Street, Dublin D07 Y9AW, Ireland. ronan.cahill@ucd.ie

## Abstract

Advances in machine learning, computer vision and artificial intelligence methods, in combination with those in processing and cloud computing capability, portend the advent of true decision support during interventions in real-time and soon perhaps in automated surgical steps. Such capability, deployed alongside technology intraoperatively, is termed digital surgery and can be delivered without the need for high-end capital robotic investment. An area close to clinical usefulness right now harnesses advances in near infrared endolaparoscopy and fluorescence guidance for tissue characterisation through the use of biophysics-inspired algorithms. This represents a potential synergistic methodology for the deep learning methods currently advancing in ophthalmology, radiology, and recently gastroenterology *via* colonoscopy. As databanks of more general surgical videos are created, greater analytic insights can be derived across the operative spectrum of gastroenterological disease and operations (including instrumentation and operative step sequencing and recognition, followed over time by surgeon and instrument performance assessment) and linked to value-based outcomes. However, issues of legality, ethics and even morality need consideration, as do the limiting effects of monopolies, cartels and isolated data silos. Furthermore, the role of the surgeon, surgical societies and healthcare institutions in this evolving field needs active deliberation, as the default risks relegation to bystander or passive recipient. This editorial provides insight into this accelerating field by illuminating the near-future and next decade evolutionary steps towards widespread clinical integration for patient and societal benefit.

**Key Words:** Digital surgery; Artificial intelligence; Gastrointestinal disease; Biophysics; Deep learning; Fluorescence-guided surgery

**Specialty type:** Gastroenterology and hepatology

**Country/Territory of origin:** Ireland

**Peer-review report's scientific quality classification**

Grade A (Excellent): 0
Grade B (Very good): B
Grade C (Good): C
Grade D (Fair): 0
Grade E (Poor): E

**Received:** April 30, 2021
**Peer-review started:** April 30, 2021
**First decision:** June 13, 2021
**Revised:** June 27, 2021
**Accepted:** October 20, 2021
**Article in press:** October 20, 2021
**Published online:** November 14, 2021

**P-Reviewer:** Hanada E, Qiang Y, Sharma J
**S-Editor:** Fan JR
**L-Editor:** Kerr C
**P-Editor:** Fan JR

**Core Tip:** Here, we introduce the concept of digital surgery and why it is important for everyone involved in the area of gastroenterological disease management. The current state and near-future of the art in this area are discussed, including the use of artificial intelligence methods to provide intraoperative real-time augmented decision making. Moral, ethical and legal challenges pertinent to digital surgery are explored, including the concerns relating to big data in health care and the transitioning role of industry in surgical development, as well as the implications such profound and imminent changes may have on the role of the clinician.

**Citation:** Hardy NP, Cahill RA. Digital surgery for gastroenterological diseases. *World J Gastroenterol* 2021; 27(42): 7240-7246
**URL:** https://www.wjgnet.com/1007-9327/full/v27/i42/7240.htm
**DOI:** https://dx.doi.org/10.3748/wjg.v27.i42.7240

## INTRODUCTION

Since the mid-20th century, our analog electromechanical world has increasingly transitioned to a digital and even automated one, leading to our current epoch being named the "Digital Age" or "Information Age". While many sectors have been rapid converts to this new order (notably retail and finance), adoption in the healthcare domain has been slower. In particular, in surgery, the focus has been on incremental iterations of existing technologies (such as upgraded displays and tweaked surgical instruments) over radical reformatting of existing methods. The reasons behind this are multifactorial, including a natural and understandable professional conservatism regarding clinical care, along with privacy concerns (patient and practitioner), ethical, moral and liability uncertainties, as well as a potential reluctance on behalf of system developers to involve themselves in intraprocedural decision making (preferring instead to leave this responsibility firmly in the hands of the human operator, for now at least). However, the evolution of surgical practice has plateaued.

Even the most sophisticated of new surgical machinery in general surgery, such as present-day robotic platforms, depend fully on the practitioner for their value and have led only to marginal benefit over the past 20 years. This is partly because of inequity in access but mostly because the fundamental distinguishing characteristic of best operative care relates to the intraoperative decision-making rather than dexterity of the practitioner. The new great hope is that the digitalisation of surgery, including the integration of artificial intelligence (AI) to the surgical workflow, will lead to better outcomes broadly; although, how exactly this will happen remains the challenge. Specifically, "digital surgery" exploits real-time analytics with technology during operations, and this editorial provides perspectives of its impact now and in the near future to all those looking after patients with digestive diseases.

## AI DECISION-PROMPTING IN GASTROENTEROLOGY

This month's landmark publication of the European Commission's "Laying Down Harmonised Rules on Artificial Intelligence" attempts to shape the future of AI's incorporation across society[1]. In this document, the enormity of the socioeconomic potential of AI is stressed, and it emphasizes that the same elements that drive benefits will bring about new risks of potential great consequence. Also this month, the first commercial AI system for endoscopy, "GI Genius" by Medtronic (Dublin, Ireland), has been approved for clinical use by the United States Food and Drug Administration (FDA)[2]. This system and others like it (*e.g.*, Olympus ENDO-AID) act as a "second pair of eyes", constantly watching the screen and alerting the user to any potential anomaly by highlighting the detected area and leaving the human to decide on its importance[3]. Unlike the endoscopist, the computer aid does not have a single point of focus on the screen and so provides accurate full field of view observation at all times, a digital safety net. While impressive results are reported regarding increased polyp detection rates by this combination (14% absolute increase in adenoma detection rate over standard endoscopy), intra-procedural polyp characterisation remains

unsupported. Detailed descriptions of AI advances in endoscopic systems have been covered elsewhere[4-6].

When assessing such applications, the FDA stratifies the risk associated with the AI tool by the device's intended use[7]. Technologies designed to treat and diagnose or to drive clinical management receive higher risk ratings than those that aim to inform clinical management. Furthermore, to date, the FDA has only approved AI or machine learning systems that are "locked" prior to marketing. In locked systems, the algorithms used must provide the same output each time the same input is provided ( *i.e.*, these systems cannot "learn" or adapt as they accrue more data, which really should be their hallmark). However, it is understandable that these early models should function within the safety of a locked domain while the appropriate regulatory and surveillance frameworks for unlocked AI are put in place. Such oversight would need to ensure that the performance and effectiveness of an unlocked system in clinical use was maintained as it evolved through learning over time. Furthermore, locking helps to ensure that the functionality of a device can be understood by clinicians and its abilities not overstated.

As more advanced systems come online, however, it is likely such "unlocked" systems will become approved for use. Therefore, the endoscopic support systems currently approved for use only represent a first pass at clinical entry, allowing sequential iterations to evolve serially with increasing degrees of data recording, analytics and self-learning, and therefore automatism.

## AI IN SURGERY

While other areas of surgical practice, such as orthopedics, neurosurgery and maxillo-facial surgery, have embraced surgical preplanning through computer-generated models and intraoperative navigational aids, the incorporation of AI methods for gastrointestinal surgery is only in its infancy[8-10]. The lack of fixed, easily identifiable landmarks and the tendency for the operator to completely shift the anatomical field intraoperatively (from the preoperatively recorded imagery) has proven difficult to accommodate in the same way as is possible with interventions in osseous lattices. Furthermore, the most valuable asset in the advancement of AI methods has been the rapid ability to accumulate, store, annotate and distribute data streams. As a result, it has been most applicable to nonsurgical uses, where enormous pre-existing archives of categorised images exist (with data points in the millions), such as breast mammography and retinal screening[11,12]. For similar impact in surgery, computer methods need integration with surgical video, which is inherently more complex and, for this reason, harder to extract meaningful datapoints from.

Indeed, the necessary technology is only now proving sufficiently capable for such analysis to be envisaged, and the main rush right now relates to the accumulation of videos for future exploitation. The major companies are, therefore, looking at placing systems alongside their core imaging technology that allow uploading of material at the operator's discretion rather than automatically. This is because, with some exceptions, the prevailing business model has been to sell imaging systems with the imagery belonging fully to the purchaser. With the current realisation that such data have real value on an aggregated basis, it is likely that the next full-service subscription models will include data sharing as a contractual clause. Therefore, the technical obstacles of video set-up, storage and distribution have already diminished in this era of big data, leaving then annotation, curation and linking to metadata to be addressed.

## IMAGE-GUIDED SURGERY

Surgical video contains more information than still images do, which means greater value but also complexity. Superimposition of more easily interpreted signals to these videos greatly enables data extraction. One such method successfully used in this manner utilizes near infrared imaging and indocyanine green, a fluorescent dye, to disclose information regarding the perfusion status of tissues in surgical videos and, hence, characterise their nature[13-15]. Indocyanine green binds to albumin within the vasculature, and real-time tracking of fluorescence intensity as it circulates can be used to create perfusion signatures of tissues. Alongside prediction of healing–nonhealing risk, this technique has been successfully piloted for the interrogation and classification of neoplasia of the colorectum using computer vision and AI with high levels

of accuracy intraoperatively based on biophysics-inspired principles (*i.e.*, perfusion within malignant tissue is fundamentally altered when compared to adjacent healthy regions in the same field of view[16]) (Figure 1).

## BEYOND FLUORESCENCE

Accurate computerised identification and labelling of structures at operation, through fluorescence or otherwise, could soon lead to better, safer surgery. Automated localisation with operator-prompting for important structures, such as the inferior mesenteric artery, ureter or neurological bundles, during left-sided colonic surgery would assist experienced surgeons in difficult cases or normalise for experience in those at the beginning of their career or with low volume practices. Deep learning models have even been demonstrated to accurately identify when the critical view of safety has been demonstrated during laparoscopic cholecystectomies[17]. Once a tissue can be accurately characterised, the next logical progression from on-screen display is the integration of such data into smart instruments to guide operative steps. This may be by way of haptic or auditory feedback or even automatic shutoff in the case of destructive instruments, such as diathermy encountering structures that require safeguarding (this already exists with some orthopaedic instrumentation).

More rudimentary systems of instrument tracking and operative step identification are already available that allow retrospective auditing of performed operations and instrument efficiency[18,19]. Parsing of operations into component steps linked with metadata including operative costs and standard (*e.g.* hospital stay and complications requiring intervention) as well as evolved patient metrics (*e.g.*, patient-reported outcome measures) could greatly facilitate our move toward value-based health care. Early-stage iterations are now emerging, with automated operative phase identification in peroral endoscopic myotomy recently being described with the hope that further technological refinement may facilitate intraoperative decision support[20].

## SURGICAL VIDEO DATA BANKS AT SCALE

Collation of surgical outcomes and surgical performance, either by the individuals wishing to gain insight into their own practice or as part of professional regulatory oversight, still presents potential controversies. The centralised deposition of surgical video followed by anonymised analysis of technique and outcomes, likely by other specialty practitioners initially and followed by AI when permissible, would provide a means for impartial appraisal and maintenance of standards (both of practitioners and indeed surgical instruments). However, there are risks to use of the data to promote commercial interests rather than the public good (this also applies to practitioners and/or institutions); as such, great transparency is needed and trust-boards should be considered. In addition, practitioners have privacy rights, as do patients, and the special status of the doctor-patient relationship prevents exploitation of this privilege, which is an important consideration alongside legality alone (*e.g.*, respect of frameworks such as General Data Protection Regulation). Furthermore, aggregation of surgical video in siloed collections, however individually large, risks lack of proper representation. Closed datasets also limit progress if others with relevant interest are excluded (frustrating the principle of reusability as a findability, accessibility, interoperability, and reusability principle), which may precipitate monopolistic practices.

## AI PITFALLS AND CONCERNS

The relatively delayed adoption of AI within surgery permits a glimpse into potential pitfalls by observing the experience of other sectors. In October 2020, the United States House Antitrust Subcommittee Report on Competition in the Digital Market noted that, in many digital domains, few dominant corporations held singular control of channels of mass distribution in a fashion that allowed them to maintain power and absorb or remove competitors with ease[21]. This is a real concern, considering the enormous financial incentives associated with success in healthcare provision. Potential safeguards include an open/shared data repository (with appropriate regulatory compliance) upon which clinicians, academics and companies could work, as well as the standardisation of policies for the acquisition and storage of digital data,

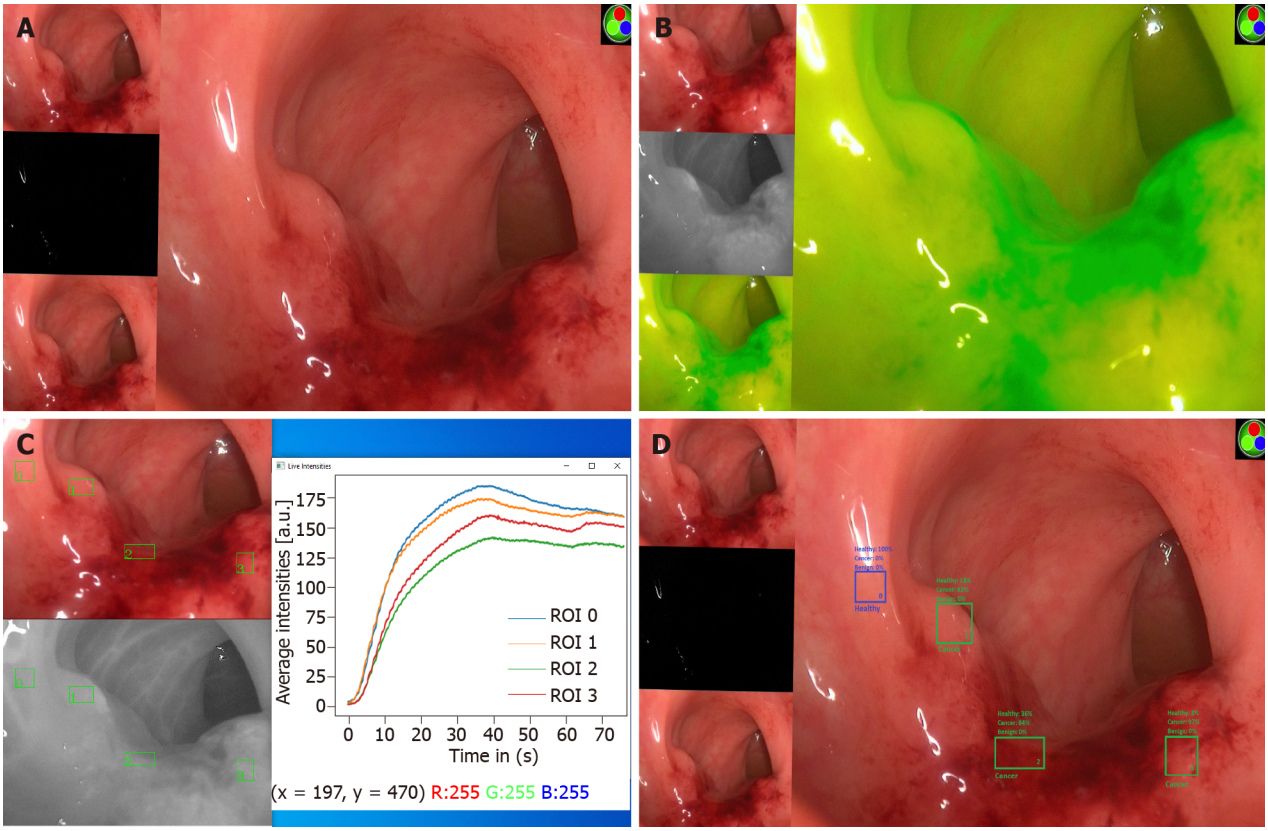


**Figure 1 Digital surgery in action–real-time discrimination of tissue nature using fluorescence imaging and artificial intelligence.** A: Transanal imaging of a rectal lesion using a Pinpoint (Novadaq, Mississauga, Canada; Stryker, Kalamazoo, MI, United States) near infrared imaging system; B: Following intravenous administration of indocyanine green (ICG) (0.25 mg/kg), fluorescence is observed within the lesion and surrounding tissue; C: In tandem with the ICG administration, regions of interest within healthy and unhealthy tissue (regions 0–3) are chosen by the surgeon for real-time assessment. Image tracking is performed using the visible light mode and light intensity readings extracted from the corresponding regions within the infrared spectrum video for each of these regions over time; D: Intensity profiles are subsequently fitted to biophysics-inspired artificial intelligence models of fluid movement in tissue to predict tissue nature with a binary outcome (healthy *vs* cancer) and a probability score (%).

to ensure universal accessibility. Additionally, the history of AI is replete with "boom and bust" cycles, and it is possible that its promised integration into surgery could prove another false dawn, especially with regard to more complex unsupervised machine learning and deep learning methods[22]. This may be a result of the complexity required to fine tune these systems to achieve acceptable results for use in humans. Controversies surrounding explainability (describing the settings used in the machine), interpretability (the degree to which a human can understand the decision) and bias within data training sets, as well as possible societal rejection of this form of technology in healthcare (akin to the criticisms of AI-based facial recognition software and recidivism predictors or any perception of data harvesting for surveillance capitalism), are other concerns.

## CONCLUSION

Nevertheless, the formative years of digital surgery have begun. As a consequence, the landscape of surgical practice as it currently stands is likely to undergo rapid and most probably frequent evolutions. Such changes will have far reaching implications on the role of professional bodies, societies and institutions and, most fundamentally, clinicians. Moore's law cautions that the limits of such changes will be neither technological nor computational. The role of implementation of digital surgery remains, for now, with the clinician in their duty as patient caregiver. Whether this role will, in time, increasingly become one of the clinician as early enabler and later bystander or clinician as co-developer and collaborator remains yet to be determined. For the latter, co-ordinated, purposeful cross-collegiate action is needed urgently.

## REFERENCES


1 **European Commission**. Laying Down Harmonised Rules on Artificial Intelligence (Artificial Intelligence Act) and Amending Certain Union Legislative Acts. Brussels. 2021. [cited 1 June 2021]. Available from: https://eur-lex.europa.eu/legal-content/EN/TXT/HTML/?uri=CELEX:52021PC0206&from=EN

2 **US Food and Drug Association (FDA)**. Gastrointestinal lesion software detection Regulation Number 21 CRF 876.1520. 2021. [cited 1 June 2021]. Available from: https://www.accessdata.fda.gov/cdrh_docs/pdf20/DEN200055.pdf

3 **Repici A**, Badalamenti M, Maselli R, Correale L, Radaelli F, Rondonotti E, Ferrara E, Spadaccini M, Alkandari A, Fugazza A, Anderloni A, Galtieri PA, Pellegatta G, Carrara S, Di Leo M, Craviotto V, Lamonaca L, Lorenzetti R, Andrealli A, Antonelli G, Wallace M, Sharma P, Rosch T, Hassan C. Efficacy of Real-Time Computer-Aided Detection of Colorectal Neoplasia in a Randomized Trial. *Gastroenterology* 2020; **159**: 512-520.e7 [PMID: 32371116 DOI: 10.1053/j.gastro.2020.04.062]

4 **Yang YJ**, Bang CS. Application of artificial intelligence in gastroenterology. *World J Gastroenterol* 2019; **25**: 1666-1683 [PMID: 31011253 DOI: 10.3748/wjg.v25.i14.1666]

5 **Abadir AP**, Ali MF, Karnes W, Samarasena JB. Artificial Intelligence in Gastrointestinal Endoscopy. *Clin Endosc* 2020; **53**: 132-141 [PMID: 32252506 DOI: 10.5946/ce.2020.038]

6 **El Hajjar A**, Rey JF. Artificial intelligence in gastrointestinal endoscopy: general overview. *Chin Med J (Engl)* 2020; **133**: 326-334 [PMID: 31929362 DOI: 10.1097/CM9.0000000000000623]

7 **US Food and Drug Association (FDA)**. Artificial Intelligence/Machine Learning (AI/ML)-Based Software as a Medical Device (SaMD) Action Plan www.fda.gov. 2021. [cited 25 June 2021]. Available from: https://www.fda.gov/files/medical%20devices/published/US-FDA-Artificial-Intelligence-and-Machine-Learning-Discussion-Paper.pdf

8 **Azagury DE**, Dua MM, Barrese JC, Henderson JM, Buchs NC, Ris F, Cloyd JM, Martinie JB, Razzaque S, Nicolau S, Soler L, Marescaux J, Visser BC. Image-guided surgery. *Curr Probl Surg* 2015; **52**: 476-520 [PMID: 26683419 DOI: 10.1067/j.cpsurg.2015.10.001]

9 **Myers TG**, Ramkumar PN, Ricciardi BF, Urish KL, Kipper J, Ketonis C. Artificial Intelligence and Orthopaedics: An Introduction for Clinicians. *J Bone Joint Surg Am* 2020; **102**: 830-840 [PMID: 32379124 DOI: 10.2106/JBJS.19.01128]

10 **Heo MS**, Kim JE, Hwang JJ, Han SS, Kim JS, Yi WJ, Park IW. Artificial intelligence in oral and maxillofacial radiology: what is currently possible? *Dentomaxillofac Radiol* 2021; **50**: 20200375 [PMID: 33197209 DOI: 10.1259/dmfr.20200375]

11 **McKinney SM**, Sieniek M, Godbole V, Godwin J, Antropova N, Ashrafian H, Back T, Chesus M, Corrado GS, Darzi A, Etemadi M, Garcia-Vicente F, Gilbert FJ, Halling-Brown M, Hassabis D, Jansen S, Karthikesalingam A, Kelly CJ, King D, Ledsam JR, Melnick D, Mostofi H, Peng L, Reicher JJ, Romera-Paredes B, Sidebottom R, Suleyman M, Tse D, Young KC, De Fauw J, Shetty S. International evaluation of an AI system for breast cancer screening. *Nature* 2020; **577**: 89-94 [PMID: 31894144 DOI: 10.1038/s41586-019-1799-6]

12 **Ting DSW**, Cheung CY, Lim G, Tan GSW, Quang ND, Gan A, Hamzah H, Garcia-Franco R, San Yeo IY, Lee SY, Wong EYM, Sabanayagam C, Baskaran M, Ibrahim F, Tan NC, Finkelstein EA, Lamoureux EL, Wong IY, Bressler NM, Sivaprasad S, Varma R, Jonas JB, He MG, Cheng CY, Cheung GCM, Aung T, Hsu W, Lee ML, Wong TY. Development and Validation of a Deep Learning System for Diabetic Retinopathy and Related Eye Diseases Using Retinal Images From Multiethnic Populations With Diabetes. *JAMA* 2017; **318**: 2211-2223 [PMID: 29234807 DOI: 10.1001/jama.2017.18152]

13 **Cahill RA**, O'Shea DF, Khan MF, Khokhar HA, Epperlein JP, Mac Aonghusa PG, Nair R, Zhuk SM. Artificial intelligence indocyanine green (ICG) perfusion for colorectal cancer intra-operative tissue classification. *Br J Surg* 2021; **108**: 5-9 [PMID: 33640921 DOI: 10.1093/bjs/znaa004]

14 **Zhuk S**, Epperlein JP, Nair R, Thirupati S, Mac Aonghusa P, Cahill R, O'Shea D. Perfusion Quantification from Endoscopic Videos: Learning to Read Tumor Signatures. 2020 Preprint. Available from: arXiv:2006.14321

15 **Park SH**, Park HM, Baek KR, Ahn HM, Lee IY, Son GM. Artificial intelligence based real-time microcirculation analysis system for laparoscopic colorectal surgery. *World J Gastroenterol* 2020; **26**: 6945-6962 [PMID: 33311942 DOI: 10.3748/wjg.v26.i44.6945]

16 **Hardy NP**, Mac Aonghusa P, Neary PM, Cahill RA. Intraprocedural Artificial Intelligence for Colorectal Cancer Detection and Characterisation in Endoscopy and Laparoscopy. *Surg Innov* 2021; 1553350621997761 [PMID: 33634722 DOI: 10.1177/1553350621997761]

17 **Mascagni P**, Alapatt D, Urade T, Vardazaryan A, Mutter D, Marescaux J, Costamagna G, Dallemagne B, Padoy N. A Computer Vision Platform to Automatically Locate Critical Events in Surgical Videos: Documenting Safety in Laparoscopic Cholecystectomy. *Ann Surg* 2021; **274**: e93-e95 [PMID: 33417329 DOI: 10.1097/SLA.0000000000004736]

18 **Aghdasi N**, Bly R, White LW, Hannaford B, Moe K, Lendvay TS. Crowd-sourced assessment of surgical skills in cricothyrotomy procedure. *J Surg Res* 2015; **196**: 302-306 [PMID: 25888499 DOI: 10.1016/j.jss.2015.03.018]

19 **Tulipan J**, Miller A, Park AG, Labrum JT 4th, Ilyas AM. Touch Surgery: Analysis and Assessment of Validity of a Hand Surgery Simulation "App". *Hand (N Y)* 2019; **14**: 311-316 [PMID: 29363359 DOI: 10.1177/1558944717751192]

20 **Ward TM**, Hashimoto DA, Ban Y, Rattner DW, Inoue H, Lillemoe KD, Rus DL, Rosman G, Meireles OR. Automated operative phase identification in peroral endoscopic myotomy. *Surg Endosc* 2021; **35**: 4008-4015 [PMID: 32720177 DOI: 10.1007/s00464-020-07833-9]
21 **United States Subcommittee on Antitrust**. Commercial and Administrative Law of the Committee on the Judiciary: Investigation of Competition in Digital Markets. 2020. [cited 1 June 2021]. Available from: https://judiciary.house.gov/uploadedfiles/competition_in_digital_markets.pdf
22 **Mitchell M**. Why AI is Harder Than We Think. 2021 Preprint. Available from: arXiv:2104.12871

World Journal of Gastroenterology

Submit a Manuscript: https://www.f6publishing.com

*World J Gastroenterol* 2021 November 14; 27(42): 7247-7270

DOI: 10.3748/wjg.v27.i42.7247

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

*REVIEW*

# Gossip in the gut: Quorum sensing, a new player in the host-microbiota interactions

Garance Coquant, Doriane Aguanno, Sandrine Pham, Nathan Grellier, Sophie Thenet, Véronique Carrière, Jean-Pierre Grill, Philippe Seksik

**ORCID number:** Garance Coquant 0000-0002-4422-8483; Doriane Aguanno 0000-0002-4154-3592; Sandrine Pham 0000-0003-0036-5862; Nathan Grellier 0000-0002-0452-3218; Sophie Thenet 0000-0002-0336-2942; Véronique Carrière 0000-0002-6263-0847; Jean-Pierre Grill 0000-0001-9434-3706; Philippe Seksik 0000-0003-3596-9893.

**Author contributions:** Coquant G and Seksik P designed the outline of the review; Coquant G, Grill JP and Seksik P wrote part I; Coquant G, Aguanno D, Thenet S, Carrière V and Seksik P wrote part II; Aguanno D, Coquant G, Pham S and Grellier N wrote part III; Aguanno D and Coquant G made the figures; Coquant G and Aguanno D harmonized and formatted the different sections; all authors have reviewed the entire manuscript.

**Supported by** Fondation pour la Recherche Médicale, No. EQU202003010171; Association François Aupetit, No. AHLs 2019 and No. AHLs 2021; Fondation pour la Recherche Médical FRM, No. ECO201806006843 (to Coquant G); and CORDDIM, Ile-de-France Region (to Aguanno D).

**Conflict-of-interest statement:** Seksik P reports consulting fees from Abbvie, Takeda, Merck-MSD,

**Garance Coquant, Doriane Aguanno, Sandrine Pham, Nathan Grellier, Sophie Thenet, Véronique Carrière, Jean-Pierre Grill, Philippe Seksik,** Centre de Recherche Saint-Antoine, INSERM, Sorbonne Université, Paris 75012, France

**Doriane Aguanno, Sandrine Pham, Sophie Thenet,** EPHE, PSL University, Paris 75014, France

**Philippe Seksik,** Department of Gastroenterology and Nutrition, Saint-Antoine Hospital, APHP, Paris 75012, France

**Corresponding author:** Philippe Seksik, MD, PhD, Professor, Department of Gastroenterology and Nutrition, Saint-Antoine Hospital, APHP, 184, rue du Faubourg Saint-Antoine, Paris 75012, France. philippe.seksik@aphp.fr

## Abstract

Bacteria are known to communicate with each other and regulate their activities in social networks by secreting and sensing signaling molecules called autoinducers, a process known as quorum sensing (QS). This is a growing area of research in which we are expanding our understanding of how bacteria collectively modify their behavior but are also involved in the crosstalk between the host and gut microbiome. This is particularly relevant in the case of pathologies associated with dysbiosis or disorders of the intestinal ecosystem. This review will examine the different QS systems and the evidence for their presence in the intestinal ecosystem. We will also provide clues on the role of QS molecules that may exert, directly or indirectly through their bacterial gossip, an influence on intestinal epithelial barrier function, intestinal inflammation, and intestinal carcinogenesis. This review aims to provide evidence on the role of QS molecules in gut physiology and the potential shared by this new player. Better understanding the impact of intestinal bacterial social networks and ultimately developing new therapeutic strategies to control intestinal disorders remains a challenge that needs to be addressed in the future.

**Key Words:** Inflammatory bowel disease; Quorum sensing; Gut microbiota; Dysbiosis; Inflammation; Intestinal barrier

Pfizer, Astellas, Janssen and Biocodex and grants from Biocodex and Janssen. These COIs are unrelated to the current presentation. All other authors declare no conflict of interests for this article.



**Specialty type:** Gastroenterology and hepatology

**Country/Territory of origin:** France

**Peer-review report's scientific quality classification**

Grade A (Excellent): 0
Grade B (Very good): B
Grade C (Good): 0
Grade D (Fair): 0
Grade E (Poor): 0

**Received:** March 16, 2021
**Peer-review started:** March 16, 2021
**First decision:** August 9, 2021
**Revised:** August 17, 2021
**Accepted:** October 27, 2021
**Article in press:** October 27, 2021
**Published online:** November 14, 2021

**P-Reviewer:** Dai YC
**S-Editor:** Gao CC
**L-Editor:** A
**P-Editor:** Gao CC

**Core Tip:** Host-microbiota interactions play a crucial role in the pathophysiology of many intestinal diseases. While biological components have been repeatedly described, a largely overlooked component is quorum sensing (QS), a density-dependent system able to coordinate bacterial responses and interact with host cells constantly exposed to bacteria. This review intends to describe the different QS systems to show evidence that QS is part of the intestinal ecosystem and highlight its impact on intestinal epithelial barrier function, inflammation, and intestinal carcinogenesis. From this report, we open up a new area of intestinal physiology.

**Citation:** Coquant G, Aguanno D, Pham S, Grellier N, Thenet S, Carrière V, Grill JP, Seksik P. Gossip in the gut: Quorum sensing, a new player in the host-microbiota interactions. *World J Gastroenterol* 2021; 27(42): 7247-7270
**URL:** https://www.wjgnet.com/1007-9327/full/v27/i42/7247.htm
**DOI:** https://dx.doi.org/10.3748/wjg.v27.i42.7247

## INTRODUCTION

Gut microbiota mutually interacts with coevolved host epithelial and immune cells in a beneficial reciprocal relationship[1]. The advent of multi-omics sequencing in the past decade has allowed researchers to investigate the complexity of the intestinal microbiota in various human disorders[2]. Many lines of evidence support a role for alteration of gut microbiota (dysbiosis) in the development or perpetuation of inflammatory and metabolic disorders; recent data pointed out the consequences of dysbiosis on host-microbiota interactions in this setting[3,4]. Currently, gut microbiota metabolites recognized as the main drivers of the impact of gut microbiota on hosts are short-chain fatty acids (SFCAs), branched-chain amino acids, trimethylamine N-oxide, bile acids, tryptophan (Trp), and indole derivatives[3,5]. A largely overlooked component is diffusible signaling molecules, which modulate the physiological response in the three domains of life[6]. A particular class of these signaling compounds is represented by bacterial quorum sensing (QS) molecules called autoinducers (AIs). QS is a density-dependent mechanism allowing bacterial populations to coordinate gene expression and physiology by modulating, for example, metabolic pathways, secretion of virulence factors, or biofilm formation in response to AIs[7]. Drawing on its density-dependent nature, it can be hypothesized that the production of bacterial signaling molecules is abundant in the highly densely populated environment of the mammalian intestinal tract.

Moreover, since several eukaryotic systems from fungi to plants and animals are known to recognize and respond to bacterial signaling compounds, it seems likely that human intestinal cells constantly exposed to bacterial compounds might also have developed response mechanisms to AIs with consequences on intestinal physiology [8]. The purpose of this current review is to provide clues to consider bacterial QS as a new actor of host-microbiota interactions. We will start by presenting the bacterial QS systems and evidence of QS in the gut. We will then provide an overview of the impact of QS molecules on host cell functions within the gut. Finally, we will investigate how modulation of the QS could be thought of as a therapeutic option, determine the key challenges, and suggest directions for future QS research.

## QS: GOSSIP IN A BACTERIAL WORLD

In the conventional view of prokaryotic existence, bacteria live as unicellular organisms, with responses to external stimuli limited to detecting chemical and physical signals of environmental origin. This view of bacteriology is now recognized as overly simplistic because bacteria communicate through small 'hormone-like' organic compounds. QS is a bacterial cell-cell communication process that involves the production, detection, and response to extracellular signaling molecules called AIs. AIs enable bacteria to perceive and respond to temporal and contiguous environments and coordinate the behavior of colonies by altering gene expression. QS controls genes that direct beneficial activities when performed by groups of bacteria acting in

synchrony. Processes controlled by QS include bioluminescence, sporulation, competence, antibiotic production, biofilm formation, and virulence factor secretion (Figure 1A). Similar to languages between humans, these signals vary between species. Some bacterial species can interpret many different signals, while others respond to a few. The first such system was described in 1979 in *Vibrio fischeri*[9], a symbiotic species that provides marine eukaryotic hosts with light. Light emission depends on transcription of the luciferase operon, which occurs when the cell population density is sufficient to produce a threshold accumulation of a secreted AI, a specific N-acyl Homoserine Lactone (AHL). It was only in 1994 that the term QS was first used to introduce the idea of a minimal population (quorum) that was needed to trigger a group behavior thanks to a signal[10]. The science of studying group behavior in microorganisms has even been named "sociomicrobiology"[11].

### *Different ways of talking*

Bacterial QS is highly complex and mediates communications thanks to the diversity of its different systems. QS systems can be divided into systems specific to species and mediating communication between Gram-positive bacteria, Gram-negative bacteria, and interspecies systems (Table 1 and Figure 1).

QS in Gram-positive species is driven in most cases by 5-17 amino acid oligopeptides (AIPs for AutoInducer Peptides), which are detected by membrane receptors belonging to the histidine kinase family and are involved in virulence or competence[12]. In addition, γ-butyrolactones are produced and integrated by *Streptomyces sp.* as signals controlling antibiotic production[13] or metabolism[14].

QS in Gram-negative bacteria relies on a high diversity of different systems, with some bacteria, such as *Pseudomonas aeruginosa (P. aeruginosa)*, possessing several QS systems (Table 1). The expression of over 300 genes is regulated by QS. The most common system is driven by AI-1 molecules belonging to the AHL family, which are constituted by a homoserine lactone ring carrying a 4-18 carbon acyl chain. The lengths and modifications of the acyl chain give each AHL its species specificity[15]. The first described model is the *Vibrio fischeri* system, in which N-3-oxohexanoyl-homoserine lactone (3-oxo-C6) is synthesized by LuxI synthase, passively diffuses out of the cell and enters another bacterium in which it binds its receptor LuxR[16] (Figure 1B). Above a threshold, the AHL-receptor complex binds a consensus DNA sequence, thus triggering luciferase expression[17]. This model applies to all AHL systems (Table 1 and Figure 1B). The system involves a positive feedback loop, thus promoting QS activation at the population scale (Figure 1B). To date, numerous homologous systems (*i.e.*, genes coding synthases and receptors) have been described in many Gram-negative bacteria, including over 70 Proteobacteria species[18] (Table 1).

Other Gram-negative QS systems involve the AI-3 molecule, initially identified in enterohemorrhagic *Escherichia coli* (*E. coli*) (EHEC) serotype O157:H7[19]. AI-3 regulates flagellar genes and pathogenicity[20,21] and is thought to be present in other enteropathogens (Table 1). A recent study[22] uncovered the structure of AI-3 and its natural analogs, including the prominent analog in mouse feces *in vivo*, which belongs to the pyrazinone family. The authors showed that various gram-negative and Gram-positive bacteria produce AI-3 analogs, thus redefining the specificity of AI-3 molecules.

Last, the third type of QS system has been identified in Gram-negative bacteria such as EHEC or *Vibrio* species and Gram-positive bacteria such as *Salmonella enterica*[23,24]. It relies on AI-2 molecules such as S-THMF-borate [for (2S,4S)-2-methyl-2,3,3',4-tetrahydroxy-tetrahydrofurane-borate][25] and R-THMF [for (2R,4S)-2-methyl-2,3,3',4-tetrahydroxy-tetrahydro furane][26]. AI-2 has now been found in various bacterial species in which it regulates many processes[27] and is proposed to mediate poly-species communication (Figure 1C).

In addition, indole is produced from Trp by Gram-negative and Gram-positive commensal and pathogenic bacteria displaying tryptophanase activity[28-31]. As the source of Trp is supplied by the diet and cannot be synthesized endogenously, either by bacteria or by the host, indole is a bacterial byproduct of Trp metabolism. However, in recent years, some authors have considered indole to be a QS molecule, as it is produced in a density-dependent manner and regulates several bacterial physiological processes, such as the formation of spores or biofilms, virulence traits, bacterial motility, and drug resistance[29,32,33].

The versatility of QS systems and their AI molecules highlights the complexity of communication and thus emphasizes the key role QS could play in a diverse ecosystem: the intestinal microbiota.

**Table 1 Examples of bacterial quorum sensing autoinducer and corresponding systems**

| | AI | Example of producing bacteria | QS system | Bacterial QS-regulated processes | Ref. |
|---|---|---|---|---|---|
| Gram + | AI peptide | *Staphylococcus aureus* | *agr* | Virulence | Novick *et al*[155] |
| | | *Listeria monocytogenes* | *agr* | Virulence | Autret *et al*[156] |
| | | *Clostridium perfringens* | *agr* | Virulence | Ohtani *et al*[157] |
| | | *Enterococcus faecalis* | *FsR* | Virulence | Sifri *et al*[158] |
| | | *Bacillus subtilis* | *com* | Competence | Magnuson *et al*[159] |
| | γ-butyrolactone | *Streptomyces* genus | *scb* | Antibiotics | Takano *et al*[13] |
| | | | *scg* | Metabolism | Du *et al*[14] |
| Gram - | AI-1 (acyl-homoserine lactones) | *Vibrio fischeri* | LuxI/LuxR | Luminescence | Engebrecht *et al*[16] |
| | | *Vibrio harveyi* | LuxLM/LuxN | Luminescence | Mok *et al*[160] |
| | | | | Virulence | Waters and Bassler[161] |
| | | *Pseudomonas aeruginosa* | LasI/LasR | Virulence and biofilm | Gambello and Iglewski[162], Gambello *et al*[163], Winson *et al*[164], and Chapon-Hervé *et al*[165] |
| | | | RhlI/RhlR | | |
| | PQS | *Pseudomonas aeruginosa* | PqsABCD/PqsR | QS regulation | Pesci *et al*[166] |
| | | | | Pyocyanin | Gallagher *et al*[167] |
| | | | | Iron homeostasis | Bredenbruch *et al*[168] and Diggle *et al*[169] |
| | | | | Virulence | Gallagher *et al*[167] and Cao *et al*[170] |
| | | | | Biofilm | Diggle *et al*[171] |
| | IQS | *Pseudomonas aeruginosa* | AmbBCDE/IqsR | Response to stress | Lee *et al*[172] |
| | CAI | *Vibrio (cholerae)* | CqsA/CqsS | Virulence | Ng *et al*[173] |
| | AI-3 | EHEC O157:H7 | Qse/QseBC | Attachment-effacement | Sperandio *et al*[19], Walters *et al*[21], and Kim *et al*[22] |
| | | EPEC O26:H11 | Qse/unknown | Unknown | Kim *et al*[22], and Kaper and Sperandio[40] |
| | | AIEC LF82 | Qse/unknown | Unknown | Kim *et al*[22] |
| | | *Escherichia coli* MG1655 | Unknown | Unknown | Kim *et al*[22] |
| | | *Escherichia coli* BW25113 | Unknown | Unknown | Kim *et al*[22] |
| | | *Salmonella enterica* | Qse/unknown | Unknown | Kim *et al*[22], Kaper and Sperandio[40], and Walters and Sperandio[174] |
| | | *Shigella flexneri* | Qse/unknown | Unknown | Kim *et al*[22], Kaper and Sperandio[40], and Walters and Sperandio[174] |
| | | *Yersinia sp.* | Qse/unknown | Unknown | Kim *et al*[22], Kaper and Sperandio[40], and Walters and Sperandio[174] |
| Gram + and - | AI-2 | *Vibrio harveyi* | LuxS/LuxPQ | Bioluminescence, TSS, protease | Surette *et al*[24], Mok *et al*[160], and Schauder *et al*[175] |
| | | *Vibrio cholerae* | LuxS/LuxPQ | Virulence and Biofilm | Schauder *et al*[175], Zhu *et al*[176], and Hammer and Bassler[177] |
| | | *Enterococcus faecalis* | LuxS/LuxPQ | Unknown | Surette *et al*[24], and Schauder *et al*[175] |
| | | *EHEC* | LuxS/LsrB (?) | Attachment-effacement | Schauder *et al*[175], and Bansal *et al*[178] |
| | | *Salmonella enterica* | LuxS/LsrB | Pathogenicity and invasion | Miller *et al*[26], Schauder *et al*[175], and Choi *et al*[179] |

AI: Autoinducer; AIEC: Adherent-invasive *Escherichia coli*; AIP: AutoInducer peptides; CAI: Cholera autoinducer-1; EHEC: Enterohemorrhagic *Escherichia coli*; EPEC: Enteropathogenic *Escherichia coli*; IQS: Integrated quorum sensing; PQS: Pseudomonas quinolone signal; QS: Quorum sensing.

## *QS in the gut*

The study of QS in the gut is still a relatively recent matter of interest, as QS is generally addressed from the pathogenic bacterium *P. aeruginosa* point of view in the lung ecosystem. However, many arguments suggest that QS is a new player in the gut ecosystem.

**AHLs in the gut:** As part of the eavesdropping mechanism, some bacteria from the human gut can sense AHLs from other species (Figure 1A). Gram-negative bacilli such as *E. coli*, *Enterobacter*, or *Klebsiella* express the receptor SdiA, which can sense AHL, without producing such a signal[34]. The opportunistic pathogen *P. aeruginosa,* which targets the digestive tract in severely immune-compromised patients[35,36], and the more common enteropathogen *Yersinia enterolitica* are known to produce AHLs[37]. Analyses from sequencing databases have shown the presence of LuxI/LuxR homologs in a few commensals: *Hafnia alvei, Edwardsiella tarda*, and *Ralstonia sp.* strain 5_7_47FAA[37]. However, the latter article did not demonstrate the presence of AHLs but only homologs of the genes encoding the synthase complex and the receptor. A cohort low sample size pediatric study ($n$ = 4) demonstrated, thanks to bacterial reporter systems, the presence of AHLs in the feces of patients without identifying them[38]. Our team investigated the question of AHLs in the human gut in the context of inflammatory bowel disease (IBD). With high-resolution mass spectrometry, we identified approximately ten AHLs in the feces of healthy patients, IBD patients in remission, and flare[39]. We also found a never-described AHL, 3-oxo-C12:2-HSL, that was less represented in IBD patients, especially in flares, than in healthy subjects[39].

**AI-2 in the gut:** AI-2 presence in the gut has been reported by several articles[19,40,41] but is mainly linked to pathogenic bacteria such as enterohemorrhagic *E. coli*[19]. As AI-2 is considered a "universal language", it is not surprising to find this AI in an ecosystem as diverse as the gut microbiota.

Thompson *et al*[42] showed that most Firmicutes contain LuxS protein orthologs, an important enzyme that allows AI-2 production. In contrast, its presence is less represented in Bacteroidetes. Mutant *E. coli* engineered to regulate AI-2 levels in the mouse gut counteract antibiotic-induced dysbiosis[42]. AI-2 produced by *E. coli* benefits Firmicutes while restraining Bacteroidetes representation[42], suggesting an important role of AI-2 in the gut ecosystem.

**Other QS signals:** Concerning other QS signals, there is less evidence of their implications in gut microbiota. A recent study showed a correlation between indole and *Clostridioides difficile* (*C. difficile*) infection (CDI) with higher indole concentrations for patients with CDI than for CDI-negative patients with diarrhea[43]. *C. difficile* induces indole production through overexpression of the tryptophanase gene tnaA in enterotoxigenic *E. coli* and other indole-producing anaerobes. This increased indole level has been shown to be detrimental to some of the beneficial bacteria and favors *C. difficile* colonization.

These mechanisms collectively suggest a complex bacteria-bacteria QS network in the gut ecosystem. The key issue is now to decode every language to fully understand its potential in host-microbiota interactions.

### *Gossip in the gut: Direct dialog with the host and indirect effects through bacterial behavior*

In an ecosystem as networked and complex as that in the gut, bacterial communication has to be seen from a large perspective, with multiple bacterial populations crosstalking to each other through eavesdropping or crosstalking between species (Figure 1A). Therefore, the question of how QS affects the host can be addressed in two ways (Figure 2).

QS modulates microorganism metabolism, which in turn can affect the host's physiology; one could consider this to be an indirect effect of QS molecules on the host (Figure 2). Bacterial metabolism modifies beneficial byproducts such as SFCAs and bile acids[42]. By modulating intestinal microbiota composition, QS can indirectly influence gut physiology by promoting deleterious or beneficial bacteria (Figure 2). Thompson *et al*[42] demonstrated that AI-2 modulates dysbiotic microbiota composition by enhancing Firmicutes growth. Several reports *in vitro* and *in vivo* describe how enteropathogens can signal through QS to commensals and trigger the expression of toxins, virulence factors, and biofilm formation[44-46]. In addition, AI-3 controls the genes that enable enterohemorrhagic *Escherichia coli* to cause lesions by the attachment and effacing process[19].

The question addressed in this review is how quorum-sensing molecules can directly affect the host, independent of the producing bacteria (Figure 2).

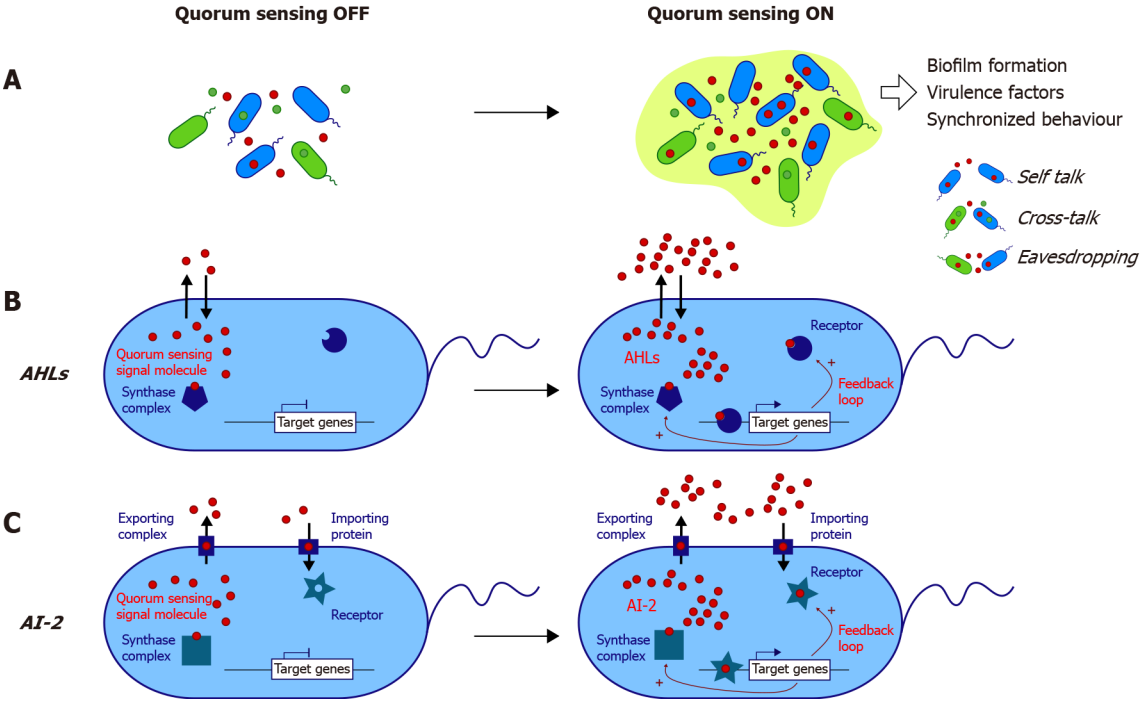


**Figure 1 Main known mechanisms of quorum sensing activation in bacteria.** A: Quorum sensing (QS) signaling depends on the release of autoinducers (AIs) in the environment. Above a threshold concentration depending on bacterial density, QS is activated and triggers gene expression. QS can be classified into three categories: self-talk (*i.e.*, one species "talking" to itself), crosstalk (*i.e.*, different species communicating using common AI), and eavesdropping, which refers to "listening" by species unable to produce AI by itself; B: The acyl-homoserine lactone (AHL) used by Gram-negative bacteria is produced by the synthase complex, and AHL can freely diffuse through the membrane. AHL is recognized by its intracellular receptor, and the complex binds to target gene regulatory elements; C: The AI-2 system is used by both Gram-negative and Gram-positive bacteria. AI-2 needs a transporter protein to exit and enter the cell. For both AHLs and AI-2, there is a positive feedback loop, allowing the expression of the synthase complex and receptor of AIs. AHL: Acyl-homoserine lactone; AI: Autoinducer.

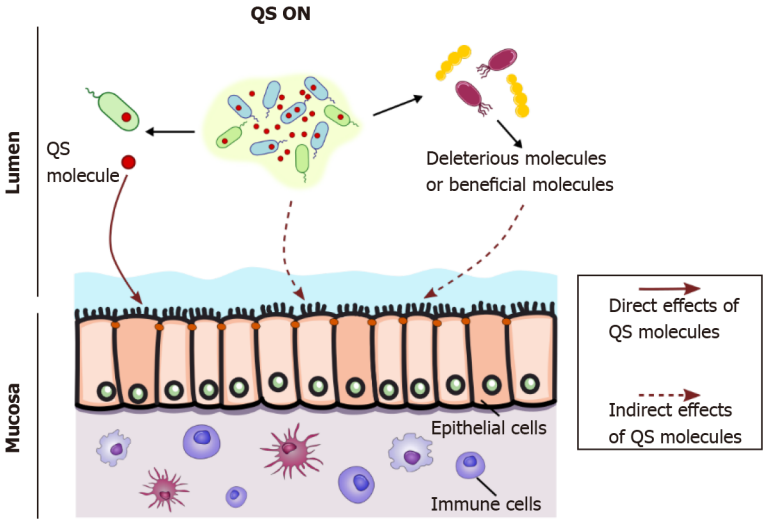


**Figure 2 Interkingdom dialog between bacteria and the host through quorum sensing molecules.** When reaching a threshold concentration within a bacterial community, quorum sensing (QS) autoinducers synchronize group behaviors such as virulence and attachment-effacement strategies as in enterohemorrhagic *Escherichia coli*, thus indirectly affecting the host (dotted line arrow, middle). QS molecules can impact the host through direct contacts (full arrow, left) with host cells, such as epithelial or immune cells, as has been extensively shown for the *Pseudomonas aeruginosa* QS molecule 3-oxo-C12-HSL, which freely enters mammalian cells. In addition, QS molecules can indirectly modify the host (dotted line arrow, right) through effects on other bacterial populations with different metabolic properties. QS: Quorum sensing.

## HOST: "YOU'VE GOT MAIL"

As discussed above, it remains largely unknown how the microbial communities hosted in the gut lumen use QS communication systems. However, there is evidence that at least several bacterial species commonly found in the gastrointestinal tract have

the capacity to synthesize QS molecules[6,8,39].

Studies on the impact of QS molecules on the biology of intestinal host cells have focused on key actors of barrier function and the immune response. Intestinal barrier function includes the ability of epithelial cells to form: (1) A selective barrier whose permeability is controlled by cell-cell junctions[47], (2) Synthesize a protective mucus layer and antimicrobial peptides[48,49], and (3) Secrete cytokines and chemokines allowing appropriate crosstalk with the underlying immune compartment. The intestinal immune system is involved in tolerogenic or inflammatory responses to the commensal microbiota or pathobionts/pathogens[50,51], and it represents the largest immune organ in the body. Intestinal epithelial cells, intraepithelial lymphocytes, and immune cells located in the lamina propria are involved in the modulation of immunity and inflammation by microbiota[52].

The impact of QS molecules on barrier function and the immune response has been mainly studied in the context of host-pathogen interactions, probably because most of the data rely on AHLs produced by *P. aeruginosa*. However, evidence of the presence of QS molecules in the healthy intestinal lumen has led to further study on their effects on the host compartment, including barrier function, inflammatory process, and carcinogenesis.

### *Effects of quorum-sensing molecules on intestinal epithelial barrier function*

**AHLs:** 3-oxo-C12-HSL produced by *P. aeruginosa* is probably the QS molecule whose effects on the barrier function of epithelial cells have been the most studied during the last two decades[53]. *P. aeruginosa* synthesizes various virulence factors, which act synergistically with QS molecules to destabilize cell-cell junctions and promote bacterial transmigration across epithelial and endothelial barriers[54].

3-oxo-C12-HSL induces an increase in paracellular permeability to ions and macromolecules[55-60] (Table 2). This deleterious effect of 3-oxo-C12-HSL on barrier function is accompanied by an alteration of tight junctions (TJs) (Figure 3). In the Caco-2 intestinal epithelial cell line, 3-oxo-C12-HSL induced a decrease in the expression, as well as mislocalization, of the TJ proteins occludin, tricellulin, ZO- 1, ZO-3, JAM-A, and of the adherent junction proteins E-cadherin and β-catenin[55,58-61] (Table 2). Loss of occludin/ZO-1 and tricellulin/ZO-1 interaction at the plasma membrane suggested the dismantling of TJ protein complexes[61]. In addition, hyperphosphorylation of occludin, ZO-1, ZO-3, JAM-A, E-cadherin, and β-catenin on tyrosine residues (as well as serine and threonine for E-cadherin and ZO-1) was reported in the presence of 3-oxo-C12-HSL, whereas the serine and threonine residues of occludin, JAM-A and β-catenin were less phosphorylated[58,59].

Several signaling mechanisms, including p38 and p42/44 MAP kinases[59,60], $Ca^{2+}$ release[57,58], matrix metalloproteinases MMP-2 and MMP-3 *via* protease-activated receptor (PAR) signaling[55] and oxidative stress[62], have been implicated in 3-oxo-C12-HSL effects on junctional proteins and on the concomitant increase in permeability (Table 2).

As discussed above, the most prominent AHL detected in the human intestinal ecosystem is unsaturated 3-oxo-C12:2-HSL[39]. Despite a high structural homology with the *P. aeruginosa* 3-oxo-C12-HSL, this intestinal AHL has recently been found to have opposite properties regarding the barrier function (Table 2). In contrast to 3-oxo-C12-HSL, 3-oxo-C12:2-HSL does not increase paracellular permeability in Caco-2/TC7 enterocytic cells[39,61]. Most importantly, 3-oxo-C12:2-HSL can limit TJ disruption induced by the proinflammatory cytokines interferon-gamma (IFN-γ) and tumor necrosis factor-α (TNF-α)[61] (Figure 3). In these conditions mimicking intestinal inflammation encountered, for example, in IBD, 3-oxo-C12:2-HSL maintains the interaction of the TJ transmembrane proteins occludin and tricellulin with their main cytoplasmic partner ZO-1. It limits cytokine-induced occludin and tricellulin ubiquitination and the interaction of these TJ proteins with the E3 ubiquitin ligase itch, suggesting stabilization of TJ complexes at the plasma membrane in inflammatory conditions. Altogether, these results show that "commensal" intestinal 3-oxo-C12:2-HSL mitigates the deleterious effects of the inflammatory environment on TJs, which are key actors in epithelial barrier function[61].

Epithelial barrier disruption may combine TJ alteration and an unrestricted passage, which occurs following epithelial damage, generated, for example, by cell apoptosis upon exposure to harmful molecules such as high doses of proinflammatory cytokines [63-65]. This TJ-independent breaking of the barrier allows translocation of large particles such as large proteins, entire bacteria, and viruses, which *a priori* cannot cross the epithelium through the paracellular route even in conditions where TJs are "open" [66]. The 3-oxo-C12-HSL produced by *P. aeruginosa* exerts cytotoxic effects, particularly through apoptosis induction, in numerous cell types, including the intestinal and

**Table 2 Effects of quorum sensing molecules on different parameters of the intestinal epithelial barrier function**

| QS molecule | Effects | Ref. |
|---|---|---|
| **Effects on the intestinal epithelial migration** | | |
| 3-oxo-C12-HSL | Increased migration at low concentrations (1.5-12 μmol/L) *vs* inhibition at 200 μmol/L | Karlsson *et al*[72] |
| | Interaction with IQGAP1 and increase in Rac1/Cdc42 (1.5-200 μmol/L) | Karlsson *et al*[72] |
| **Effects on the intestinal epithelial permeability and intercellular junctions** | | |
| 3-oxo-C12-HSL | Increased permeability to ions and macromolecules (100-400 μmol/L) | Eum *et al*[55], Vikström *et al*[58-60], and Aguanno *et al*[61] |
| | Activation of p38 and p42/44 and calcium signaling (100-200 μmol/L) | Vikström *et al*[58-60] |
| | Decreased expression levels of tight junction genes (100-400 μmol/L); Disassembly of tight and adherens junctions (modification of their phosphorylation status and involvement of MMP-2 and -3) | Eum *et al*[55], Vikström *et al*[58-60], and Aguanno *et al*[61] |
| | Decreased levels of tight junction proteins occludin and tricellulin (100-400 μmol/L) | Eum *et al*[55] |
| | Decreased protein levels of extracellular matrix and tight junction proteins (400 μmol/L) | Tao *et al*[62] |
| 3-oxo-C12:2-HSL | No deleterious effects on permeabilityProtection of tight junction integrity and maintenance of junctional complexes at the plasma membrane under pro-inflammatory conditions | Landman *et al*[39] and Aguanno *et al*[61] |
| 3-oxo-C14-HSL | Decreased protein levels of extracellular matrix and tight junction proteins (400 μmol/L) | Tao *et al*[62] |
| Indole and indole derivatives | Decreased permeability to ions and increased expression of genes coding tight junction and cytoskeleton proteins | Bansal *et al*[76] and Shimada *et al*[77] |
| | Decreased permeability to macromolecules | Venkatesh *et al*[79] |
| | Increased transcripts levels of genes coding tight junction proteins | Shin *et al*[78] |
| **Effects on the mucus layer components** | | |
| 3-oxo-C12-HSL | Decreased MUC3 mRNA levels (30 μmol/L) | Taguchi *et al*[70] |
| | Decrease in Muc2 production in goblet cell-like cell line (100 μmol/L) *vs* increase in colonic cell line (400 μmol/L) | Tao *et al*[67] |
| Indole | Increased expression of genes involved in the production of mucins | Bansal *et al*[76] |
| **Effects on intestinal epithelial cell viability** | | |
| 3-oxo-C12-HSL | Mitochondrial dysfunction and induction of apoptosis in goblet cell-like cell line (100 μmol/L) and in colonic cell line (30-100 μmol/L) | Tao *et al*[67-69], and Taguchi *et al*[70] |
| | Induction of apoptosis, mitochondrial dysfunction, oxidative stress and blocking of cell cycle (400 μmol/L) | Tao *et al*[62] |
| 3-oxo-C14-HSL | Induction of apoptosis, mitochondrial dysfunction, oxidative stress and blocking of cell cycle (400 μmol/L) | Eum *et al*[55], Vikström *et al*[58-60], Aguanno *et al*[61], and Tao *et al*[62] |
| CSF | Reduction of oxidative stress-induced cell death and loss of the epithelial barrier (involving HSP27 and p38/MAPK pathway) | Fujiya *et al*[74] |

CSF: Competence and sporulation factor; HSL: Homoserine lactones; HSP27: Heat shock protein 27; IQGAP1: IQ motif containing GTPase activating protein 1; MAPK: Mitogen-activated protein kinase; MMP-2/-3: Matrix metalloproteinase-2/-3; MUC: Mucin; QS: Quorum sensing; Rac1/Cdc42: Ras-related C3 botulinum toxin substrate 1/cell division control protein 42 homolog.

colonic epithelial cell lines LS174T[67-69], Caco-2[70], and CT26[62] (Table 2 and Figure 3). Apoptosis triggered by 3-oxo-C12-HSL relies on oxidative stress and caspase-dependent processes[62,69], whereas short-chain C4-HSL does not exert any apoptotic effects[67]. Interestingly, the increase in paracellular permeability to macromolecules induced by 3-oxo-C12-HSL was dramatically exacerbated in Caco-2/TC7 cells cultured in the presence of IFN-γ and TNF-α or cocultured with THP-1 activated monocytic cells[61]. These synergistic effects on barrier disruption probably rely on epithelial cell apoptosis, as they are abolished by a caspase inhibitor (unpublished results). In contrast, intestinal AHL 3-oxo-C12:2-HSL neither exerts cytotoxic effects nor synergizes with proinflammatory cytokines to disrupt the epithelial barrier[61].

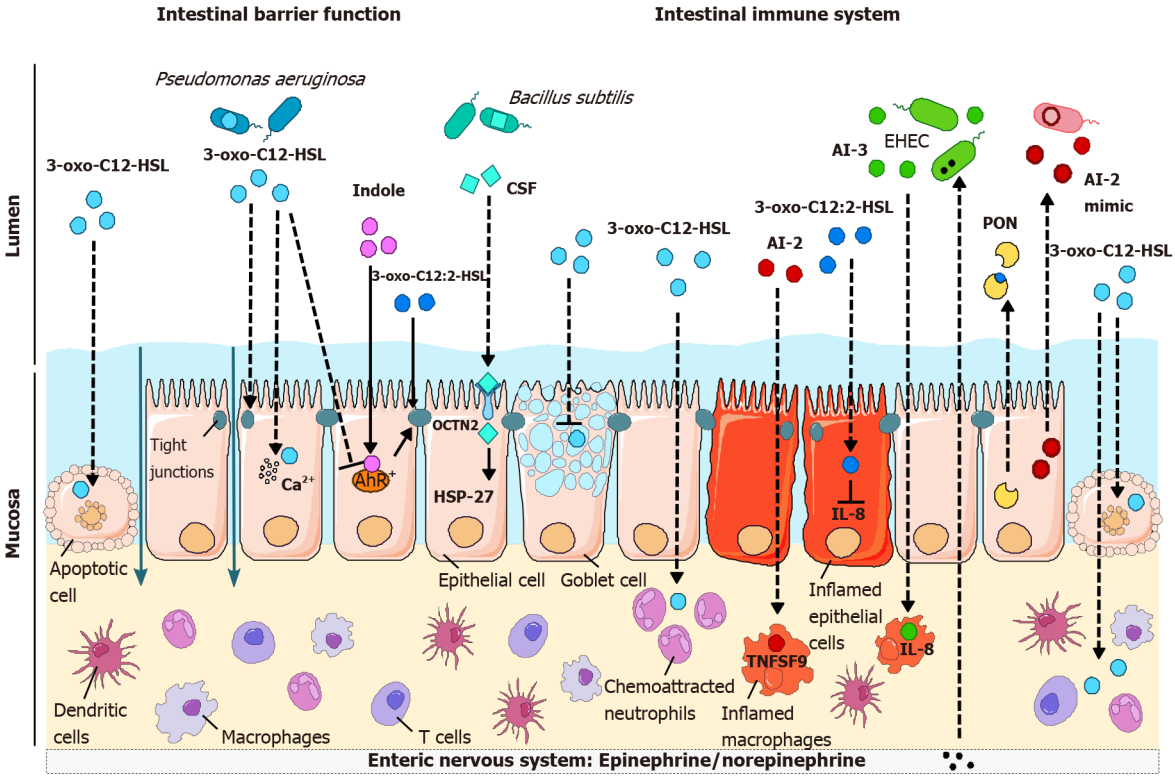


**Figure 3 Effects of quorum sensing molecules on intestinal barrier function (see Table 2) and on the immune response (see Table 3).** The *Pseudomonas aeruginosa* quorum sensing (QS) molecule 3-oxo-C12-HSL induces apoptosis in various cell types, including epithelial cells, promoting a breach in the intestinal barrier. In addition, 3-oxo-C12-HSL disrupts tight junctions, thus leading to increased paracellular permeability, and affects mucin production. Conversely, intestinal acyl-homoserine lactone 3-oxo-C12:2-HSL and the tryptophan metabolite indole protect tight junctions. *Bacillus subtilis* CSF, which binds to OCTN2, also promotes intestinal barrier integrity by reducing cell death through activation of HSP27 signaling. While 3-oxo-C12-HSL stimulates chemoattraction and phagocytosis in neutrophils and induces cell death, its pro- or anti-inflammatory effects on immune cells are more complex (see Table 3). Autoinducers (AI)-2 and AI-3 both exert proinflammatory effects on macrophages by inducing the expression of the immune mediators TNSF9 and interleukin (IL)-8, respectively, whereas 3-oxo-C12:2-HSL reduces IL-8 production by epithelial cells. It remains to be clarified how all these QS molecules could cross the intestinal barrier and/or reach immune cells *in vivo* in a physiological context, as illustrated by dotted lines. Last, just as QS molecules can impact eukaryotic cells, the host can interfere with QS: the hormones epinephrine/norepinephrine bind to the AI-3 receptor in EHEC; intestinal epithelial cells secrete an AI-2 mimic in addition to paraoxonase (PON) enzymes degrading homoserine lactones. CSF: Competence and sporulation factor; AI: Autoinducer; PON: Paraoxonase; AhR: Aryl hydrocarbon receptor; IL: Interleukin.

Epithelial injury accompanying acute inflammatory conditions is followed by a re-epithelialization phase, during which cell migration plays an important role[71]. Interestingly, 3-oxo-C12-HSL has been shown to dose-dependently modulate Caco-2 cell migration in a wound-healing assay and interact directly with the GTPase activating protein IQGAP1, stressing a potential role of AHL in cytoskeletal reorganization[72] (Table 2).

Another key actor of the intestinal physical barrier is the mucus layer, which is essential to maintain segregation between luminal microorganisms and the epithelium [49]. 3-oxo-C12-HSL induces reduced expression and production of Mucin2 in LS174T cells[67], as well as a decrease in the levels of MUC3 mRNA in the Caco-2 cell line cultivated in an undifferentiated state[70] (Table 2 and Figure 3). Interestingly, differentiated Caco-2 cells, which express higher levels of mucin 3, showed less sensitivity to 3-oxo-C12-HSL-induced apoptosis in the latter study, and the addition of mucin dose-dependently protected cells from apoptosis induced by this AHL[70].

It must be specified that all these studies on barrier function were carried out with high concentrations of AHLs (100-400 μmol/L) (Table 2), knowing that the concentration of 3-oxo-C12-HSL has been estimated to reach 600 μmol/L in biofilms of *P. aeruginosa*[73].

**Gram-positive QS peptides:** The effects of AIP (found in Gram-positive bacteria) on intestinal barrier function are much less documented than those of Gram-negative QS molecules. Whereas most of the studies on the effects of AIP on host inflammation describe the indirect effects of AI through the modulation of bacterial metabolism, one article reported the direct effects of AIP. Fujiya *et al*[74] reported that *Bacillus subtilis* AIP, named competence and sporulation factor (CSF), induces HSP27 expression and the p38/MAPK pathway and reduces cell death and the loss of the epithelial barrier

induced by oxidative stress (Table 2 and Figure 3). Inducible HSPs are needed under stress and help stabilize proteins to prevent denaturation[75]. *B. subtilis* is part of the normal microbiota; it is also used as a commercial probiotic and is beneficial to the host. Moreover, CSF seems to signal through a receptor named OCTN2 (organic cation/carnitine transporter 2), and polymorphisms of the gene encoding this receptor are part of the susceptibility locus of Crohn's disease[74].

**Indole:** Indole exerts a beneficial role on TJ protein expression in several intestinal epithelial cells[76-79] (Table 2). Oral administration of indole to germ-free mice, which display very low indole fecal levels, increased the expression of TJ and adherens junction-associated proteins in the colonic epithelium and improved their resistance to dextran sulfate sodium (DSS)-induced colitis[77]. Indole has been identified as an endogenous agonist of aryl hydrocarbon receptor (AhR), which can compete for receptor binding with well-known AhR ligands[80]; several studies have also stressed the key role of the AhR pathway in indole derivative protective effects[81-83]. Accordingly, several studies have shown that AhR activation strengthens the epithelial barrier by protecting TJs[82,84-87] (Figure 3) or by stimulating antimicrobial peptide production *via* interleukin (IL)-22[88,89].

### *Effects of QS molecules on immune response*

In addition to their effects on the intestinal barrier, QS molecules were analyzed on different actors of the immune compartment of the intestine, which is involved in a complex crosstalk with the epithelial compartment to maintain an appropriate immune response toward the content of the intestinal lumen.

**AHLs:** Our group described that 3-oxo-C12:2-HSL, an AHL recently discovered in the human gut[39], exerts anti-inflammatory effects on intestinal epithelial cells. During inflammation, intestinal epithelial cells can secrete some cytokines, among which the chemokine IL-8 promotes the recruitment of neutrophils in the mucosa and participates in the acute-phase response[90,91]. In a study comparing the effect of 3-oxo-C12:2-HSL to 3-oxo-C12-HSL produced by *P. aeruginosa*, our group demonstrated in the human enterocytic Caco-2/TC7 cell line that 3-oxo-C12:2-HSL, but not 3-oxo-C12-HSL, attenuated the induction of IL-8 secretion induced by the proinflammatory cytokine IL-1β[39,92] (Table 3 and Figure 3). This potential anti-inflammatory effect of 3-oxo-C12:2-HSL is consistent with the hypothesis of a beneficial role of this AHL in gut ecosystems[39], as are its protective effects on TJ integrity. The impact of intestinal 3-oxo-C12:2-HSL on immune cells remains largely unknown.

The effects of 3-oxo-C12-HSL depend on the concentration and cell type studied[6,93]. Telford *et al*[94] showed that 3-oxo-C12-HSL inhibits the production of TNF-α and IL-12 [a cytokine involved in the T helper cell-1 type response (Th1-type response)] by lipopolysaccharide-activated macrophages at high concentrations and stimulates the production of antibodies, particularly immunoglobulin G1, which is an indicator of a Th2-type response at lower concentrations (Table 3). Conversely, Smith *et al*[95] showed that 3-oxo-C12-HSL activates and promotes the differentiation of naive T lymphocytes toward a Th1-like phenotype, while Ritchie *et al*[96] observed that 3-oxo-C12-HSL inhibits the differentiation of both Th1 and Th2 T lymphocytes (Table 3). Altogether, these results demonstrated that 3-oxo-C12-HSL is an immunomodulator of the Th1/Th2 response. 3-oxo-C12-HSL and two other QS molecules from *P. aeruginosa*, PQS (*Pseudomonas* quinolone signal), and HHQ (4-hydroxy-2-heptylquinoline), suppress both innate and adaptive immune responses acting on lymphoid cells, dendritic cells, and neutrophil monocytes/macrophages[22,97,98]. 3-oxo-C12-HSL and PQS decreased the production of the cytokines IL-12 and IFN-γ by activated dendritic cells, which in turn decreased T-cell proliferation and activity[98-100] while promoting the induction of regulatory T-cells[99] (Table 3). 3-oxo-C12-HSL provoked apoptosis of macrophages, neutrophils, and T lymphocytes through activation of caspases and the mitochondrial apoptosis pathway[101,102]. Several reports described inhibition of the nuclear factor-kappa B (NF-κB) pathway by QS molecules from *P. aeruginosa*[93,103-106] and/or the activation of signaling pathways such as p38 MAPK[105,107] (Table 3). It has been recently demonstrated that only long-chain AHLs such as 3-oxo-C12-HSL modulate the phenotype of dendritic cells and the type 2 immune response through mechanisms involving retinoic acid signaling and the protein kinase AKT [106].

The molecular mechanisms involved in the effects of QS molecules from *P. aeruginosa* on immune cells are independent of the Toll-like receptor pathway[105], which is a classical cell process involved in recognizing pathogen fragments. Some reports have indicated that the perception of AHL by mammalian cells involves the

**Table 3 Effects of quorum sensing molecules on inflammation in different cell types**

| Cell type | QS molecule | Effects | Ref. |
|---|---|---|---|
| **Effects on innate immune cells** | | | |
| Macrophages | 3-oxo-C12-HSL | Anti-inflammatory effects on IL-12 and TNF-α (0.1-100 μmol/L) | Telford *et al*[94] |
| | | Increased TLR2 and TLR4 expression and decreased TNF-α production (1-100 μmol/L) | Bao *et al*[180] |
| | | Pro-apoptotic effects (12-50 μmol/L) | Tateda *et al*[102] |
| | | Increased phagocytosis (100 μmol/L) | Vikström *et al*[107] |
| | | NF-κB inhibition (4.7 μmol/L) | Kravchenko *et al*[104] |
| | | Dose-dependent anti-inflammatory effects (1-50 μmol/L) | Kravchenko *et al*[105] |
| | | Involvement in p38/MAPK signaling (1-100 μmol/L) | Kravchenko *et al*[105], Vikström *et al*[107], Glucksam-Galnoy *et al*[181] |
| | | Activation of the Unfolded Protein Response (6.25-100 μmol/L) | Zhang *et al*[182] |
| | | Change in cell volume and shape (10-50 μmol/L) | Holm *et al*[183] |
| | Indole derivatives | Prevents the induction of pro-inflammatory cytokines | Krishnan *et al*[184] |
| | AI-2 | Induction of the expression of cytokines, chemokines and TNFSF9 | Li *et al*[41] |
| Monocytes | AI-3 and analogues | Increase in IL-8 secretion | Kim *et al*[22] |
| Dendritic cells | 3-oxo-C12-HSL | Pro-apoptotic effects (100 μmol/L) | Boontham *et al*[185] |
| | | No effect on IL-10 secretion (5-30 μmol/L) | Skindersoe *et al*[100] |
| | | Increased IL-10 production (5-100 μmol/L) | Li *et al*[99] |
| | | Decreased IL-12 secretion (5-100 μmol/L) | Li *et al*[99] and Skindersoe *et al*[100] |
| | | Increased induction of Treg (5-100 μmol/L) | Li *et al*[99] |
| Neutrophils | 3-oxo-C12-HSL | Chemoattraction (0.01-100 μmol/L) | Karlsson *et al*[186] and Zimmermann *et al*[187] |
| | | Activation of MAPK signaling (12-50 μmol/L) | Tateda *et al*[102] and Singh *et al*[188] |
| | | Increased phagocytosis (10 μmol/L) | Wagner *et al*[189] |
| | | Pro-apoptotic effects (12-50 μmol/L) | Tateda *et al*[102] |
| **Effects on adaptive immune cells** | | | |
| T cells | 3-oxo-C12-HSL | Inhibition of proliferation and activation (0.1-100 μmol/L) | Telford *et al*[94], Boontham *et al*[185], Gupta *et al*[190], and Hooi *et al*[191] |
| | | Activation of naïve T cells towards Th1 phenotype (5 μmol/L) | Smith *et al*[95] |
| | | Decreased secretion of IL-4 and IFN-γ (5 μmol/L) | Ritchie *et al*[96] |
| | | Induction of apoptosis *via* the mitochondria pathway (100 μmol/L) | Jacobi *et al*[101] |
| | | Induction of Treg (1-50 μmol/L) | Li *et al*[99] |
| | Indole derivatives | Re-programming into tolerogenic T cells | Cervantes-Barragan *et al*[192] |
| | | Promotion of differentiation towards a regulatory type 1 phenotype | Aoki *et al*[193] |
| B cells | 3-oxo-C12-HSL | Modulation of immunoglobulin production (0.1-100 μmol/L) | Telford *et al*[94] and Ritchie *et al*[194] |
| ILC | Indole derivatives | Promotion of IL-22 production | Zelante *et al*[83] |
| **Effects on epithelial cells** | | | |
| Pulmonary tract epithelial | 3-oxo-C12-HSL | Induction of IL-8 production and NF-B activation | Smith *et al*[195] |

| | | | |
|---|---|---|---|
| cells | | (100 μmol/L) | |
| | | Increased expression levels of pro-inflammatory cytokines | Jahoor *et al*[115] |
| Intestinal epithelial cells | 3-oxo-C12-HSL | Mitigation (1-10 μmol/L) or aggravation (> 50 μmol/L) of IL-8 expression induction | Peyrottes *et al*[92] |
| | 3-oxo-C12:2-HSL | Attenuation of the induction of IL-8 expression (5-50 μmol/L) | Landman *et al*[39] |

AI: Autoinducer; B cells: Lymphocytes B; HSL: Homoserine lactones; IFN-γ: Interferon-γ; IL: Interleukin; ILC: Innate lymphoid cells; MAPK: Mitogen-activated protein kinase; NF-κB: Nuclear factor-kappa B; QS: Quorum sensing; T cells: Lymphocytes T; Th: T helper; TLR: Toll like receptors; TNF-α: Tumor necrosis factor-α; Treg: Regulatory T cells.

bitter taste receptor T2R38[108-110], which is widely expressed in the human digestive tract from the tongue to the colon[111]. Polymorphisms in the *TAS2R38* gene may increase susceptibility to infections and colorectal cancer (CRC)[112]. It has been shown that these receptors use inflammatory pathways, which differ according to the cell type and their localization[113]. 3-oxo-C12-HSL binds to the transcription factor peroxisome proliferator-activated receptor γ[114], which has been proposed as a potential receptor for AHL and seems to be involved in AHL proinflammatory effects [115]. Recently, Moura-Alves *et al*[116] showed that QS molecules produced by *P. aeruginosa* modulated the activity of the transcription factor AhR, which plays an important role in regulating innate and adaptive immunity[117,118].

Overall, it has been demonstrated that QS molecules from *P. aeruginosa* have an immunosuppressive effect, allowing the pathogen to evade the immune system during infection. It remains to be determined whether endogenous intestinal 3-oxo-C12:2-HSL participates in controlling intestinal immunity in health and diseases and to decipher the underlying mechanisms.

**AI-2 and AI-3:** AI-2 is produced by both Gram-negative and Gram-positive bacteria and is mainly studied for its role in bacteria-bacteria communication and the virulence of pathogenic strains[8]. However, little is known about the effect of AI-2 on immune cells. In mice, AI-2 administration has no effect by itself on cytokine expression but aggravates lung inflammation during *P. aeruginosa* infection by interfering with QS molecules produced by this pathogen[119]. In cultured macrophages, AI-2 induces the expression of several cytokines and chemokines as well as the expression of TNF superfamily member 9 (TNFSF9), a protein involved in the immune response[41] (Figure 3).

The AI-3 system is mainly described in enterohemorrhagic *E. coli* and is therefore linked to the development of intestinal epithelial lesions, suggesting its proinflammatory activity[120] (Table 3 and Figure 3). Indeed, AI-3 and its analogs increase IL-8 secretion by THP-1 monocytes[22]. Given that the AI-3 structure has only been uncovered recently[22], the direct effect of this molecule on the host is poorly known so far. In addition, since the AI-3 bacterial receptor can recognize host-synthesized epinephrine/norepinephrine (Figure 3), one could suggest that adrenergic receptors could recognize AI-3[19]. However, it has been shown that AI-3 and its analogs do not activate or modulate adrenergic signaling[22].

**Gram-positive AIP:** The effects of Gram-positive AIP bacteria on inflammation are far less documented than those of Gram-negative QS. Moreover, most of the studies on the effects of AIP on host inflammation describe the indirect effects of AIs through the modulation of bacterial metabolism.

A study described that AIP could selectively cross intestinal epithelial cell Caco-2 cell monolayers[121]. Additionally, it has been reported that AIP can cross the highly selective blood-brain barrier *in vivo*[122]. These processes seem to be peptide-specific: *Clostridium acetobutylicum* AIP easily penetrates the blood-brain barrier, while *Streptococcus pneumonia*'s AIP crosses it poorly[122]. This shows that small molecules such as AIs affect the host's physiology beyond the gastrointestinal tract. For instance, it has been described that AIP has various effects on muscle inflammation as part of the gut-muscle axis. De Spiegeleer *et al*[121] performed an extensive screening of 75 QS molecules on muscle cells. They demonstrated both pro- and anti-inflammatory effects of four peptides from the genera *Staphylococcus*, *Streptococcus*, *Lactobacillus*, and *Bacillus*, and some of those peptides have been described in the gut.

**Indole:** Several studies have reported that indole exerts anti-inflammatory effects in the intestine and protects against pathogenic infection[123] (Table 3). AhR, an important contributor to the maintenance of innate and adaptive immunity, drives most of these effects, particularly in the intestinal mucosa[117,118,124].

Several reports have shown altered Trp metabolism in gut inflammation in humans and mice [81,125-127]. A decrease in endogenous indole was observed in human feces from subjects with celiac disease or IBD[127]. This was associated with a decrease in AhR activity in the intestinal mucosa. In parallel, an increase in Trp levels in the same samples suggested that the gut microbiota-dependent metabolism of Trp was altered [127]. In mouse models of celiac disease and IBD, the implantation of indole-producing bacteria increases AhR activity and protects them from gut inflammation[81]. Interestingly, Moura-Alves *et al*[116] showed that 3-oxo-C12-HSL and HHQ had an inhibitory effect on AhR activity and could compete with well-known activators of AhR. This observation raises the question of potential competition between several QS molecules for AhR-dependent modulation of innate and adaptive immunity.

### *Effects of QS molecules on carcinogenesis*

There is growing evidence that gut microbiota dysbiosis plays a major role in CRC development[128]. Indeed, modifications of commensal gut microbiota in favor of opportunist bacteria promote intestinal inflammation, which is well known as a driver event in CRC onset[129,130]. Thus, the concept of the "bacterial driver-passenger model" highlights the crosstalk between host immunity and colonic microbiota[131]. For example, some driver pathogens, such as *Bacteroides fragilis,* have been proposed to promote a strong Th17 inflammatory response[132]. This proinflammatory microenvironment might favor colonization by opportunist pathogens such as *Fusobacterium spp.* Accordingly, Fusobacteria-dominant biofilms were associated with human CRC [133-135]. Altogether, these findings support that polymicrobial interactions and intercellular communications might play an important role in CRC development[136]. Nevertheless, how bacteria communicate with themselves and with the host during CRC remains poorly understood.

Recent findings provide new insights into the role of the QS molecule AI-2 in intercellular communication during CRC. First, the AI-2 concentration is increased in tumors compared to the surrounding normal tissue in human CRC[41]. These levels also correlate with the progression of the disease according to the CRC TNM (tumor node and metastasis) score[41]. Regarding the tumor immune microenvironment, the AI-2 concentration positively correlates with TNFSF9 expression, which is mainly expressed by tumor-associated macrophages, and negatively correlates with the CD4/CD8 ratio, suggesting that AI-2 associates with the antitumor response[41]. At the molecular level, it was demonstrated that AI-2 induces *in vitro* M1 polarization of U987-derived macrophages through the TNFSF9 signaling pathway[137]. These findings reveal that AI-2 could be an important factor linked to the immune tumor microenvironment and shed light on the role of the quorum-sensing system during CRC development and progression. Interestingly, mammalian epithelial cells are able to produce AI-2 analog molecules that mimic AI-2 effects (Figure 3), illustrating the complexity of bacteria-host crosstalk[45]. Thus, a better characterization of QS molecules involved in tumorigenesis might be an opportunity to improve our knowledge of the mechanisms underlying CRC development.

### *The host strikes back to QS*

Interkingdom signaling works in two ways, as host cells are able to counterattack the QS system using several strategies.

As described above, as part of AI-3 signaling, host hormones such as epinephrine and norepinephrine can be recognized by EHEC and lead to the expression of virulence genes[19]. This AI-3/epinephrine/norepinephrine signaling is not restricted to EHEC, and the receptor QseC is also expressed, for example, by the intestinal pathogenic *Salmonella enterica* serovar Typhimurium[138] (Table 1). Recently, *in silico* analysis suggested that another catecholamine neurotransmitter, dopamine, can bind to QseC. However, no effect *in vitro* was measured[139]. This study of interkingdom signaling through hormones has been named "microbial endocrinology"[140].

Interestingly, there is evidence that human epithelial cells can produce AI-2 mimicking molecules. The study was conducted on Caco-2 intestinal epithelial cells, and the authors showed that an AI-2 mimic is produced not only when cells are in contact with bacteria but also after TJ disruption by calcium deprivation or DSS treatment[45]. This emphasizes how much AI-2 is a universal language between Gram-positive and Gram-negative bacteria and the host (Figure 3).

Hosts have also developed defense tools against QS, leading to a mechanism named quorum quenching. Mammals can synthesize enzymes named paraoxonases (PONs) that hydrolyze the lactone ring of long-chain AHLs[141] (Figure 3). There are three types of PONs (PON 1, PON2, PON3) that are highly conserved across species, and PON2 has greater activity on AHLs[142]. It has been demonstrated that PON2 is more highly expressed in the human jejunum than in other parts of the intestine[143]. Interestingly, PON1 and PON3 are expressed at lower levels in patients with Crohn's disease and ulcerative colitis than in healthy subjects[144]. A case-control study has also shown that carriage of the PON1 R192 allele in Ashkenazi Jewish may confer protection against the development of IBD. This allele was significantly less common among IBD Ashkenazi patients, with a significant odds ratio of 0.61[145].

## QS IN THE GUT: FUTURE DIRECTIONS FOR THIS NEW PLAYER

### *Using QS to modulate gut microbiota: Application in gut ecosystem disorders*

Gut dysbiosis is an imbalance in the composition of microorganisms inside the digestive tract, especially described with bacteria. This dysregulation has been shown to be a preponderant risk factor in several digestive and extra digestive diseases[146-149]. For example, in IBD and recurrent *C. difficile* infections, it is well known that the over- and underrepresentation of certain phyla can lead to a pathologic state[150,151]. Modulating the gut microbiota may be the key to treating or even preventing such diseases by restoring normobiosis. Fecal microbiota transplantation is now commonly used in the setting of *C. difficile* infections[152,153]. However, the lack of standardization and the safety and quality issues of this procedure call for the development of new strategies.

Theoretically, AHLs remain good candidates in this approach using natural molecules from QS to modulate microbiota composition and gut inflammation. As seen above, AHL signaling may involve different pathways that contribute to controlling intestinal inflammation, such as inhibition of NF-κB, modulation, inhibition of MAPK activation, increase in regulatory T cell induction, decrease in proinflammatory cytokines, and modulation of junctional complexes in the epithelial barrier. Indeed, using QS molecules could play a role in both components (gut microbiota and host responses) of gut ecosystem disorders observed in metabolic and inflammatory diseases. AHL-based QS devices already exist as therapeutic applications for the dynamic control of Gram-negative bacterial populations, especially in infectious diseases. Other QS molecules could be extended as potential clinical therapies for diseases related to the gut microbiota that involve biofilm formation and antibiotic resistance[154]. Research efforts must investigate the potential of this new trial.

In addition to therapeutic applications, one could consider QS molecules as reliable biomarkers for dysbiosis-related chronic diseases such as IBD or CRC. Indeed, it has been shown that the presence of some AI-1 QS molecules in the gut ecosystem directly correlates with bacterial group size[39]. AHLs could represent a biomarker of the bacterial level population acting as a magnifying glass for dysbiosis. In addition, AI-2 concentration increased during adenomas to colorectal transition and CRC progression [41]. This opens the perspective for using the QS system as a biomarker for the prevention and follow-up of chronic diseases.

### *For the future*

Knowing which commensal bacteria carry QS systems, their site of production, their ability to be mobilized during dysbiosis, and their effect on the luminal or mucosal microenvironment are as many unresolved questions. The scientific community, together with gastroenterologists, needs to tackle these issues to pave the way for translation into clinical use. Future directions also involve designing dedicated QS derivatives targeting either the host cells or the bacterial compartment. Such QS derivatives have already been reported to control the epithelial cell inflammation pathway with a wider effect than natural 3-oxoC12:2 without bacterial-activating properties[92]. Using QS molecules as an approach to tackle the gut microbiota compartment has already been proven to be a successful strategy[42], thus leading to interesting perspectives. Considering the QS system as a new player in the gut ecosystem, it represents a control platform to shape the host's gut microbiota population and/or major physiological pathways.

## CONCLUSION

In conclusion, the intestinal microbiota interacts mutually with epithelial and immune cells of the coevolved host in a beneficial, reciprocal relationship. The QS signaling of bacteria probably contributes substantially to establishing symbiotic interactions in certain dynamics of interaction between the different kingdoms. A better understanding of QS systems by researchers and gastroenterologists involved in describing and managing ecological disorders of the intestinal ecosystem is a new approach that opens up fascinating therapeutic opportunities.

## ACKNOWLEDGEMENTS

We thank Rainteau D for his support and knowledge on quorum sensing. We acknowledge the Association François Aupetit for its unwavering support.

## REFERENCES

1 **Le Chatelier E**, Nielsen T, Qin J, Prifti E, Hildebrand F, Falony G, Almeida M, Arumugam M, Batto JM, Kennedy S, Leonard P, Li J, Burgdorf K, Grarup N, Jørgensen T, Brandslund I, Nielsen HB, Juncker AS, Bertalan M, Levenez F, Pons N, Rasmussen S, Sunagawa S, Tap J, Tims S, Zoetendal EG, Brunak S, Clément K, Doré J, Kleerebezem M, Kristiansen K, Renault P, Sicheritz-Ponten T, de Vos WM, Zucker JD, Raes J, Hansen T; MetaHIT consortium, Bork P, Wang J, Ehrlich SD, Pedersen O. Richness of human gut microbiome correlates with metabolic markers. *Nature* 2013; **500**: 541-546 [PMID: 23985870 DOI: 10.1038/nature12506]

2 **Sokol H**, Seksik P. The intestinal microbiota in inflammatory bowel diseases: time to connect with the host. *Curr Opin Gastroenterol* 2010; **26**: 327-331 [PMID: 20445446 DOI: 10.1097/MOG.0b013e328339536b]

3 **Agus A**, Clément K, Sokol H. Gut microbiota-derived metabolites as central regulators in metabolic disorders. *Gut* 2021; **70**: 1174-1182 [PMID: 33272977 DOI: 10.1136/gutjnl-2020-323071]

4 **Qin J**, Li R, Raes J, Arumugam M, Burgdorf KS, Manichanh C, Nielsen T, Pons N, Levenez F, Yamada T, Mende DR, Li J, Xu J, Li S, Li D, Cao J, Wang B, Liang H, Zheng H, Xie Y, Tap J, Lepage P, Bertalan M, Batto JM, Hansen T, Le Paslier D, Linneberg A, Nielsen HB, Pelletier E, Renault P, Sicheritz-Ponten T, Turner K, Zhu H, Yu C, Jian M, Zhou Y, Li Y, Zhang X, Qin N, Yang H, Wang J, Brunak S, Doré J, Guarner F, Kristiansen K, Pedersen O, Parkhill J, Weissenbach J; MetaHIT Consortium, Bork P, Ehrlich SD, Wang J. A human gut microbial gene catalogue established by metagenomic sequencing. *Nature* 2010; **464**: 59-65 [PMID: 20203603 DOI: 10.1038/nature08821]

5 **Jandhyala SM**, Talukdar R, Subramanyam C, Vuyyuru H, Sasikala M, Nageshwar Reddy D. Role of the normal gut microbiota. *World J Gastroenterol* 2015; **21**: 8787-8803 [PMID: 26269668 DOI: 10.3748/wjg.v21.i29.8787]

6 **Coquant G**, Grill JP, Seksik P. Impact of *N*-Acyl-Homoserine Lactones, Quorum Sensing Molecules, on Gut Immunity. *Front Immunol* 2020; **11**: 1827 [PMID: 32983093 DOI: 10.3389/fimmu.2020.01827]

7 **Prescott RD**, Decho AW. Flexibility and Adaptability of Quorum Sensing in Nature. *Trends Microbiol* 2020; **28**: 436-444 [PMID: 32001099 DOI: 10.1016/j.tim.2019.12.004]

8 **Wu L**, Luo Y. Bacterial Quorum-Sensing Systems and Their Role in Intestinal Bacteria-Host Crosstalk. *Front Microbiol* 2021; **12**: 611413 [PMID: 33584614 DOI: 10.3389/fmicb.2021.611413]

9 **Nealson KH**, Hastings JW. Bacterial bioluminescence: its control and ecological significance. *Microbiol Rev* 1979; **43**: 496-518 [PMID: 396467 DOI: 10.1128/mr.43.4.496-518.1979]

10 **Fuqua WC**, Winans SC, Greenberg EP. Quorum sensing in bacteria: the LuxR-LuxI family of cell density-responsive transcriptional regulators. *J Bacteriol* 1994; **176**: 269-275 [PMID: 8288518 DOI: 10.1128/jb.176.2.269-275.1994]

11 **Parsek MR**, Greenberg EP. Sociomicrobiology: the connections between quorum sensing and biofilms. *Trends Microbiol* 2005; **13**: 27-33 [PMID: 15639629 DOI: 10.1016/j.tim.2004.11.007]

12 **Ng WL**, Bassler BL. Bacterial quorum-sensing network architectures. *Annu Rev Genet* 2009; **43**: 197-222 [PMID: 19686078 DOI: 10.1146/annurev-genet-102108-134304]

13 **Takano E**, Chakraburtty R, Nihira T, Yamada Y, Bibb MJ. A complex role for the gamma-butyrolactone SCB1 in regulating antibiotic production in Streptomyces coelicolor A3(2). *Mol Microbiol* 2001; **41**: 1015-1028 [PMID: 11555283 DOI: 10.1046/j.1365-2958.2001.02562.x]

14 **Du YL**, Shen XL, Yu P, Bai LQ, Li YQ. Gamma-butyrolactone regulatory system of Streptomyces chattanoogensis links nutrient utilization, metabolism, and development. *Appl Environ Microbiol* 2011; **77**: 8415-8426 [PMID: 21948843 DOI: 10.1128/AEM.05898-11]

15 **Galloway WR**, Hodgkinson JT, Bowden SD, Welch M, Spring DR. Quorum sensing in Gram-negative bacteria: small-molecule modulation of AHL and AI-2 quorum sensing pathways. *Chem Rev* 2011; **111**: 28-67 [PMID: 21182299 DOI: 10.1021/cr100109t]

16 **Engebrecht J**, Silverman M. Identification of genes and gene products necessary for bacterial bioluminescence. *Proc Natl Acad Sci U S A* 1984; **81**: 4154-4158 [PMID: 6377310 DOI: 10.1073/pnas.81.13.4154]

17 **Stevens AM**, Dolan KM, Greenberg EP. Synergistic binding of the Vibrio fischeri LuxR transcriptional activator domain and RNA polymerase to the lux promoter region. *Proc Natl Acad Sci U S A* 1994; **91**: 12619-12623 [PMID: 7809088 DOI: 10.1073/pnas.91.26.12619]

18 **Case RJ**, Labbate M, Kjelleberg S. AHL-driven quorum-sensing circuits: their frequency and function among the Proteobacteria. *ISME J* 2008; **2**: 345-349 [PMID: 18273067 DOI: 10.1038/ismej.2008.13]

19 **Sperandio V**, Torres AG, Jarvis B, Nataro JP, Kaper JB. Bacteria-host communication: the language of hormones. *Proc Natl Acad Sci U S A* 2003; **100**: 8951-8956 [PMID: 12847292 DOI: 10.1073/pnas.1537100100]

20 **Carlson-Banning KM**, Sperandio V. Enterohemorrhagic Escherichia coli outwits hosts through sensing small molecules. *Curr Opin Microbiol* 2018; **41**: 83-88 [PMID: 29258058 DOI: 10.1016/j.mib.2017.12.002]

21 **Walters M**, Sircili MP, Sperandio V. AI-3 synthesis is not dependent on luxS in Escherichia coli. *J Bacteriol* 2006; **188**: 5668-5681 [PMID: 16885435 DOI: 10.1128/JB.00648-06]

22 **Kim CS**, Gatsios A, Cuesta S, Lam YC, Wei Z, Chen H, Russell RM, Shine EE, Wang R, Wyche TP, Piizzi G, Flavell RA, Palm NW, Sperandio V, Crawford JM. Characterization of Autoinducer-3 Structure and Biosynthesis in *E. coli*. *ACS Cent Sci* 2020; **6**: 197-206 [PMID: 32123737 DOI: 10.1021/acscentsci.9b01076]

23 **Schauder S**, Bassler BL. The languages of bacteria. *Genes Dev* 2001; **15**: 1468-1480 [PMID: 11410527 DOI: 10.1101/gad.899601]

24 **Surette MG**, Miller MB, Bassler BL. Quorum sensing in Escherichia coli, Salmonella typhimurium, and Vibrio harveyi: a new family of genes responsible for autoinducer production. *Proc Natl Acad Sci U S A* 1999; **96**: 1639-1644 [PMID: 9990077 DOI: 10.1073/pnas.96.4.1639]

25 **Chen X**, Schauder S, Potier N, Van Dorsselaer A, Pelczer I, Bassler BL, Hughson FM. Structural identification of a bacterial quorum-sensing signal containing boron. *Nature* 2002; **415**: 545-549 [PMID: 11823863 DOI: 10.1038/415545a]

26 **Miller ST**, Xavier KB, Campagna SR, Taga ME, Semmelhack MF, Bassler BL, Hughson FM. Salmonella typhimurium recognizes a chemically distinct form of the bacterial quorum-sensing signal AI-2. *Mol Cell* 2004; **15**: 677-687 [PMID: 15350213 DOI: 10.1016/j.molcel.2004.07.020]

27 **Pereira CS**, Thompson JA, Xavier KB. AI-2-mediated signalling in bacteria. *FEMS Microbiol Rev* 2013; **37**: 156-181 [PMID: 22712853 DOI: 10.1111/j.1574-6976.2012.00345.x]

28 **Defoirdt T**. Amino acid-derived quorum sensing molecules controlling the virulence of vibrios (and beyond). *PLoS Pathog* 2019; **15**: e1007815 [PMID: 31295324 DOI: 10.1371/journal.ppat.1007815]

29 **Lee JH**, Lee J. Indole as an intercellular signal in microbial communities. *FEMS Microbiol Rev* 2010; **34**: 426-444 [PMID: 20070374 DOI: 10.1111/j.1574-6976.2009.00204.x]

30 **Pena RT**, Blasco L, Ambroa A, González-Pedrajo B, Fernández-García L, López M, Bleriot I, Bou G, García-Contreras R, Wood TK, Tomás M. Relationship Between Quorum Sensing and Secretion Systems. *Front Microbiol* 2019; **10**: 1100 [PMID: 31231316 DOI: 10.3389/fmicb.2019.01100]

31 **Rumbaugh KP**, Kaufmann GF. Exploitation of host signaling pathways by microbial quorum sensing signals. *Curr Opin Microbiol* 2012; **15**: 162-168 [PMID: 22204809 DOI: 10.1016/j.mib.2011.12.003]

32 **Wang G**, Huang S, Wang Y, Cai S, Yu H, Liu H, Zeng X, Zhang G, Qiao S. Bridging intestinal immunity and gut microbiota by metabolites. *Cell Mol Life Sci* 2019; **76**: 3917-3937 [PMID: 31250035 DOI: 10.1007/s00018-019-03190-6]

33 **Defoirdt T**. Quorum-Sensing Systems as Targets for Antivirulence Therapy. *Trends Microbiol* 2018; **26**: 313-328 [PMID: 29132819 DOI: 10.1016/j.tim.2017.10.005]

34 **Tobias NJ**, Brehm J, Kresovic D, Brameyer S, Bode HB, Heermann R. New Vocabulary for Bacterial Communication. *Chembiochem* 2020; **21**: 759-768 [PMID: 31709676 DOI: 10.1002/cbic.201900580]

35 **Agodi A**, Barchitta M, Cipresso R, Giaquinta L, Romeo MA, Denaro C. Pseudomonas aeruginosa carriage, colonization, and infection in ICU patients. *Intensive Care Med* 2007; **33**: 1155-1161 [PMID: 17503016 DOI: 10.1007/s00134-007-0671-6]

36 **Bertrand X**, Thouverez M, Talon D, Boillot A, Capellier G, Floriot C, Hélias JP. Endemicity, molecular diversity and colonisation routes of Pseudomonas aeruginosa in intensive care units. *Intensive Care Med* 2001; **27**: 1263-1268 [PMID: 11511937 DOI: 10.1007/s001340100979]

37 **Swearingen MC**, Sabag-Daigle A, Ahmer BM. Are there acyl-homoserine lactones within mammalian intestines? *J Bacteriol* 2013; **195**: 173-179 [PMID: 23144246 DOI: 10.1128/JB.01341-12]

38 **Kumari A**, Pasini P, Deo SK, Flomenhoft D, Shashidhar H, Daunert S. Biosensing systems for the detection of bacterial quorum signaling molecules. *Anal Chem* 2006; **78**: 7603-7609 [PMID: 17105149 DOI: 10.1021/ac061421n]

39 **Landman C**, Grill JP, Mallet JM, Marteau P, Humbert L, Le Balc'h E, Maubert MA, Perez K, Chaara W, Brot L, Beaugerie L, Sokol H, Thenet S, Rainteau D, Seksik P, Quévrain E; Saint Antoine IBD Network. Inter-kingdom effect on epithelial cells of the N-Acyl homoserine lactone 3-oxo-C12:2, a major quorum-sensing molecule from gut microbiota. *PLoS One* 2018; **13**: e0202587 [PMID: 30157234 DOI: 10.1371/journal.pone.0202587]

40 **Kaper JB**, Sperandio V. Bacterial cell-to-cell signaling in the gastrointestinal tract. *Infect Immun* 2005; **73**: 3197-3209 [PMID: 15908344 DOI: 10.1128/iai.73.6.3197-3209.2005]

41 **Li Q**, Peng W, Wu J, Wang X, Ren Y, Li H, Peng Y, Tang X, Fu X. Autoinducer-2 of gut microbiota, a potential novel marker for human colorectal cancer, is associated with the activation of TNFSF9 signaling in macrophages. *Oncoimmunology* 2019; **8**: e1626192 [PMID: 31646072 DOI: 10.1080/2162402X.2019.1626192]

42 **Thompson JA**, Oliveira RA, Djukovic A, Ubeda C, Xavier KB. Manipulation of the quorum sensing signal AI-2 affects the antibiotic-treated gut microbiota. *Cell Rep* 2015; **10**: 1861-1871 [PMID: 25801025 DOI: 10.1016/j.celrep.2015.02.049]

43 **Darkoh C**, Plants-Paris K, Bishoff D, DuPont HL. Clostridium difficile Modulates the Gut Microbiota by Inducing the Production of Indole, an Interkingdom Signaling and Antimicrobial Molecule. *mSystems* 2019; **4** [PMID: 30944877 DOI: 10.1128/mSystems.00346-18]

44 **Chen J**, Ma M, Uzal FA, McClane BA. Host cell-induced signaling causes Clostridium perfringens to upregulate production of toxins important for intestinal infections. *Gut Microbes* 2014; **5**: 96-107 [PMID: 24061146 DOI: 10.4161/gmic.26419]

45 **Ismail AS**, Valastyan JS, Bassler BL. A Host-Produced Autoinducer-2 Mimic Activates Bacterial Quorum Sensing. *Cell Host Microbe* 2016; **19**: 470-480 [PMID: 26996306 DOI: 10.1016/j.chom.2016.02.020]

46 **Pinheiro J**, Lisboa J, Pombinho R, Carvalho F, Carreaux A, Brito C, Pöntinen A, Korkeala H, Dos Santos NMS, Morais-Cabral JH, Sousa S, Cabanes D. MouR controls the expression of the Listeria monocytogenes Agr system and mediates virulence. *Nucleic Acids Res* 2018; **46**: 9338-9352 [PMID: 30011022 DOI: 10.1093/nar/gky624]

47 **Turner JR**. Intestinal mucosal barrier function in health and disease. *Nat Rev Immunol* 2009; **9**: 799-809 [PMID: 19855405 DOI: 10.1038/nri2653]

48 **Bevins CL**, Salzman NH. Paneth cells, antimicrobial peptides and maintenance of intestinal homeostasis. *Nat Rev Microbiol* 2011; **9**: 356-368 [PMID: 21423246 DOI: 10.1038/nrmicro2546]

49 **Johansson ME**, Hansson GC. Immunological aspects of intestinal mucus and mucins. *Nat Rev Immunol* 2016; **16**: 639-649 [PMID: 27498766 DOI: 10.1038/nri.2016.88]

50 **Hooper LV**, Macpherson AJ. Immune adaptations that maintain homeostasis with the intestinal microbiota. *Nat Rev Immunol* 2010; **10**: 159-169 [PMID: 20182457 DOI: 10.1038/nri2710]

51 **Peterson LW**, Artis D. Intestinal epithelial cells: regulators of barrier function and immune homeostasis. *Nat Rev Immunol* 2014; **14**: 141-153 [PMID: 24566914 DOI: 10.1038/nri3608]

52 **Wang L**, Zhu L, Qin S. Gut Microbiota Modulation on Intestinal Mucosal Adaptive Immunity. *J Immunol Res* 2019; **2019**: 4735040 [PMID: 31687412 DOI: 10.1155/2019/4735040]

53 **Holm A**, Vikström E. Quorum sensing communication between bacteria and human cells: signals, targets, and functions. *Front Plant Sci* 2014; **5**: 309 [PMID: 25018766 DOI: 10.3389/fpls.2014.00309]

54 **Golovkine G**, Reboud E, Huber P. *Pseudomonas aeruginosa* Takes a Multi-Target Approach to Achieve Junction Breach. *Front Cell Infect Microbiol* 2017; **7**: 532 [PMID: 29379773 DOI: 10.3389/fcimb.2017.00532]

55 **Eum SY**, Jaraki D, Bertrand L, András IE, Toborek M. Disruption of epithelial barrier by quorum-sensing N-3-(oxododecanoyl)-homoserine lactone is mediated by matrix metalloproteinases. *Am J Physiol Gastrointest Liver Physiol* 2014; **306**: G992-G1001 [PMID: 24742991 DOI: 10.1152/ajpgi.00016.2014]

56 **Halldorsson S**, Gudjonsson T, Gottfredsson M, Singh PK, Gudmundsson GH, Baldursson O. Azithromycin maintains airway epithelial integrity during Pseudomonas aeruginosa infection. *Am J Respir Cell Mol Biol* 2010; **42**: 62-68 [PMID: 19372247 DOI: 10.1165/rcmb.2008-0357OC]

57 **Schwarzer C**, Ravishankar B, Patanwala M, Shuai S, Fu Z, Illek B, Fischer H, Machen TE. Thapsigargin blocks Pseudomonas aeruginosa homoserine lactone-induced apoptosis in airway epithelia. *Am J Physiol Cell Physiol* 2014; **306**: C844-C855 [PMID: 24598360 DOI: 10.1152/ajpcell.00002.2014]

58 **Vikström E**, Bui L, Konradsson P, Magnusson KE. Role of calcium signalling and phosphorylations in disruption of the epithelial junctions by Pseudomonas aeruginosa quorum sensing molecule. *Eur J Cell Biol* 2010; **89**: 584-597 [PMID: 20434232 DOI: 10.1016/j.ejcb.2010.03.002]

59 **Vikström E**, Bui L, Konradsson P, Magnusson KE. The junctional integrity of epithelial cells is modulated by Pseudomonas aeruginosa quorum sensing molecule through phosphorylation-dependent mechanisms. *Exp Cell Res* 2009; **315**: 313-326 [PMID: 19038248 DOI: 10.1016/j.yexcr.2008.10.044]

60 **Vikström E**, Tafazoli F, Magnusson KE. Pseudomonas aeruginosa quorum sensing molecule N-(3 oxododecanoyl)-l-homoserine lactone disrupts epithelial barrier integrity of Caco-2 cells. *FEBS Lett* 2006; **580**: 6921-6928 [PMID: 17157842 DOI: 10.1016/j.febslet.2006.11.057]

61 **Aguanno D**, Coquant G, Postal BG, Osinski C, Wieckowski M, Stockholm D, Grill JP, Carrière V, Seksik P, Thenet S. The intestinal quorum sensing 3-oxo-C12:2 Acyl homoserine lactone limits cytokine-induced tight junction disruption. *Tissue Barriers* 2020; **8**: 1832877 [PMID: 33100129 DOI: 10.1080/21688370.2020.1832877]

62 **Tao S**, Xiong Y, Han D, Pi Y, Zhang H, Wang J. N-(3-oxododecanoyl)-l-homoserine lactone disrupts intestinal epithelial barrier through triggering apoptosis and collapsing extracellular matrix and tight junction. *J Cell Physiol* 2021; **236**: 5771-5784 [PMID: 33400297 DOI: 10.1002/jcp.30261]

63 **Buckley A**, Turner JR. Cell Biology of Tight Junction Barrier Regulation and Mucosal Disease.

*Cold Spring Harb Perspect Biol* 2018; **10** [PMID: 28507021 DOI: 10.1101/cshperspect.a029314]

64 **Su L**, Nalle SC, Shen L, Turner ES, Singh G, Breskin LA, Khramtsova EA, Khramtsova G, Tsai PY, Fu YX, Abraham C, Turner JR. TNFR2 activates MLCK-dependent tight junction dysregulation to cause apoptosis-mediated barrier loss and experimental colitis. *Gastroenterology* 2013; **145**: 407-415 [PMID: 23619146 DOI: 10.1053/j.gastro.2013.04.011]

65 **Thoo L**, Noti M, Krebs P. Keep calm: the intestinal barrier at the interface of peace and war. *Cell Death Dis* 2019; **10**: 849 [PMID: 31699962 DOI: 10.1038/s41419-019-2086-z]

66 **France MM**, Turner JR. The mucosal barrier at a glance. *J Cell Sci* 2017; **130**: 307-314 [PMID: 28062847 DOI: 10.1242/jcs.193482]

67 **Tao S**, Luo Y, Bin He, Liu J, Qian X, Ni Y, Zhao R. Paraoxonase 2 modulates a proapoptotic function in LS174T cells in response to quorum sensing molecule N-(3-oxododecanoyl)-L-homoserine lactone. *Sci Rep* 2016; **6**: 28778 [PMID: 27364593 DOI: 10.1038/srep28778]

68 **Tao S**, Niu L, Cai L, Geng Y, Hua C, Ni Y, Zhao R. N-(3-oxododecanoyl)-l-homoserine lactone modulates mitochondrial function and suppresses proliferation in intestinal goblet cells. *Life Sci* 2018; **201**: 81-88 [PMID: 29596921 DOI: 10.1016/j.lfs.2018.03.049]

69 **Tao S**, Sun Q, Cai L, Geng Y, Hua C, Ni Y, Zhao R. Caspase-1-dependent mechanism mediating the harmful impacts of the quorum-sensing molecule N-(3-oxo-dodecanoyl)-l-homoserine lactone on the intestinal cells. *J Cell Physiol* 2019; **234**: 3621-3633 [PMID: 30471106 DOI: 10.1002/jcp.27132]

70 **Taguchi R**, Tanaka S, Joe GH, Maseda H, Nomura N, Ohnishi J, Ishizuka S, Shimizu H, Miyazaki H. Mucin 3 is involved in intestinal epithelial cell apoptosis *via* N-(3-oxododecanoyl)-L-homoserine lactone-induced suppression of Akt phosphorylation. *Am J Physiol Cell Physiol* 2014; **307**: C162-C168 [PMID: 24848113 DOI: 10.1152/ajpcell.00271.2013]

71 **Sturm A**, Dignass AU. Epithelial restitution and wound healing in inflammatory bowel disease. *World J Gastroenterol* 2008; **14**: 348-353 [PMID: 18200658 DOI: 10.3748/wjg.14.348]

72 **Karlsson T**, Turkina MV, Yakymenko O, Magnusson KE, Vikström E. The Pseudomonas aeruginosa N-acylhomoserine lactone quorum sensing molecules target IQGAP1 and modulate epithelial cell migration. *PLoS Pathog* 2012; **8**: e1002953 [PMID: 23071436 DOI: 10.1371/journal.ppat.1002953]

73 **Charlton TS**, de Nys R, Netting A, Kumar N, Hentzer M, Givskov M, Kjelleberg S. A novel and sensitive method for the quantification of N-3-oxoacyl homoserine lactones using gas chromatography-mass spectrometry: application to a model bacterial biofilm. *Environ Microbiol* 2000; **2**: 530-541 [PMID: 11233161 DOI: 10.1046/j.1462-2920.2000.00136.x]

74 **Fujiya M**, Musch MW, Nakagawa Y, Hu S, Alverdy J, Kohgo Y, Schneewind O, Jabri B, Chang EB. The Bacillus subtilis quorum-sensing molecule CSF contributes to intestinal homeostasis *via* OCTN2, a host cell membrane transporter. *Cell Host Microbe* 2007; **1**: 299-308 [PMID: 18005709 DOI: 10.1016/j.chom.2007.05.004]

75 **Petrof EO**, Ciancio MJ, Chang EB. Role and regulation of intestinal epithelial heat shock proteins in health and disease. *Chin J Dig Dis* 2004; **5**: 45-50 [PMID: 15612656 DOI: 10.1111/j.1443-9573.2004.00154.x]

76 **Bansal T**, Alaniz RC, Wood TK, Jayaraman A. The bacterial signal indole increases epithelial-cell tight-junction resistance and attenuates indicators of inflammation. *Proc Natl Acad Sci U S A* 2010; **107**: 228-233 [PMID: 19966295 DOI: 10.1073/pnas.0906112107]

77 **Shimada Y**, Kinoshita M, Harada K, Mizutani M, Masahata K, Kayama H, Takeda K. Commensal bacteria-dependent indole production enhances epithelial barrier function in the colon. *PLoS One* 2013; **8**: e80604 [PMID: 24278294 DOI: 10.1371/journal.pone.0080604]

78 **Shin JH**, Lee YK, Shon WJ, Kim B, Jeon CO, Cho JY, Morse HC 3rd, Choi EY, Shin DM. Gut microorganisms and their metabolites modulate the severity of acute colitis in a tryptophan metabolism-dependent manner. *Eur J Nutr* 2020; **59**: 3591-3601 [PMID: 32055962 DOI: 10.1007/s00394-020-02194-4]

79 **Venkatesh M**, Mukherjee S, Wang H, Li H, Sun K, Benechet AP, Qiu Z, Maher L, Redinbo MR, Phillips RS, Fleet JC, Kortagere S, Mukherjee P, Fasano A, Le Ven J, Nicholson JK, Dumas ME, Khanna KM, Mani S. Symbiotic bacterial metabolites regulate gastrointestinal barrier function *via* the xenobiotic sensor PXR and Toll-like receptor 4. *Immunity* 2014; **41**: 296-310 [PMID: 25065623 DOI: 10.1016/j.immuni.2014.06.014]

80 **Vyhlídalová B**, Krasulová K, Pečinková P, Marcalíková A, Vrzal R, Zemánková L, Vančo J, Trávníček Z, Vondráček J, Karasová M, Mani S, Dvořák Z. Gut Microbial Catabolites of Tryptophan Are Ligands and Agonists of the Aryl Hydrocarbon Receptor: A Detailed Characterization. *Int J Mol Sci* 2020; **21** [PMID: 32283770 DOI: 10.3390/ijms21072614]

81 **Lamas B**, Richard ML, Leducq V, Pham HP, Michel ML, Da Costa G, Bridonneau C, Jegou S, Hoffmann TW, Natividad JM, Brot L, Taleb S, Couturier-Maillard A, Nion-Larmurier I, Merabtene F, Seksik P, Bourrier A, Cosnes J, Ryffel B, Beaugerie L, Launay JM, Langella P, Xavier RJ, Sokol H. CARD9 impacts colitis by altering gut microbiota metabolism of tryptophan into aryl hydrocarbon receptor ligands. *Nat Med* 2016; **22**: 598-605 [PMID: 27158904 DOI: 10.1038/nm.4102]

82 **Natividad JM**, Agus A, Planchais J, Lamas B, Jarry AC, Martin R, Michel ML, Chong-Nguyen C, Roussel R, Straube M, Jegou S, McQuitty C, Le Gall M, da Costa G, Lecornet E, Michaudel C, Modoux M, Glodt J, Bridonneau C, Sovran B, Dupraz L, Bado A, Richard ML, Langella P, Hansel B, Launay JM, Xavier RJ, Duboc H, Sokol H. Impaired Aryl Hydrocarbon Receptor Ligand Production by the Gut Microbiota Is a Key Factor in Metabolic Syndrome. *Cell Metab* 2018; **28**:

737-749.e4 [PMID: 30057068 DOI: 10.1016/j.cmet.2018.07.001]
83 **Zelante T**, Iannitti RG, Cunha C, De Luca A, Giovannini G, Pieraccini G, Zecchi R, D'Angelo C, Massi-Benedetti C, Fallarino F, Carvalho A, Puccetti P, Romani L. Tryptophan catabolites from microbiota engage aryl hydrocarbon receptor and balance mucosal reactivity *via* interleukin-22. *Immunity* 2013; **39**: 372-385 [PMID: 23973224 DOI: 10.1016/j.immuni.2013.08.003]
84 **Han B**, Sheng B, Zhang Z, Pu A, Yin J, Wang Q, Yang K, Sun L, Yu M, Qiu Y, Xiao W, Yang H. Aryl Hydrocarbon Receptor Activation in Intestinal Obstruction Ameliorates Intestinal Barrier Dysfunction Via Suppression of MLCK-MLC Phosphorylation Pathway. *Shock* 2016; **46**: 319-328 [PMID: 26939041 DOI: 10.1097/SHK.0000000000000594]
85 **Postal BG**, Ghezzal S, Aguanno D, André S, Garbin K, Genser L, Brot-Laroche E, Poitou C, Soula H, Leturque A, Clément K, Carrière V. AhR activation defends gut barrier integrity against damage occurring in obesity. *Mol Metab* 2020; **39**: 101007 [PMID: 32360426 DOI: 10.1016/j.molmet.2020.101007]
86 **Yu M**, Wang Q, Ma Y, Li L, Yu K, Zhang Z, Chen G, Li X, Xiao W, Xu P, Yang H. Aryl Hydrocarbon Receptor Activation Modulates Intestinal Epithelial Barrier Function by Maintaining Tight Junction Integrity. *Int J Biol Sci* 2018; **14**: 69-77 [PMID: 29483826 DOI: 10.7150/ijbs.22259]
87 **Ziegler N**, Awwad K, Fisslthaler B, Reis M, Devraj K, Corada M, Minardi SP, Dejana E, Plate KH, Fleming I, Liebner S. β-Catenin Is Required for Endothelial Cyp1b1 Regulation Influencing Metabolic Barrier Function. *J Neurosci* 2016; **36**: 8921-8935 [PMID: 27559173 DOI: 10.1523/JNEUROSCI.0148-16.2016]
88 **Taleb S**. Tryptophan Dietary Impacts Gut Barrier and Metabolic Diseases. *Front Immunol* 2019; **10**: 2113 [PMID: 31552046 DOI: 10.3389/fimmu.2019.02113]
89 **Wang J**, Wang P, Tian H, Tian F, Zhang Y, Zhang L, Gao X, Wang X. Aryl hydrocarbon receptor/IL-22/Stat3 signaling pathway is involved in the modulation of intestinal mucosa antimicrobial molecules by commensal microbiota in mice. *Innate Immun* 2018; **24**: 297-306 [PMID: 29976114 DOI: 10.1177/1753425918785016]
90 **Cotton JA**, Platnich JM, Muruve DA, Jijon HB, Buret AG, Beck PL. Interleukin-8 in gastrointestinal inflammation and malignancy: induction and clinical consequences. *Int J Interferon Cytokine Mediat Res* 2016; **8**: 13-34 [DOI: 10.2147/IJICMR.S63682]
91 **Ghezzal S**, Postal BG, Quevrain E, Brot L, Seksik P, Leturque A, Thenet S, Carrière V. Palmitic acid damages gut epithelium integrity and initiates inflammatory cytokine production. *Biochim Biophys Acta Mol Cell Biol Lipids* 2020; **1865**: 158530 [PMID: 31647994 DOI: 10.1016/j.bbalip.2019.158530]
92 **Peyrottes A**, Coquant G, Brot L, Rainteau D, Seksik P, Grill JP, Mallet JM. Anti-Inflammatory Effects of Analogues of *N*-Acyl Homoserine Lactones on Eukaryotic Cells. *Int J Mol Sci* 2020; **21** [PMID: 33322538 DOI: 10.3390/ijms21249448]
93 **Turkina MV**, Vikström E. Bacteria-Host Crosstalk: Sensing of the Quorum in the Context of Pseudomonas aeruginosa Infections. *J Innate Immun* 2019; **11**: 263-279 [PMID: 30428481 DOI: 10.1159/000494069]
94 **Telford G**, Wheeler D, Williams P, Tomkins PT, Appleby P, Sewell H, Stewart GS, Bycroft BW, Pritchard DI. The Pseudomonas aeruginosa quorum-sensing signal molecule N-(3-oxododecanoyl)-L-homoserine lactone has immunomodulatory activity. *Infect Immun* 1998; **66**: 36-42 [PMID: 9423836 DOI: 10.1128/IAI.66.1.36-42.1998]
95 **Smith RS**, Harris SG, Phipps R, Iglewski B. The Pseudomonas aeruginosa quorum-sensing molecule N-(3-oxododecanoyl)homoserine lactone contributes to virulence and induces inflammation in vivo. *J Bacteriol* 2002; **184**: 1132-1139 [PMID: 11807074 DOI: 10.1128/jb.184.4.1132-1139.2002]
96 **Ritchie AJ**, Jansson A, Stallberg J, Nilsson P, Lysaght P, Cooley MA. The Pseudomonas aeruginosa quorum-sensing molecule N-3-(oxododecanoyl)-L-homoserine lactone inhibits T-cell differentiation and cytokine production by a mechanism involving an early step in T-cell activation. *Infect Immun* 2005; **73**: 1648-1655 [PMID: 15731065 DOI: 10.1128/iai.73.3.1648-1655.2005]
97 **Kim K**, Kim SH, Lépine F, Cho YH, Lee GR. Global gene expression analysis on the target genes of PQS and HHQ in J774A.1 monocyte/macrophage cells. *Microb Pathog* 2010; **49**: 174-180 [PMID: 20595074 DOI: 10.1016/j.micpath.2010.05.009]
98 **Lin J**, Cheng J, Wang Y, Shen X. The *Pseudomonas* Quinolone Signal (PQS): Not Just for Quorum Sensing Anymore. *Front Cell Infect Microbiol* 2018; **8**: 230 [PMID: 30023354 DOI: 10.3389/fcimb.2018.00230]
99 **Li Y**, Zhou H, Zhang Y, Chen C, Huang B, Qu P, Zeng J, Shunmei E, Zhang X, Liu J. N-3-(oxododecanoyl)-L-homoserine lactone promotes the induction of regulatory T-cells by preventing human dendritic cell maturation. *Exp Biol Med (Maywood)* 2015; **240**: 896-903 [PMID: 25749498 DOI: 10.1177/1535370214564742]
100 **Skindersoe ME**, Zeuthen LH, Brix S, Fink LN, Lazenby J, Whittall C, Williams P, Diggle SP, Froekiaer H, Cooley M, Givskov M. Pseudomonas aeruginosa quorum-sensing signal molecules interfere with dendritic cell-induced T-cell proliferation. *FEMS Immunol Med Microbiol* 2009; **55**: 335-345 [PMID: 19187218 DOI: 10.1111/j.1574-695X.2008.00533.x]
101 **Jacobi CA**, Schiffner F, Henkel M, Waibel M, Stork B, Daubrawa M, Eberl L, Gregor M, Wesselborg S. Effects of bacterial N-acyl homoserine lactones on human Jurkat T lymphocytes-OdDHL induces apoptosis *via* the mitochondrial pathway. *Int J Med Microbiol* 2009; **299**: 509-519 [PMID: 19464950 DOI: 10.1016/j.ijmm.2009.03.005]

102 **Tateda K**, Ishii Y, Horikawa M, Matsumoto T, Miyairi S, Pechere JC, Standiford TJ, Ishiguro M, Yamaguchi K. The Pseudomonas aeruginosa autoinducer N-3-oxododecanoyl homoserine lactone accelerates apoptosis in macrophages and neutrophils. *Infect Immun* 2003; **71**: 5785-5793 [PMID: 14500500 DOI: 10.1128/iai.71.10.5785-5793.2003]

103 **Kim K**, Kim YU, Koh BH, Hwang SS, Kim SH, Lépine F, Cho YH, Lee GR. HHQ and PQS, two Pseudomonas aeruginosa quorum-sensing molecules, down-regulate the innate immune responses through the nuclear factor-kappaB pathway. *Immunology* 2010; **129**: 578-588 [PMID: 20102415 DOI: 10.1111/j.1365-2567.2009.03160.x]

104 **Kravchenko VV**, Kaufmann GF, Mathison JC, Scott DA, Katz AZ, Grauer DC, Lehmann M, Meijler MM, Janda KD, Ulevitch RJ. Modulation of gene expression *via* disruption of NF-kappaB signaling by a bacterial small molecule. *Science* 2008; **321**: 259-263 [PMID: 18566250 DOI: 10.1126/science.1156499]

105 **Kravchenko VV**, Kaufmann GF, Mathison JC, Scott DA, Katz AZ, Wood MR, Brogan AP, Lehmann M, Mee JM, Iwata K, Pan Q, Fearns C, Knaus UG, Meijler MM, Janda KD, Ulevitch RJ. N-(3-oxo-acyl)homoserine lactones signal cell activation through a mechanism distinct from the canonical pathogen-associated molecular pattern recognition receptor pathways. *J Biol Chem* 2006; **281**: 28822-28830 [PMID: 16893899 DOI: 10.1074/jbc.M606613200]

106 **Wu R**, Li X, Ma N, Jin X, Yuan X, Qu C, Tang H, Liu Z, Zhang Z. Bacterial Quorum Sensing Molecules Promote Allergic Airway Inflammation by Activating the Retinoic Acid Response. *iScience* 2020; **23**: 101288 [PMID: 32622265 DOI: 10.1016/j.isci.2020.101288]

107 **Vikström E**, Magnusson KE, Pivoriūnas A. The Pseudomonas aeruginosa quorum-sensing molecule N-(3-oxododecanoyl)-L-homoserine lactone stimulates phagocytic activity in human macrophages through the p38 MAPK pathway. *Microbes Infect* 2005; **7**: 1512-1518 [PMID: 16039899 DOI: 10.1016/j.micinf.2005.05.012]

108 **Gaida MM**, Dapunt U, Hänsch GM. Sensing developing biofilms: the bitter receptor T2R38 on myeloid cells. *Pathog Dis* 2016; **74** [PMID: 26782143 DOI: 10.1093/femspd/ftw004]

109 **Lee RJ**, Xiong G, Kofonow JM, Chen B, Lysenko A, Jiang P, Abraham V, Doghramji L, Adappa ND, Palmer JN, Kennedy DW, Beauchamp GK, Doulias PT, Ischiropoulos H, Kreindler JL, Reed DR, Cohen NA. T2R38 taste receptor polymorphisms underlie susceptibility to upper respiratory infection. *J Clin Invest* 2012; **122**: 4145-4159 [PMID: 23041624 DOI: 10.1172/JCI64240]

110 **Tizzano M**, Gulbransen BD, Vandenbeuch A, Clapp TR, Herman JP, Sibhatu HM, Churchill ME, Silver WL, Kinnamon SC, Finger TE. Nasal chemosensory cells use bitter taste signaling to detect irritants and bacterial signals. *Proc Natl Acad Sci U S A* 2010; **107**: 3210-3215 [PMID: 20133764 DOI: 10.1073/pnas.0911934107]

111 **Rozengurt E**. Taste receptors in the gastrointestinal tract. I. Bitter taste receptors and alpha-gustducin in the mammalian gut. *Am J Physiol Gastrointest Liver Physiol* 2006; **291**: G171-G177 [PMID: 16710053 DOI: 10.1152/ajpgi.00073.2006]

112 **Carrai M**, Steinke V, Vodicka P, Pardini B, Rahner N, Holinski-Feder E, Morak M, Schackert HK, Görgens H, Stemmler S, Betz B, Kloor M, Engel C, Büttner R, Naccarati A, Vodickova L, Novotny J, Stein A, Hemminki K, Propping P, Försti A, Canzian F, Barale R, Campa D. Association between TAS2R38 gene polymorphisms and colorectal cancer risk: a case-control study in two independent populations of Caucasian origin. *PLoS One* 2011; **6**: e20464 [PMID: 21674048 DOI: 10.1371/journal.pone.0020464]

113 **Cohen LJ**, Esterhazy D, Kim SH, Lemetre C, Aguilar RR, Gordon EA, Pickard AJ, Cross JR, Emiliano AB, Han SM, Chu J, Vila-Farres X, Kaplitt J, Rogoz A, Calle PY, Hunter C, Bitok JK, Brady SF. Commensal bacteria make GPCR ligands that mimic human signalling molecules. *Nature* 2017; **549**: 48-53 [PMID: 28854168 DOI: 10.1038/nature23874]

114 **Cooley M**, Chhabra SR, Williams P. N-Acylhomoserine lactone-mediated quorum sensing: a twist in the tail and a blow for host immunity. *Chem Biol* 2008; **15**: 1141-1147 [PMID: 19022174 DOI: 10.1016/j.chembiol.2008.10.010]

115 **Jahoor A**, Patel R, Bryan A, Do C, Krier J, Watters C, Wahli W, Li G, Williams SC, Rumbaugh KP. Peroxisome proliferator-activated receptors mediate host cell proinflammatory responses to Pseudomonas aeruginosa autoinducer. *J Bacteriol* 2008; **190**: 4408-4415 [PMID: 18178738 DOI: 10.1128/JB.01444-07]

116 **Moura-Alves P**, Puyskens A, Stinn A, Klemm M, Guhlich-Bornhof U, Dorhoi A, Furkert J, Kreuchwig A, Protze J, Lozza L, Pei G, Saikali P, Perdomo C, Mollenkopf HJ, Hurwitz R, Kirschhoefer F, Brenner-Weiss G, Weiner J 3rd, Oschkinat H, Kolbe M, Krause G, Kaufmann SHE. Host monitoring of quorum sensing during *Pseudomonas aeruginosa* infection. *Science* 2019; **366** [PMID: 31857448 DOI: 10.1126/science.aaw1629]

117 **Lamas B**, Natividad JM, Sokol H. Aryl hydrocarbon receptor and intestinal immunity. *Mucosal Immunol* 2018; **11**: 1024-1038 [PMID: 29626198 DOI: 10.1038/s41385-018-0019-2]

118 **Trikha P**, Lee DA. The role of AhR in transcriptional regulation of immune cell development and function. *Biochim Biophys Acta Rev Cancer* 2020; **1873**: 188335 [PMID: 31816350 DOI: 10.1016/j.bbcan.2019.188335]

119 **Li H**, Li X, Song C, Zhang Y, Wang Z, Liu Z, Wei H, Yu J. Autoinducer-2 Facilitates *Pseudomonas aeruginosa* PAO1 Pathogenicity *in Vitro* and *in Vivo*. *Front Microbiol* 2017; **8**: 1944 [PMID: 29089927 DOI: 10.3389/fmicb.2017.01944]

120 **Walters M**, Sperandio V. Autoinducer 3 and epinephrine signaling in the kinetics of locus of enterocyte effacement gene expression in enterohemorrhagic Escherichia coli. *Infect Immun* 2006;

**74**: 5445-5455 [PMID: 16988219 DOI: 10.1128/IAI.00099-06]

121 **De Spiegeleer A**, Elewaut D, Van Den Noortgate N, Janssens Y, Debunne N, Van Langenhove S, Govindarajan S, De Spiegeleer B, Wynendaele E. Quorum sensing molecules as a novel microbial factor impacting muscle cells. *Biochim Biophys Acta Mol Basis Dis* 2020; **1866**: 165646 [PMID: 31870715 DOI: 10.1016/j.bbadis.2019.165646]

122 **Wynendaele E**, Verbeke F, Stalmans S, Gevaert B, Janssens Y, Van De Wiele C, Peremans K, Burvenich C, De Spiegeleer B. Quorum Sensing Peptides Selectively Penetrate the Blood-Brain Barrier. *PLoS One* 2015; **10**: e0142071 [PMID: 26536593 DOI: 10.1371/journal.pone.0142071]

123 **Gao J**, Xu K, Liu H, Liu G, Bai M, Peng C, Li T, Yin Y. Impact of the Gut Microbiota on Intestinal Immunity Mediated by Tryptophan Metabolism. *Front Cell Infect Microbiol* 2018; **8**: 13 [PMID: 29468141 DOI: 10.3389/fcimb.2018.00013]

124 **Roager HM**, Licht TR. Microbial tryptophan catabolites in health and disease. *Nat Commun* 2018; **9**: 3294 [PMID: 30120222 DOI: 10.1038/s41467-018-05470-4]

125 **Alexeev EE**, Lanis JM, Kao DJ, Campbell EL, Kelly CJ, Battista KD, Gerich ME, Jenkins BR, Walk ST, Kominsky DJ, Colgan SP. Microbiota-Derived Indole Metabolites Promote Human and Murine Intestinal Homeostasis through Regulation of Interleukin-10 Receptor. *Am J Pathol* 2018; **188**: 1183-1194 [PMID: 29454749 DOI: 10.1016/j.ajpath.2018.01.011]

126 **Dong F**, Perdew GH. The aryl hydrocarbon receptor as a mediator of host-microbiota interplay. *Gut Microbes* 2020; **12**: 1859812 [PMID: 33382356 DOI: 10.1080/19490976.2020.1859812]

127 **Lamas B**, Hernandez-Galan L, Galipeau HJ, Constante M, Clarizio A, Jury J, Breyner NM, Caminero A, Rueda G, Hayes CL, McCarville JL, Bermudez Brito M, Planchais J, Rolhion N, Murray JA, Langella P, Loonen LMP, Wells JM, Bercik P, Sokol H, Verdu EF. Aryl hydrocarbon receptor ligand production by the gut microbiota is decreased in celiac disease leading to intestinal inflammation. *Sci Transl Med* 2020; **12** [PMID: 33087499 DOI: 10.1126/scitranslmed.aba0624]

128 **Sears CL**, Garrett WS. Microbes, microbiota, and colon cancer. *Cell Host Microbe* 2014; **15**: 317-328 [PMID: 24629338 DOI: 10.1016/j.chom.2014.02.007]

129 **Greten FR**, Grivennikov SI. Inflammation and Cancer: Triggers, Mechanisms, and Consequences. *Immunity* 2019; **51**: 27-41 [PMID: 31315034 DOI: 10.1016/j.immuni.2019.06.025]

130 **Schwabe RF**, Jobin C. The microbiome and cancer. *Nat Rev Cancer* 2013; **13**: 800-812 [PMID: 24132111 DOI: 10.1038/nrc3610]

131 **Tjalsma H**, Boleij A, Marchesi JR, Dutilh BE. A bacterial driver-passenger model for colorectal cancer: beyond the usual suspects. *Nat Rev Microbiol* 2012; **10**: 575-582 [PMID: 22728587 DOI: 10.1038/nrmicro2819]

132 **Wu S**, Rhee KJ, Albesiano E, Rabizadeh S, Wu X, Yen HR, Huso DL, Brancati FL, Wick E, McAllister F, Housseau F, Pardoll DM, Sears CL. A human colonic commensal promotes colon tumorigenesis *via* activation of T helper type 17 T cell responses. *Nat Med* 2009; **15**: 1016-1022 [PMID: 19701202 DOI: 10.1038/nm.2015]

133 **Castellarin M**, Warren RL, Freeman JD, Dreolini L, Krzywinski M, Strauss J, Barnes R, Watson P, Allen-Vercoe E, Moore RA, Holt RA. Fusobacterium nucleatum infection is prevalent in human colorectal carcinoma. *Genome Res* 2012; **22**: 299-306 [PMID: 22009989 DOI: 10.1101/gr.126516.111]

134 **Dejea CM**, Wick EC, Hechenbleikner EM, White JR, Mark Welch JL, Rossetti BJ, Peterson SN, Snesrud EC, Borisy GG, Lazarev M, Stein E, Vadivelu J, Roslani AC, Malik AA, Wanyiri JW, Goh KL, Thevambiga I, Fu K, Wan F, Llosa N, Housseau F, Romans K, Wu X, McAllister FM, Wu S, Vogelstein B, Kinzler KW, Pardoll DM, Sears CL. Microbiota organization is a distinct feature of proximal colorectal cancers. *Proc Natl Acad Sci U S A* 2014; **111**: 18321-18326 [PMID: 25489084 DOI: 10.1073/pnas.1406199111]

135 **Kostic AD**, Gevers D, Pedamallu CS, Michaud M, Duke F, Earl AM, Ojesina AI, Jung J, Bass AJ, Tabernero J, Baselga J, Liu C, Shivdasani RA, Ogino S, Birren BW, Huttenhower C, Garrett WS, Meyerson M. Genomic analysis identifies association of Fusobacterium with colorectal carcinoma. *Genome Res* 2012; **22**: 292-298 [PMID: 22009990 DOI: 10.1101/gr.126573.111]

136 **Li S**, Konstantinov SR, Smits R, Peppelenbosch MP. Bacterial Biofilms in Colorectal Cancer Initiation and Progression. *Trends Mol Med* 2017; **23**: 18-30 [PMID: 27986421 DOI: 10.1016/j.molmed.2016.11.004]

137 **Wu J**, Li K, Peng W, Li H, Li Q, Wang X, Peng Y, Tang X, Fu X. Autoinducer-2 of Fusobacterium nucleatum promotes macrophage M1 polarization *via* TNFSF9/IL-1β signaling. *Int Immunopharmacol* 2019; **74**: 105724 [PMID: 31272064 DOI: 10.1016/j.intimp.2019.105724]

138 **Bearson BL**, Bearson SM. The role of the QseC quorum-sensing sensor kinase in colonization and norepinephrine-enhanced motility of Salmonella enterica serovar Typhimurium. *Microb Pathog* 2008; **44**: 271-278 [PMID: 17997077 DOI: 10.1016/j.micpath.2007.10.001]

139 **Luqman A**, Kharisma VD, Ruiz RA, Götz F. In Silico and in Vitro Study of Trace Amines (TA) and Dopamine (DOP) Interaction with Human Alpha 1-Adrenergic Receptor and the Bacterial Adrenergic Receptor QseC. *Cell Physiol Biochem* 2020; **54**: 888-898 [PMID: 32930525 DOI: 10.33594/000000276]

140 **Lyte M**. The role of microbial endocrinology in infectious disease. *J Endocrinol* 1993; **137**: 343-345 [PMID: 8371072 DOI: 10.1677/joe.0.1370343]

141 **Grandclément C**, Tannières M, Moréra S, Dessaux Y, Faure D. Quorum quenching: role in nature and applied developments. *FEMS Microbiol Rev* 2016; **40**: 86-116 [PMID: 26432822 DOI: 10.1093/femsre/fuv038]

142 **Teiber JF**, Horke S, Haines DC, Chowdhary PK, Xiao J, Kramer GL, Haley RW, Draganov DI. Dominant role of paraoxonases in inactivation of the Pseudomonas aeruginosa quorum-sensing signal N-(3-oxododecanoyl)-L-homoserine lactone. *Infect Immun* 2008; **76**: 2512-2519 [PMID: 18347034 DOI: 10.1128/IAI.01606-07]

143 **Levy E**, Trudel K, Bendayan M, Seidman E, Delvin E, Elchebly M, Lavoie JC, Precourt LP, Amre D, Sinnett D. Biological role, protein expression, subcellular localization, and oxidative stress response of paraoxonase 2 in the intestine of humans and rats. *Am J Physiol Gastrointest Liver Physiol* 2007; **293**: G1252-G1261 [PMID: 17916643 DOI: 10.1152/ajpgi.00369.2007]

144 **Rothem L**, Hartman C, Dahan A, Lachter J, Eliakim R, Shamir R. Paraoxonases are associated with intestinal inflammatory diseases and intracellularly localized to the endoplasmic reticulum. *Free Radic Biol Med* 2007; **43**: 730-739 [PMID: 17664137 DOI: 10.1016/j.freeradbiomed.2007.05.003]

145 **Karban A**, Hartman C, Eliakim R, Waterman M, Nesher S, Barnett-Griness O, Shamir R. Paraoxonase (PON)1 192R allele carriage is associated with reduced risk of inflammatory bowel disease. *Dig Dis Sci* 2007; **52**: 2707-2715 [PMID: 17436100 DOI: 10.1007/s10620-006-9700-5]

146 **Britton RA**, Young VB. Role of the intestinal microbiota in resistance to colonization by Clostridium difficile. *Gastroenterology* 2014; **146**: 1547-1553 [PMID: 24503131 DOI: 10.1053/j.gastro.2014.01.059]

147 **Maeda Y**, Kurakawa T, Umemoto E, Motooka D, Ito Y, Gotoh K, Hirota K, Matsushita M, Furuta Y, Narazaki M, Sakaguchi N, Kayama H, Nakamura S, Iida T, Saeki Y, Kumanogoh A, Sakaguchi S, Takeda K. Dysbiosis Contributes to Arthritis Development *via* Activation of Autoreactive T Cells in the Intestine. *Arthritis Rheumatol* 2016; **68**: 2646-2661 [PMID: 27333153 DOI: 10.1002/art.39783]

148 **Quévrain E**, Maubert MA, Michon C, Chain F, Marquant R, Tailhades J, Miquel S, Carlier L, Bermúdez-Humarán LG, Pigneur B, Lequin O, Kharrat P, Thomas G, Rainteau D, Aubry C, Breyner N, Afonso C, Lavielle S, Grill JP, Chassaing G, Chatel JM, Trugnan G, Xavier R, Langella P, Sokol H, Seksik P. Identification of an anti-inflammatory protein from Faecalibacterium prausnitzii, a commensal bacterium deficient in Crohn's disease. *Gut* 2016; **65**: 415-425 [PMID: 26045134 DOI: 10.1136/gutjnl-2014-307649]

149 **Sokol H**, Seksik P, Furet JP, Firmesse O, Nion-Larmurier I, Beaugerie L, Cosnes J, Corthier G, Marteau P, Doré J. Low counts of Faecalibacterium prausnitzii in colitis microbiota. *Inflamm Bowel Dis* 2009; **15**: 1183-1189 [PMID: 19235886 DOI: 10.1002/ibd.20903]

150 **Sokol H**, Jegou S, McQuitty C, Straub M, Leducq V, Landman C, Kirchgesner J, Le Gall G, Bourrier A, Nion-Larmurier I, Cosnes J, Seksik P, Richard ML, Beaugerie L. Specificities of the intestinal microbiota in patients with inflammatory bowel disease and Clostridium difficile infection. *Gut Microbes* 2018; **9**: 55-60 [PMID: 28786749 DOI: 10.1080/19490976.2017.1361092]

151 **Vich Vila A**, Imhann F, Collij V, Jankipersadsing SA, Gurry T, Mujagic Z, Kurilshikov A, Bonder MJ, Jiang X, Tigchelaar EF, Dekens J, Peters V, Voskuil MD, Visschedijk MC, van Dullemen HM, Keszthelyi D, Swertz MA, Franke L, Alberts R, Festen EAM, Dijkstra G, Masclee AAM, Hofker MH, Xavier RJ, Alm EJ, Fu J, Wijmenga C, Jonkers DMAE, Zhernakova A, Weersma RK. Gut microbiota composition and functional changes in inflammatory bowel disease and irritable bowel syndrome. *Sci Transl Med* 2018; **10** [PMID: 30567928 DOI: 10.1126/scitranslmed.aap8914]

152 **Cammarota G**, Ianiro G, Kelly CR, Mullish BH, Allegretti JR, Kassam Z, Putignani L, Fischer M, Keller JJ, Costello SP, Sokol H, Kump P, Satokari R, Kahn SA, Kao D, Arkkila P, Kuijper EJ, Vehreschild MJG, Pintus C, Lopetuso L, Masucci L, Scaldaferri F, Terveer EM, Nieuwdorp M, López-Sanromán A, Kupcinskas J, Hart A, Tilg H, Gasbarrini A. International consensus conference on stool banking for faecal microbiota transplantation in clinical practice. *Gut* 2019; **68**: 2111-2121 [PMID: 31563878 DOI: 10.1136/gutjnl-2019-319548]

153 **van Nood E**, Vrieze A, Nieuwdorp M, Fuentes S, Zoetendal EG, de Vos WM, Visser CE, Kuijper EJ, Bartelsman JF, Tijssen JG, Speelman P, Dijkgraaf MG, Keller JJ. Duodenal infusion of donor feces for recurrent Clostridium difficile. *N Engl J Med* 2013; **368**: 407-415 [PMID: 23323867 DOI: 10.1056/NEJMoa1205037]

154 **Wu S**, Liu J, Liu C, Yang A, Qiao J. Quorum sensing for population-level control of bacteria and potential therapeutic applications. *Cell Mol Life Sci* 2020; **77**: 1319-1343 [PMID: 31612240 DOI: 10.1007/s00018-019-03326-8]

155 **Novick RP**, Projan SJ, Kornblum J, Ross HF, Ji G, Kreiswirth B, Vandenesch F, Moghazeh S. The agr P2 operon: an autocatalytic sensory transduction system in Staphylococcus aureus. *Mol Gen Genet* 1995; **248**: 446-458 [PMID: 7565609 DOI: 10.1007/BF02191645]

156 **Autret N**, Raynaud C, Dubail I, Berche P, Charbit A. Identification of the agr locus of Listeria monocytogenes: role in bacterial virulence. *Infect Immun* 2003; **71**: 4463-4471 [PMID: 12874326 DOI: 10.1128/IAI.71.8.4463-4471.2003]

157 **Ohtani K**, Yuan Y, Hassan S, Wang R, Wang Y, Shimizu T. Virulence gene regulation by the agr system in Clostridium perfringens. *J Bacteriol* 2009; **191**: 3919-3927 [PMID: 19363118 DOI: 10.1128/JB.01455-08]

158 **Sifri CD**, Mylonakis E, Singh KV, Qin X, Garsin DA, Murray BE, Ausubel FM, Calderwood SB. Virulence effect of Enterococcus faecalis protease genes and the quorum-sensing locus fsr in Caenorhabditis elegans and mice. *Infect Immun* 2002; **70**: 5647-5650 [PMID: 12228293 DOI: 10.1128/iai.70.10.5647-5650.2002]

159 **Magnuson R**, Solomon J, Grossman AD. Biochemical and genetic characterization of a competence pheromone from B. subtilis. *Cell* 1994; **77**: 207-216 [PMID: 8168130 DOI:

10.1016/0092-8674(94)90313-1]
160 **Mok KC**, Wingreen NS, Bassler BL. Vibrio harveyi quorum sensing: a coincidence detector for two autoinducers controls gene expression. *EMBO J* 2003; **22**: 870-881 [PMID: 12574123 DOI: 10.1093/emboj/cdg085]
161 **Waters CM**, Bassler BL. The Vibrio harveyi quorum-sensing system uses shared regulatory components to discriminate between multiple autoinducers. *Genes Dev* 2006; **20**: 2754-2767 [PMID: 17015436 DOI: 10.1101/gad.1466506]
162 **Gambello MJ**, Iglewski BH. Cloning and characterization of the Pseudomonas aeruginosa lasR gene, a transcriptional activator of elastase expression. *J Bacteriol* 1991; **173**: 3000-3009 [PMID: 1902216 DOI: 10.1128/jb.173.9.3000-3009.1991]
163 **Gambello MJ**, Kaye S, Iglewski BH. LasR of Pseudomonas aeruginosa is a transcriptional activator of the alkaline protease gene (apr) and an enhancer of exotoxin A expression. *Infect Immun* 1993; **61**: 1180-1184 [PMID: 8454322 DOI: 10.1128/iai.61.4.1180-1184.1993]
164 **Winson MK**, Camara M, Latifi A, Foglino M, Chhabra SR, Daykin M, Bally M, Chapon V, Salmond GP, Bycroft BW. Multiple N-acyl-L-homoserine lactone signal molecules regulate production of virulence determinants and secondary metabolites in Pseudomonas aeruginosa. *Proc Natl Acad Sci U S A* 1995; **92**: 9427-9431 [PMID: 7568146 DOI: 10.1073/pnas.92.20.9427]
165 **Chapon-Hervé V**, Akrim M, Latifi A, Williams P, Lazdunski A, Bally M. Regulation of the xcp secretion pathway by multiple quorum-sensing modulons in Pseudomonas aeruginosa. *Mol Microbiol* 1997; **24**: 1169-1178 [PMID: 9218766 DOI: 10.1046/j.1365-2958.1997.4271794.x]
166 **Pesci EC**, Milbank JB, Pearson JP, McKnight S, Kende AS, Greenberg EP, Iglewski BH. Quinolone signaling in the cell-to-cell communication system of Pseudomonas aeruginosa. *Proc Natl Acad Sci U S A* 1999; **96**: 11229-11234 [PMID: 10500159 DOI: 10.1073/pnas.96.20.11229]
167 **Gallagher LA**, McKnight SL, Kuznetsova MS, Pesci EC, Manoil C. Functions required for extracellular quinolone signaling by Pseudomonas aeruginosa. *J Bacteriol* 2002; **184**: 6472-6480 [PMID: 12426334 DOI: 10.1128/jb.184.23.6472-6480.2002]
168 **Bredenbruch F**, Geffers R, Nimtz M, Buer J, Häussler S. The Pseudomonas aeruginosa quinolone signal (PQS) has an iron-chelating activity. *Environ Microbiol* 2006; **8**: 1318-1329 [PMID: 16872396 DOI: 10.1111/j.1462-2920.2006.01025.x]
169 **Diggle SP**, Matthijs S, Wright VJ, Fletcher MP, Chhabra SR, Lamont IL, Kong X, Hider RC, Cornelis P, Cámara M, Williams P. The Pseudomonas aeruginosa 4-quinolone signal molecules HHQ and PQS play multifunctional roles in quorum sensing and iron entrapment. *Chem Biol* 2007; **14**: 87-96 [PMID: 17254955 DOI: 10.1016/j.chembiol.2006.11.014]
170 **Cao H**, Krishnan G, Goumnerov B, Tsongalis J, Tompkins R, Rahme LG. A quorum sensing-associated virulence gene of Pseudomonas aeruginosa encodes a LysR-like transcription regulator with a unique self-regulatory mechanism. *Proc Natl Acad Sci U S A* 2001; **98**: 14613-14618 [PMID: 11724939 DOI: 10.1073/pnas.251465298]
171 **Diggle SP**, Winzer K, Chhabra SR, Worrall KE, Cámara M, Williams P. The Pseudomonas aeruginosa quinolone signal molecule overcomes the cell density-dependency of the quorum sensing hierarchy, regulates rhl-dependent genes at the onset of stationary phase and can be produced in the absence of LasR. *Mol Microbiol* 2003; **50**: 29-43 [PMID: 14507361 DOI: 10.1046/j.1365-2958.2003.03672.x]
172 **Lee J**, Wu J, Deng Y, Wang J, Wang C, Chang C, Dong Y, Williams P, Zhang LH. A cell-cell communication signal integrates quorum sensing and stress response. *Nat Chem Biol* 2013; **9**: 339-343 [PMID: 23542643 DOI: 10.1038/nchembio.1225]
173 **Ng WL**, Perez LJ, Wei Y, Kraml C, Semmelhack MF, Bassler BL. Signal production and detection specificity in Vibrio CqsA/CqsS quorum-sensing systems. *Mol Microbiol* 2011; **79**: 1407-1417 [PMID: 21219472 DOI: 10.1111/j.1365-2958.2011.07548.x]
174 **Walters M**, Sperandio V. Quorum sensing in Escherichia coli and Salmonella. *Int J Med Microbiol* 2006; **296**: 125-131 [PMID: 16487745 DOI: 10.1016/j.ijmm.2006.01.041]
175 **Schauder S**, Shokat K, Surette MG, Bassler BL. The LuxS family of bacterial autoinducers: biosynthesis of a novel quorum-sensing signal molecule. *Mol Microbiol* 2001; **41**: 463-476 [PMID: 11489131 DOI: 10.1046/j.1365-2958.2001.02532.x]
176 **Zhu J**, Miller MB, Vance RE, Dziejman M, Bassler BL, Mekalanos JJ. Quorum-sensing regulators control virulence gene expression in Vibrio cholerae. *Proc Natl Acad Sci U S A* 2002; **99**: 3129-3134 [PMID: 11854465 DOI: 10.1073/pnas.052694299]
177 **Hammer BK**, Bassler BL. Quorum sensing controls biofilm formation in Vibrio cholerae. *Mol Microbiol* 2003; **50**: 101-104 [PMID: 14507367 DOI: 10.1046/j.1365-2958.2003.03688.x]
178 **Bansal T**, Jesudhasan P, Pillai S, Wood TK, Jayaraman A. Temporal regulation of enterohemorrhagic Escherichia coli virulence mediated by autoinducer-2. *Appl Microbiol Biotechnol* 2008; **78**: 811-819 [PMID: 18256823 DOI: 10.1007/s00253-008-1359-8]
179 **Choi J**, Shin D, Kim M, Park J, Lim S, Ryu S. LsrR-mediated quorum sensing controls invasiveness of Salmonella typhimurium by regulating SPI-1 and flagella genes. *PLoS One* 2012; **7**: e37059 [PMID: 22623980 DOI: 10.1371/journal.pone.0037059]
180 **Bao L**, Yu J, Zhong H, Huang D, Lu Q. Expression of toll-like receptors in T lymphocytes stimulated with N-(3-oxododecanoyl)-L-homoserine lactone from Pseudomonas aeruginosa. *APMIS* 2017; **125**: 553-557 [PMID: 28418096 DOI: 10.1111/apm.12690]
181 **Glucksam-Galnoy Y**, Sananes R, Silberstein N, Krief P, Kravchenko VV, Meijler MM, Zor T. The bacterial quorum-sensing signal molecule N-3-oxo-dodecanoyl-L-homoserine lactone reciprocally

modulates pro- and anti-inflammatory cytokines in activated macrophages. *J Immunol* 2013; **191**: 337-344 [PMID: 23720811 DOI: 10.4049/jimmunol.1300368]

182 **Zhang J**, Gong F, Li L, Zhao M, Song J. *Pseudomonas aeruginosa* quorum-sensing molecule N-(3-oxododecanoyl) homoserine lactone attenuates lipopolysaccharide-induced inflammation by activating the unfolded protein response. *Biomed Rep* 2014; **2**: 233-238 [PMID: 24649102 DOI: 10.3892/br.2014.225]

183 **Holm A**, Magnusson KE, Vikström E. Pseudomonas aeruginosa N-3-oxo-dodecanoyl-homoserine Lactone Elicits Changes in Cell Volume, Morphology, and AQP9 Characteristics in Macrophages. *Front Cell Infect Microbiol* 2016; **6**: 32 [PMID: 27047801 DOI: 10.3389/fcimb.2016.00032]

184 **Krishnan S**, Ding Y, Saedi N, Choi M, Sridharan GV, Sherr DH, Yarmush ML, Alaniz RC, Jayaraman A, Lee K. Gut Microbiota-Derived Tryptophan Metabolites Modulate Inflammatory Response in Hepatocytes and Macrophages. *Cell Rep* 2018; **23**: 1099-1111 [PMID: 29694888 DOI: 10.1016/j.celrep.2018.03.109]

185 **Boontham P**, Robins A, Chandran P, Pritchard D, Cámara M, Williams P, Chuthapisith S, McKechnie A, Rowlands BJ, Eremin O. Significant immunomodulatory effects of Pseudomonas aeruginosa quorum-sensing signal molecules: possible link in human sepsis. *Clin Sci (Lond)* 2008; **115**: 343-351 [PMID: 18363571 DOI: 10.1042/CS20080018]

186 **Karlsson T**, Musse F, Magnusson KE, Vikström E. N-Acylhomoserine lactones are potent neutrophil chemoattractants that act *via* calcium mobilization and actin remodeling. *J Leukoc Biol* 2012; **91**: 15-26 [PMID: 21807742 DOI: 10.1189/jlb.0111034]

187 **Zimmermann S**, Wagner C, Müller W, Brenner-Weiss G, Hug F, Prior B, Obst U, Hänsch GM. Induction of neutrophil chemotaxis by the quorum-sensing molecule N-(3-oxododecanoyl)-L-homoserine lactone. *Infect Immun* 2006; **74**: 5687-5692 [PMID: 16988244 DOI: 10.1128/iai.01940-05]

188 **Singh PK**, Yadav VK, Kalia M, Sharma D, Pandey D, Agarwal V. Pseudomonas aeruginosa quorum-sensing molecule N-(3-oxo-dodecanoyl)-L-homoserine lactone triggers mitochondrial dysfunction and apoptosis in neutrophils through calcium signaling. *Med Microbiol Immunol* 2019; **208**: 855-868 [PMID: 31377870 DOI: 10.1007/s00430-019-00631-8]

189 **Wagner C**, Zimmermann S, Brenner-Weiss G, Hug F, Prior B, Obst U, Hänsch GM. The quorum-sensing molecule N-3-oxododecanoyl homoserine lactone (3OC12-HSL) enhances the host defence by activating human polymorphonuclear neutrophils (PMN). *Anal Bioanal Chem* 2007; **387**: 481-487 [PMID: 16906383 DOI: 10.1007/s00216-006-0698-5]

190 **Gupta RK**, Chhibber S, Harjai K. Acyl homoserine lactones from culture supernatants of Pseudomonas aeruginosa accelerate host immunomodulation. *PLoS One* 2011; **6**: e20860 [PMID: 21698201 DOI: 10.1371/journal.pone.0020860]

191 **Hooi DS**, Bycroft BW, Chhabra SR, Williams P, Pritchard DI. Differential immune modulatory activity of Pseudomonas aeruginosa quorum-sensing signal molecules. *Infect Immun* 2004; **72**: 6463-6470 [PMID: 15501777 DOI: 10.1128/IAI.72.11.6463-6470.2004]

192 **Cervantes-Barragan L**, Chai JN, Tianero MD, Di Luccia B, Ahern PP, Merriman J, Cortez VS, Caparon MG, Donia MS, Gilfillan S, Cella M, Gordon JI, Hsieh CS, Colonna M. *Lactobacillus reuteri* induces gut intraepithelial $CD4^+CD8\alpha\alpha^+$ T cells. *Science* 2017; **357**: 806-810 [PMID: 28775213 DOI: 10.1126/science.aah5825]

193 **Aoki R**, Aoki-Yoshida A, Suzuki C, Takayama Y. Indole-3-Pyruvic Acid, an Aryl Hydrocarbon Receptor Activator, Suppresses Experimental Colitis in Mice. *J Immunol* 2018; **201**: 3683-3693 [PMID: 30429284 DOI: 10.4049/jimmunol.1701734]

194 **Ritchie AJ**, Yam AO, Tanabe KM, Rice SA, Cooley MA. Modification of *in vivo* and *in vitro* T- and B-cell-mediated immune responses by the Pseudomonas aeruginosa quorum-sensing molecule N-(3-oxododecanoyl)-L-homoserine lactone. *Infect Immun* 2003; **71**: 4421-4431 [PMID: 12874321 DOI: 10.1128/iai.71.8.4421-4431.2003]

195 **Smith RS**, Fedyk ER, Springer TA, Mukaida N, Iglewski BH, Phipps RP. IL-8 production in human lung fibroblasts and epithelial cells activated by the Pseudomonas autoinducer N-3-oxododecanoyl homoserine lactone is transcriptionally regulated by NF-kappa B and activator protein-2. *J Immunol* 2001; **167**: 366-374 [PMID: 11418672 DOI: 10.4049/jimmunol.167.1.366]

World Journal of Gastroenterology

Submit a Manuscript: https://www.f6publishing.com

*World J Gastroenterol* 2021 November 14; 27(42): 7271-7284

DOI: 10.3748/wjg.v27.i42.7271

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

*REVIEW*

# Hepatitis B virus/hepatitis D virus epidemiology: Changes over time and possible future influence of the SARS-CoV-2 pandemic

Caterina Sagnelli, Mariantonietta Pisaturo, Caterina Curatolo, Alessio Vinicio Codella, Nicola Coppola, Evangelista Sagnelli

**ORCID number:** Caterina Sagnelli 0000-0002-6413-7810; Mariantonietta Pisaturo 0000-0001-5870-0956; Caterina Curatolo 0000-0000-4829-6336; Alessio Vinicio Codella 0000-0003-1657-6613; Nicola Coppola 0000-0001-5897-4949; Evangelista Sagnelli 0000-0003-2817-8436.

**Author contributions:** Sagnelli C, Pisaturo M, Curatolo C, and Codella AV acquired, collected, extracted data, drafted, and made the final approval; Sagnelli C, and Sagnelli E designed the study, interpreted the data, drafted the article; Sagnelli C, Coppola N, and Sagnelli E, revised the article, and made the final approval.

**Conflict-of-interest statement:** All the authors of the manuscript declare they have no conflict of interest in connection with this paper.



**Caterina Sagnelli, Evangelista Sagnelli,** Department of Mental Health and Public Medicine, Section of Infectious Diseases, University of Campania Luigi Vanvitelli, Naples 80131, Italy

**Mariantonietta Pisaturo, Caterina Curatolo, Alessio Vinicio Codella, Nicola Coppola,** Department of Mental Health and Public Medicine, University of Campania, Naples 80135, Italy

**Corresponding author:** Evangelista Sagnelli, MD, PhD, Chief Doctor, Full Professor, Senior Researcher, Department of Mental Health and Public Medicine, Section of Infectious Diseases, University of Campania Luigi Vanvitelli, Via: L. Armanni 5, Naples 80131, Italy.
evangelista.sagnelli@unicampania.it

## Abstract

Hepatitis D virus (HDV) is a defective liver-tropic virus that needs the helper function of hepatitis B virus (HBV) to infect humans and replicate. HDV is transmitted sexually or by a parenteral route, in co-infection with HBV or by super-infection in HBV chronic carriers. HDV infection causes acute hepatitis that may progress to a fulminant form (7%-14% by super-infection and 2%-3% by HBV/HDV co-infection) or to chronic hepatitis (90% by HDV super-infection and 2%-5% by HBV/HDV co-infection), frequently and rapidly progressing to cirrhosis or hepatocellular carcinoma (HCC). Peg-interferon alfa the only recommended therapy, clears HDV in only 10%-20% of cases and, consequently, new treatment strategies are being explored. HDV endemicity progressively decreased over the 50 years from the identification of the virus, due to improved population lifestyles and economic levels, to the use of HBV nuclei(t)side analogues to suppress HBV replication and to the application of universal HBV vaccination programs. Further changes are expected during the severe acute respiratory syndrome coronavirus-2 pandemic, unfortunately towards increased endemicity due to the focus of healthcare towards coronavirus disease 2019 and the consequently lower possibility of screening and access to treatments, lower care for patients with severe liver diseases and a reduced impulse to the HBV vaccination policy.

**Key Words:** Hepatitis B virus/hepatitis delta virus; SARS-CoV-2; COVID-19; Hepatitis delta virus infection; Hepatitis D; Hepatitis delta virus epidemiology

**Specialty type:** Gastroenterology and hepatology

**Country/Territory of origin:** Italy

**Peer-review report's scientific quality classification**

Grade A (Excellent): 0
Grade B (Very good): B, B, B, B
Grade C (Good): C
Grade D (Fair): 0
Grade E (Poor): 0

**Received:** May 2, 2021
**Peer-review started:** May 2, 2021
**First decision:** June 12, 2021
**Revised:** June 20, 2021
**Accepted:** October 25, 2021
**Article in press:** October 25, 2021
**Published online:** November 14, 2021

**P-Reviewer:** Azarbakhsh H, Cai J, Papazafiropoulou A, Samadder S, Sun C
**S-Editor:** Fan JR
**L-Editor:** Filipodia
**P-Editor:** Fan JR

**Core Tip:** There has been a tendency to a reduction in hepatitis D virus (HDV) endemicity in most countries in recent decades, mostly due to an improvement in population lifestyles and economic levels, to an extensive use of hepatitis B virus (HBV) nuclei(t)side analogues to suppress HBV replication and to the application of universal HBV vaccination programs. However, an increase in HDV endemicity is expected during the severe acute respiratory syndrome coronavirus-2 pandemic since healthcare is mostly diverted towards coronavirus disease 2019, with a reduced attention to liver disease, screening, access to treatment, care for patients with severe liver disease and the HBV vaccination policy.

**Citation:** Sagnelli C, Pisaturo M, Curatolo C, Codella AV, Coppola N, Sagnelli E. Hepatitis B virus/hepatitis D virus epidemiology: Changes over time and possible future influence of the SARS-CoV-2 pandemic. *World J Gastroenterol* 2021; 27(42): 7271-7284
**URL:** https://www.wjgnet.com/1007-9327/full/v27/i42/7271.htm
**DOI:** https://dx.doi.org/10.3748/wjg.v27.i42.7271

## INTRODUCTION

A new antigenic reactivity was identified in hepatocytic nuclei by immunofluorescence technique on paraffinized liver sections of hepatitis B surface antigen (HBsAg) positive patients by Rizzetto *et al*[1] in the early 1970s in Turin, Italy, and called Delta. The Authors understood that this new reactivity was different from that expressed by the hepatitis B virus (HBV) core antigen (HBcAg) and hypothesized that it could be part of a new virus; with a careful interlacing of antisera on cryostat-cut biopsy sections, Delta reactivity was identified as a new antigen localized in the nucleus of the hepatocytes and yielding a fluorescent staining similar but not equal to HBcAg[1]. In Bethesda, United States, Rizzetto *et al*[2] developed a radioimmunoassay for the delta antigen-antibody system in serum and performed transmission studies in chimpanzees demonstrating the existence of a new a liver tropic virus [hepatitis D virus (HDV)][2]. Further studies mentioned below defined HDV as a defective virus that needs a helper function of HBV to infect humans and replicate.

This virus consists of the HDV antigen (HDAg, small and large antigen) and a circular single-strand RNA (HDV-RNA). It uses a human RNA polymerase II and a rolling-cycle mechanism like that of plant viroid to replicate. Being a defective virus, HDV needs the S-HBsAg, M-HBsAg and L-HBsAg proteins of HBV to produce its envelope and propagate. Coated with the HBV envelope, HDV uses the pre-S1 domain of L-HBsAg to bind NCTP (sodium taurocholate co-transporting polypeptide) and enter differentiated hepatocytes[3].

Eight genotypes of HDV have been identified at present, distributed differently in the world: Type 1 is present worldwide and prevalent in western Europe and North America, type 2 mainly in the Far East, Egypt and Iran, type 3 in the Amazon Basin, type 4 in Japan and Taiwan, types 5 to 8 in Africa but now spreading also in Western regions due to immigration flows[4]. In Western countries, HDV infection at present occurs mostly in elderly adults with chronic HBV infection or in young immigrants from endemic areas[5].

HDV is parenterally and sexually transmitted in co-infection with HBV or by super-infection in HBV chronic carriers. Both types of transmission cause acute hepatitis, self-limited with a clearance of both viruses in 95% of subjects simultaneously infected, while in the case of HDV super-infection in HBV chronic carriers the disease progresses to chronicity in nearly 90% of cases[6].

Acute hepatitis delta is clinically indistinguishable from other types of acute viral hepatitis and is usually mistaken for HBV acute hepatitis if the specific diagnostic tests [search for HDV RNA and immunoglobulin M (IgM) to HDV-Ag] are not performed. HDV super-infection in HBsAg chronic carriers causes massive acute exacerbation with a fulminant clinical course in 7%-14% of patients, two thirds of whom die of liver failure. The outcome is less severe in subjects acquiring HBV and HDV in co-infection. They may develop fulminant hepatitis in 2%-3% of cases and progression to chronicity in 1%-5%.

Chronic HDV infection is the most severe and rapidly progressive form of chronic hepatitis, leading to cirrhosis in 70% of patients within 5-10 years[7]. Furthermore, as compared with HBV mono-infection, HBV/HDV co-infection increases the risk up to three times of cirrhotic patients developing hepatocellular carcinoma[4,8,9].

Despite the importance of HDV infection, no treatment has been so far approved by the Food and Drug Administration. However, treatment with pegylated interferon alfa (Peg-IFN) is recommended by the major scientific societies for the study of the liver because it may clear HDV infection in 10%-20% of patients and may reduce liver fibrosis and the risk of hepatic decompensation and mortality. Because of its limited efficacy, its significant side effects, and the prolonged time of administration (1-2 years) Peg-IFN was used also in combination with ribavirin and/or HBV nucleoside analogues, but no improvement was obtained. Therefore, new treatment strategies are now under study with the use of peginterferon lambda, bulevirtide (entry inhibitor that targets NCTP), nucleic acid polymers (HBsAg secretion inhibitors), and lonafarnib (virus assembly inhibitor)[10].

In end-stage liver disease, HCC and fulminant hepatitis, liver transplant is the only therapeutic option. Comparing HBV mono-infected patients with the HBV/HDV co-infected, the latter show a better outcome if reinfection is prevented by the continuous administration nucleos(t)ide analogues and of hepatitis B immune globulins[11,12].

The epidemiology of HDV infection has changed a lot in nearly every country since its first identification 55 years ago[13-20]. This is related to changes in population lifestyle habits, variations in economic levels, extension of the use of HBV nucleos(t)ide analogues to treat HBV chronic carriers and, most importantly, to the effectiveness of universal vaccination campaigns against HBV infection in most countries. Further changes are expected in this era of the coronavirus disease 2019 (COVID-19) pandemic, which might involve, among others, a lower possibility of screening and access to treatment and a reduced effectiveness of the vaccination policy against HBV infection and of the management of patients with severe chronic hepatitis.

These epidemiological changes have great impact on the health quality of life of human beings and are the subject of this review.

## HBV/HDV EPIDEMIOLOGY BEFORE SEVERE ACUTE RESPIRATORY SYNDROME CORONAVIRUS-2 PANDEMIC

The epidemiology of HDV infection has been mainly studied by assessing the prevalence of subjects with HDV infection among HBV chronic carriers because acute HDV hepatitis, either acquired in co-infection with HBV or by HDV super-infection in HBV chronic carriers, easily identifiable during outbreaks, often remains undiagnosed when it occurs in single subjects. In fact, the clinical presentation of HBV and HDV acute hepatitis is similar, and only specific tests (anti-HDV IgM and quantitative HDV RNA), infrequently performed in general practice, allow the differentiation between these etiologies. These tests, however, do not provide accurate results in epidemiological studies on chronic HDV infection, because their titers, elevated in acute phase of the infection, decrease markedly in the chronic phase. In addition, HDV RNA is typically determined home made only in some specialized laboratories and its quantification is not standardized. For these reasons, commercial standardized kits to detect serum anti-HD IgG have been used in almost all epidemiological studies on HDV chronic hepatitis.

HDV has spread all over the world, with a global disease burden around 62-72 million HBV/HDV co-infected subjects[13] and with an HDV seroprevalence among HBV carriers widely varying in different geographic areas[14,15].

## EPIDEMIOLOGICAL INFORMATION OF HDV/HBV ACUTE HEPATITIS

Outbreaks of fulminant hepatitis have occurred in the Amazon Basin. in French Guyana, in Yucpa Indians of Venezuela from 1934 to 1955, and in the Central African Republic in 1997 as documented by the presence of Delta antigen in hepatocytic nuclei of autoptic liver specimens; small but similar outbreaks have occurred from 1955 to 1985 in northern Colombia and designated as hepatitis of Santa Marta (de Sierra Nevada)[16-24].

In patients with fulminant hepatitis, morphologic studies showed marked liver steatosis with characteristic "morular hepatocytes", cells with small droplets of fat and

central pycnotic nuclei. Worthy of note, some similarities in the clinical presentation of fulminant hepatitis Delta with that of yellow fever has led to the hypothesis that some of the outbreaks occurring in the past in the Amazon basin and attributed to yellow fever, may have been due to HBV/HDV co-infection[25].

In the mid-1980s, several cases of severe acute liver failure were described in Bangui, in the Central African Republic, associated with an HDV outbreak. At least 124 cases of fulminant hepatitis were observed among 154 jaundiced patients, 88% of whom died[23]. The disease was associated with specific pathological lesions (spongiocyte-associated hepatitis) involving lympho-plasmocytoid infiltrates, eosinophilic necrosis and massive macro- or micro-vacuolar steatosis[23] resembling the "morula-like" cells previously described in South American HDV outbreaks[24-26]. In Italy, two collections of sera from patients with acute hepatitis B collected in Naples in 1972 and 1974 were retrospectively evaluated and for the first time the IgM antibody to HDV (anti-HDV IgM) was detected in over 90% of cases. Most anti-HDV IgM-positive patients were also anti-HBc IgM-positive, suggesting an outbreak of HBV/HDV coinfection in Naples in those years[27].

## THE INCIDENCE OF ACUTE HDV HEPATITIS IN HOSPITALIZED PATIENTS

Italy was estimated by a system of hospital surveillance in the period 1987-2010[28]. The incidence of primary HDV infection in the general population progressively decreased from 3.2/100000 inhabitants in 1987 to 0.2/100000 in 2010. The decrease in the incidence of HDV acute hepatitis and the parallel similar trend observed for HBV acute hepatitis in the same study were most likely an effect of the mass vaccination, which in 2010 had covered all Italian citizens aged from 0 to 31 years. In this period, however, three peaks of HDV epidemic were registered, in 1990 and 1993 in the age groups 14-25 and 25 or more and in 1997 only in the 15-24 age group, all due to the spread of HDV infection among intravenous drug users[29]. Apart from intravenous drug use (IVDU), other putative risk factors associated with the development of HDV acute hepatitis were family contacts with a chronic HBsAg carrier, promiscuous sexual activity, and cosmetic treatments with percutaneous exposure in 1992-1994, whereas those identified from 2008 to 2010 were dental therapy, cosmetic treatment with percutaneous exposure and promiscuous sexual activity.

Between December 2004 and January 2005, out of 110 patients with acute hepatitis in 8 city hospitals of Mongolia, 1.8% had HDV etiology by co-infection with HBV and 27.3% by HDV super-infection[30]. Again, in Mongolia from January 2012 to December 2014, 546 consecutive patients with acute hepatitis were observed, of whom 6% had HDV etiology by co-infection with HBV and 10.8% by HDV super-infection [31].

In Spain, 398 patients with HDV infection were observed from 1983 to 2008, 182 with acute hepatitis, of whom 84% by co-infection and 16% by super-infection, and 216 had chronic HDV infection[28]. The number of patients with acute hepatitis Delta strongly decreased across this study, while the number of those with chronic Delta infection showed a small decline. In addition, patients observed in the 1983–1995 period compared to those examined from 1996-2008 were significantly younger, born in Spain, mainly intravenous drug users, and more frequently had acute hepatitis. In this study, the percentage of immigrants (prevalently from Africa and Eastern Europe) increased from 1% of the first period to 28% in the second.

## EPIDEMIOLOGICAL INFORMATION OF HDV/HBV CHRONIC INFECTION

In 2018, the World Health Organization considered these geographic areas HBV/HDV endemic regions: Central and West Africa, Central and Northern Asia, Vietnam, Mongolia, Pakistan, Japan, Chinese Taipei, the Pacific Islands (Kiribati and Nauru), the Middle East and Eastern Europe, Eastern Mediterranean regions, Turkey, the Amazonian Basin, and Greenland[32].

To offer the readers an overview of the worldwide variability of the prevalence of anti-HDV among HBV chronic carriers, we report data recorded at different times in some countries: 10.6%–37.5% in India (1985-2005)[33,34], 3.5%–13.1% in China (1987-1993)[35,36], 17.7% in Tunisia (1987-1994)[37], 8.3%–23.0% in Italy (1987 and 1997)[13,38], 21.8%–65.5% in Thailand (1988)[39], 48%-5.7% in Iran (1991-2005)[40], 8%–10.9% in Germany (1992-2006)[41], 16.6%–58.6% in Pakistan (1994 to 2001)[42], 8.6% in Saudi Arabia (1996-1997)[43], 4.4%–15.3% in Taiwan (1997-2012)[13,44], 56.5% in Mongolia

(2004)[13,44], 41.9% in Brazil (2005-2006)[45], 7%–45.5% in Turkey (2006-2009)[46], and 9.9% in Egypt (2013)[13,44] (Table 1).

A lower prevalence was reported for non-endemic areas, ranging from 6% in Japan (1991)[47], 2% in Jordan (1978-1985)[48], 4.1%-4.8% in Australia (1997-2016)[49,50], 4.2% in Greece (1997)[51], and 2.6%–8.5% in England (2000-2006)[13,44]. Notably, in the United States, the prevalence of HDV among HBV chronic carriers has been reported to range from 2% in native citizens to 50% in some foreign populations[52-54] (Table 1).

There was a gradual reduction in the percentage of HBsAg-positive subjects and a parallel decrease in the prevalence of cases with HBV/HDV chronic infection in several countries in the last 3 decades, primarily due to the application of universal vaccination programs. As an example, the prevalence of HDV infection in HBsAg-positive chronic carriers was very high in some Mediterranean countries (Greece, southern Spain, southern Italy) 5 decades ago[55], a prevalence that gradually declined, like in Italy, where the prevalence of HDV infection in HBV chronic carriers decreased from 24% in 1990 to 8.5% in 2006[38].

About 35 million people are infected with human immunodeficiency virus (HIV) in the world and among these the percentage of those with HBV/HDV co-infection varies from 1.4% to 19.7% in relation to the epidemiological diversity of these infections in individual nations[56-60]. Considering the high number of subjects with triple HBV, HDV and HIV infections, it is highly likely that some of them have contracted severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) infection, asymptomatic or symptomatic, although there are no data in the literature to the best of our knowledge.

## REASONS FOR REDUCED PREVALENCE RATE OF HDV/HBV CHRONIC INFECTION

The impact of risk factors for acquiring HBV infection has changed over the past five decades around the world. Vertical transmission is no longer a risk factor for the transmission of HBV infection, due to the introduction of mass passive/active immunization of newborn babies. Furthermore, the impact of risk factors for HBV transmission through household contacts with HBsAg infected subjects has dropped in Western countries with the application of universal HBV vaccination and the reduction in family size. The epidemiological impact of other risk factors, such as the use of improperly sterilized medical and surgical instruments, transfusion of contaminated blood and blood products, the use of glass syringes and for men shaving at a barber's shop have dramatically decreased worldwide. IVDU and sexual transmission of HBV infection plays a major role in HBV transmission nowadays due to the exchange between drug addicts of syringes or other objects used for the preparation of drugs and to the infrequent or incorrect use of condoms in unsafe sexual activity. A marginal role is still played by cosmetic treatment with percutaneous exposure (piercing, tattooing, manicure, pedicure, and acupuncture) and dental treatment[61].

The current residual reservoir of HDV in Western countries comprises aging patients with advanced liver disease, young immigrants with chronic hepatitis Delta from areas where HDV infection remains endemic and drug users. The increase in the spread of HDV has recently been observed in some countries of Western Europe mostly regarding IV drug users and immigrants from Eastern Europe and Sub-Saharan Africa[44,62]. The recently increased HDV prevalence observed in the United Kingdom, France, and Germany reflects the increased prevalence in young immigrants from regions at high HBV/HDV prevalence. A high prevalence has been reported for immigrants from Equatorial Guinea living in Spain (20.9% among 1220 HBV chronic carriers).

Drug use remains a major risk factor, as seen by a study performed in Baltimore (United States) showing 50% HBV/HDV co-infection in IV drug users[54].

In some other geographic areas, however, the prevalence of HDV infection in HBV chronic carriers has remained high in the last two decades, such as in some eastern countries of the Mediterranean basin, the Middle East, central and northern Asia, western and central Africa, the Amazonian basin (Brazil, Peru, Venezuela, and Colombia), some Pacific islands[63] and Vietnam[64]. In a recent study in the western Brazilian Amazon region, 41.9% of HBV chronic carriers were found to be coinfected with HDV, with a prevalence of over 60% among individuals aged 20–39 years[45], suggesting the likelihood of sexual transmission. In a 2013 study from West Africa (Mauritania), 30% of HBV chronic carriers had the anti-HDV antibody[65] and among

**Table 1 Worldwide variability of anti-hepatitis D virus in hepatitis B surface antigen chronic carriers**

| Country | Anti-HDV in HBsAg chronic carriers, % (years of study) | Ref. |
|---|---|---|
| India | 10.6 (1985); 37.5 (2005) | [33,34] |
| China | 3.5 (1987); 13.1 (1993) | [35,36] |
| Tunisia | 17.7 (1987-1994) | [37] |
| Italy | 23.0 (1987); 8.3 (2006) | [13,38] |
| Thailand[1] | 8.3; 11.1; 65.5 (1988) | [39] |
| Iran | 48.0 (1991); 5.7 (2005) | [40] |
| Germany | 8.0 (1992); 10.9 (2006) | [41] |
| Pakistan | 16.6 (1994); 58.6( 2001) | [42] |
| Saudi Arabia | 8.6 (1996-1997) | [43] |
| Taiwan | 4.4 (1997); 15.3 (2012) | [13,44] |
| Mongolia | 56.5 (2004) | [13,44] |
| Brazil | 41.9 (2005-2006) | [45] |
| Turkey | 7 (2006); 45.5 (2009) | [46] |
| Egypt | 9.9 ( 2013) | [13,44] |
| Japan | 6.0 (1991) | [47] |
| Jordan | 2.0 (1978-1985) | [48] |
| Australia | 4.1 (1997); 4.8 (2016) | [49,50] |
| Greece | 4.2 (1997) | [51] |
| England | 2.6 (2000); 8.5(2006) | [13,44] |
| United States | 2 .0 (1950); 50.0 (2016) | [52-54] |

[1]65.5% in intravenous drug abusers, 11.1% in chronic active hepatitis, and 8.3% in cirrhosis patients.
HDV: Hepatitis D virus; HBsAg: Hepatitis B surface antigen.

these, 62.2% were HDV-RNA-positive[66-68]. A recent study from Iran showed that in Zahedan, 17% of HBV chronic carriers were anti-HDV-positive[69]. A meta-analysis showed that 14.8% of asymptomatic HBsAg-positive patients in the eastern Mediterranean region were coinfected with HDV and that cirrhosis was common in these patients[70]. A study from Vietnam showed an anti-HDV prevalence of 10.7% in HBV chronic carriers[64], a prevalence much higher than expected based on previous reports[71]. These studies confirm the continuing high prevalence of HDV infection in the Amazonian basin, the Middle East, the eastern Mediterranean region, and western Africa, and show an unexpectedly increased prevalence in Vietnam. Instead, a low anti-HDV prevalence in HBV chronic carriers was reported from Egypt (4.7%)[72] and India (4.8%)[73,74].

The reports on the epidemiology of HDV infection are numerous, but at the same time are inadequate to have a complete overview of the spread of the Delta infection around the world[75,76].

First, HDV acute infection is infrequently recognized outside the outbreaks and the rate of chronic forms are likely to be underestimated for the unsatisfactory use of laboratory tests to identify HDV infection in HBsAg chronic carriers. In a nationwide study in the United States only 8.5% of the 25603 HBV patients investigated were ever tested for HDV. Manesis *et al*[51] reported that only one third of Greek patients with HBV chronic hepatitis have been tested for HDV infection[51]. Similarly, El Bouzidi *et al*[77] reported that only 40% of British patients with HBV chronic infection have been tested for anti-HDV[77]. In addition, most epidemiologic studies used anti-HDV to identify the seroprevalence of HDV infection, whereas HDV RNA was performed in some other studies to confirm the etiology and was used for epidemiological investigations.

Second, studies from the same country have reported a discrepant prevalence, probably due to significant geographic variations even within the same country. For

example, a meta-analysis from Turkey reported an anti-HDV seroprevalence of 4.8% in west Turkey, and 46.3% in south-east Turkey[78].

Finally, the time when the epidemiological survey was carried out is of considerable importance, due to the frequent variations over time in the lifestyle habits of the population of a geographic area and, even more, in the progress of anti-hepatitis B vaccination in the same area. As an example, HDV seroprevalence in chronic HBV carriers has largely declined in Italy over the last 50 years (from 23% in 1987, to 14% in 1992 and to 8.3% in 1997), indicating a substantial, progressive reduction in the endemicity level, due to improved socioeconomic conditions and the associated increase in the standards of hygiene, to a substantial reduction in family size, and, most importantly, to the mass vaccination campaign against HBV started in 1991[79-82]. More recent data, however, suggest that the prevalence of HDV in Italy has remained stable at nearly 8%[83].

Overall, HDV infection is still a major burden for public healthcare management in many countries. In recent times we have accumulated evidence that the prevalence of HDV has remained stable or has increased in many endemic and non-endemic geographic areas, due to an increase in migratory flows and to the persistent impact of other relevant risk factors like IVDU, intra-family spread and unsafe sexual activity, homosexual, and heterosexual[13,38,44,84,85]. Furthermore, outbreaks of HDV acute hepatitis are still occurring[80]. In this regard, it is useful to recall the important role of sex workers in the spread of HBV and HDV infections, as well as HIV and other sexually transmitted diseases. The prevalence of HBsAg among migrant sex workers in Chiangmai, Thailand, was 11.4%, but none of them showed anti-HDV antibodies [86].

Of note, most of these sex workers were migrants from Myanmar, born before the introduction of the universal HBV vaccination program in their native country. A meta-analysis on five studies, 4 from China and one from Vietnam, showed that Commercial sex workers have a greater risk to be anti-HDV positive than the external comparator population from the same geographic areas, with a pooled OR of 18.7 (95%CI: 6.70-51.17). In the same meta-analysis, a pooled OD ratio of 16.00 (95%CI: 3.94-64.92) indicates that MSM from Italy and Indonesia are at a greater risk to be anti-HDV positive than the comparator population from the same geographic areas, asymptomatic HBsAg carriers for Italy and normal subjects for Indonesia. No positive case was observed in MSM from a Burkina Faso study analyzed in the same contest [44].

## SARS-COV-2 PANDEMICS: REDUCED OPPORTUNITY FOR SCREENING, MANAGEMENT, AND TREATMENT

Much of the assistance not linked to the SARS-CoV-2 pandemic has been severely compromised and/or downsized during this pandemic, a drastic reduction also affecting liver diseases. In Italy, 26% of hepatology wards have been converted to SARS-CoV-2 wards and another 33% have suffered a substantial reduction in the availability of beds. Screening and day hospital activities for liver diseases have been significantly reduced and the start of treatments for "new" HBV chronic hepatitis have been postponed in 23% of the open liver units[87]. Overall, hepatology activities, including the management of patients with compensated or decompensated liver disease, liver transplantation or HCC have been significantly reduced and/or suspended[34].

In April 2020, health centers in Burkina Faso, Gambia, and Tanzania, respectively, recorded a 71%, 83% and 95% decline in the numbers of "new" patients attending liver clinics, compared to the numbers recorded at the beginning of the same year, mainly due to the patient's fear of accessing health services[88].

A limitation or closure of health centers offering harm reduction services (such as needle exchange and opioid replacement therapy) due to SARS-CoV-2 pandemic have been reported from South Africa, with an increased risk in overdose and transmission of blood-borne infections[89].

In other countries in Sub-Saharan Africa, essential diagnostic tests to manage chronic hepatitis, rapid tests for HBsAg, alanine aminotransferase and hematological tests, have always been available but there has been a serious shortage of nucleic acid tests to detect HBV and HDV replications; therefore, people newly identified as HBsAg carriers may have missed the opportunity to be evaluated for viral load, connected to treatments, and finally treated[89]. For Sub-Saharan African countries, there has also been a strong reduction in tenofovir supply[89], with an increased risk

of an exacerbation of liver cell necrosis, the onset of drug resistance and transmission of HBV infection.

In Japan, Singapore and the United States, the number of physical examinations for HBV chronic hepatitis decreased significantly in 2020, compared to 2019, especially for more elderly subjects[90].

Numbers from England give clear information on the influence of the SARS-CoV-2 pandemic on screening for liver diseases, 2478 tests in 2019 and 1224 in 2020[91].

COVID-19 has also led to an increase in economic and public health disparities. The pandemic has caused a global recession, driving millions of people below the poverty line, not only in countries with low economic levels[92]. In the United States, 6.2 million people have lost their health insurance since the start of the pandemic, due to job losses, leading to a greater risk of acquiring viral hepatitis as well as other infectious diseases[93].

The World Hepatitis Alliance conducted a worldwide survey to assess the impact of the SARS-CoV-2 pandemic on patients with viral hepatitis: Job loss during this pandemic resulted in a strong impact on household monetary income in low- and middle-income countries, with a frequent lack of access to drugs. Indigenous communities in some developing countries were the most affected both by traffic restrictions with the inability to reach healthcare centers and to the difficulties in earning a living and paying for a physical examination[93,94].

In high-income countries, remote assistance through telemedicine and other virtual platforms has been strongly encouraged to maintain continuity of care[95]. However, in sub-Saharan Africa, many patients and healthcare providers do not have access to telemedicine solutions[88].

There is also an increased risk of HBV vertical transmission during the SARS-CoV-2 pandemic, due to a higher frequency of home births without prophylactic measures [92]. Childhood prophylaxis against HBV infection performed at birth is estimated to have prevented 310 million new HBV infections between 1990 and 2020[96], including 14 million HBV/HDV infections[44]. Preliminary data from the Institute for Health Metrics and Evaluation indicate that global levels of vaccination coverage progressively increased from 1990 to the beginning of 2020, but during 2020 it fell to the levels of the 1990s[97]. The reduction in vaccination coverage may lead to a high increase in the incidence of HBV infection in childhood, and to an increased risk of acquiring HDV infection in subsequent years.

## CONCEIVABLE DEVELOPMENTS IN HBV/HDV EPIDEMIOLOGY DUE TO THE SARS COV-2 PANDEMIC

The SARS-CoV-2 pandemic has had a negative impact on the global health system and on the development of assistance programs for other diseases. Considering that 290 million people live with chronic HBV, it is easy to calculate that the unidirectionality of healthcare towards SARS-CoV-2 has strongly damaged the programs for screening, management, and treatment of HBV and HDV associated diseases worldwide.

No epidemiological data on HBV/HDV coinfection during the SARS-CoV-2 pandemic is available at present and few data on HBV infection are reported in the literature, although HBsAg positive subjects are at high risk of acquiring SARS-CoV-2 infection. Guan *et al*[98] reported a 2.1% HBsAg positivity in 1099 SARS-CoV-2 Chinese patients[98] while Chen *et al*[99] found a 4% HBsAg prevalence in 274 SARS-CoV-2 Chinese patients[99]. Instead, the HBV prevalence was 0.1% in 5700 (8/5700) SARS-CoV-2 patients in the United States[100].

Even in the absence of data on the epidemiology of HBV/HDV co-infection during the SARS-CoV-2 pandemic, mostly due to the short period elapsed, it seems useful to make some predictions based on the sudden great variation in lifestyle worldwide, imposed by the spread of this life-threatening pandemic.

The multiplicity of factors, favoring or hindering the spread of HBV and HDV infections, mostly created by the measures put in place by the Political or Healthcare Authorities of different countries to hinder the spread of SARS-CoV-2, makes it difficult to predict the epidemiological variations of these two infections in the coming years. In fact, the restrictions in movement imposed in many countries may have decreased HBV transmission due to the reduction, outside the family, of physical contact between infected subjects and those exposed. These restrictions, however, have induced a depression in large segments of the population, which has led to an increase in alcohol consumption, drug use and unprotected sex. Another adverse effect of the restrictions is the increased risk of child deliveries at home, which may involve the

lack of screening for HBV infection for mothers and an inadequacy in the prophylaxis of the newborn.

In addition, a significant part of the resources usually reserved for the different fields of medicine have been devolved to the screening of SARS-CoV-2 infection and to the assistance of COVID-19 patients. As a result, there has been a downsizing of care for liver diseases: A lower number of hepatic wards and day hospitals, a lower number of beds in hepatic wards still open, difficulty in managing patients with serious liver diseases and the recipients of liver graft. In addition, screening for HBV and HDV infections have been significantly reduced, and the start of treatment for "new" patients frequently postponed. Another important role in the reduction of healthcare facilities for liver patients was played by their fear of contracting SARS-CoV-2 infection in healthcare structures and in centers giving assistance to drug addicts. Moreover, many "new" cases of HDV infection may have gone undiagnosed, since the determination of HBV DNA, HDV DNA and IgM to HD-Ag has undergone a significant reduction in several countries for the considerable commitment of many laboratories in the diagnosis of SARS-CoV-2 infection and for the fear of patients attending them. Therefore, several HBsAg carriers and HBV/HDV patients may not have been identified, and if identified may not have received appropriate treatment.

The sum of these contrasting effects exerted by the measures restricting mobility on the spread of HBV and HDV infections makes it difficult to predict the extent and modalities of their spread in coming years. However, we have no doubt in advancing pessimistic forecasts, because in the continuing course of the SARS-CoV-2 pandemic, foreseeable for about another 2 years, all the negative elements reported above will remain, while the positive effects of movement restriction will gradually decrease, because such restrictions, already not respected in many countries, will be even less so in the future. We only trust in a more rapid and intense production of vaccines against SARS-CoV-2 infection and in universal vaccination campaigns for a future return to normality[101].

## CONCLUSION

The impact of the SARS-CoV-2 pandemic on essential health services is of great concern. In fact, it is feared that most improvements in public health achieved in recent years could be wiped out, with negative repercussions on the provision of health services to the public, with a consequent reduction in the prevention of infectious diseases, diagnostic efficacy, and the application of therapeutic and rehabilitative treatments, and overall, of healthcare promotion. The reduction of essential healthcare services, due to the focus towards COVID-19, risks having serious negative effects on the health of the most vulnerable populations, such as children, the elderly, patients with chronic diseases, viral (HBV, HDV and hepatitis C virus hepatitis, HIV infection), inflammatory, degenerative, oncological, cardiac, autoimmune, *etc.*

The COVID-19 pandemic has required the use of enormous healthcare resources and, consequently, reduces the care of patients with other diseases, particularly those who need continuous clinical monitoring to avoid clinical deterioration such as patients with chronic HBV/HDV infection. For these patients, there has already been, and will occur even more in the next few years, a lack of diagnosis for some, delayed access to treatment or lack of continuity of antiviral therapy for others, and finally a lack in the surveillance for the possible emergence of liver cirrhosis and HCC and the need for liver transplantation. When the COVID-19 emergency draws to a close, there will be a resumption of health activities for all diseases neglected during the pandemic, including screening for HBV and HDV infection, and a false increase in the prevalence of these infections will occur because of the effect of the recovery of cases undiagnosed in 2020, 2021 and, for some countries with reduced vaccination activity against COVID-19 even in some subsequent years.

We remind those in charge of treating patients with chronic hepatitis B and those who have the related administrative responsibility that the interruption of drugs suppressing HBV replication in patients with HBV chronic hepatitis frequently leads to the exacerbation of liver cell necrosis, with a risk of death from acute liver failure. Furthermore, patients with chronic HBV/HDV infection are at risk of developing cirrhosis, HCC, and serious decompensation of the disease, and therefore require close clinical, biochemical and ultrasound monitoring, now made difficult by the COVID-19 emergency.

There is also the need for strategic adjustments to ensure specialist healthcare services to identify, monitor and treat patients with chronic HBV infection and those

with HBV/HDV coinfection.

## REFERENCES


1 **Rizzetto M**, Canese MG, Aricò S, Crivelli O, Trepo C, Bonino F, Verme G. Immunofluorescence detection of new antigen-antibody system (delta/anti-delta) associated to hepatitis B virus in liver and in serum of HBsAg carriers. *Gut* 1977; **18**: 997-1003 [PMID: 75123 DOI: 10.1136/gut.18.12.997]

2 **Rizzetto M**, Canese MG, Gerin JL, London WT, Sly LD, Purcell R. H. Transmission of the hepatitis B virus-associated delta antigen to chimpanzees. *J Infect Dis* 1980; **141**: 590-602

3 **Sureau C**, Negro F. The hepatitis delta virus: Replication and pathogenesis. *J Hepatol* 2016; **64**: S102-S116 [PMID: 27084031 DOI: 10.1016/j.jhep.2016.02.013]

4 **Sagnelli C**, Sagnelli E, Russo A, Pisaturo M, Occhiello L, Coppola N. HBV/HDV Co-Infection: Epidemiological and Clinical Changes, Recent Knowledge and Future Challenges. *Life (Basel)* 2021; **11** [PMID: 33671730 DOI: 10.3390/Life11020169]

5 **Nguyen MH**, Wong G, Gane E, Kao JH, Dusheiko G. Hepatitis B Virus: Advances in Prevention, Diagnosis, and Therapy. *Clin Microbiol Rev* 2020; **33** [PMID: 32102898 DOI: 10.1128/CMR.00046-19]

6 **Negro F**. Hepatitis D virus coinfection and superinfection. *Cold Spring Harb Perspect Med* 2014; **4**: a021550 [PMID: 25368018 DOI: 10.1101/cshperspect.a021550]

7 **Farci P**, Niro GA. Clinical features of hepatitis D. *Semin Liver Dis* 2012; **32**: 228-236 [PMID: 22932971 DOI: 10.1055/s-0032-1323628]

8 **Fattovich G**, Giustina G, Christensen E, Pantalena M, Zagni I, Realdi G, Schalm SW. Influence of hepatitis delta virus infection on morbidity and mortality in compensated cirrhosis type B. The European Concerted Action on Viral Hepatitis (Eurohep). *Gut* 2000; **46**: 420-426 [PMID: 10673308 DOI: 10.1136/gut.46.3.420]

9 **Stroffolini T**, Ciancio A, Furlan C, Vinci M, Niro GA, Russello M, Colloredo G, Morisco F, Coppola N, Babudieri S, Ferrigno L, Sagnelli C, Sagnelli E; Collaborating group. Chronic hepatitis B virus infection in Italy during the twenty-first century: an updated survey in 2019. *Eur J Clin Microbiol Infect Dis* 2021; **40**: 607-614 [PMID: 33029767 DOI: 10.1007/s10096-020-04065-6]

10 **Koh C**, Da BL, Glenn JS. HBV/HDV Coinfection: A Challenge for Therapeutics. *Clin Liver Dis* 2019; **23**: 557-572 [PMID: 31266627 DOI: 10.1016/j.cld.2019.04.005]

11 **Muhammad H**, Tehreem A, Hammami MB, Ting PS, Idilman R, Gurakar A. Hepatitis D virus and liver transplantation: Indications and outcomes. *World J Hepatol* 2021; **13**: 291-299 [PMID: 33815673 DOI: 10.4254/wjh.v13.i3.291]

12 **Rifai K**, Wedemeyer H, Rosenau J, Klempnauer J, Strassburg CP, Manns MP, Tillmann HL. Longer survival of liver transplant recipients with hepatitis virus coinfections. *Clin Transplant* 2007; **21**: 258-264 [PMID: 17425755 DOI: 10.1111/j.1399-0012.2006.00636.x]

13 **Chen HY**, Shen DT, Ji DZ, Han PC, Zhang WM, Ma JF, Chen WS, Goyal H, Pan S, Xu HG. Prevalence and burden of hepatitis D virus infection in the global population: a systematic review and meta-analysis. *Gut* 2019; **68**: 512-521 [PMID: 30228220 DOI: 10.1136/gutjnl-2018-316601]

14 **Wedemeyer H**, Manns MP. Epidemiology, pathogenesis and management of hepatitis D: update and challenges ahead. *Nat Rev Gastroenterol Hepatol* 2010; **7**: 31-40 [PMID: 20051970 DOI: 10.1038/nrgastro.2009.205]

15 **Chang MS**, Nguyen MH. Epidemiology of hepatitis B and the role of vaccination. *Best Pract Res Clin Gastroenterol* 2017; **31**: 239-247 [PMID: 28774405 DOI: 10.1016/j.bpg.2017.05.008]

16 **Gayotto LC**. Hepatitis Delta in South America and especially in the Amazon basin. in "The Hepatitis Delta Virus. Wley-liss, Inc. 1991: 123-135

17 **Costa E**. Black fever of the Purus River. Some Observations about an outbreak in the '"Infectious beach" mouth of the acre-amazons. *Gaz Medica da Bahia* 1970; **70**: 148 [DOI: 10.34117/bjdv7n4-364]

18 **Andrade ZA**, Santos JB, Prata A, Dourado H. Histopathology of Labrea hepatitis. *Rev Soc Bras Med Trop* 1983; **16**: 31-40 [DOI: 10.1590/s0037-86821983000100005]

19 **Bensabath G**, Dias LB. [Labrea hepatitis (Labrea black fever) and other fulminant forms of hepatitis in Sena Madureira, Acre and Boca do Acre, Amazonas, Brazil]. *Rev Inst Med Trop Sao Paulo* 1983; **25**: 182-194 [PMID: 6658300]

20 **de Fonseca JC**, Gayotto LC, Ferreira LC, Araújo JR, Alecrim WD, Santos RT, Simonetti JP, Alves VA. Labrea hepatitis--hepatitis B and delta antigen expression in liver tissue: report of three autopsy cases. *Rev Inst Med Trop Sao Paulo* 1985; **27**: 224-227 [PMID: 3832342 DOI: 10.1590/s0036-46651985000400011]

21 **Gast-Galvis A**. Febrile hepatitis from Santa Marta. *Salubridad* 1955; **1**: 45-52 [DOI: 10.1093/oxfordjournals.aje.a119138]

22 **Aguilera A**, Morales A, Buitrago B, Guzmán M, Peña C, Marquez G. Hepatitis fulminante epidé mica de la Sierra Nevada de Santa Marta I. Estudio de un brote en la localidad de Julio Zawady, Cié naga, Magdalena Colombia. *Biomédica* 1981; **1**: 187 [DOI: 10.7705/biomedica.v1i4.1801]

23 **Lesbordes JL**, Ravisse P, Georges AJ, Chevallier P, Pichoud C, Vitvitski L, Trepo C. Studies on the role of HDV in an outbreak of fulminant hepatitis in Bangui (Central African Republic). *Prog Clin*

*Biol Res* 1987; **234**: 451-459 [PMID: 3628412]
24 **Gomes-Gouvêa MS**, Soares MCP, Bensabath G, de Carvalho-Mello IMVG, Brito EMF, Souza OSC, Queiroz ATL, Carrilho FJ, Pinho JRR. Hepatitis B virus and hepatitis delta virus genotypes in outbreaks of fulminant hepatitis (Labrea black fever) in the western Brazilian Amazon region. *J Gen Virol* 2009; **90**: 2638-2643 [PMID: 19605587 DOI: 10.1099/vir.0.013615-0]
25 **Popper H**, Thung SN, Gerber MA, Hadler SC, de Monzon M, Ponzetto A, Anzola E, Rivera D, Mondolfi A, Bracho A. Histologic studies of severe delta agent infection in Venezuelan Indians. *Hepatology* 1983; **3**: 906-912 [PMID: 6629319 DOI: 10.1002/hep.1840030603]
26 **Andrade ZA**, Lesbordes JL, Ravisse P, Paraná R, Prata A, Barberino JS, Trepo C. Fulminant hepatitis with microvesicular steatosis (a histologic comparison of cases occurring in Brazil--Labrea hepatitis--and in central Africa--Bangui hepatitis). *Rev Soc Bras Med Trop* 1992; **25**: 155-160 [PMID: 1308946 DOI: 10.1590/s0037-86821992000300001]
27 **Smedile A**, Dentico P, Zanetti A, Sagnelli E, Nordenfelt E, Actis GC, Rizzetto M. Infection with the delta agent in chronic HBsAg carriers. *Gastroenterology* 1981; **81**: 992-997 [PMID: 7286594]
28 **Buti M**, Homs M, Rodriguez-Frias F, Funalleras G, Jardí R, Sauleda S, Tabernero D, Schaper M, Esteban R. Clinical outcome of acute and chronic hepatitis delta over time: a long-term follow-up study. *J Viral Hepat* 2011; **18**: 434-442 [PMID: 20546496 DOI: 10.1111/j.1365-2893.2010.01324.x]
29 **SEIEVA**. Istituto Superiore di Sanità (ISS) National health institute. [cited 10 April 2021]. Available from: https://www.iss.it/seieva/chi-siamo
30 **Tsatsralt-Od B**, Takahashi M, Endo K, Buyankhuu O, Baatarkhuu O, Nishizawa T, Okamoto H. Infection with hepatitis A, B, C, and delta viruses among patients with acute hepatitis in Mongolia. *J Med Virol* 2006; **78**: 542-550 [PMID: 16555292 DOI: 10.1002/jmv.20574]
31 **Baatarkhuu O**, Lee HW, George J, Munkh-Orshikh D, Enkhtuvshin B, Ariunaa S, Eslam M, Ahn SH, Han KH, Kim DY. Acute hepatitis A, B and C but not D is still prevalent in Mongolia: a time trend analysis. *Clin Mol Hepatol* 2017; **23**: 147-153 [PMID: 28535669 DOI: 10.3350/cmh.2016.0055]
32 **WHO**. Hepatitis D. [cited 10 April 2021]. Available from: https://www.who.int/news-room/fact-sheets/detail/hepatitis-d
33 **Chakraborty P**, Kailash U, Jain A, Goyal R, Gupta RK, Das BC, Kar P. Seroprevalence of hepatitis D virus in patients with hepatitis B virus-related liver diseases. *Indian J Med Res* 2005; **122**: 254-257 [PMID: 16251784]
34 **Banker DD**, Desai P, Brawner TA, Decker RH. Hepatitis delta virus infection in Bombay. *Trans R Soc Trop Med Hyg* 1992; **86**: 424-425 [PMID: 1440825 DOI: 10.1016/0035-9203(92)90251-7]
35 **Chen X**, Xuan M, Yin Y. [Study of HDV infection in Shandong province]. *Zhonghua Liu Xing Bing Xue Za Zhi* 1998; **19**: 138-140 [PMID: 10322728]
36 **Zhang JY**, Jin ZH, Wang CJ. [A seroepidemiological study on hepatitis D virus (HDV) infection in Henan Province, China]. *Zhonghua Liu Xing Bing Xue Za Zhi* 1995; **16**: 365-368 [PMID: 8728959]
37 **Triki H**, Said N, Ben Salah A, Arrouji A, Ben Ahmed F, Bouguerra A, Hmida S, Dhahri R, Dellagi K. Seroepidemiology of hepatitis B, C and delta viruses in Tunisia. *Trans R Soc Trop Med Hyg* 1997; **91**: 11-14 [PMID: 9093616 DOI: 10.1016/s0035-9203(97)90374-6]
38 **Sagnelli E**, Sagnelli C, Pisaturo M, Macera M, Coppola N. Epidemiology of acute and chronic hepatitis B and delta over the last 5 decades in Italy. *World J Gastroenterol* 2014; **20**: 7635-7643 [PMID: 24976701 DOI: 10.3748/wjg.v20.i24.7635]
39 **Louisirirotchanakul S**, Wasi C, Uneklabh C, Phutiprawan T, Suwanagool S, Chainuvati T, Thongcharoen P. High prevalence of delta virus infection in Thai intravenous drug abusers. *Southeast Asian J Trop Med Public Health* 1988; **19**: 191-195 [PMID: 3227398]
40 **Sadeghian H**, Varasteh N, Esmaeelzadeh A, Nomani H, Alimardani M, Davoodnejad M, Meshkat M, Ahadi M, Sepahi S, Rostami S, Meshkat Z. Distribution of hepatitis delta virus genotypes in mashhad, northeast iran. *Jundishapur J Microbiol* 2015; **8**: e14908 [PMID: 25793092 DOI: 10.5812/jjm.14908]
41 **Heidrich B**, Deterding K, Tillmann HL, Raupach R, Manns MP, Wedemeyer H. Virological and clinical characteristics of delta hepatitis in Central Europe. *J Viral Hepat* 2009; **16**: 883-894 [PMID: 19566789 DOI: 10.1111/j.1365-2893.2009.01144.x]
42 **Mumtaz K**, Hamid SS, Adil S, Afaq A, Islam M, Abid S, Shah HA, Jafri W. Epidemiology and clinical pattern of hepatitis delta virus infection in Pakistan. *J Gastroenterol Hepatol* 2005; **20**: 1503-1507 [PMID: 16174065 DOI: 10.1111/j.1440-1746.2005.03857.x]
43 **Al-Traif I**, Ali A, Dafalla M, Al-Tamimi W, Qassem L. Prevalence of hepatitis delta antibody among HBsAG carriers in Saudi Arabia. *Ann Saudi Med* 2004; **24**: 343-344 [PMID: 15573844 DOI: 10.5144/0256-4947.2004.343]
44 **Stockdale AJ**, Kreuels B, Henrion MYR, Giorgi E, Kyomuhangi I, de Martel C, Hutin Y, Geretti AM. The global prevalence of hepatitis D virus infection: Systematic review and meta-analysis. *J Hepatol* 2020; **73**: 523-532 [PMID: 32335166 DOI: 10.1016/j.jhep.2020.04.008]
45 **Braga WS**, Castilho Mda C, Borges FG, Leão JR, Martinho AC, Rodrigues IS, Azevedo EP, Barros Júnior GM, Paraná R. Hepatitis D virus infection in the Western Brazilian Amazon - far from a vanishing disease. *Rev Soc Bras Med Trop* 2012; **45**: 691-695 [PMID: 23295870 DOI: 10.1590/s0037-86822012000600007]
46 **Mese S**, Nergiz S, Tekes S, Gul K. Seroprevalence of serum HBsAg positivity and hepatitis delta virus infection among blood donors in Southeastern Turkey. *Clin Ter* 2014; **165**: 95-98 [PMID: 24770811 DOI: 10.7471/CT.2014.1683]

47 **Tamura I**, Kurimura O, Koda T, Ichimura H, Katayama S, Kurimura T, Inaba Y. Risk of liver cirrhosis and hepatocellular carcinoma in subjects with hepatitis B and delta virus infection: a study from Kure, Japan. *J Gastroenterol Hepatol* 1993; **8**: 433-436 [PMID: 8218990 DOI: 10.1111/j.1440-1746.1993.tb01543.x]

48 **Toukan AU**, Abu-el-Rub OA, Abu-Laban SA, Tarawneh MS, Kamal MF, Hadler SC, Krawczynski K, Margolis HS, Maynard JE. The epidemiology and clinical outcome of hepatitis D virus (delta) infection in Jordan. *Hepatology* 1987; **7**: 1340-1345 [PMID: 2824316 DOI: 10.1002/hep.1840070627]

49 **Coghill S**, McNamara J, Woods M, Hajkowicz K. Epidemiology and clinical outcomes of hepatitis delta (D) virus infection in Queensland, Australia. *Int J Infect Dis* 2018; **74**: 123-127 [PMID: 30003953 DOI: 10.1016/j.ijid.2018.07.005]

50 **Shadur B**, MacLachlan J, Cowie B. Hepatitis D virus in Victoria 2000-2009. *Intern Med J* 2013; **43**: 1081-1087 [PMID: 23869436 DOI: 10.1111/imj.12247]

51 **Manesis EK**, Vourli G, Dalekos G, Vasiliadis T, Manolaki N, Hounta A, Koutsounas S, Vafiadis I, Nikolopoulou G, Giannoulis G, Germanidis G, Papatheodoridis G, Touloumi G. Prevalence and clinical course of hepatitis delta infection in Greece: a 13-year prospective study. *J Hepatol* 2013; **59**: 949-956 [PMID: 23850875 DOI: 10.1016/j.jhep.2013.07.005]

52 **Hershow RC**, Chomel BB, Graham DR, Schyve PM, Mandel EJ, Kane MA, Fields HA, Hadler SC. Hepatitis D virus infection in Illinois state facilities for the developmentally disabled. Epidemiology and clinical manifestations. *Ann Intern Med* 1989; **110**: 779-785 [PMID: 2712461 DOI: 10.7326/0003-4819-110-10-779]

53 **Patel EU**, Thio CL, Boon D, Thomas DL, Tobian AAR. Prevalence of Hepatitis B and Hepatitis D Virus Infections in the United States, 2011-2016. *Clin Infect Dis* 2019; **69**: 709-712 [PMID: 30605508 DOI: 10.1093/cid/ciz001]

54 **Kucirka LM**, Farzadegan H, Feld JJ, Mehta SH, Winters M, Glenn JS, Kirk GD, Segev DL, Nelson KE, Marks M, Heller T, Golub ET. Prevalence, correlates, and viral dynamics of hepatitis delta among injection drug users. *J Infect Dis* 2010; **202**: 845-852 [PMID: 20701536 DOI: 10.1086/655808]

55 **Pascarella S**, Negro F. Hepatitis D virus: an update. *Liver Int* 2011; **31**: 7-21 [PMID: 20880077 DOI: 10.1111/j.1478-3231.2010.02320.x]

56 **Motamedifar M PhD**, Taheri M MSc, Lankarani KB Md, Gholami M Bc, Lari MA Md, Faramarzi H Md, Sarvari J PhD. The Prevalence and Risk Factors of Hepatitis Delta Virus in HIV/HBV Co-Infected Patients in Shiraz, Iran, 2012. *Iran J Med Sci* 2015; **40**: 448-453 [PMID: 26379352]

57 **Chambal LM**, Gudo ES, Carimo A, Corte Real R, Mabunda N, Maueia C, Vubil A, Zicai AF, Bhatt N, Antunes F. HBV infection in untreated HIV-infected adults in Maputo, Mozambique. *PLoS One* 2017; **12**: e0181836 [PMID: 28759595 DOI: 10.1371/journal.pone.0181836]

58 **Salpini R**, Fokam J, Ceccarelli L, Santoro MM, Nanfack A, Sosso SM, Kowo M, Cento V, Torimiro J, Sarmati L, Andreoni M, Colizzi V, Perno CF, Njoya O. High Burden of HBV-Infection and Atypical HBV Strains among HIV-infected Cameroonians. *Curr HIV Res* 2016; **14**: 165-171 [PMID: 26419862 DOI: 10.2174/1570162x13666150930114742]

59 **Ogwu-Richard SO**, Ojo DA, Akingbade OA, Okonko IO. Triple positivity of HBsAg, anti-HCV antibody, and HIV and their influence on CD4+ lymphocyte levels in the highly HIV infected population of Abeokuta, Nigeria. *Afr Health Sci* 2015; **15**: 719-727 [PMID: 26957958 DOI: 10.4314/ahs.v15i3.4]

60 **Coffie PA**, Tchounga BK, Bado G, Kabran M, Minta DK, Wandeler G, Gottlieb GS, Dabis F, Eholie SP, Ekouevi DK. Prevalence of hepatitis B and delta according to HIV-type: a multi-country cross-sectional survey in West Africa. *BMC Infect Dis* 2017; **17**: 466 [PMID: 28676076 DOI: 10.1186/s12879-017-2568-5]

61 **Mele A**, Catapano R, Ferrigno L, Marzolini A, Stazi M, Martelli A, Pasquini P. Integrated Epidemiological System of Acute Viral Hepatitis. 1991 annual report. Roma: National Health Institute, 1993

62 **William Tong CY**, Asher R, Toby M, Ngui SL, Tettmar K, Ijaz S, Tedder R, Kulasegaram R, Wilkinson M, Wong T. A re-assessment of the epidemiology and patient characteristics of hepatitis D virus infection in inner city London. *J Infect* 2013; **66**: 521-527 [PMID: 23466596 DOI: 10.1016/j.jinf.2013.02.006]

63 **Alvarado-Mora MV**, Locarnini S, Rizzetto M, Pinho JR. An update on HDV: virology, pathogenesis and treatment. *Antivir Ther* 2013; **18**: 541-548 [PMID: 23792471 DOI: 10.3851/IMP2598]

64 **Dunford L**, Carr MJ, Dean J, Nguyen LT, Ta Thi TH, Nguyen BT, Connell J, Coughlan S, Nguyen HT, Hall WW, Thi LA. A multicentre molecular analysis of hepatitis B and blood-borne virus coinfections in Viet Nam. *PLoS One* 2012; **7**: e39027 [PMID: 22720022 DOI: 10.1371/journal.pone.0039027]

65 **Lunel-Fabiani F**, Mansour W, Amar AO, Aye M, Le Gal F, Malick FZ, Baïdy L, Brichler S, Veillon P, Ducancelle A, Gordien E, Rosenheim M. Impact of hepatitis B and delta virus co-infection on liver disease in Mauritania: a cross sectional study. *J Infect* 2013; **67**: 448-457 [PMID: 23796871 DOI: 10.1016/j.jinf.2013.06.008]

66 **Ciccozzi M**, Lai A, Zehender G, Borsetti A, Cella E, Ciotti M, Sagnelli E, Sagnelli C, Angeletti S. The phylogenetic approach for viral infectious disease evolution and epidemiology: An updating review. *J Med Virol* 2019; **91**: 1707-1724 [PMID: 31243773 DOI: 10.1002/jmv.25526]

67 **Lai A**, Sagnelli C, Presti AL, Cella E, Angeletti S, Spoto S, Costantino S, Sagnelli E, Ciccozzi M. What is changed in HBV molecular epidemiology in Italy? *J Med Virol* 2018; **90**: 786-795 [PMID: 29315661 DOI: 10.1002/jmv.25027]

68 **Stroffolini T**, Sagnelli E, Sagnelli C, Russello M, De Luca M, Rosina F, Cacopardo B, Brancaccio G, Furlan C, Gaeta GB, Licata A, Almasio PL; behalf of EPACRON study group. Hepatitis delta infection in Italian patients: towards the end of the story? *Infection* 2017; **45**: 277-281 [PMID: 27817147 DOI: 10.1007/s15010-016-0956-1]

69 **Bakhshipour A**, Mashhadi M, Mohammadi M, Nezam SK. ORIGINAL REPORT Seroprevalence and Risk Factors of Hepatitis Delta Virus in Chronic Hepatitis B Virus Infection in Zahedan. *Acta Med Iran* 2013; **51**: 260-264 [DOI: 10.1001/archinte.1993.00410140095011]

70 **Amini N**, Alavian SM, Kabir A, Aalaei-Andabili SH, Saiedi Hosseini SY, Rizzetto M. Prevalence of hepatitis d in the eastern mediterranean region: systematic review and meta analysis. *Hepat Mon* 2013; **13**: e8210 [PMID: 23554822 DOI: 10.5812/hepatmon.8210]

71 **Nguyen VT**, McLaws ML, Dore GJ. Highly endemic hepatitis B infection in rural Vietnam. *J Gastroenterol Hepatol* 2007; **22**: 2093-2100 [PMID: 17645465 DOI: 10.1111/j.1440-1746.2007.05010.x]

72 **Gomaa NI**, Metwally LA, Nemr N, Younis S. Seroprevalence of HDV infection in HBsAg positive population in Ismailia, Egypt. *Egypt J Immunol* 2013; **20**: 23-28 [PMID: 23888554]

73 **Saravanan S**, Madhavan V, Velu V, Murugavel KG, Waldrop G, Solomon SS, Balakrishnan P, Kumarasamy N, Smith DM, Mayer KH, Solomon S, Thyagarajan SP. High prevalence of hepatitis delta virus among patients with chronic hepatitis B virus infection and HIV-1 in an intermediate hepatitis B virus endemic region. *J Int Assoc Provid AIDS Care* 2014; **13**: 85-90 [PMID: 23722085 DOI: 10.1177/2325957413488166]

74 **Rivas P**, Herrero MD, Poveda E, Madejón A, Treviño A, Gutiérrez M, Ladrón de Guevara C, Lago M, de Mendoza C, Soriano V, Puente S. Hepatitis B, C, and D and HIV infections among immigrants from Equatorial Guinea living in Spain. *Am J Trop Med Hyg* 2013; **88**: 789-794 [PMID: 23339201 DOI: 10.4269/ajtmh.12-0319]

75 **Palumbo E**, Scotto G, Faleo G, Cibelli DC, Saracino A, Angarano G. Prevalence of HBV-genotypes in immigrants affected by HBV-related chronic active hepatitis. *Arq Gastroenterol* 2007; **44**: 54-57 [PMID: 17639184 DOI: 10.1590/s0004-28032007000100012]

76 **Sagnelli C**, Ciccozzi M, Alessio L, Cella E, Gualdieri L, Pisaturo M, Minichini C, Di Caprio G, Starace M, Onorato L, Capoprese M, Occhiello L, Angeletti S, Scotto G, Macera M, Sagnelli E, Coppola N. HBV molecular epidemiology and clinical condition of immigrants living in Italy. *Infection* 2018; **46**: 523-531 [PMID: 29796738 DOI: 10.1007/s15010-018-1153-1]

77 **El Bouzidi K**, Elamin W, Kranzer K, Irish DN, Ferns B, Kennedy P, Rosenberg W, Dusheiko G, Sabin CA, Smith BC, Nastouli E. Hepatitis delta virus testing, epidemiology and management: a multicentre cross-sectional study of patients in London. *J Clin Virol* 2015; **66**: 33-37 [PMID: 25866333 DOI: 10.1016/j.jcv.2015.02.011]

78 **Değertekin H**, Yalçin K, Yakut M, Yurdaydin C. Seropositivity for delta hepatitis in patients with chronic hepatitis B and liver cirrhosis in Turkey: a meta-analysis. *Liver Int* 2008; **28**: 494-498 [PMID: 18339076 DOI: 10.1111/j.1478-3231.2008.01673.x]

79 **Crovari P**. Epidemiology of viral hepatitis B in Italy. *Vaccine* 1995; **13** Suppl 1: S26-S30 [PMID: 7571823 DOI: 10.1016/0264-410X(95)80043-D]

80 **Romanò L**, Paladini S, Galli C, Raimondo G, Pollicino T, Zanetti AR. Hepatitis B vaccination. *Hum Vaccin Immunother* 2015; **11**: 53-57 [PMID: 25483515 DOI: 10.4161/hv.34306]

81 **Smedile A**, Lavarini C, Farci P, Aricò S, Marinucci G, Dentico P, Giuliani G, Cargnel A, Del Vecchio Blanco C, Rizzetto M. Epidemiologic patterns of infection with the hepatitis B virus-associated delta agent in Italy. *Am J Epidemiol* 1983; **117**: 223-229 [PMID: 6829551 DOI: 10.1093/oxfordjournals.aje.a113533]

82 **Romanò L**, Paladini S, Zanetti AR. Twenty years of universal vaccination against hepatitis B in Italy: achievements and challenges. *J Public Health Res* 2012; **1**: 126-129 [PMID: 25170454 DOI: 10.4081/jphr.2012.e18]

83 **La Torre G**, Mannocci A, Saulle R, Colamesta V, Meggiolaro A, Mipatrini D, Sinopoli A. Economic evaluation of HBV vaccination: A systematic review of recent publications (2000-2013). *Hum Vaccin Immunother* 2016; **12**: 2299-2311 [PMID: 27105443 DOI: 10.1080/21645515.2016.1166328]

84 **Servant-Delmas A**, Le Gal F, Gallian P, Gordien E, Laperche S. Increasing prevalence of HDV/HBV infection over 15 years in France. *J Clin Virol* 2014; **59**: 126-128 [PMID: 24365475 DOI: 10.1016/j.jcv.2013.11.016]

85 **Komas NP**, Ghosh S, Abdou-Chekaraou M, Pradat P, Al Hawajri N, Manirakiza A, Laghoe GL, Bekondi C, Brichler S, Ouavéné JO, Sépou A, Yambiyo BM, Gody JC, Fikouma V, Gerber A, Abeywickrama Samarakoon N, Alfaiate D, Scholtès C, Martel N, Le Gal F, Lo Pinto H, Amri I, Hantz O, Durantel D, Lesbordes JL, Gordien E, Merle P, Drugan T, Trépo C, Zoulim F, Cortay JC, Kay AC, Dény P. Hepatitis B and hepatitis D virus infections in the Central African Republic, twenty-five years after a fulminant hepatitis outbreak, indicate continuing spread in asymptomatic young adults. *PLoS Negl Trop Dis* 2018; **12**: e0006377 [PMID: 29698488 DOI: 10.1371/journal.pntd.0006377]

86 **Hongjaisee S**, Khamduang W, Sripan P, Choyrum S, Thepbundit V, Ngo-Giang-Huong N, Tangmunkongvorakul A. Prevalence and factors associated with hepatitis B and D virus infections

among migrant sex workers in Chiangmai, Thailand: A cross-sectional study in 2019. *Int J Infect Dis* 2020; **100**: 247-254 [PMID: 32898671 DOI: 10.1016/j.ijid.2020.09.004]

87 **Aghemo A**, Masarone M, Montagnese S, Petta S, Ponziani FR, Russo FP; Associazione Italiana Studio Fegato (AISF). Assessing the impact of COVID-19 on the management of patients with liver diseases: A national survey by the Italian association for the study of the Liver. *Dig Liver Dis* 2020; **52**: 937-941 [PMID: 32703730 DOI: 10.1016/j.dld.2020.07.008]

88 **Lemoine M**, Kim JU, Ndow G, Bah S, Forrest K, Rwegasha J, Bouyou M, Napon D, Somda S, Sawadogo A, Sombie R, Shimakawa Y. Effect of the COVID-19 pandemic on viral hepatitis services in sub-Saharan Africa. *Lancet Gastroenterol Hepatol* 2020; **5**: 966-967 [PMID: 32950107 DOI: 10.1016/S2468-1253(20)30305-8]

89 **Stowe MJ**, Scheibe A, Shelly S, Marks M. COVID-19 restrictions and increased risk of overdose for street-based people with opioid dependence in South Africa. *S Afr Med J* 2020; **110**: 12939 [PMID: 32880539 DOI: 10.7196/SAMJ.2020.v110i6.14832]

90 **Toyoda H**, Huang DQ, Le MH, Nguyen MH. Liver Care and Surveillance: The Global Impact of the COVID-19 Pandemic. *Hepatol Commun* 2020 [PMID: 32838107 DOI: 10.1002/hep4.1579]

91 **Ratna N**, Mitchell H, Vilaplana T, Harb A, Glancy M, Shah A, Kuyumdzhieva G, Talebi A, Duffell S, Prochazka M, Thorn L, Charles H, Kirsebom F, Penman C, Costella A, Balogun K, Wilkinson R, Simmons R, Croxford S, Edmundson C, Brown A, McCall M, Logan L, Winter A, Harris H, Folkard K, Delpech V, Phipps E, Mohammed H, Sinka K, Mandal S, Hughes G. The impact of the COVID-19 pandemic on prevention, testing, diagnosis and care for sexually transmitted infections, HIV and viral hepatitis in England. *Public Health England* 2020 [DOI: 10.1136/sextrans-2021-055262]

92 **Freije-Rodríguez S**, Woolcock M, Castañeda RA, Cojocaru A, Howton E, Lakner C, Nguyen MC, Schoch M, Yang J, Yonzan N. Poverty and Shared Prosperity 2020: Reversals of Fortune. Washington, DC: World Bank, 2020

93 **Pley CM**, McNaughton AL, Matthews PC, Lourenço J. The global impact of the COVID-19 pandemic on the prevention, diagnosis and treatment of hepatitis B virus (HBV) infection. *BMJ Glob Health* 2021; **6** [PMID: 33402334 DOI: 10.1136/bmjgh-2020-004275]

94 **Karimi-Sari H**, Rezaee-Zavareh MS. COVID-19 and viral hepatitis elimination programs: Are we stepping backward? *Liver Int* 2020; **40**: 2042 [PMID: 32319207 DOI: 10.1111/liv.14486]

95 **Fix OK**, Hameed B, Fontana RJ, Kwok RM, McGuire BM, Mulligan DC, Pratt DS, Russo MW, Schilsky ML, Verna EC, Loomba R, Cohen DE, Bezerra JA, Reddy KR, Chung RT. Clinical Best Practice Advice for Hepatology and Liver Transplant Providers During the COVID-19 Pandemic: AASLD Expert Panel Consensus Statement. *Hepatology* 2020; **72**: 287-304 [PMID: 32298473 DOI: 10.1002/hep.31281]

96 **Cooke GS**, Andrieux-Meyer I, Applegate TL, Atun R, Burry JR, Cheinquer H, Dusheiko G, Feld JJ, Gore C, Griswold MG, Hamid S, Hellard ME, Hou J, Howell J, Jia J, Kravchenko N, Lazarus JV, Lemoine M, Lesi OA, Maistat L, McMahon BJ, Razavi H, Roberts T, Simmons B, Sonderup MW, Spearman CW, Taylor BE, Thomas DL, Waked I, Ward JW, Wiktor SZ; Lancet Gastroenterology & Hepatology Commissioners. Accelerating the elimination of viral hepatitis: a Lancet Gastroenterology & Hepatology Commission. *Lancet Gastroenterol Hepatol* 2019; **4**: 135-184 [PMID: 30647010 DOI: 10.1016/S2468-1253(18)30270-X]

97 **Gates B**, Gates M. Covid-19 a global perspective 2020 golakeepers report. 2020. [cited 10 April 2021]. Available from: https://www.gatesfoundation.org/goalkeepers/downloads/2020-report/report_a4_en.pdf

98 **Guan WJ**, Ni ZY, Hu Y, Liang WH, Ou CQ, He JX, Liu L, Shan H, Lei CL, Hui DSC, Du B, Li LJ, Zeng G, Yuen KY, Chen RC, Tang CL, Wang T, Chen PY, Xiang J, Li SY, Wang JL, Liang ZJ, Peng YX, Wei L, Liu Y, Hu YH, Peng P, Wang JM, Liu JY, Chen Z, Li G, Zheng ZJ, Qiu SQ, Luo J, Ye CJ, Zhu SY, Zhong NS; China Medical Treatment Expert Group for Covid-19. Clinical Characteristics of Coronavirus Disease 2019 in China. *N Engl J Med* 2020; **382**: 1708-1720 [PMID: 32109013 DOI: 10.1056/NEJMoa2002032]

99 **Chen T**, Wu D, Chen H, Yan W, Yang D, Chen G, Ma K, Xu D, Yu H, Wang H, Wang T, Guo W, Chen J, Ding C, Zhang X, Huang J, Han M, Li S, Luo X, Zhao J, Ning Q. Clinical characteristics of 113 deceased patients with coronavirus disease 2019: retrospective study. *BMJ* 2020; **368**: m1091 [PMID: 32217556 DOI: 10.1136/bmj.m1091]

100 **Richardson S**, Hirsch JS, Narasimhan M, Crawford JM, McGinn T, Davidson KW; the Northwell COVID-19 Research Consortium, Barnaby DP, Becker LB, Chelico JD, Cohen SL, Cookingham J, Coppa K, Diefenbach MA, Dominello AJ, Duer-Hefele J, Falzon L, Gitlin J, Hajizadeh N, Harvin TG, Hirschwerk DA, Kim EJ, Kozel ZM, Marrast LM, Mogavero JN, Osorio GA, Qiu M, Zanos TP. Presenting Characteristics, Comorbidities, and Outcomes Among 5700 Patients Hospitalized With COVID-19 in the New York City Area. *JAMA* 2020; **323**: 2052-2059 [PMID: 32320003 DOI: 10.1001/jama.2020.6775]

101 **Gupta N**, Desalegn H, Ocama P, Lacombe K, Njouom R, Afihene M, Cunha L, Spearman CW, Sonderup MW, Kateera F. Converging pandemics: implications of COVID-19 for the viral hepatitis response in sub-Saharan Africa. *Lancet Gastroenterol Hepatol* 2020; **5**: 634-636 [PMID: 32553140 DOI: 10.1016/S2468-1253(20)30155-2]

World Journal of Gastroenterology

Submit a Manuscript: https://www.f6publishing.com

*World J Gastroenterol* 2021 November 14; 27(42): 7285-7298

DOI: 10.3748/wjg.v27.i42.7285

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

*MINIREVIEWS*

# Hemostasis testing in patients with liver dysfunction: Advantages and caveats

Guillaume Nguyen, Manon Lejeune, Benjamin Crichi, Corinne Frere

**ORCID number:** Guillaume Nguyen 0000-0002-6836-288X; Manon Lejeune 0000-0001-7960-8622; Benjamin Crichi 0000-0003-4562-766X; Corinne Frere 0000-0001-6303-4732.

**Author contributions:** Nguyen G and Frere C performed the literature search, analyzed the data and wrote the paper; all authors contributed to editing, reviewing and final approval of manuscript.

**Conflict-of-interest statement:** Authors declare that they have no competing interests.



**Specialty type:** Gastroenterology and hepatology

**Country/Territory of origin:** France

**Guillaume Nguyen,** Department of Hematology, Trousseau Hospital, Assistance Publique Hôpitaux de Paris, Paris 75012, France

**Manon Lejeune, Corinne Frere,** Department of Hematology, Pitié-Salpêtrière Hospital, Assistance Publique Hôpitaux de Paris, Paris 75013, France

**Benjamin Crichi,** Department of Internal Medicine, Saint-Louis Hospital, Assistance Publique Hôpitaux de Paris, Paris 75010, France

**Corinne Frere,** Inserm UMRS_1166, Institute of Cardiometabolism and Nutrition, Sorbonne Université, Paris 75013, France

**Corresponding author:** Corinne Frere, MD, PhD, Assistant Professor, Department of Hematology, Pitié-Salpêtrière Hospital, Assistance Publique Hôpitaux de Paris, 47-83 Boulevard de l'Hôpital, Paris 75013, France. corinne.frere@aphp.fr

## Abstract

Due to concomitant changes in pro- and anti-coagulant mechanisms, patients with liver dysfunction have a "rebalanced hemostasis", which can easily be tipped toward either a hypo- or a hypercoagulable phenotype. Clinicians are often faced with the question whether patients with chronic liver disease undergoing invasive procedures or surgery and those having active bleeding require correction of the hemostasis abnormalities. Conventional coagulation screening tests, such as the prothrombin time/international normalized ratio and the activated partial thromboplastin time have been demonstrated to have numerous limitations in these patients and do not predict the risk of bleeding prior to high-risk procedures. The introduction of global coagulation assays, such as viscoelastic testing (VET), has been an important step forward in the assessment of the overall hemostasis profile. A growing body of evidence now suggests that the use of VET might be of significant clinical utility to prevent unnecessary infusion of blood products and to improve outcomes in numerous settings. The present review discusses the advantages and caveats of both conventional and global coagulation assays to assess the risk of bleeding in patients with chronic liver disease as well as the current role of transfusion and hemostatic agents to prevent or manage bleeding.

**Key Words:** Hemostasis; Bleeding risk; Conventional tests; Thrombin generation; Viscoelastic tests; Hemostatic agents

**Peer-review report's scientific quality classification**
Grade A (Excellent): 0
Grade B (Very good): B, B
Grade C (Good): 0
Grade D (Fair): 0
Grade E (Poor): 0

**Received:** March 17, 2021
**Peer-review started:** March 17, 2021
**First decision:** May 1, 2021
**Revised:** May 8, 2021
**Accepted:** October 20, 2021
**Article in press:** October 20, 2021
**Published online:** November 14, 2021

**P-Reviewer:** Chawla S, Skok P
**S-Editor:** Zhang H
**L-Editor:** A
**P-Editor:** Zhang H



**Core Tip:** Patients with liver dysfunction have a "rebalanced hemostasis" which can easily be tipped toward either a hypo- or a hypercoagulable phenotype. Clinicians are often faced with the question whether patients with liver dysfunction undergoing invasive procedures or surgery and those having active bleeding require correction of hemostasis abnormalities. While conventional coagulation screening tests have numerous limitations and do not predict the risk of bleeding prior to high-risk procedures or during surgery, a growing body of evidence suggests that viscoelastic testing might be of significant clinical utility in this setting. The present review discusses the advantages and caveats of both conventional and global coagulation assays in patients with chronic liver disease and the current role of hemostatic agents to prevent and manage bleeding.

**Citation:** Nguyen G, Lejeune M, Crichi B, Frere C. Hemostasis testing in patients with liver dysfunction: Advantages and caveats. *World J Gastroenterol* 2021; 27(42): 7285-7298
**URL:** https://www.wjgnet.com/1007-9327/full/v27/i42/7285.htm
**DOI:** https://dx.doi.org/10.3748/wjg.v27.i42.7285

## INTRODUCTION

The liver plays a crucial role in synthesizing numerous plasma proteins, including most of the clotting factors except factor VIII and von Willebrand factor (VWF)[1-3]. Liver cells also produce thrombopoietin (TPO), which is the primary regulator of megakaryopoiesis, accounting for approximately 90% of the overall platelet production[4]. Therefore, liver dysfunction results in decreased levels of circulating pro- and anticoagulant factors, decreased levels of circulating pro- and antifibrinolytic factors, and thrombocytopenia, which all worsen with disease severity. Alterations in platelet number and function are, however, partially compensated by elevated levels of VWF and decreased levels of ADAMTS13.

For many years, liver dysfunction has been considered as an acquired bleeding disorder, and patients with acute or chronic liver disease thought to be naturally "autoanticoagulated". Nevertheless, recent advances in the understanding of changes occurring in the hemostasis balance during liver dysfunction (summarized in Figure 1) support a paradigm shift from "liver disease-associated coagulopathy" to "rebalanced hemostasis"[1-3].

This overall "rebalanced hemostasis" is nevertheless fragile and can easily be tipped toward either a hypo- or a hypercoagulable phenotype, leading to either bleeding or thrombotic complications[5].

Clinicians are often faced with the question whether patients with liver dysfunction undergoing invasive procedures or surgery and those having active bleeding require correction of hemostasis abnormalities. While conventional coagulation tests do not accurately predict the risk of bleeding, a growing body of evidence now supports a role for viscoelastic testing (VET) to monitor hemostasis in this setting.

The present review will discuss: (1) the advantages and caveats of both conventional and global coagulation assays to assess the risk of bleeding in patients with chronic liver disease; and (2) the current role of transfusion and hemostatic agents to prevent and manage bleeding in patients with liver dysfunction.

## EVALUATION OF THE HEMOSTASIS PROFILE OF PATIENTS WITH LIVER DYSFUNCTION

### *Platelet count*

Thrombocytopenia, as defined by a platelet count < 150 × $10^9$/L, is common in patients with chronic liver disease (CLD), particularly in those with portal hypertension. Its prevalence varies widely according to the underlying disease and its severity[6-8]. However, data regarding the association between thrombocytopenia and the risk of

**Hemostasis changes that may favor thrombosis or bleeding in patients with liver dysfunction**

| | Favors thrombosis | Favors bleeding |
|---|---|---|
| Primary hemostasis | Increased levels of VWF<br>Decreased levels of ADAMTS-13 | Thrombocytopenia<br>Platelet dysfunction<br>Increased levels of NO and prostacyclin |
| Coagulation | Increased levels of factor VIII. Decreased levels of antithrombin, protein C and protein S | Dysfibrinogenemia<br>Decreased levels of fibrinogen. Decreased levels of factor II, V, VII, IX, X, XI XII, XIII |
| Fibrinolysis | Decreased levels of plasminogen<br>Increased levels of PAI-1 | Decreased levels of α2-antiplasmin & TAFI<br>Increased levels of t-PA |

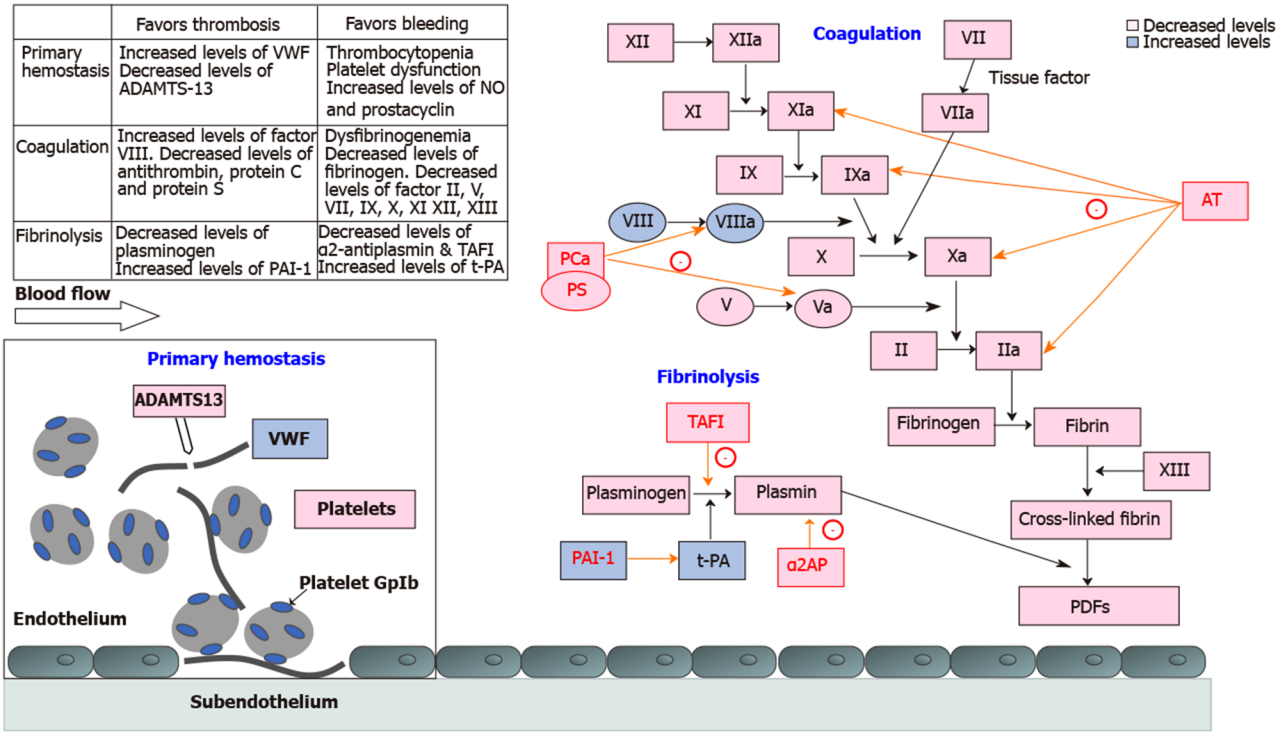


**Figure 1 "Rebalanced" Hemostasis in patients with liver dysfunction.** AT: Antithrombin; ADAMTS13: a disintegrin and metalloprotease with thrombospondin type I repeats-13; α2AP: α2-antiplasmin; PAI-1: Plasminogen activator inhibitor-1; PDFs: Fibrin degradation products; PC: Protein C; PS: Protein S; TAFI: Thrombin-activatable fibrinolysis inhibitor; t-PA: Tissue plasminogen activator; VWF: Von Willebrand factor.

bleeding in patients with liver dysfunction are conflicting.

In the PRO-LIVER study, 280 patients with cirrhosis were followed up for a median duration of 3 years. One hundred eighty-one (66%) patients had thrombocytopenia at inclusion while 23 (8%) of them had severe thrombocytopenia (defined as a platelet count < 50 × $10^9$/L). During the follow-up, bleeding occurred in 52 (18.6%) patients. However, platelet count did not predict the onset of unprovoked bleeding[9]. Similarly, platelet count did not predict the risk of bleeding in a case series of cirrhotic patients undergoing invasive procedure[10]. Conversely, severe thrombocytopenia was associated with a high risk of periprocedural bleeding in patients with advanced liver disease undergoing invasive procedure[11]. Severe thrombocytopenia also independently predicted the onset of major bleeding [odds ratio (OR) 6.476, 95%CI: 1.386-30.255, $P$ < 0.05] in a prospective study of 1493 critically ill patients with cirrhosis admitted to intensive care unit[12].

According to the 7th International Coagulation in Liver Disease Conference[13], platelet count alone is not sufficient to assess the risk of bleeding in patients with CLD. However, a platelet count < 50 × $10^9$/L may be associated with a high risk of bleeding.

### *Global platelet functional tests*

Global platelet functional tests are of low clinical utility in patients with liver dysfunction. Their use is limited by several factors including pre-analytical and analytical constraints, lack of standardization and their time-consuming nature. Moreover, none of them has been demonstrated to be clinically useful for stratifying the risk of bleeding in patients with liver dysfunction. The platelet function analyzer (PFA)-100® or PFA-200® closure time (CT) is the most widely used test to assess primary hemostasis. It measures the CT of an aperture within a membrane coated with either collagen/adenosine diphosphate or collagen/epinephrine under flow conditions. Importantly, this test is highly influenced by platelet count, VWF level and hematocrit, making the results challenging to interpret in many cases. Patients with stable cirrhosis or those with end-stage liver disease have been reported to have prolonged CT[14-16]. However, the prognostic value of CT in predicting bleeding complications in patients with liver dysfunction has yet never been investigated. Other functional platelet tests, such as light transmission aggregometry or whole blood aggregometry, have been extensively reviewed elsewhere[17-19].

The use of global platelet functional tests to assess the risk of bleeding in patients with CLD is not recommended by current guidelines.

### Conventional coagulation tests

Conventional coagulation tests, such as the prothrombin time (PT)/international normalized ratio (INR) and the activated partial thromboplastin time (aPTT), are commonly used for screening of inherited bleeding disorders and for monitoring of anticoagulant therapy. However, they are of limited value in patients with liver dysfunction since they predict neither the risk of bleeding complications nor the efficacy of blood cell product transfusion or hemostatic agent infusion[1-3].

PT and aPTT are both performed on platelet-poor plasma by measuring the time for fibrin clot formation after addition of phospholipids, calcium, and a trigger agent (tissue factor for the extrinsic pathway and kaolin, celite or ellagic acid for the intrinsic pathway). These tests only evaluate the time to the start of fibrin polymerization, knowing that 90% to 95% of thrombin generation occurs after this step. Importantly, they are insensitive to coagulation inhibitors (*e.g.*, antithrombin, protein C and protein S), which are also decreased in patients with liver dysfunction who have a "rebalanced" hemostasis. Furthermore, they contain low amount of thrombomodulin which is required to activate protein C. Finally, they do not provide any insight on clot lysis.

PT and aPTT are influenced by the level of procoagulant factors [*e.g.* factors I (fibrinogen), II, V, VII and X for the PT and factors I (fibrinogen), II, V, VIII, IX, X, XI and XII for the aPTT]. With the exception of factor VIII, procoagulant factors are heterogeneously decreased in patients with liver dysfunction. Therefore, PT and aPTT are frequently prolonged in patients with acute or CLD. Nevertheless, thrombin generation has been demonstrated to be preserved in patients with liver dysfunction and prolonged PT/aPTT[20], due to a concomitant decrease in anticoagulant levels.

Although the INR is currently used as a prognostic factor when calculating the model for end stage liver disease (MELD) score (which includes serum bilirubin, INR, serum creatinine, dialysis, and serum sodium[21]), it is important to emphasize that it was exclusively designed to monitor vitamin K antagonist therapy. Several studies have shown that its use is not appropriate for patients with liver dysfunction since it may vary for a single sample according to the reagent used[22,23]. A prospective study of 29 consecutive patients listed for liver transplantation (LT) reported that the between-laboratory variability in INR determination had a significant impact on the calculated MELD score[24], further suggesting the need to establish a "modified INR" specific for liver diseases[25].

According to the 7$^{th}$ International Coagulation in Liver Disease Conference[13], the American Association for the study of liver diseases (AASLD)[26], and the American Gastroenterology Association (AGA)[27], the PT/INR and the aPTT should not be used for assessing the risk of bleeding or for guiding blood products transfusion in patients with CLD.

Fibrinogen levels (Clauss assay) are determined by measuring the time for fibrin clot formation in the presence of excess thrombin. The clotting time expressed in seconds is converted to mg/dL by using a calibration curve prepared by serial dilution of a reference plasma of known fibrinogen concentration. Fibrinogen levels may either be normal, increased or decreased in patients with liver dysfunction[3]. Dysfibrinogenemia has been observed in up to 76% of patients with cirrhosis, 78% of those with chronic active liver disease and 86% of those with acute liver failure[28]. In a prospective cohort study of 165 patients with cirrhosis, low fibrinogen levels were associated with decreased survival in univariate analysis[29]. Fibrinogen levels < 60 mg/dL were a strong independent predictor for major bleeding (OR 11.129, 95%CI: 1.189-104.173, $P < 0.05$) in critically ill patients admitted to intensive care unit[12]. In a series of 109 cirrhotic patients undergoing endoscopic variceal band ligation, patients who bled had significant lower median fibrinogen levels compared to those who did not bleed (146 mg *vs* 230 mg/dL, $P = 0.009$)[30]. A fibrinogen level cut-off of 179 mg/dL predicted bleeding with a sensitivity of 83.3% and a specificity of 73.0%[30]. Finally, in a retrospective study of 322 patients undergoing orthotopic LT, baseline fibrinogen levels were found to predict excessive transfusion[31]. Additional studies are warranted to determine the best fibrinogen level cut-off for predicting the risk of bleeding.

### Global coagulation assay

Compared to conventional coagulation tests, global coagulation assays such as thrombin generation assays (TGA) or VET, which take into account the interactions between procoagulants, anticoagulants, platelet function and the fibrinolytic system,

are expected to better reflect the overall hemostasis profile, especially in patients with liver dysfunction who have a "rebalanced" hemostasis.

### Thrombin generation assays

TGAs have been developed in the 1950s. The most widely used method is the calibrated automated thrombogram[32], which dynamically quantifies the total amount of thrombin generated in platelet-poor plasma or in platelet-rich plasma after initiating the coagulation by the addition of tissue factor, phospholipids and calcium. Tripodi *et al*[20] reported that the profile of thrombin generation measured without thrombomodulin was decreased in cirrhosis patients compared to healthy controls, while it was normal in the presence of thrombomodulin (which is required for protein C activation), demonstrating for the first time that patients with liver dysfunction have a "rebalanced hemostasis". Recent TGA studies (reviewed elsewhere by Lebreton *et al* [33]) further reported that patients with liver dysfunction may have an hypercoagulable phenotype which seems to correlate with the disease severity[33]. Studies assessing the ability of TGA in predicting bleeding or thrombotic complications in patients with liver dysfunction are yet lacking. For now, there is still a need for standardization and automation of TGA methods.

According to the 7th International Coagulation in Liver Disease Conference[13], the use of TGA in patients with CLD should be restricted to clinical research studies.

### Viscoelastic tests

VETs, which are performed in whole blood, evaluate the *in vivo* dynamics of clot formation, clot stabilization and fibrinolysis. They are expected to provide a more accurate assessment of the overall hemostasis profile, and therefore to better predict the risk of bleeding and thrombosis. Nowadays, VETs are used as point-of-care testing and provide faster results than standard coagulation tests, which constitute an additional advantage.

Three methods are currently available, *i.e.*, thromboelastography (TEG, Hemonetics Corporation, Braintree, MA), thromboelastometry (ROTEM, TEM International GmbH, Munich, Germany) and sonorheometry (Quantra QPlus System, HemoSonics, LLC, Charlottesville, VA).

In patients with CLD, VET parameters correlate well with platelet count and fibrinogen levels (except in patients with fibrinogen levels < 100 mg/dL) but not with PT/INR and aPTT [34-42].

VETs have been mainly studied in patients with end-stage liver disease undergoing LT, but numerous studies have also been conducted in patients with less severe liver disease, including those well compensated.

### Viscoelastic tests in liver transplantation

Historically, the first orthotopic LT was performed by Starzl *et al*[43] in 1963. The recipient was a 3-year-old boy with congenital biliary atresia. Interestingly, coagulation was monitored by performing serial thromboelastograms[43]. Twenty years later, Kang *et al*[44] first suggested that VET-guided transfusion could substantially reduce blood products administration during LT. In their series of 66 patients undergoing LT, VET-monitoring was associated with a significant reduction in the administration of red blood cell units (17 ± 12.9 units *vs* 26.7 ± 23.8 units in the historical cohort, $P < 0.05$), fresh frozen plasma (FFP) units (18.3 ± 12.5 units *vs* 26.7 ± 24.1 units, $P < 0.05$) and total volume infused (20.2 ± 11.2 L *vs* 31.4 ± 19.2 L, $P < 0.05$). However, VET-guided transfusion led to a significant increase in the administration of platelets (20.8 ± 12.8 units *vs* 14.1 ± 13.7 units, $P < 0.05$) and cryoprecipitate units (17.2 ± 8.5 units *vs* 10.2 ± 4.5 units; $P < 0.05$). Numerous non-randomized studies further confirmed these findings[45-47].

Only two small randomized controlled trials (RCTs) compared VET-guided transfusion to the standard of care (SOC) during LT (Table 1)[48,49]. Importantly, they used different VET methodologies and various algorithms for VET-guided transfusion. Wang *et al*[48] randomized 28 adult patients with cirrhosis undergoing LT in either a TEG-guided transfusion arm or a SOC arm. Intraoperative administration of FFP was significantly reduced in the TEG-guided transfusion arm (12.8 ± 7.0 units *vs* 21.5 ± 12.7 units in the SOC arm, $P < 0.05$), without significant difference in the administration of other blood products between the two arms. In the TEG-guided transfusion arm, there was a trend towards reduction in blood loss (4775 ± 4264 mL *vs* 6348 ± 3704 mL in the SOC arm, $P$ = ns). More recently, Bonnet *et al*[49] randomized 82 adult patients with cirrhosis undergoing orthotopic LT in either a ROTEM-guided transfusion arm or a SOC arm. Median [interquartile range] intraoperative administration of blood

**Table 1 Randomized controlled trials assessing viscoelastic (tests) in patients with cirrhosis undergoing liver transplantation or invasive procedures and for the management of active bleeding**

| Ref. | VET | Population | *n* | Exclusion criteria | Intervention in the VET arm | Intervention in the SOC arm | Blood product | Bleeding |
|---|---|---|---|---|---|---|---|---|
| Liver transplantation | | | | | | | | |
| Wang *et al*[48], 2010 | TEG | Adult patients with cirrhosis undergoing liver transplantation | 28 patients (14 in each arm) | Unspecified | FFP titrated to maintain R time < 10 min; 6-8 pooled platelet units if MA < 55 mm; 5 pooled units of cryoprecipitate if alpha angle < 45 degrees | FFP titrated to maintain PT and APTT at less than one and a half times control; Platelets to maintain a platelet count ≥ 50 × $10^9$ /L; Cryoprecipitate to maintain fibrinogen > 1 g/L | FFP use: 12.8 units in the TEG arm *vs* 21.5 units in the SOC arm ( $P < 0.05$); RBC: no difference; Platelets use: no difference; Cryoprecipitate use: no difference | Trend towards reduction in blood loss in the TEG arm (not statistically significant) |
| Bonnet *et al*[49], 2019 | ROTEM | Adult patients with cirrhosis undergoing orthotopic liver transplantation | 82 patients (41 in each arm) | Pregnancy; congenital coagulopathy; patients participating in another study | 2 FFP units if EXTEM CT < 110 s; 1 platelet unit if EXTEM MCF < 40 mm or A10 < 35 mm and FIBTEM A10 or MCF > 8 mm; Fibrinogen 3 g if FIBTEM A10 < 8 mm | 2 FFP units if PT < 40% at baseline or an hepatic phase or hemorrhage; PT < 30% at declamping or end of surgery and no hemorrhage; 1 platelet unit if platelet count < 50 × $10^9$/L at baseline or an hepatic phase or hemorrhageor if platelet count < 30 × $10^9$/L at declamping or end of surgery and no hemorrhage; Fibrinogen 3 g if fibrinogen ≤ 1 g/L | FFP use: 6 patients in the TEG arm *vs* 19 patients in the SOC arm ($P = 0.002$); RBC use: no difference; Platelets use: no difference; Cryoprecipitate use: 29 patients in the TEG arm *vs* 12 patients in the SOC arm ($P < 0.001$) | No difference in revision surgery or postoperative hemorrhage at 24 and 48 h |
| Invasive procedure | | | | | | | | |
| De Pietri *et al*[51], 2016 | TEG | Adult patients with cirrhosis undergoing invasive procedures with an INR > 1.8 and/or platelet count < 50 × $10^9$ /L | 60 patients (30 in each arm) | Ongoing bleeding; current thrombotic events; antiplatelets or anticoagulants use; infection or sepsis; hemodialysis | FFP 10 mL/kg if R > 40 min; Platelets if MA < 30 mm | FFP10 mL/kg if INR > 1.8; Platelets if platelet count < 50 × $10^9$/L | 16.7% in the TEG arm *vs* 100% in the SOC arm ($P < 0.0001$) | 1 post procedure bleeding after large volume paracentesis in the SOC arm |
| Vuyyuru *et al*[52], 2019 | TEG | Adult patients with cirrhosis undergoing invasive liver-related procedures with INR > 1.8 and/or < 50 × $10^9$/L | 58 patients(29 in each arm) | Cancer; hemophilia; DIC; antiplatelets use; pregnancy; renal failure; blood products in the previous 7 d | FFP if R > 14 min; 6-8 pooled platelet units; if MA < 30 mm | FFP if INR > 1.8; 6-8 pooled platelet units; if platelet count < 50 × $10^9$/L | 31% in the TEG arm *vs* 100% in the SOC arm ($P < 0.001$) | No bleeding in any group |
| Rocha *et al*[53], 2020 | ROTEM | Adult critically ill patients with cirrhosis undergoing CVC insertion | 57 patients (19 per arm) | Acute liver failure; vonWillebrand's disease; anticoagulants use; patients participating in another study | FFP10 mL/kg if CT EXTEM > 80 s; 1 apheresis platelets unit if A10 EXTEM < 40 mm and A10 FIBTEM ≥ 10 mm; 1 unit/kg of cryoprecipitate if A10 ESTEM < 40 mm and A10 EXTEM < 10 mm | SOC arm: FFP 10 mL/kg if INR > 1.5 or aPTT > 50 s 1 unit/kg of platelets if platelet count < 50 × $10^9$ /L; 1 unit/kg of cryoprecipitate if fibrinogen < 150 mg/dL; Restrictive arm: FFP 10 mL/kg if INR > 5; 1 unit/kg of platelets if | Significantly lower in the restrictive arm (15.8% *vs* 68.4% in the ROTEM arm; $P < 0.006$ and *vs* 73.7%; $P < 0.002$) in the SOC arm. No difference between ROTEM and SOC arms | No major bleeding in any group |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | | | | | platelet count < 25 × $10^9$/L | | |
| Active bleeding | | | | | | | |
| Kumar *et al*[55], 2020 | TEG | Adult patients with advanced liver cirrhosis presenting with nonvariceal upper gastrointestinal bleeding with INR > 1.8 and/or platelet count < 50 × $10^9$/L | 96 patients (49 in the TEG arm, 47 in the SOC arm) | Variceal bleed; postvariceal ligation; ulcer bleed, previous or current thrombotic events; anticoagulant therapy at the time of enrollment or that had been discontinued less than 7 d before evaluation for the study; hemodialysis in the previous 7 d; pregnancy; significant cardiopulmonary disease | FFP 10 mL/kg if R > 10 min; 6-8 pooled platelet units if MA < 55 mm; 5 pooled units of cryoprecipitate if α-angle < 45 degrees | FFP 10 mL/kg if INR > 1.8; 6-8 pooled platelet units if plateletcount < 50 × $10^9$/L 5 pooled units of cryoprecipitate if fibrinogen < 80 mg/dL | Patients transfused with all three blood components: 26.5% in TEG *vs* 87.2% SOC ($P$ < 0.001) | No difference in failure to control bleeding or rebleeding on day 5. No difference in mortality on day 5 and on day 42 |
| Rout *et al* [56],2020 | TEG | Adult patients with cirrhosis presenting with acute variceal bleeding with INR > 1.8 and/or plateletcount < 50 × $10^9$/L | 60 patients (30 in each arm) | Malignancy; hemophilia; DIC; antiplatelets use; pregnancy; blood products in the previous 7 d; shock; sepsis; acute-on-chronic liver failure, renal failure, encephalopathy | FFP 5mL/kg if R > 15 min; 3 pooled units of platelets if MA < 30 mm | FFP if INR > 1.8; Platelets if platelet count < 50 × $10^9$/L | 13.3% TEG *vs* 100% SOC ($P$ < 0.001) | No difference in control of bleeding or rebleeding on day 5 between the two groups.Rebleeding on day 42 less in TEG (10%) than SOC (36.7% ; $P$ = 0.012) |

A10: Amplitude at 10 min; ACLF: Acute-on-chronic liver failure; aPTT: activated partial thromboplastin time; CCT: Conventional coagulation test; CT: Clotting time; CVC: Central venous catheter; DIC: Disseminated intravascular coagulation; INR: International normalized ratio; FFP: Fresh frozen plasma; MA: Maximum amplitude; R: Reaction time; ROTEM: Rotational thromboelastometry; SOC: Standard of care; TEG: Thromboelastography; VET: Viscoelastic test.

products was significantly decreased in the ROTEM-guided transfusion arm [3 (2–4) *vs* 7 (4–10) units in the SOC arm, $P$ = 0.005]. FFP was administered less frequently in the ROTEM-guided transfusion arm (15% *vs* 46.3% in the SOC arm, $P$ = 0.002). However, fibrinogen concentrates were administered more frequently (72.5% *vs* 29.3% in the SOC arm, $P$ < 0.001). There was no difference in revision surgery or postoperative bleeding at 24 and 48 h.

Overall, the body of evidence supporting the use of VET to guide transfusion during LT remains poor. Nevertheless, VETs have been widely adopted in most large-volume academic transplant centers, as reported in a recent survey[50].

### *Viscoelastic tests during invasive procedures*

Three small RCTs assessed the benefit of VET-guided prophylactic transfusion during invasive procedures (Table 1)[51-53]. De Pietri *et al*[51] randomized 60 adult cirrhosis patients with an INR > 1.8 and/or a platelet count < 50 × $10^9$/L undergoing invasive procedures in either a TEG-guided prophylaxis transfusion arm or a SOC arm. Nearly half of patients (47%) underwent low-risk procedures such as paracentesis or thoracentesis. FFP alone (0% *vs* 53.3%, $P$ < 0.001), platelets alone (6.7% *vs* 33.3%, $P$ = 0.021) and overall blood products (16.7% *vs* 100%, $P$ < 0.0001) were administered less frequently in the TEG-guided arm compared to the SOC arm. Post-procedure bleeding occurred in only 1 patient in the SOC arm after large-volume paracentesis.

A second RCT[52] randomized 58 adult cirrhosis patients with an INR > 1.8 and/or a platelet count < 50 × $10^9$/L undergoing high-risk invasive procedures (83% of liver biopsy) in either a TEG-guided prophylaxis transfusion arm or a SOC arm. Transfusions of any blood products (31% *vs* 100%, $P$ < 0.0001), and platelet transfusions (6.9% *vs* 42.4%, $P$ < 0.001) were administered less frequently in the TEG-guided arm. There was no difference in the rate of patients who received FFP. No procedure-related bleeding was observed.

Finally, Rocha *et al*[53] randomized 57 adult critically ill patients with cirrhosis undergoing central venous catheterization in three arms, *i.e.*, a restrictive strategy arm,

a SOC arm, or a ROTEM-guided arm. The restrictive strategy decreased the rates of any blood component administration compared to the SOC (OR 0.07, 95%CI: 0.01-0.45, $P$ = 0.002) and to the ROTEM-guided strategy (OR 0.09, 95%CI: 0.01-0.56, $P$ = 0.006). There was no difference in bleeding, length of stay, mortality, and transfusion□related adverse events between the three arms.

Overall, the current evidence suggests that the use of VET-guided transfusion during invasive procedures might significantly decrease the prophylactic infusion of blood products. However, the benefit of VTE monitoring during low bleeding risk procedures remains debated.

### *Viscoelastic tests in patients with liver dysfunction and active bleeding*

In a prospective study of 20 cirrhotic patients with active variceal bleeding, serial TEGs were performed daily for 7 d to assess the association between changes occurring in TEG profile and early rebleeding[54]. Patients who presented early rebleeding had longer median R (42 mm *vs* 24 mm, $P$ < 0.001) and median K (48 mm *vs* 13 mm, $P$ < 0.001) and smaller median alpha (12 *vs* 38, $P$ < 0.001) on the day of rebleeding compared with the mean of all daily results in patients who did not rebleed[54].

Two small RCTs evaluated the benefit of using VETs to monitor transfusion in cirrhotic patients with active upper gastrointestinal bleeding (Table 1)[55,56]. The first one randomized 96 adult patients with advanced liver cirrhosis, coagulopathy (as defined by an INR > 1.8 and/or platelet count < 50 × $10^9$/L) and active non-variceal upper gastrointestinal bleeding in either a TEG-guided transfusion arm or a SOC arm. The volume of FFP infused per patient was significantly lower in the TEG-guided arm (440 mL *vs* 800 mL in the SOC arm, $P$ < 0.001). Moreover, the rate of patients who received blood products was significantly lower in the TEG-guided arm (26.5% *vs* 87.2% in the SOC arm, $P$ < 0.001). The rates of control of bleeding, rebleeding, and mortality did not differ between the two arms[55]. The second RCT[56] randomized 60 adult patients with cirrhosis, coagulopathy (as defined by an INR > 1.8 and/or platelet count < 50 × $10^9$/L) and active variceal upper gastrointestinal bleeding in either a TEG-guided transfusion arm or a SOC arm. A significant reduction in the rate of blood product infusion was observed in the TEG-guided arm (13.3% *vs* 100% in the SOC arm, $P$ < 0.001). The rate of initial control of bleeding at the primary endoscopy and the rate of rebleeding at 5 d did not differ between the two arms.

Overall, a growing body of evidence indicates that VETs may be useful to guide transfusion, particularly in patients with active bleeding. Further studies to establish standardized algorithms and cut-off values to guide transfusion are warranted.

Nevertheless, some limitations of VTE leading to underestimate the true hemostatic potential should be acknowledged[57]. First, some VET parameters such as the maximum amplitude are frequently hypocoagulable in patients with liver dysfunction. This is mainly due to thrombocytopenia. However, it is important to notice that VETs are insensitive to VWF levels which are increased in patients with liver dysfunction, promoting platelet adhesion and partly compensating thrombocytopenia. Second, VETs are insensitive to the protein C system which requires the transmembrane protein thrombomodulin expressed on the luminal surface of endothelial cells to be activated. Third, VETs do not capture clot quality. Finally, most VETs parameters are obtained after the addition of an activator of the intrinsic pathway, which might not be relevant to study the physiological initiation of coagulation.

According to the 7$^{th}$ International Coagulation in Liver Disease Conference[13], the use of VETs in patients with CLD should be restricted to clinical research studies.

## CURRENT ROLE OF HEMOSTATIC AGENTS IN PATIENTS WITH LIVER DYSFUNCTION

### *Platelet transfusion and thrombopoietin agonists*

Platelet transfusion is the standard of care to transiently increase platelet count in patients undergoing invasive procedures or in those with active bleeding. A conventional threshold of 50 × $10^9$/L, which is exclusively based on expert opinion, is recommended by the AASLD[26]and the AGA[27]. However, *in vitro* studies have suggested that platelet-dependent thrombin generation should be preserved in patients with cirrhosis having a platelet count above 56 × $10^9$/L[58].

Due to platelet transfusion detrimental side effects, treatments stimulating endogenous production of functional platelets are increasingly used in CLD patients undergoing invasive procedures. Two second generation TPO receptor agonists

(avatrombopag and lusutrombopag) were recently approved by the Food and Drug Administration for the treatment of severe thrombocytopenia in this setting.

The ADAPT-1 and ADAPT-2 trials randomized a total of 435 CLD patients with severe thrombocytopenia (platelet count < 50 × $10^9$/L) undergoing a diagnostic or therapeutic invasive procedure to receive either avatrombopag ($n$ = 277) or placebo ($n$ = 158) daily for 5 d[59]. The primary endpoint was the rate of patients not requiring platelet transfusions or rescue therapy for bleeding within 7 d following procedure. In ADAPT-1, the rate of patients who met the primary endpoint was significantly higher in patients receiving avatrombopag (66% in patients receiving 60 mg avatrombopag *vs* 23% in the placebo arm, and 88% in patients receiving 40 mg avatrombopag *vs* 38% in the placebo arm). Similarly, in ADAPT-2, the rate of patients who met the primary endpoint was significantly higher in patients receiving avatrombopag (69% in patients receiving 60 mg avatrombopag *vs* 35% in the placebo arm, and 88% in patients receiving 40 mg avatrombopag *vs* 33% in the placebo arm). There was no difference in serious adverse events between the two arms.

The L-PLUS 1 trial randomized 96 CLD patients with severe thrombocytopenia (platelet count < 50 × $10^9$/L) undergoing an invasive procedure to receive either lusutrombopag ($n$ = 48) or placebo ($n$ = 48) daily for up to 7 d[60]. The primary efficacy endpoint was the rate of patients not requiring platelet transfusion before the invasive procedure. Seventy-nine percent of patients met the primary endpoint in the lusutrombopag arm *vs* 12% in the placebo arm ($P$ < 0.001). Lusutrombopag was well tolerated. The L-PLUS 2 trial randomized 215 CLD patients with severe thrombocytopenia (platelet count < 50 × $10^9$/L) undergoing an invasive procedure to receive either lusutrombopag ($n$ = 108) or placebo ($n$ = 107) daily for up to 7 d[61]. The procedure was scheduled 2 to 7 d after the last dose of lusutrombopag or placebo. The primary efficacy endpoint was the rate of patients not requiring platelet transfusion before the invasive procedure or rescue therapy for bleeding. Sixty-five percent of patients met the primary endpoint in the lusutrombopag arm *vs* 29 % in the placebo arm ($P$ < 0.001). There was no difference in serious adverse events between the two arms.

Importantly, TPO receptor agonists have a slow onset of action (5 d or more) and are indicated only in stable CLD patients having severe thrombocytopenia t without active bleeding who undergo planned elective procedures. Performing platelet count on the day of the procedure is warranted.

### FFP

FFP is commonly used to correct coagulation factor deficiencies in patients with active bleeding when coagulation tests are abnormal. However, there is currently insufficient evidence to support the prophylactic use of FFP in patients with CLD.

In an *in vitro* study of 58 patients with advanced cirrhosis (Child–Pugh B and C), addition of normal pooled plasma to plasmas from cirrhotic patients shortened both PT and aPTT ($P$ < 0.0001), without significant change in endogenous thrombin potential (ETP) in presence of thrombomodulin ($P$ = ns)[62]. In line with these results, a small sample study ($n$ = 53) found that infusion of FFP increased the ETP by only 5.7%[63]. Two out of 53 (3.8%) patients had baseline ETP below normal values and infusion of FFP corrected thrombin generation in only one out of two patients.

The only large multicenter, RCT aiming to assess the efficacy and safety of prophylactic FFP infusion in CLD patients undergoing invasive procedures[64] was unfortunately interrupted due to inadequate enrollment.

Potential detrimental side effects of FFP, such as transfusion-related acute lung injury or volume expansion leading to portal hypertension exacerbation, should be considered.

Based on available evidence, the AASLD[26] and the AGA[27] do not recommend using prophylactic infusion of FFP in CLD patients undergoing invasive procedures or surgery.

### Fibrinogen

Cryoprecipitates or fibrinogen concentrates are generally preferred over FFP to correct hypofibrinogenemia due to lower volume to infuse and best standardized fibrinogen content. Cryoprecipitates contain fibrinogen, von Willebrand factor and factor VIII. One unit of cryoprecipitate per 10 kg of body weight increases fibrinogen levels by approximately 50 mg/dL. In a randomized, multicenter, double-blind, controlled trial comparing preemptive fibrinogen administration to placebo during LT, preemptive fibrinogen infusion did not reduce blood products transfusion (RR 0.80, 95%CI: 0.57-1.13)[65]. In cirrhosis patients with active bleeding, maintaining fibrinogen levels above 120 mg/dL is generally required.

### Recombinant activated factor VII

The use of activated recombinant factor VII (rFVIIa) is restricted to patients presenting massive bleeding.

Two RCTs aiming to assess the efficacy and safety of rFVIIa on upper gastrointestinal or variceal bleeding in cirrhosis patients reported no difference between the rFVIIa and the placebo arms in the composite primary endpoint of failure to control acute bleeding, rebleeding within the first 5 d, and death within the first 5 d [66,67]. However, patients with variceal bleeding receiving rFVIIa had a significantly lower rate of 42-d mortality compared to those receiving placebo (15% *vs* 29%, OR 0.31, 95%CI: 0.13-0.74)[67].

Consensus guidelines on the use of rFVIIa as an adjuvant treatment for massive bleeding do not recommend using rFVIIa in patients with Child-Pugh A cirrhosis (grade B evidence)[68]. Furthermore, they consider its benefit as uncertain in patients with more advanced liver disease (grade C evidence)[68].

### Prothrombin complex concentrates

Data on the benefit of prothrombin complex concentrates (PCC) in patients with cirrhosis are limited to retrospective studies and case reports[69,70]. PCC infusion was reported to significantly reduce INR in critically ill cirrhosis patients, with lower amount of blood transfusion requirements compared to FFP. The ongoing double-blind, multicenter, placebo-controlled randomized PROTON trial comparing infusion of PCC *vs* placebo prior to surgery to reduce transfusion requirements in cirrhotic patients undergoing LT is expected to provide more data on the benefit of PCC in the near future[71].

### Desmopressin

The 1-deamino-8-D-arginine vasopressin (DDAVP) is commonly used to prevent blood loss in a variety of bleeding disorders. It has also been demonstrated to improve platelet function in patients with severe renal failure. An early study reported that intranasal DDAVP was effective and safe in cirrhotic patients undergoing dental extraction and having thrombocytopenia < 50 × $10^9$/L. However, later RCTs found no benefit of DDAVP administration in controlling acute variceal bleeding[72] or in preventing blood loss in CLD patients undergoing LT[73]. In line with these results, DDAVP administration did not improve VWF-dependent platelet adhesion in patients with cirrhosis in a flow-based model[74]. According to the AGA clinical practice guidelines, the use of DDVP in CLD patients should be restricted to those with concomitant end-stage renal disease[27].

### Antifibrinolytic agents

Data regarding the benefits and risks of antifibrinolytic agents to prevent or manage bleeding in patients with CLD are conflicting. A metanalysis pooled the results of several RCTs assessing their efficacy and safety to prevent blood loss in patients undergoing LT[75]. In the pooled analysis, aprotinin or tranexamic acid (TXA) reduced red blood cell and FFP transfusion requirements compared with placebo, without increasing the rates of hepatic artery and venous thromboembolism[75]. The recent international, randomized, double-blind, placebo-controlled hemorrhage alleviation with tranexamic acid-intestinal system (HALT-IT) trial randomized 12009 patients with gastrointestinal bleeding to receive either TXA or matching placebo[76]. TXA did not decrease death from gastrointestinal bleeding [risk ratio (RR) 0.99, 95%CI: 0.82–1.18] but it increased the risk of venous thromboembolism (RR 1.85; 95%CI: 1.15-2.98)[76]. Conversely a recent pooled analysis of 11 RCT including the HALT-IT trial found that TXA significantly decreased the risk of mortality compared to control (RR 0.75, 95%CI: 0.57-0.96)[77].

Based on available evidence, the AGA suggests using antifibrinolytic agents for a short duration to control periprocedural bleeding in case of hyperfibrinolysis[27].

## CONCLUSION

In patients with liver dysfunction, hemostasis is rebalanced due to concomitant changes in pro- and anti-coagulant mechanisms. In these patients, current conventional coagulation screening tests, such as the PT/INR and the aPTT, have numerous limitations and should not be used to predict the risk of bleeding prior to high-risk procedures. The introduction of global coagulation tests has been an important step

forward in the assessment of the overall hemostasis profile. There is nowadays a growing body of evidence suggesting that they might be of significant clinical utility to prevent unnecessary infusion of blood products and to improve outcomes in numerous settings. Further studies are, however, required to develop standardized algorithms and establish clinical practice guidelines for VET-guided transfusion in patients with liver dysfunction.

## REFERENCES


1 **Tripodi A**, Mannucci PM. The coagulopathy of chronic liver disease. *N Engl J Med* 2011; **365**: 147-156 [PMID: 21751907 DOI: 10.1056/NEJMra1011170]
2 **Lisman T**, Porte RJ. Rebalanced hemostasis in patients with liver disease: evidence and clinical consequences. *Blood* 2010; **116**: 878-885 [PMID: 20400681 DOI: 10.1182/blood-2010-02-261891]
3 **Kujovich JL**. Coagulopathy in liver disease: a balancing act. *Hematology Am Soc Hematol Educ Program* 2015; **2015**: 243-249 [PMID: 26637729 DOI: 10.1182/asheducation-2015.1.243]
4 **Wolber EM**, Jelkmann W. Thrombopoietin: the novel hepatic hormone. *News Physiol Sci* 2002; **17**: 6-10 [PMID: 11821529 DOI: 10.1152/physiologyonline.2002.17.1.6]
5 **Roberts LN**, Bernal W. Incidence of Bleeding and Thrombosis in Patients with Liver Disease. *Semin Thromb Hemost* 2020; **46**: 656-664 [PMID: 32757184 DOI: 10.1055/s-0040-1714205]
6 **Giannini EG**. Review article: thrombocytopenia in chronic liver disease and pharmacologic treatment options. *Aliment Pharmacol Ther* 2006; **23**: 1055-1065 [PMID: 16611265 DOI: 10.1111/j.1365-2036.2006.02889.x]
7 **Peck-Radosavljevic M**. Thrombocytopenia in chronic liver disease. *Liver Int* 2017; **37**: 778-793 [PMID: 27860293 DOI: 10.1111/liv.13317]
8 **Nilles KM**, Caldwell SH, Flamm SL. Thrombocytopenia and Procedural Prophylaxis in the Era of Thrombopoietin Receptor Agonists. *Hepatol Commun* 2019; **3**: 1423-1434 [PMID: 31701067 DOI: 10.1002/hep4.1423]
9 **Basili S**, Raparelli V, Napoleone L, Talerico G, Corazza GR, Perticone F, Sacerdoti D, Andriulli A, Licata A, Pietrangelo A, Picardi A, Raimondo G, Violi F; PRO-LIVER Collaborators. Platelet Count Does Not Predict Bleeding in Cirrhotic Patients: Results from the PRO-LIVER Study. *Am J Gastroenterol* 2018; **113**: 368-375 [PMID: 29257146 DOI: 10.1038/ajg.2017.457]
10 **Napolitano G**, Iacobellis A, Merla A, Niro G, Valvano MR, Terracciano F, Siena D, Caruso M, Ippolito A, Mannuccio PM, Andriulli A. Bleeding after invasive procedures is rare and unpredicted by platelet counts in cirrhotic patients with thrombocytopenia. *Eur J Intern Med* 2017; **38**: 79-82 [PMID: 27989373 DOI: 10.1016/j.ejim.2016.11.007]
11 **Giannini EG**, Greco A, Marenco S, Andorno E, Valente U, Savarino V. Incidence of bleeding following invasive procedures in patients with thrombocytopenia and advanced liver disease. *Clin Gastroenterol Hepatol* 2010; **8**: 899-902; quiz e109 [PMID: 20601131 DOI: 10.1016/j.cgh.2010.06.018]
12 **Drolz A**, Horvatits T, Roedl K, Rutter K, Staufer K, Kneidinger N, Holzinger U, Zauner C, Schellongowski P, Heinz G, Perkmann T, Kluge S, Trauner M, Fuhrmann V. Coagulation parameters and major bleeding in critically ill patients with cirrhosis. *Hepatology* 2016; **64**: 556-568 [PMID: 27124745 DOI: 10.1002/hep.28628]
13 **Intagliata NM**, Argo CK, Stine JG, Lisman T, Caldwell SH, Violi F; faculty of the 7th International Coagulation in Liver Disease. Concepts and Controversies in Haemostasis and Thrombosis Associated with Liver Disease: Proceedings of the 7th International Coagulation in Liver Disease Conference. *Thromb Haemost* 2018; **118**: 1491-1506 [PMID: 30060258 DOI: 10.1055/s-0038-1666861]
14 **Kujovich JL**. Hemostatic defects in end stage liver disease. *Crit Care Clin* 2005; **21**: 563-587 [PMID: 15992673 DOI: 10.1016/j.ccc.2005.03.002]
15 **Escolar G**, Cases A, Viñas M, Pino M, Calls J, Cirera I, Ordinas A. Evaluation of acquired platelet dysfunctions in uremic and cirrhotic patients using the platelet function analyzer (PFA-100 ): influence of hematocrit elevation. *Haematologica* 1999; 84: 614-619 [PMID: 10406903]
16 **Pihusch R**, Rank A, Göhring P, Pihusch M, Hiller E, Beuers U. Platelet function rather than plasmatic coagulation explains hypercoagulable state in cholestatic liver disease. *J Hepatol* 2002; **37**: 548-555 [PMID: 12399218 DOI: 10.1016/s0168-8278(02)00239-8]
17 **Violi F**, Basili S, Raparelli V, Chowdary P, Gatt A, Burroughs AK. Patients with liver cirrhosis suffer from primary haemostatic defects? *J Hepatol* 2011; **55**: 1415-1427 [PMID: 21718668 DOI: 10.1016/j.jhep.2011.06.008]
18 **Witters P**, Freson K, Verslype C, Peerlinck K, Hoylaerts M, Nevens F, Van Geet C, Cassiman D. Review article: blood platelet number and function in chronic liver disease and cirrhosis. *Aliment Pharmacol Ther* 2008; **27**: 1017-1029 [PMID: 18331464 DOI: 10.1111/j.1365-2036.2008.03674.x]
19 **van Dievoet MA**, Eeckhoudt S, Stephenne X. Primary Hemostasis in Chronic Liver Disease and Cirrhosis: What Did We Learn over the Past Decade? *Int J Mol Sci* 2020; **21** [PMID: 32384725 DOI: 10.3390/ijms21093294]
20 **Tripodi A**, Salerno F, Chantarangkul V, Clerici M, Cazzaniga M, Primignani M, Mannuccio Mannucci P. Evidence of normal thrombin generation in cirrhosis despite abnormal conventional

coagulation tests. *Hepatology* 2005; **41**: 553-558 [PMID: 15726661 DOI: 10.1002/hep.20569]
21 **Wiesner R**, Edwards E, Freeman R, Harper A, Kim R, Kamath P, Kremers W, Lake J, Howard T, Merion RM, Wolfe RA, Krom R; United Network for Organ Sharing Liver Disease Severity Score Committee. Model for end-stage liver disease (MELD) and allocation of donor livers. *Gastroenterology* 2003; **124**: 91-96 [PMID: 12512033 DOI: 10.1053/gast.2003.50016]
22 **Kovacs MJ**, Wong A, MacKinnon K, Weir K, Keeney M, Boyle E, Cruickshank M. Assessment of the validity of the INR system for patients with liver impairment. *Thromb Haemost* 1994; **71**: 727-730 [PMID: 7605424]
23 **Lee JH**, Kweon OJ, Lee MK, Lee HW, Kim HJ, Kim HR. Clinical usefulness of international normalized ratio calibration of prothrombin time in patients with chronic liver disease. *Int J Hematol* 2015; **102**: 163-169 [PMID: 26068391 DOI: 10.1007/s12185-015-1820-2]
24 **Trotter JF**, Brimhall B, Arjal R, Phillips C. Specific laboratory methodologies achieve higher model for endstage liver disease (MELD) scores for patients listed for liver transplantation. *Liver Transpl* 2004; **10**: 995-1000 [PMID: 15390325 DOI: 10.1002/Lt.20195]
25 **Bellest L**, Eschwège V, Poupon R, Chazouillères O, Robert A. A modified international normalized ratio as an effective way of prothrombin time standardization in hepatology. *Hepatology* 2007; **46**: 528-534 [PMID: 17654598 DOI: 10.1002/hep.21680]
26 **Garcia-Tsao G**, Abraldes JG, Berzigotti A, Bosch J. Portal hypertensive bleeding in cirrhosis: Risk stratification, diagnosis, and management: 2016 practice guidance by the American Association for the study of liver diseases. *Hepatology* 2017; **65**: 310-335 [PMID: 27786365 DOI: 10.1002/hep.28906]
27 **O'Leary JG**, Greenberg CS, Patton HM, Caldwell SH. AGA Clinical Practice Update: Coagulation in Cirrhosis. *Gastroenterology* 2019; **157**: 34-43. e1 [PMID: 30986390 DOI: 10.1053/j.gastro.2019.03.070]
28 **Francis JL**, Armstrong DJ. Acquired dysfibrinogenaemia in liver disease. *J Clin Pathol* 1982; **35**: 667-672 [PMID: 7085917 DOI: 10.1136/jcp.35.6.667]
29 **Violi F**, Ferro D, Basili S, Cimminiello C, Saliola M, Vezza E, Cordova C. Prognostic value of clotting and fibrinolytic systems in a follow-up of 165 liver cirrhotic patients. CALC Group. *Hepatology* 1995; **22**: 96-100 [PMID: 7601439]
30 **Giannini EG**, Giambruno E, Brunacci M, Plaz Torres MC, Furnari M, Bodini G, Zentilin P, Savarino V. Low Fibrinogen Levels Are Associated with Bleeding After Varices Ligation in Thrombocytopenic Cirrhotic Patients. *Ann Hepatol* 2018; **17**: 830-835 [PMID: 30145561 DOI: 10.5604/01.3001.0012.0775]
31 **Thakrar SV**, Mallett SV. Thrombocytopenia in cirrhosis: Impact of fibrinogen on bleeding risk. *World J Hepatol* 2017; **9**: 318-325 [PMID: 28293381 DOI: 10.4254/wjh.v9.i6.318]
32 **Hemker HC**, Giesen P, AlDieri R, Regnault V, de Smed E, Wagenvoord R, Lecompte T, Béguin S. The calibrated automated thrombogram (CAT): a universal routine test for hyper- and hypocoagulability. *Pathophysiol Haemost Thromb* 2002; **32**: 249-253 [PMID: 13679651 DOI: 10.1159/000073575]
33 **Lebreton A**, Sinegre T, Lecompte T, Talon L, Abergel A, Lisman T. Thrombin Generation and Cirrhosis: State of the Art and Perspectives. *Semin Thromb Hemost* 2020; **46**: 693-703 [PMID: 32820480 DOI: 10.1055/s-0040-1715102]
34 **Shin KH**, Kim IS, Lee HJ, Kim HH, Chang CL, Hong YM, Yoon KT, Cho M. Thromboelastographic Evaluation of Coagulation in Patients With Liver Disease. *Ann Lab Med* 2017; **37**: 204-212 [PMID: 28224766 DOI: 10.3343/alm.2017.37.3.204]
35 **Hugenholtz GCG**, Lisman T, Stravitz RT. Thromboelastography does not predict outcome in different etiologies of cirrhosis. *Res Pract Thromb Haemost* 2017; **1**: 275-285 [PMID: 30046697 DOI: 10.1002/rth2.12037]
36 **Veronese L**, Miller M, Spearman W. Thromboelastography in mild, chronic liver disease: challenging conventional coagulation tests preceding liver biopsy. *Southern African Journal of Anaesthesia and Analgesia* 2018; **24**: 135-139 [DOI: 10.1080/22201181.2018.1510234]
37 **Yang Lu S**, Tanaka KA, Abuelkasem E, Planinsic RM, Sakai T. Clinical applicability of rapid thrombelastography and functional fibrinogen thrombelastography to adult liver transplantation. *Liver Transpl* 2014; **20**: 1097-1105 [PMID: 24889921 DOI: 10.1002/lt.23923]
38 **Song JG**, Jeong SM, Jun IG, Lee HM, Hwang GS. Five-minute parameter of thromboelastometry is sufficient to detect thrombocytopenia and hypofibrinogenaemia in patients undergoing liver transplantation. *Br J Anaesth* 2014; **112**: 290-297 [PMID: 24065728 DOI: 10.1093/bja/aet325]
39 **Tripodi A**, Primignani M, Chantarangkul V, Viscardi Y, Dell'Era A, Fabris FM, Mannucci PM. The coagulopathy of cirrhosis assessed by thromboelastometry and its correlation with conventional coagulation parameters. *Thromb Res* 2009; **124**: 132-136 [PMID: 19135704 DOI: 10.1016/j.thromres.2008.11.008]
40 **Vucelic D**, Jesic R, Jovicic S, Zivotic M, Grubor N, Trajkovic G, Canic I, Elezovic I, Antovic A. Comparison of standard fibrinogen measurement methods with fibrin clot firmness assessed by thromboelastometry in patients with cirrhosis. *Thromb Res* 2015; **135**: 1124-1130 [PMID: 25900310 DOI: 10.1016/j.thromres.2015.04.003]
41 **Jeong SM**, Song JG, Seo H, Choi JH, Jang DM, Hwang GS. Quantification of Both Platelet Count and Fibrinogen Concentration Using Maximal Clot Firmness of Thromboelastometry During Liver Transplantation. *Transplant Proc* 2015; **47**: 1890-1895 [PMID: 26293068 DOI: 10.1016/j.transproceed.2015.02.019]

42 **Seo H**, Choi JH, Moon YJ, Jeong SM. FIBTEM of Thromboelastometry does not Accurately Represent Fibrinogen Concentration in Patients with Severe Hypofibrinogenemia During Liver Transplantation. *Ann Transplant* 2015; **20**: 342-350 [PMID: 26084863 DOI: 10.12659/AOT.893741]

43 **Starzl TE**, Marchioro TL, Vonkaulla KN, Hermann G, Brittain RS, Waddell WR. Homotransplantation of the liver in humans. *Surg Gynecol Obstet* 1963; **117**: 659-676 [PMID: 14100514]

44 **Kang YG**, Martin DJ, Marquez J, Lewis JH, Bontempo FA, Shaw BW Jr, Starzl TE, Winter PM. Intraoperative changes in blood coagulation and thrombelastographic monitoring in liver transplantation. *Anesth Analg* 1985; **64**: 888-896 [PMID: 38960]

45 **Alamo JM**, León A, Mellado P, Bernal C, Marín LM, Cepeda C, Suárez G, Serrano J, Padillo J, Gómez MÁ. Is "intra-operating room" thromboelastometry useful in liver transplantation? *Transplant Proc* 2013; **45**: 3637-3639 [PMID: 24314981 DOI: 10.1016/j.transproceed.2013.11.008]

46 **Smart L**, Mumtaz K, Scharpf D, Gray NO, Traetow D, Black S, Michaels AJ, Elkhammas E, Kirkpatrick R, Hanje AJ. Rotational Thromboelastometry or Conventional Coagulation Tests in Liver Transplantation: Comparing Blood Loss, Transfusions, and Cost. *Ann Hepatol* 2017; **16**: 916-923 [PMID: 29055918 DOI: 10.5604/01.3001.0010.5283]

47 **Nascimento JCR**, Neto EBL, da Silva EL, Nunes RR, Marinho DS, Muniz FN, Praciano AM, Brasil IRC. Analysis of the hemostatic therapy in liver transplantation guided by rotational thromboelastometry or conventional laboratory tests. *Eur J Gastroenterol Hepatol* 2020; **32**: 1452-1457 [PMID: 32118854 DOI: 10.1097/MEG.0000000000001660]

48 **Wang SC**, Shieh JF, Chang KY, Chu YC, Liu CS, Loong CC, Chan KH, Mandell S, Tsou MY. Thromboelastography-guided transfusion decreases intraoperative blood transfusion during orthotopic liver transplantation: randomized clinical trial. *Transplant Proc* 2010; **42**: 2590-2593 [PMID: 20832550 DOI: 10.1016/j.transproceed.2010.05.144]

49 **Bonnet A**, Gilquin N, Steer N, Gazon M, Quattrone D, Pradat P, Maynard M, Mabrut JY, Aubrun F. The use of a thromboelastometry-based algorithm reduces the need for blood product transfusion during orthotopic liver transplantation: A randomised controlled study. *Eur J Anaesthesiol* 2019; **36**: 825-833 [PMID: 31567574 DOI: 10.1097/EJA.0000000000001084]

50 **Schumann R**, Mandell MS, Mercaldo N, Michaels D, Robertson A, Banerjee A, Pai R, Klinck J, Pandharipande P, Walia A. Anesthesia for liver transplantation in United States academic centers: intraoperative practice. *J Clin Anesth* 2013; **25**: 542-550 [PMID: 23994704 DOI: 10.1016/j.jclinane.2013.04.017]

51 **De Pietri L**, Bianchini M, Montalti R, De Maria N, Di Maira T, Begliomini B, Gerunda GE, di Benedetto F, Garcia-Tsao G, Villa E. Thrombelastography-guided blood product use before invasive procedures in cirrhosis with severe coagulopathy: A randomized, controlled trial. *Hepatology* 2016; **63**: 566-573 [PMID: 26340411 DOI: 10.1002/hep.28148]

52 **Vuyyuru SK**, Singh AD, Gamanagatti SR, Rout G, Gunjan D, Shalimar. A Randomized Control Trial of Thromboelastography-Guided Transfusion in Cirrhosis for High-Risk Invasive Liver-Related Procedures. *Dig Dis Sci* 2020; **65**: 2104-2111 [PMID: 31720889 DOI: 10.1007/s10620-019-05939-2]

53 **Rocha LL**, Neto AS, Pessoa CMS, Almeida MD, Juffermans NP, Crochemore T, Rodrigues RR, Filho RR, de Freitas Chaves RC, Cavalheiro AM, Prado RR, Assunção MSC, Guardia BD, Silva E, Corrêa TD. Comparison of three transfusion protocols prior to central venous catheterization in patients with cirrhosis: A randomized controlled trial. *J Thromb Haemost* 2020; **18**: 560-570 [PMID: 31667992 DOI: 10.1111/jth.14672]

54 **Chau TN**, Chan YW, Patch D, Tokunaga S, Greenslade L, Burroughs AK. Thrombelastographic changes and early rebleeding in cirrhotic patients with variceal bleeding. *Gut* 1998; **43**: 267-271 [PMID: 10189856 DOI: 10.1136/gut.43.2.267]

55 **Kumar M**, Ahmad J, Maiwall R, Choudhury A, Bajpai M, Mitra LG, Saluja V, Mohan Agarwal P, Bihari C, Shasthry SM, Jindal A, Bhardwaj A, Kumar G, Sarin SK. Thromboelastography-Guided Blood Component Use in Patients With Cirrhosis With Nonvariceal Bleeding: A Randomized Controlled Trial. *Hepatology* 2020; **71**: 235-246 [PMID: 31148204 DOI: 10.1002/hep.30794]

56 **Rout G**, Shalimar, Gunjan D, Mahapatra SJ, Kedia S, Garg PK, Nayak B. Thromboelastography-guided Blood Product Transfusion in Cirrhosis Patients With Variceal Bleeding: A Randomized Controlled Trial. *J Clin Gastroenterol* 2020; **54**: 255-262 [PMID: 31008867 DOI: 10.1097/MCG.0000000000001214]

57 **Lisman T**. Interpreting Hemostatic Profiles Assessed With Viscoelastic Tests in Patients With Cirrhosis. *J Clin Gastroenterol* 2020; **54**: 389-391 [PMID: 32028285 DOI: 10.1097/MCG.0000000000001327]

58 **Tripodi A**, Primignani M, Chantarangkul V, Clerici M, Dell'Era A, Fabris F, Salerno F, Mannucci PM. Thrombin generation in patients with cirrhosis: the role of platelets. *Hepatology* 2006; **44**: 440-445 [PMID: 16871542 DOI: 10.1002/hep.21266]

59 **Terrault N**, Chen YC, Izumi N, Kayali Z, Mitrut P, Tak WY, Allen LF, Hassanein T. Avatrombopag Before Procedures Reduces Need for Platelet Transfusion in Patients With Chronic Liver Disease and Thrombocytopenia. *Gastroenterology* 2018; **155**: 705-718 [PMID: 29778606 DOI: 10.1053/j.gastro.2018.05.025]

60 **Hidaka H**, Kurosaki M, Tanaka H, Kudo M, Abiru S, Igura T, Ishikawa T, Seike M, Katsube T, Ochiai T, Kimura K, Fukuhara T, Kano T, Nagata T, Tanaka K, Kurokawa M, Yamamoto K, Osaki Y, Izumi N, Imawari M. Lusutrombopag Reduces Need for Platelet Transfusion in Patients With Thrombocytopenia Undergoing Invasive Procedures. *Clin Gastroenterol Hepatol* 2019; **17**: 1192-

1200 [PMID: 30502505 DOI: 10.1016/j.cgh.2018.11.047]
61 **Peck-Radosavljevic M**, Simon K, Iacobellis A, Hassanein T, Kayali Z, Tran A, Makara M, Ben Ari Z, Braun M, Mitrut P, Yang SS, Akdogan M, Pirisi M, Duggal A, Ochiai T, Motomiya T, Kano T, Nagata T, Afdhal N. Lusutrombopag for the Treatment of Thrombocytopenia in Patients With Chronic Liver Disease Undergoing Invasive Procedures (L-PLUS 2). *Hepatology* 2019; **70**: 1336-1348 [PMID: 30762895 DOI: 10.1002/hep.30561]
62 **Tripodi A**, Chantarangkul V, Primignani M, Clerici M, Dell'era A, Aghemo A, Mannucci PM. Thrombin generation in plasma from patients with cirrhosis supplemented with normal plasma: considerations on the efficacy of treatment with fresh-frozen plasma. *Intern Emerg Med* 2012; **7**: 139-144 [PMID: 21298360 DOI: 10.1007/s11739-011-0528-4]
63 **Rassi AB**, d'Amico EA, Tripodi A, da Rocha TRF, Migita BY, Ferreira CM, Carrilho FJ, Farias AQ. Fresh frozen plasma transfusion in patients with cirrhosis and coagulopathy: Effect on conventional coagulation tests and thrombomodulin-modified thrombin generation. *J Hepatol* 2020; **72**: 85-94 [PMID: 31536747 DOI: 10.1016/j.jhep.2019.09.008]
64 **National Heart**, Lung, and Blood Institute (NHLBI). Fresh-frozen plasma infusions to reduce risk of bleeding related to invasive procedures. In: ClinicalTrials.gov [Internet]. Available from: http://clinicaltrials.gov/ct2/show/NCT00233246 ClinicalTrials.gov Identifier: NCT00233246
65 **Sabate A**, Gutierrez R, Beltran J, Mellado P, Blasi A, Acosta F, Costa M, Reyes R, Torres F. Impact of Preemptive Fibrinogen Concentrate on Transfusion Requirements in Liver Transplantation: A Multicenter, Randomized, Double-Blind, Placebo-Controlled Trial. *Am J Transplant* 2016; **16**: 2421-2429 [PMID: 26880105 DOI: 10.1111/ajt.13752]
66 **Bosch J**, Thabut D, Bendtsen F, D'Amico G, Albillos A, González Abraldes J, Fabricius S, Erhardtsen E, de Franchis R; European Study Group on rFVIIa in UGI Haemorrhage. Recombinant factor VIIa for upper gastrointestinal bleeding in patients with cirrhosis: a randomized, double-blind trial. *Gastroenterology* 2004; **127**: 1123-1130 [PMID: 15480990 DOI: 10.1053/j.gastro.2004.07.015]
67 **Bosch J**, Thabut D, Albillos A, Carbonell N, Spicak J, Massard J, D'Amico G, Lebrec D, de Franchis R, Fabricius S, Cai Y, Bendtsen F; International Study Group on rFVIIa in UGI Hemorrhage. Recombinant factor VIIa for variceal bleeding in patients with advanced cirrhosis: A randomized, controlled trial. *Hepatology* 2008; **47**: 1604-1614 [PMID: 18393319 DOI: 10.1002/hep.22216]
68 **Vincent JL**, Rossaint R, Riou B, Ozier Y, Zideman D, Spahn DR. Recommendations on the use of recombinant activated factor VII as an adjunctive treatment for massive bleeding--a European perspective. *Crit Care* 2006; **10**: R120 [PMID: 16919168 DOI: 10.1186/cc5026]
69 **Laubham M**, Kallwitz E. Coagulation in chronic liver disease and the use of prothrombin complex concentrate for an emergent procedure: a case report and review of literature. *J Community Hosp Intern Med Perspect* 2018; **8**: 138-141 [PMID: 29915653 DOI: 10.1080/20009666.2018.1466600]
70 **Kwon JO**, MacLaren R. Comparison of Fresh-Frozen Plasma, Four-Factor Prothrombin Complex Concentrates, and Recombinant Factor VIIa to Facilitate Procedures in Critically Ill Patients with Coagulopathy from Liver Disease: A Retrospective Cohort Study. *Pharmacotherapy* 2016; **36**: 1047-1054 [PMID: 27547916 DOI: 10.1002/phar.1827]
71 **Arshad F**, Ickx B, van Beem RT, Polak W, Grüne F, Nevens F, Ilmakunnas M, Koivusalo AM, Isoniemi H, Strengers PF, Groen H, Hendriks HG, Lisman T, Pirenne J, Porte RJ. Prothrombin complex concentrate in the reduction of blood loss during orthotopic liver transplantation: PROTON-trial. *BMC Surg* 2013; **13**: 22 [PMID: 23815798 DOI: 10.1186/1471-2482-13-22]
72 **de Franchis R**, Arcidiacono PG, Carpinelli L, Andreoni B, Cestari L, Brunati S, Zambelli A, Battaglia G, Mannucci PM. Randomized controlled trial of desmopressin plus terlipressin vs. terlipressin alone for the treatment of acute variceal hemorrhage in cirrhotic patients: a multicenter, double-blind study. New Italian Endoscopic Club. *Hepatology* 1993; **18**: 1102-1107 [PMID: 8225214 DOI: 10.1016/0270-9139(93)90464-x]
73 **Wong AY**, Irwin MG, Hui TW, Fung SK, Fan ST, Ma ES. Desmopressin does not decrease blood loss and transfusion requirements in patients undergoing hepatectomy. *Can J Anaesth* 2003; **50**: 14-20 [PMID: 12514144 DOI: 10.1007/BF03020180]
74 **Arshad F**, Stoof SC, Leebeek FW, Ruitenbeek K, Adelmeijer J, Blokzijl H, van den Berg AP, Porte RJ, Kruip MJ, Lisman T. Infusion of DDAVP does not improve primary hemostasis in patients with cirrhosis. *Liver Int* 2015; **35**: 1809-1815 [PMID: 25522671 DOI: 10.1111/liv.12765]
75 **Molenaar IQ**, Warnaar N, Groen H, Tenvergert EM, Slooff MJ, Porte RJ. Efficacy and safety of antifibrinolytic drugs in liver transplantation: a systematic review and meta-analysis. *Am J Transplant* 2007; **7**: 185-194 [PMID: 17227567 DOI: 10.1111/j.1600-6143.2006.01591.x]
76 **HALT-IT Trial Collaborators**. Effects of a high-dose 24-h infusion of tranexamic acid on death and thromboembolic events in patients with acute gastrointestinal bleeding (HALT-IT): an international randomised, double-blind, placebo-controlled trial. *Lancet* 2020; **395**: 1927-1936 [PMID: 32563378 DOI: 10.1016/S0140-6736(20)30848-5]
77 **Ho YW**, Lee PL, Kang YN. Is Tranexamic Acid for Upper Gastrointestinal Bleeding Under Debate? *Ann Emerg Med* 2021; **77**: 279-281 [PMID: 33487326 DOI: 10.1016/j.annemergmed.2020.09.441]

World Journal of Gastroenterology

Submit a Manuscript: https://www.f6publishing.com

*World J Gastroenterol* 2021 November 14; 27(42): 7299-7310

DOI: 10.3748/wjg.v27.i42.7299

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

MINIREVIEWS

# Colonoscopy-related colonic ischemia

Sinan Sadalla, Andrea Lisotti, Lorenzo Fuccio, Pietro Fusaroli

**ORCID number:** Sinan Sadalla 0000-0001-5336-317X; Andrea Lisotti 0000-0002-7724-7402; Lorenzo Fuccio 0000-0001-8618-2447; Pietro Fusaroli 0000-0002-4397-9314.

**Author contributions:** Fusaroli P designed the research study; Sadalla L and Lisotti A performed the research and wrote the article; Fuccio L analyzed the data for relevant intellectual content; All authors have read and approved the final manuscript.

**Conflict-of-interest statement:** All authors declare that there are no conflicts of interest.



**Specialty type:** Gastroenterology and hepatology

**Country/Territory of origin:** Italy

**Peer-review report's scientific**

**Sinan Sadalla,** Unità Operativa Complessa di Gastroenterologia e Endoscopia Digestiva, Università di Bologna/ Ospedale di Imola, Imola (BO) 40024, Italy

**Andrea Lisotti, Pietro Fusaroli,** Unità Operativa Complessa di Gastroenterologia e Endoscopia Digestiva, Università di Bologna/ Ospedale di Imola, Imola (BO) 40026, Italy

**Lorenzo Fuccio,** Divisione di Gastroenterologia, Dipartimento di Scienze Medico-Chirurgiche (DIMEC), IRCSS- Azienda Ospedaliero-Universitaria di Bologna, Bologna 40138, Italy

**Corresponding author:** Pietro Fusaroli, MD, Associate Professor, Unità Operativa Complessa di Gastroenterologia e Endoscopia Digestiva, Università di Bologna/ Ospedale di Imola, Via Montericco 4, Imola (BO) 40026, Italy. pietro.fusaroli@unibo.it

## Abstract

Colonoscopy is a risk factor for colon ischemia. The colon is susceptible to ischemia due to its minor blood flow compared to other abdominal organs; the etiology of colon ischemia after colonoscopy is multifactorial. The causative mechanisms include splanchnic circulation impairment, bowel preparation, drugs used for sedation, bowel wall ischemia due to insufflation/barotrauma, and introduction of the endoscope. Gastroenterologists must be aware of this condition and its risk factors for risk minimization, early diagnosis, and proper treatment.

**Key Words:** Endoscopy; Colon ischemia; Colonoscopy; Bowel preparation; Mesenteric circulation; Ischemic colitis



**Core Tip:** Colonic ischemia is the main form of vascular injury to the gastrointestinal tract and is characterized by sudden onset of nausea and abdominal pain, followed by bloody diarrhea. Among the different etiologies, colonoscopy has been proposed as a risk factor for colon ischemia.

**Citation:** Sadalla S, Lisotti A, Fuccio L, Fusaroli P. Colonoscopy-related colonic ischemia. *World J Gastroenterol* 2021; 27(42): 7299-7310

**URL:** https://www.wjgnet.com/1007-9327/full/v27/i42/7299.htm

**DOI:** https://dx.doi.org/10.3748/wjg.v27.i42.7299

**quality classification**
Grade A (Excellent): 0
Grade B (Very good): B
Grade C (Good): C
Grade D (Fair): 0
Grade E (Poor): 0

**Received:** March 30, 2021
**Peer-review started:** March 30, 2021
**First decision:** June 23, 2021
**Revised:** July 6, 2021
**Accepted:** October 20, 2021
**Article in press:** October 20, 2021
**Published online:** November 14, 2021

**P-Reviewer:** Gkolfakis P, Imaeda H
**S-Editor:** Ma YJ
**L-Editor:** Filipodia
**P-Editor:** Ma YJ

## INTRODUCTION

At the end of 2018, an elderly retired physician came to our attention because of worsening post-prandial epigastric pain, hyporexia and weight loss. Complete blood cell count showed moderate anemia (hemoglobin 10.2 g/dL). The patient had sought medical attention in the emergency room of our hospital 1 mo prior for the same symptoms. At that time, doctors mainly focused on premature atrial complexes and hypokalemia, as the patient was suffering from hypertensive cardiopathy and had undergone percutaneous transluminal coronary angioplasty. Comorbidities were mild chronic renal failure, type-2 diabetes mellitus, and stable metastatic hormone-sensitive prostate cancer.

Upon presentation to our unit, no remarkable physical findings were noted. Medications included low-dose aspirin, beta-blockers, anti-hypertensive agents (candesartan and amlodipine), simvastatin, glibenclamide, bicalutamide, and omeprazole. We performed percutaneous abdominal ultrasound and esophago-gastroduodenoscopy, which were unremarkable. As the patient was strongly determined not to neglect any diagnostic possibility, consent to undergo colonoscopy was given too. Being a fragile individual, we elected to administer the bowel preparation in our in-hospital facility with a 2-liter same-day ascorbate-based polyethylene glycol regimen. After the first laxative dose, the patient had syncope and was brought to the emergency room. No notable findings emerged; serum sodium was 147 mmol/L (136-145) while potassium concentration was normal. The patient was able to complete the preparation and was still determined to undergo a colonoscopy. The exam went smoothly and failed to show any relevant findings. The patient was discharged home in good condition and relieved as no malignancy was found.

However, 48 hours after the exam, disaster struck. The patient came back to the hospital suffering from severe abdominal pain of sudden onset. Urgent computed tomography showed complete thrombosis of the superior mesenteric artery, ileocolic arteries, and common hepatic artery (Figure 1). Large bowel loops were over-distended with a lack of parietal perfusion as per severe ischemic changes. Due to the rapidly deteriorating conditions, the patient died 24 h later despite intensive care.

Colonic ischemia, also described as ischemic colitis if an inflammatory component is present[1], is usually a benign condition that occurs when there is sudden and temporary hypoperfusion of the large bowel wall[2]. Colonic ischemia can usually be managed conservatively and must be differentiated from other causes of acute and chronic ischemic injury, such as emboli and thrombosis[3], which often require urgent intervention[4].

Ischemia occurs when blood supply is inadequate to fulfill the metabolic requirements of tissues and can occur in every district of the body[5]. When there is a decrease in blood flow, the affected tissues initially react with vasodilation. However, if hypoperfusion persists, vasoconstriction may ensue[6].

The colon is susceptible to ischemia due to its minor blood flow compared to other abdominal organs and to its decrease in perfusion when functionally inactive[7]. In the colon, ischemic injury displays a wide spectrum of manifestations. Reversible ischemia determines the rupture of the sub-epithelial microvasculature, which evolves in a colitis phase when the mucosa ulcerates and subepithelial hemorrhage is reabsorbed [8]. On the other hand, irreversible damage leads to necrosis of the bowel wall, gangrene, strictures, and ultimately to fulminant colitis[9].

Colonic ischemia represents the predominant form of vascular injury to the gastrointestinal tract with an estimated incidence ranging from 7.2 to 15.6 per 100000 population[10]. It is also one of the main causes of lower gastrointestinal bleeding[8]. Women seem affected more than men, especially among patients < 40 years of age [10]. However, the incidence of colonic ischemia is probably underestimated because many patients never seek medical attention due to its frequent presentation with mild symptoms[2].

### *Etiology*

Colonic ischemia is multifactorial. Previous studies identified risk factors for colonic ischemia such as cardiovascular, pulmonary, and renal comorbidities, history of irritable bowel syndrome, and surgical interventions[10,11]. As far as drugs are concerned, immunomodulators and constipation-inducing drugs have been associated more frequently with colonic ischemia[12]. The use of vasopressors, alfa-adrenergic agents, diuretics as well as cocaine has also been described as contributing factors[8]. Additionally, in most cases, no specific cause of colonic ischemia can be determined [13].

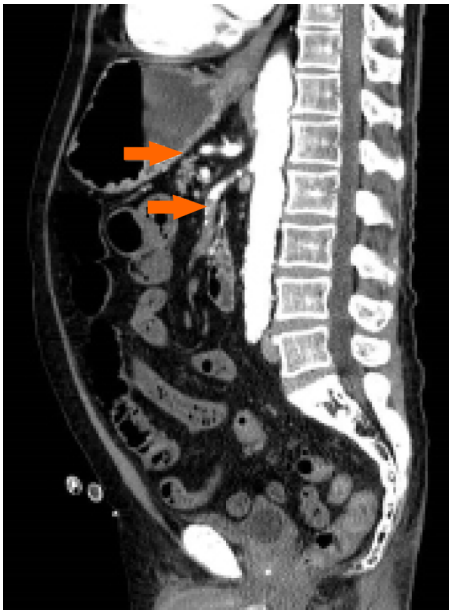

**Figure 1 Abdominal computed tomography with intravenous contrast, sagittal scan showing thrombosis of the superior mesenteric artery and the common hepatic artery (arrows).**

Colonoscopy has been proposed as a risk factor for colonic ischemia although only few reports are available in this respect[14]. To our knowledge, no current epidemiologic data exist about colonic ischemia after colonoscopy.

### *Splanchnic vascular anatomy*

Visceral blood supply is strictly connected to the function of the gastrointestinal organs. The celiac trunk, the superior mesenteric artery (SMA), and the inferior mesenteric artery (IMA), all of which originate from the abdominal aorta (Figure 2) [15], represent the main abdominal vessels.

The SMA and IMA sustain colon perfusion. The SMA is the largest abdominal artery supplying the inferior part of the duodenum, the entire jejunum and ileum, the proximal colon, and a portion of the pancreas[16]. The IMA feeds the distal colon through three different branches and the rectum with its terminal branch, the superior rectal artery[17].

These vessels are deeply interconnected through collateral branches to ensure visceral perfusion also in case of deficiency of a single vessel[18]. Despite this network, some segments of the colon can be more frequently affected by ischemia due to their location between two different vascular supplies. These are known as "water-shed areas", the most important being the splenic flexure (Griffith's point) and the sigmoid colon (Sudeck's point)[2].

## COLONIC ISCHEMIA AFTER COLONOSCOPY

Colonoscopy is the gold standard for colorectal screening programs and the number of colonoscopies is progressively increasing worldwide[19]. This exam allows direct visualization of the colonic mucosa and allows resecting of pre-neoplastic lesions. Colonoscopy is considered relatively safe being associated with a perforation rate of about 4 in 10000 procedures, a major bleeding rate of 8 in 10000 procedures, and an estimated overall mortality of 0.007-0.07%[20,21].

An extensive search of the literature showed 25 reports of colonoscopy-related colonic ischemia[22]. The first report in 1990 described a young woman with a history of systemic lupus erythematosus who developed abdominal pain and bloody diarrhea after a diagnostic colonoscopy. Arteriography showed no abnormalities of mesenteric vessels while sigmoidoscopy showed severe inflammation with biopsies suggestive for necrotic areas, compatible with ischemic colitis. The authors suggested that the impairment in small circulation due to the connective tissue disease combined with the mechanical stress of the colonoscopy probably led to colonic ischemia[14]. Subsequently, more cases of ischemic colitis were published[23]. More recent literature

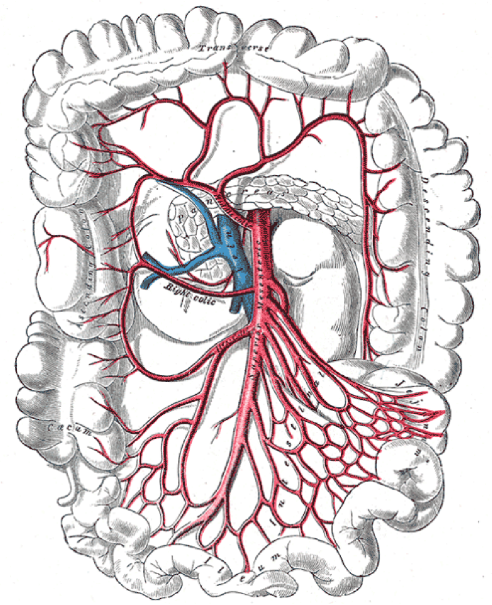

**Figure 2 Splanchnic vascular anatomy, detail of colonic arteries (Case courtesy of Assoc Prof Craig Hacking, Radiopaedia.org, rID: 54523.**

reported one case of SMA thrombosis and one case of SMA embolism[24]. Other case reports of colonic ischemia after colonoscopy described patients with cardiovascular comorbidities[25] or without any known risk factor[26].

Based on the previous considerations, we propose five different mechanisms by which colonoscopy-related colonic ischemia might arise.

### *Splanchnic circulation impairment*

Colonic ischemia can develop in patients with bowel circulation impairment. Chronic mesenteric ischemia is predominantly related to atherosclerotic disease, patients with this condition usually being elderly and with a history of smoking[27]. A study reported an 18% prevalence of asymptomatic stenoses of abdominal vessels in individuals over 65 years[28]. Studies with magnetic resonance showed a significant postprandial reduction of mesenteric flow in patients with vascular stenoses compared to healthy subjects[29,30].

Moreover, conditions such as antiphospholipid syndrome, systemic lupus erythematosus and diabetes can determine an endothelial dysfunction of the colonic vasculature[8]. In these conditions, the involvement of the splanchnic vessels can range from mesenteric thrombosis in the largest veins to mild ischemic injury in the mesenteric microcirculation[23,31]. In all these cases, the colon suffers a condition of chronic ischemia. Therefore, when subjected to gas insufflation and mechanical compression during colonoscopy, the previously impaired blood flow can easily drop to a critical level leading to ischemic injury[14].

### *Bowel preparation*

Adequate bowel cleansing is fundamental to perform a high-quality colonoscopy. Various combinations of volume, timing, and adjuvants have been validated for bowel preparations[32].

The isotonic polyethylene glycol (PEG) bowel preparations are generally considered safe as they minimize electrolyte and water loss[33]. Hypertonic bowel preparations include PEG solutions combined with osmotically active molecules to reduce the volume of total fluid, oral sulfate solutions, and magnesium picosulfate solutions. It is generally assumed that hypertonic preparations have potential risks of inducing serum electrolyte imbalance. Furthermore, as they bind with water molecules, PEG monomers cause fluid retention that can increase intraluminal pressure[34]. This effect, combined with an inadequate assumption of fluids during the preparation, can thus lead to dehydration[35] and potentially precipitate an ischemic injury. We found on the Federal Drug Administration Adverse Events Report System a study from Bielefeldt *et al*[36] describing bowel preparations as the suspected causative agents of 60 cases of colonic ischemia. Adjunctive drugs such as bisacodyl and magnesium

citrate are often administered with low volume preparations to improve colon cleansing. Of note, case reports of ischemic colitis have been reported following the intake of bisacodyl[32].

### Drugs used for endoscopy

Sedation during colonoscopy aims to reduce discomfort and improve outcomes. Narcotics such as meperidine and fentanyl, benzodiazepines (midazolam) and propofol represent the most common drugs. Although considered safe, sedation has been associated with cardiovascular and pulmonary adverse events such as hypotension and hypoxemia with an overall frequency of 1.1 per 100 procedures[37].

Midazolam, alone or in combination with opioids, is frequently used in colonoscopy as it has a rapid onset and a short duration of action. However, it can induce cardiovascular effects such as vasodilation, depression of myocardial contractility, and hypotension. In a recent study, Kim *et al*[38] demonstrated a reduction of heart rate, systolic and diastolic blood pressure in patients undergoing colonoscopy with midazolam compared to no sedation. Given that hypoperfusion plays a key role in the multifactorial pathogenesis of colonic ischemia, the risk of anesthesia-related cardiovascular depression deserves consideration. In a recent prospective study, Wernli *et al*[39] found that the overall risk of complications associated with colonoscopy increases in patients receiving anesthesia services. Moreover, propofol is often used in combination with benzodiazepines and/or opioids with the combination effects on cardiovascular and respiratory depression still being under investigation.

### Bowel wall ischemia due to insufflation/barotrauma

Gas insufflation fills the colon up to the cecum, which is the highest point when the patient is in the left lateral decubitus[40,41]. It is necessary to correctly inspect the mucosa but it leads to an increase in luminal pressure and consequently in vascular resistance. As a result, barotrauma should be considered the main cause of reduction in parietal blood flow during colonoscopy.

When the pressure on the vessels reaches 30-60 mmHg, it causes a reduction in parietal blood flow that can cause mucosal damage in about 20 min[26,42]. Light source air pumps generate a maximal pressure of 375 mmHg, which is reduced by 30%-40% when measured at the tip of the endoscope because of air leakage[43]. In a seminal study, Kozarek *et al*[44] showed that in human cadaver colon air pressures leading to serosal tears, pneumatosis and transmural rupture ranged between 52 and 226 mmHg. Caecum rupture was observed with 81 mmHg of air pressure while tearing of the sigma required pressure of 169 mmHg. In the same study, the authors measured the intraluminal pressure during routine colonoscopies. Intraluminal pressure ranged from 9 to 57 mmHg when the tip of the endoscope was free, while it reached a maximum of 138 mmHg when the tip was impacted against the bowel wall.

### Advancement of the scope

Scope manipulation can traumatize the vascular pedicles and lead to mesocolon injury [45]. Anatomical conditions of the colon such as length, mobility, and redundancy can affect procedures such as colonoscopy[46]. The ascending and the descending colon are fixed and retroperitoneal, while the sigmoid and transverse colon are suspended in a double layer of peritoneum, the mesentery or mesocolon[47]. Excessive traction and torsion on the mesocolon can occur in the context of abdominal adhesions or during pull-back maneuvers, slide-by advancement, alpha maneuver, and straightening of the sigmoid loop[48]. The stress on the mesocolon can reduce blood flow, impair microcirculation and activate the inflammatory cascade, eventually leading to vascular thrombosis. Cases of splenic trauma due to colonoscopy are also described in the literature[49,50], suggesting caution with straightening maneuvers and manual compression of the abdominal wall.

## CLINICAL PRESENTATION

The symptoms of acute colonic ischemia after colonoscopy do not differ from those of colonic ischemia per se. These include the sudden onset of abdominal pain associated with nausea, vomiting, bloating, and passage of bright bloody diarrhea within 24 h, which often evolve in melena[8]. Zizzo *et al*[22] found that abdominal pain and bloody diarrhea were present in 95% of patients reported to have colonic ischemia after colonoscopy. Moreover, it is assumed that a non-negligible number of cases may be paucisymptomatic and thereby go undetected.

### Diagnosis

Given the scant literature focusing on the association between colonoscopy and colonic ischemia, we rely on the general evidence of ischemic colitis per se. In a patient with progressive abdominal pain and/or bloody diarrhea after colonoscopy, colonic ischemia related to the procedure must be suspected along with other possible adverse events (*e.g.*, perforation, post-polypectomy syndrome)[51]. Laboratory tests such as complete blood count, albumin, and acid-base status should be requested to help predict the severity of ischemia.

Computed tomography with intravenous and oral contrast can show signs of ischemia such as mesenteric fat stranding, bowel wall thickening, and abnormal wall enhancement, regardless of the disease severity[52].

Colonoscopy is also the gold standard for the optical diagnosis of colonic ischemia along with biopsies for histological confirmation. When signs of colonic ischemia are found, the endoscopic procedure should be halted at the most distal part of the affected segment[8]. In the existing reports of post-colonoscopy colonic ischemia, the diagnosis was confirmed with a second colonoscopy with biopsies in 85% of the cases [22]. Nevertheless, we believe that the indication to repeat colonoscopy must be weighed against clinical and radiological presentation in order not to worsen existing conditions.

Typical endoscopic signs of ischemia include patchy petechial hemorrhages, erosions, and edematous mucosal inflammation (Figure 3)[26]. Zuckerman *et al*[53] described a typical single linear ulcer running longitudinally along the anti-mesenteric left colonic wall[54]. Notably, Cheema *et al*[55] described a dynamic in-vivo appearance of bowel ischemia, where an intermittent blanching of the colonic mucosa was observed.

### Treatment

Treatment of colonic ischemia after colonoscopy should be based on general knowledge of a disease that may vary from a mild to a severe and life-threatening condition. In the available literature, the majority of patients suffered from transient reversible ischemic colitis, which required just a conservative therapy[2]. In such cases, therapeutic options included general supportive measures, intravenous hydration, bowel rest, and correction of possible precipitating conditions including withdrawal of putative causative medications[36]. On the other hand, emergent surgery was needed in two cases of irreversible ischemia[22].

## PREVENTION

### Double-check colonoscopy indications and risk factors

Physicians must be aware of risk factors for colonic ischemia such as advanced age, recent vascular surgery, atherosclerotic and vascular disease such as a peripheral artery, cerebrovascular, coronary artery, and renovascular disease[8]. Additionally, a careful drug history should be obtained with particular attention to constipation-inducing agents[12]. A history of either coronary heart disease, peripheral obliterative arteriopathy, or ischemic stroke could be suggestive for the presence of mesenteric thrombosis while abdominal angina, as well as gastroparesis and gastric ulcers, could be indirect signs of chronic ischemia[4]. Doppler ultrasound and/or computed tomography angiography of the major abdominal arteries can be helpful when suspicion arises. Antiplatelet and anticoagulant agents should be managed according to the available clinical guidelines. As a result, whether to perform a colonoscopy in high-risk cases must always be weighed in every case by accurately balancing colonoscopy indications with the individual risk factors (Table 1).

### Optimize bowel preparation

Bowel laxatives determine a large loss of fluids that can lead to volume depletion. Therefore, physicians should assess the presence of cardiovascular and renal comorbidities before recommending a specific bowel preparation, above all in elderly and fragile patients.

Phosphate-based preparations should be avoided due to their adverse effects on kidney function[32]. On the contrary, high volume PEG-based regimens account for a higher safety profile due to their osmotically balanced formulation and reduced sodium load[56]. However, the high volume accounts for poor tolerability in elderly people. Alternatively, low-volume PEG-based regimens should be considered[56]. In

**Table 1 Summary of proposed pathogenetic factors for colonoscopy-related mesenteric ischemia and suggested interventions to reduce the risk**

| Pathogenetic factor | Mechanism of action | Type of ischemia | Prevention |
|---|---|---|---|
| Splanchnic circulation impairment | Chronic mesenteric ischemia (atherosclerosis, smoking habits), parietal vessels inflammation (connective tissue diseases, LES, antiphospholipid syndrome) | Vascular thrombosis; microcirculatory mild ischemic injury | Careful evaluation of medical history. Specific and indirect symptoms assessment. Antiplatelet agents according to guidelines. Consider pre-colonoscopy assessments (serum electrolyte, color-Doppler ultrasound) |
| Bowel preparation (hypertonic, isotonic, laxative) | Serum electrolyte imbalance, dehydration. Potential additional risk if laxative were used (*i.e.*, bisacodyl) | Multifactorial | Give specific information. Consider high-volume isotonic formulations; split-dose regimens; avoid bisacodyl-containing preparations |
| Sedation (midazolam, opioids, propofol) | Vasodilation, depression of myocardial contractility and hypotension | Multifactorial | Minimal sedation protocol (response to verbal stimulation, patent airways, spontaneous ventilation, and normal cardiovascular function) in high-risk patients. Consider prophylactic fluid infusion |
| Air insufflation/barotrauma | Increased luminal pressure and consequent vascular resistance | Non-occlusive mesenteric ischemia | Use carbon dioxide ($CO_2$) insufflation. Consider water-exchange colonoscopy technique |
| Scope manipulation | Mechanical stress on mesocolon, blood flow reduction, microcirculatory damage, and inflammatory cascade activation | Vascular thrombosis | Procedure interruption in case of intense discomfort or endoscopic findings of ischemia. Consider pediatric or "ultra-slim" colonoscopes. Reschedule or reconsider indication in case of complex exams |

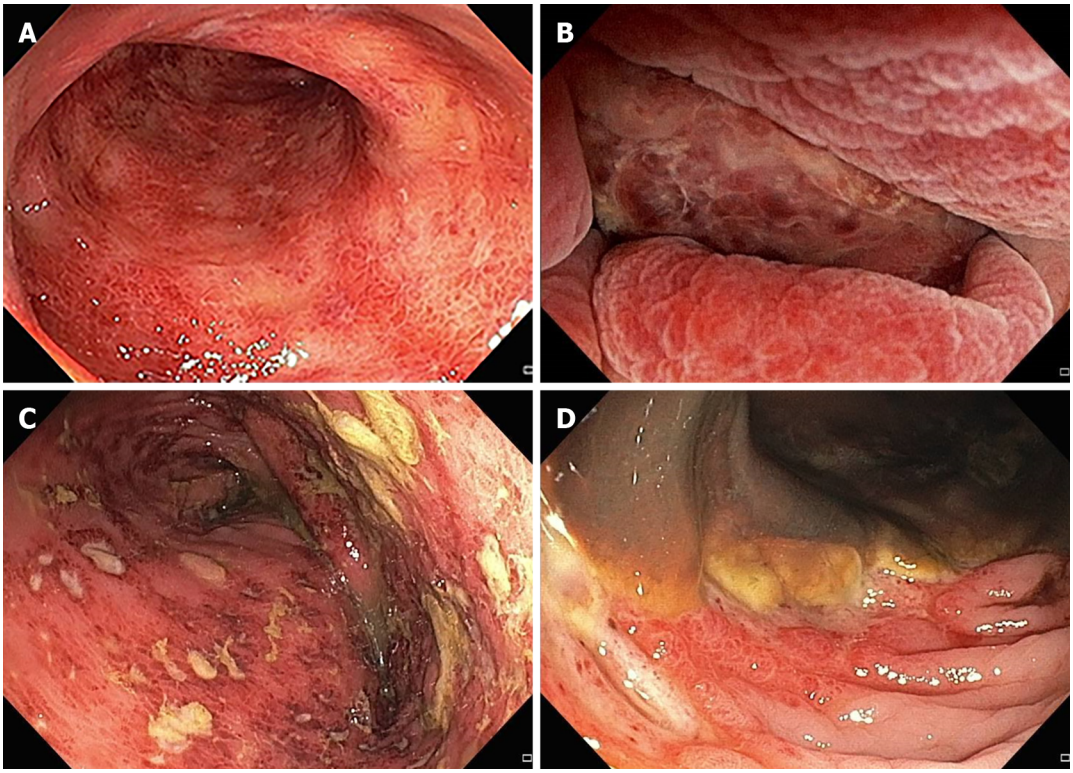


**Figure 3 Endoscopic signs of ischemia, showing moderate diffuse erythema (A), severe erythema with mucosal edema and erosions (B), multiple ulcerations and inflammatory exudate (C), necrosis (D).**

both cases, split regimens should always be advised with written and oral information to achieve the best patients' adherence and optimize the results[32].

As all bowel preparations can determine electrolyte imbalance, baseline serum testing should be obtained in patients with risk factors. Finally, to prevent dehydration induced by osmotic diarrhea, patients should be instructed to drink enough replacement water prior, during, and after colonoscopy[57]. Because of reports about bisacodyl-associated colonic ischemia, this drug should be avoided in patients with multiple risk factors[58].

### Check sedation protocol

Sedation in colonoscopy is generally safe. However, sedation-related adverse events such as hypotension and hypoxemia could act as precipitating factors in individuals at risk for colonic ischemia. Therefore, physicians should understand the pharmacokinetics, pharmacodynamics, and the potential adverse effects of each sedative agent [59]. Patients should be evaluated before colonoscopy to assess their cardiopulmonary risk to better gauge the sedation protocol.

Minimal sedation, defined as a normal response to verbal stimulation and ability to maintain patent airways, spontaneous ventilation, and cardiovascular function[59] should be preferred in patients at risk for colonic ischemia. Unsedated endoscopy could also be considered in selected patients.

Monitoring during colonoscopy includes repeated assessment of blood pressure, heart rate, and pulse oximetry. Oxygen should be routinely administered to reduce the risk of hypoxemia.

Hypotension is considered a key factor for the development of colon hypoperfusion. Tang *et al*[60] found that, in patients undergoing sedated colonoscopy with fentanyl and midazolam, the presence of pre-procedural low blood pressure was a primary risk factor for the development of hypotension during the procedure. Moreover, there was no evidence to suspend antihypertensive drugs in the morning before colonoscopy to prevent procedural hypotension. Even if prophylactic intravenous fluid infusion did not prevent procedural hypotension in a study by Leslie *et al*[61], it could be considered in patients with pre-existent low blood pressure.

### Minimize air insufflation

Although air insufflation through endoscopic light source air pumps has been the most common technique for luminal distension, it is now recognized that this method can potentially damage the colon[43]. On the other hand, carbon dioxide ($CO_2$) is rapidly absorbed from the bowel and eliminated through the lungs. In an animal study, Brandt *et al*[62] demonstrated that $CO_2$ insufflation minimized the reduction in blood flow and intraluminal pressure compared to air insufflation during colonoscopy. Moreover, it has been demonstrated that $CO_2$ insufflation is more advantageous than air in terms of reduced procedural and post-procedural pain[63,64]. Other studies showed less residual gas with $CO_2$ insufflation at 1 h after colonoscopy compared to air insufflation[65], and even suggested that $CO_2$ has a potential dilating effect on colonic microvasculature when insufflated at low pressure[66].

Water-assisted colonoscopy may represent another way to minimize air insufflation [40,67]. Moreover, as procedural pain is also reduced with this technique, the amount of sedation can be minimized[68]. Therefore, $CO_2$ insufflation and water-assisted colonoscopy should be adopted in patients with multiple risk factors for colonic ischemia.

### Minimize mechanical trauma

The sudden observation of a dry and pale mucosa during a colonoscopy could represent a real-time sign of ischemia[55,69]. In these cases, interruption of the procedure should be always considered. Special attention should be taken in patients undergoing combined procedures such as same-session gastroscopy and colonoscopy.

Experienced endoscopists often rely on torqueing maneuvers to pass the sigmoid tract, thus stretching the mesentery. Using left/right controls can reduce the mechanical stress on the mesentery, as well as patient discomfort. Caution with straightening maneuvers and manual compression of the abdominal wall is also suggested[49]. Change of decubitus to right or supine position can be useful in difficult segments[19].

Consider switching to pediatric or ultraslim endoscopes in difficult cases as thinner instruments may allow negotiation of fixed angulations. Finally, if the patient still manifests discomfort, consider rescheduling the procedure with a more experienced operator and/or anesthesia care.

## CONCLUSION

In our patient, the abdominal computed tomography performed after colonoscopy showed thrombosis of the main abdominal arteries. We suppose that in this fragile person, bowel preparation led to general dehydration and hypoperfusion. Subsequently, colonoscopy-related procedural factors (sedation, advancement of the scope, gas insufflation) could have precipitated a pre-existing state of chronic

mesenteric ischemia, leading to thrombosis of the colonic vascular supply, and to the fatal acute colonic injury.

In conclusion, colonoscopy-related colonic ischemia is a rare complication that needs to be considered in the differential diagnosis of abdominal pain after colonoscopy, particularly in elderly, fragile, and comorbid patients. Given the increasing number of colonoscopies performed every year in an aging population with multiple comorbidities, endoscopists must be aware of this dreaded adverse event and should adopt all the possible preventive measures.

## REFERENCES


1 **Trotter JM**, Hunt L, Peter MB. Ischaemic colitis. *BMJ* 2016; **355**: i6600 [PMID: 28007701 DOI: 10.1136/bmj.i6600]

2 **Feuerstadt P**, Brandt LJ. Update on Colon Ischemia: Recent Insights and Advances. *Curr Gastroenterol Rep* 2015; **17**: 45 [PMID: 26446556 DOI: 10.1007/s11894-015-0469-6]

3 **Oldenburg WA**, Lau LL, Rodenberg TJ, Edmonds HJ, Burger CD. Acute mesenteric ischemia: a clinical review. *Arch Intern Med* 2004; **164**: 1054-1062 [PMID: 15159262 DOI: 10.1001/archinte.164.10.1054]

4 **Herbert GS**, Steele SR. Acute and chronic mesenteric ischemia. *Surg Clin North Am* 2007; **87**: 1115-1134, ix [PMID: 17936478 DOI: 10.1016/j.suc.2007.07.016]

5 **Clair DG**, Beach JM. Mesenteric Ischemia. *N Engl J Med* 2016; **374**: 959-968 [PMID: 26962730 DOI: 10.1056/NEJMra1503884]

6 **Boley SJ**, Brandt LJ, Sammartano RJ. History of mesenteric ischemia. The evolution of a diagnosis and management. *Surg Clin North Am* 1997; **77**: 275-288 [PMID: 9146712 DOI: 10.1016/s0039-6109(05)70548-x]

7 Intestinal ischemia. In: Feldman M, Friedman L, Brandt LJ. Sleisenger and Fordtran's Gastrointestinal and Liver Disease: Pathophysiology/diagnosis/management. 10th ed. Philadelphia: Saunders/Elsevier, 2016

8 **Brandt LJ**, Feuerstadt P, Longstreth GF, Boley SJ; American College of Gastroenterology. ACG clinical guideline: epidemiology, risk factors, patterns of presentation, diagnosis, and management of colon ischemia (CI). *Am J Gastroenterol* 2015; **110**: 18-44; quiz 45 [PMID: 25559486 DOI: 10.1038/ajg.2014.395]

9 **Corcos O**, Nuzzo A. Gastro-intestinal vascular emergencies. *Best Pract Res Clin Gastroenterol* 2013; **27**: 709-725 [PMID: 24160929 DOI: 10.1016/j.bpg.2013.08.006]

10 **Longstreth GF**, Yao JF. Epidemiology, clinical features, high-risk factors, and outcome of acute large bowel ischemia. *Clin Gastroenterol Hepatol* 2009; **7**: 1075-80.e1 [PMID: 19500689 DOI: 10.1016/j.cgh.2009.05.026]

11 **Yadav S**, Dave M, Edakkanambeth Varayil J, Harmsen WS, Tremaine WJ, Zinsmeister AR, Sweetser SR, Melton LJ 3rd, Sandborn WJ, Loftus EV Jr. A population-based study of incidence, risk factors, clinical spectrum, and outcomes of ischemic colitis. *Clin Gastroenterol Hepatol* 2015; **13**: 731-8.e1 [PMID: 25130936 DOI: 10.1016/j.cgh.2014.07.061]

12 **Chang L**, Chey WD, Harris L, Olden K, Surawicz C, Schoenfeld P. Incidence of ischemic colitis and serious complications of constipation among patients using alosetron: systematic review of clinical trials and post-marketing surveillance data. *Am J Gastroenterol* 2006; **101**: 1069-1079 [PMID: 16606352 DOI: 10.1111/j.1572-0241.2006.00459.x]

13 **Mosli M**, Parfitt J, Gregor J. Retrospective analysis of disease association and outcome in histologically confirmed ischemic colitis. *J Dig Dis* 2013; **14**: 238-243 [PMID: 23419044 DOI: 10.1111/1751-2980.12045]

14 **Wheeldon NM**, Grundman MJ. Ischaemic colitis as a complication of colonoscopy. *BMJ* 1990; **301**: 1080-1081 [PMID: 2249072 DOI: 10.1136/bmj.301.6760.1080]

15 **Gray**. Superior Mesenteric Artery, Case courtesy of Associate Professor Craig Hacking, Radiopaedia.org, rID: 54523. Available from: https://upload.wikimedia.org/wikipedia/commons/6/62/Gray534.png

16 **Cappell MS**. Intestinal (mesenteric) vasculopathy. I. Acute superior mesenteric arteriopathy and venopathy. *Gastroenterol Clin North Am* 1998; **27**: 783-825, vi [PMID: 9890114 DOI: 10.1016/s0889-8553(05)70033-9]

17 **Geboes K**, Geboes KP, Maleux G. Vascular anatomy of the gastrointestinal tract. *Best Pract Res Clin Gastroenterol* 2001; **15**: 1-14 [PMID: 11355897 DOI: 10.1053/bega.2000.0152]

18 **Rosenblum JD**, Boyle CM, Schwartz LB. The mesenteric circulation. Anatomy and physiology. *Surg Clin North Am* 1997; **77**: 289-306 [PMID: 9146713 DOI: 10.1016/s0039-6109(05)70549-1]

19 **Lee SH**, Park YK, Lee DJ, Kim KM. Colonoscopy procedural skills and training for new beginners. *World J Gastroenterol* 2014; **20**: 16984-16995 [PMID: 25493011 DOI: 10.3748/wjg.v20.i45.16984]

20 **Lin JS**, Piper MA, Perdue LA, Rutter CM, Webber EM, O'Connor E, Smith N, Whitlock EP. Screening for Colorectal Cancer: Updated Evidence Report and Systematic Review for the US Preventive Services Task Force. *JAMA* 2016; **315**: 2576-2594 [PMID: 27305422 DOI: 10.1001/jama.2016.3332]

21 **Kim SY**, Kim HS, Park HJ. Adverse events related to colonoscopy: Global trends and future

challenges. *World J Gastroenterol* 2019; **25**: 190-204 [PMID: 30670909 DOI: 10.3748/wjg.v25.i2.190]
22 **Zizzo M**, Castro Ruiz C, Ugoletti L, Giunta A, Bonacini S, Manzini L, Aguzzoli F, Colognesi A, Pedrazzoli C. Transmural Colonic Infarction after Routine Colonoscopy in a Young Patient without Risk Factors. *Case Rep Gastroenterol* 2016; **10**: 479-488 [PMID: 27721736 DOI: 10.1159/000448884]
23 **Versaci A**, Macrì A, Scuderi G, Bartolone S, Familiari L, Lupattelli T, Famulari C. Ischemic colitis following colonoscopy in a systemic lupus erythematosus patient: report of a case. *Dis Colon Rectum* 2005; **48**: 866-869 [PMID: 15750796 DOI: 10.1007/s10350-004-0837-z]
24 **Sanchez-Aldehuelo R**, Martinez-Ortega A, García García de Paredes A, Martin-Mateos RM. An infrequent cause of abdominal pain following a colonoscopy. *Dig Liver Dis* 2019; **51**: 1346 [PMID: 31239215 DOI: 10.1016/j.dld.2019.05.026]
25 **Nozawa H**, Akiyama Y, Sunaga S, Tsurita G. Ischemic colitis following colonoscopy in an elderly patient on cardiovascular medication. *Endoscopy* 2007; **39** Suppl 1: E344-E345 [PMID: 18273789 DOI: 10.1055/s-2007-995329]
26 **Lee SO**, Kim SH, Jung SH, Park CW, Lee MJ, Lee JA, Koo HC, Kim A, Han HY, Kang DW. Colonoscopy-induced ischemic colitis in patients without risk factors. *World J Gastroenterol* 2014; **20**: 3698-3702 [PMID: 24707156 DOI: 10.3748/wjg.v20.i13.3698]
27 **van Dijk LJ**, van Noord D, de Vries AC, Kolkman JJ, Geelkerken RH, Verhagen HJ, Moelker A, Bruno MJ. Clinical management of chronic mesenteric ischemia. *United European Gastroenterol J* 2019; **7**: 179-188 [PMID: 31080602 DOI: 10.1177/2050640618817698]
28 **Roobottom CA**, Dubbins PA. Significant disease of the celiac and superior mesenteric arteries in asymptomatic patients: predictive value of Doppler sonography. *AJR Am J Roentgenol* 1993; **161**: 985-988 [PMID: 8273642 DOI: 10.2214/ajr.161.5.8273642]
29 **Li KC**, Whitney WS, McDonnell CH, Fredrickson JO, Pelc NJ, Dalman RL, Jeffrey RB Jr. Chronic mesenteric ischemia: evaluation with phase-contrast cine MR imaging. *Radiology* 1994; **190**: 175-179 [PMID: 8259400 DOI: 10.1148/radiology.190.1.8259400]
30 **Burkart DJ**, Johnson CD, Reading CC, Ehman RL. MR measurements of mesenteric venous flow: prospective evaluation in healthy volunteers and patients with suspected chronic mesenteric ischemia. *Radiology* 1995; **194**: 801-806 [PMID: 7862982 DOI: 10.1148/radiology.194.3.7862982]
31 **England RJ**, Woodcock B, Zeiderman MR. Superior mesenteric artery thrombosis in a patient with the antiphospholipid syndrome. *Eur J Vasc Endovasc Surg* 1995; **10**: 372-373 [PMID: 7552543 DOI: 10.1016/s1078-5884(05)80061-9]
32 **Hassan C**, East J, Radaelli F, Spada C, Benamouzig R, Bisschops R, Bretthauer M, Dekker E, Dinis-Ribeiro M, Ferlitsch M, Fuccio L, Awadie H, Gralnek I, Jover R, Kaminski MF, Pellisé M, Triantafyllou K, Vanella G, Mangas-Sanjuan C, Frazzoni L, Van Hooft JE, Dumonceau JM. Bowel preparation for colonoscopy: European Society of Gastrointestinal Endoscopy (ESGE) Guideline - Update 2019. *Endoscopy* 2019; **51**: 775-794 [PMID: 31295746 DOI: 10.1055/a-0959-0505]
33 **Anastassopoulos K**, Farraye FA, Knight T, Colman S, Cleveland MV, Pelham RW. A Comparative Study of Treatment-Emergent Adverse Events Following Use of Common Bowel Preparations Among a Colonoscopy Screening Population: Results from a Post-Marketing Observational Study. *Dig Dis Sci* 2016; **61**: 2993-3006 [PMID: 27278957 DOI: 10.1007/s10620-016-4214-2]
34 **Dabaja A**, Dabaja A, Abbas M. Polyethylene Glycol. In: StatPearls. Treasure Island (FL): StatPearls Publishing, 2021
35 **Ishii R**, Sakai E, Nakajima K, Matsuhashi N, Ohata K. Non-occlusive mesenteric ischemia induced by a polyethylene glycol with ascorbate-based colonic bowel preparation. *Clin J Gastroenterol* 2019; **12**: 403-406 [PMID: 30937697 DOI: 10.1007/s12328-019-00970-2]
36 **Bielefeldt K**. Ischemic Colitis as a Complication of Medication Use: An Analysis of the Federal Adverse Event Reporting System. *Dig Dis Sci* 2016; **61**: 2655-2665 [PMID: 27073073 DOI: 10.1007/s10620-016-4162-x]
37 **Sharma VK**, Nguyen CC, Crowell MD, Lieberman DA, de Garmo P, Fleischer DE. A national study of cardiopulmonary unplanned events after GI endoscopy. *Gastrointest Endosc* 2007; **66**: 27-34 [PMID: 17591470 DOI: 10.1016/j.gie.2006.12.040]
38 **Kim YH**, Kim JW, Lee KL, Joo SK, Lee J, Koh SJ, Kim BG, Park CK. Effect of midazolam on cardiopulmonary function during colonoscopy with conscious sedation. *Dig Endosc* 2014; **26**: 417-423 [PMID: 24164632 DOI: 10.1111/den.12189]
39 **Wernli KJ**, Brenner AT, Rutter CM, Inadomi JM. Risks Associated With Anesthesia Services During Colonoscopy. *Gastroenterology* 2016; **150**: 888-94; quiz e18 [PMID: 26709032 DOI: 10.1053/j.gastro.2015.12.018]
40 **Cadoni S**, Leung FW. Water-Assisted Colonoscopy. *Curr Treat Options Gastroenterol* 2017; **15**: 135-154 [PMID: 28205108 DOI: 10.1007/s11938-017-0119-1]
41 **Duncan JE**, McNally MP, Sweeney WB, Gentry AB, Barlow DS, Jensen DW, Cash BD. CT colonography predictably overestimates colonic length and distance to polyps compared with optical colonoscopy. *AJR Am J Roentgenol* 2009; **193**: 1291-1295 [PMID: 19843744 DOI: 10.2214/AJR.09.2365]
42 **Boley SJ**, Agrawal GP, Warren AR, Veith FJ, Levowitz BS, Treiber W, Dougherty J, Schwartz SS, Gliedman ML. Pathophysiologic effects of bowel distention on intestinal blood flow. *Am J Surg* 1969; **117**: 228-234 [PMID: 5773936 DOI: 10.1016/0002-9610(69)90308-0]
43 **GE Technology Assessment Committee**. Maple JT, Banerjee S, Barth BA, Bhat YM, Desilets DJ,

Gottlieb KT, Pfau PR, Pleskow DK, Siddiqui UD, Tokar JL, Wang A, Song LM, Rodriguez SA. Methods of luminal distention for colonoscopy. *Gastrointest Endosc* 2013; **77**: 519-525 [PMID: 23415258 DOI: 10.1016/j.gie.2012.09.025]

44 **Kozarek RA**, Earnest DL, Silverstein ME, Smith RG. Air-pressure-induced colon injury during diagnostic colonoscopy. *Gastroenterology* 1980; **78**: 7-14 [PMID: 7350038 DOI: 10.1016/0016-5085(80)90185-7]

45 **Friedman KB**, Cohen B, DePalo LR, Aisenberg J. Stirring the embers: mesenteric panniculitis provoked by colonoscopy. *Endoscopy* 2015; **47** Suppl 1 UCTN: E470 [PMID: 26465189 DOI: 10.1055/s-0034-1392864]

46 **Saunders BP**, Halligan S, Jobling C, Fukumoto M, Moussa ME, Williams CB, Bartram CI. Can barium enema indicate when colonoscopy will be difficult? *Clin Radiol* 1995; **50**: 318-321 [PMID: 7743720 DOI: 10.1016/s0009-9260(05)83424-5]

47 **Phillips M**, Patel A, Meredith P, Will O, Brassett C. Segmental colonic length and mobility. *Ann R Coll Surg Engl* 2015; **97**: 439-444 [PMID: 26274737 DOI: 10.1308/003588415X14181254790527]

48 **Choi YJ**, Park JS, Kim GE, Han JY, Nah SY, Bang BW. Mesocolon laceration following colonoscopy. *Korean J Gastroenterol* 2014; **63**: 313-315 [PMID: 24870304 DOI: 10.4166/kjg.2014.63.5.313]

49 **Andrade EG**, Olufajo OA, Drew EL, Bochicchio GV, Punch LJ. Blunt splenic injury during colonoscopy: Is it as rare as we think? *Am J Surg* 2018; **215**: 1042-1045 [PMID: 29776642 DOI: 10.1016/j.amjsurg.2018.05.010]

50 **Zhang AN**, Sherigar JM, Guss D, Mohanty SR. A delayed presentation of splenic laceration and hemoperitoneum following an elective colonoscopy: A rare complication with uncertain risk factors. *SAGE Open Med Case Rep* 2018; **6**: 2050313X18791069 [PMID: 30083323 DOI: 10.1177/2050313X18791069]

51 **Jehangir A**, Bennett KM, Rettew AC, Fadahunsi O, Shaikh B, Donato A. Post-polypectomy electrocoagulation syndrome: a rare cause of acute abdominal pain. *J Community Hosp Intern Med Perspect* 2015; **5**: 29147 [PMID: 26486121 DOI: 10.3402/jchimp.v5.29147]

52 **Cruz C**, Abujudeh HH, Nazarian RM, Thrall JH. Ischemic colitis: spectrum of CT findings, sites of involvement and severity. *Emerg Radiol* 2015; **22**: 357-365 [PMID: 25732355 DOI: 10.1007/s10140-015-1304-y]

53 **Parikh MP**, Satiya J, Berger-Saunderson M, Gupta NM, Sanaka MR. The Colonic Single Stripe Sign: A Case of Ischemic Colitis. *Cureus* 2019; **11**: e4622 [PMID: 31281764 DOI: 10.7759/cureus.4622]

54 **Zuckerman GR**, Prakash C, Merriman RB, Sawhney MS, DeSchryver-Kecskemeti K, Clouse RE. The colon single-stripe sign and its relationship to ischemic colitis. *Am J Gastroenterol* 2003; **98**: 2018-2022 [PMID: 14499781 DOI: 10.1111/j.1572-0241.2003.07633.x]

55 **Cheema U**, Ali A. Right colon blanching: endoscopic epiphany for mesenteric ischemia. *Dig Endosc* 2012; **24**: 391 [PMID: 22925307 DOI: 10.1111/j.1443-1661.2012.01304.x]

56 **Ho SB**, Hovsepians R, Gupta S. Optimal Bowel Cleansing for Colonoscopy in the Elderly Patient. *Drugs Aging* 2017; **34**: 163-172 [PMID: 28214970 DOI: 10.1007/s40266-017-0436-z]

57 **Lichtenstein GR**, Cohen LB, Uribarri J. Review article: Bowel preparation for colonoscopy--the importance of adequate hydration. *Aliment Pharmacol Ther* 2007; **26**: 633-641 [PMID: 17697197 DOI: 10.1111/j.1365-2036.2007.03406.x]

58 **Ajani S**, Hurt RT, Teeters DA, Bellmore LR. Ischaemic colitis associated with oral contraceptive and bisacodyl use. *BMJ Case Rep* 2012; **2012** [PMID: 22843752 DOI: 10.1136/bcr-12-2011-5451]

59 **American Association for the Study of Liver Diseases**; American College of Gastroenterology; American Gastroenterological Association Institute; American Society for Gastrointestinal Endoscopy; Society for Gastroenterology Nurses and Associates, Vargo JJ, DeLegge MH, Feld AD, Gerstenberger PD, Kwo PY, Lightdale JR, Nuccio S, Rex DK, Schiller LR. Multisociety sedation curriculum for gastrointestinal endoscopy. *Gastroenterology* 2012; **143**: e18-e41 [PMID: 22624720 DOI: 10.1053/j.gastro.2012.05.001]

60 **Tang DM**, Simmons K, Friedenberg FK. Anti-hypertensive therapy and risk factors associated with hypotension during colonoscopy under conscious sedation. *J Gastrointestin Liver Dis* 2012; **21**: 165-170 [PMID: 22720305]

61 **Leslie K**, Tay T, Neo E. Intravenous fluid to prevent hypotension in patients undergoing elective colonoscopy. *Anaesth Intensive Care* 2006; **34**: 316-321 [PMID: 16802483 DOI: 10.1177/0310057X0603400314]

62 **Brandt LJ**, Boley SJ, Sammartano R. Carbon dioxide and room air insufflation of the colon. Effects on colonic blood flow and intraluminal pressure in the dog. *Gastrointest Endosc* 1986; **32**: 324-329 [PMID: 3095168 DOI: 10.1016/s0016-5107(86)71876-2]

63 **Sajid MS**, Caswell J, Bhatti MI, Sains P, Baig MK, Miles WF. Carbon dioxide insufflation vs conventional air insufflation for colonoscopy: a systematic review and meta-analysis of published randomized controlled trials. *Colorectal Dis* 2015; **17**: 111-123 [PMID: 25393051 DOI: 10.1111/codi.12837]

64 **Serrani M**, Lisotti A, Spada A, Sferrazza S, Calvanese C, Fusaroli P. $CO_2$ vs. air insufflation in endoscopic ultrasonography: a prospective study. *Endosc Int Open* 2019; **7**: E317-E321 [PMID: 30834290 DOI: 10.1055/a-0809-4912]

65 **Rogers AC**, Van De Hoef D, Sahebally SM, Winter DC. A meta-analysis of carbon dioxide versus room air insufflation on patient comfort and key performance indicators at colonoscopy. *Int J Colorectal Dis* 2020; **35**: 455-464 [PMID: 31900583 DOI: 10.1007/s00384-019-03470-4]

66 **Yasumasa K**, Nakajima K, Endo S, Ito T, Matsuda H, Nishida T. Carbon dioxide insufflation attenuates parietal blood flow obstruction in distended colon: potential advantages of carbon dioxide insufflated colonoscopy. *Surg Endosc* 2006; **20**: 587-594 [PMID: 16437273 DOI: 10.1007/s00464-005-0252-0]

67 **Maida M**, Sferrazza S, Murino A, Lisotti A, Lazaridis N, Vitello A, Fusaroli P, de Pretis G, Sinagra E. Effectiveness and safety of underwater techniques in gastrointestinal endoscopy: a comprehensive review of the literature. *Surg Endosc* 2021; **35**: 37-51 [PMID: 32856154 DOI: 10.1007/s00464-020-07907-8]

68 **Siau K**, Beintaris I. My approach to water-assisted colonoscopy. *Frontline Gastroenterol* 2019; **10**: 194-197 [PMID: 31205663 DOI: 10.1136/flgastro-2018-101143]

69 **Cheng YC**, Wu CC, Lee CC, Lee TY, Hsiao KC. Rare complication following screening colonoscopy: ischemic colitis. *Dig Endosc* 2012; **24**: 379 [PMID: 22925295 DOI: 10.1111/j.1443-1661.2011.01231.x]

World Journal of Gastroenterology

Submit a Manuscript: https://www.f6publishing.com

*World J Gastroenterol* 2021 November 14; 27(42): 7311-7323

DOI: 10.3748/wjg.v27.i42.7311

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

ORIGINAL ARTICLE

Basic Study

# *Fusobacterium nucleatum* colonization is associated with decreased survival of *helicobacter pylori*-positive gastric cancer patients

Yung-Yu Hsieh, Shui-Yi Tung, Hung-Yu Pan, Te-Sheng Chang, Kuo-Liang Wei, Wei-Ming Chen, Yi-Fang Deng, Chung-Kuang Lu, Yu-Hsuan Lai, Cheng-Shyong Wu, Chin Li

**ORCID number:** Yung-Yu Hsieh 0000-0001-8465-5204; Shui-Yi Tung 0000-0002-8399-4754; Hung-Yu Pan 0000-0002-4036-3047; Te-Sheng Chang 0000-0002-1581-6948; Kuo-Liang Wei 0000-0002-7209-5411; Wei-Ming Chen 0000-0002-4949-393X; Yi-Fang Deng 0000-0003-0093-4228; Chung-Kuang Lu 0000-0002-3688-5002; Yu-Hsuan Lai 0000-0002-8576-2236; Cheng-Shyong Wu 0000-0002-5917-2400; Chin Li 0000-0002-5457-9623.

**Author contributions:** Hsieh YY and Tung SY contributed equally to this work; Hsieh YY, Tung SY, Chang TS, Wei KL, Chen WM, and Wu CS recruited patients for the study and collected biopsies; Deng YF performed *in vitro* and sequencing experiments; Lai YH and Pan HY performed data and statistical analyses; Wu CS and Li C designed and supervised the study; Li C wrote the manuscript; All authors reviewed the manuscript.

**Supported by** Chiayi Chang Gung Memorial Hospital grants, including No. CORPG6G0021, No. CORPG6G0022 and No. CORPG6G0023 to Wu CS; No. CORPG6G0061, No. CORPG6G0062 and No. CORPG6G0063 to Tung SY; No. CORPG6G0071, No.

**Yung-Yu Hsieh, Shui-Yi Tung, Te-Sheng Chang, Kuo-Liang Wei, Wei-Ming Chen, Yi-Fang Deng, Chung-Kuang Lu, Yu-Hsuan Lai, Cheng-Shyong Wu,** Department of Gastroenterology and Hepatology, Chiayi Chang Gung Memorial Hospital, Chiayi 61301, Taiwan

**Yung-Yu Hsieh, Shui-Yi Tung, Te-Sheng Chang, Kuo-Liang Wei, Wei-Ming Chen, Cheng-Shyong Wu,** College of Medicine, Chang Gung University, Taoyuan 33302, Taiwan

**Hung-Yu Pan,** Department of Applied Mathematics, National Chiayi University, Chiayi 60035, Taiwan

**Chin Li,** Department of Biomedical Sciences, National Chung Cheng University, Chiayi 62130, Taiwan

**Corresponding author:** Chin Li, PhD, Associate Professor, Department of Biomedical Sciences, National Chung Cheng University, No. 168, Section 1, University Road, Minhsiung, Chiayi 62130, Taiwan. biocl@ccu.edu.tw

## Abstract

***BACKGROUND***

An increased amount of *Fusobacterium nucleatum* (*F. nucleatum*) is frequently detected in the gastric cancer-associated microbiota of the Taiwanese population. *F. nucleatum* is known to exert cytotoxic effects and play a role in the progression of colorectal cancer, though the impact of *F. nucleatum* colonization on gastric cancer cells and patient prognosis has not yet been examined.

***AIM***

To identify *F. nucleatum*-dependent molecular pathways in gastric cancer cells and to determine the impact of *F. nucleatum* on survival in gastric cancer.

***METHODS***

Coculture of *F. nucleatum* with a gastric cancer cell line was performed, and changes in gene expression were investigated. Genes with significant changes in expression were identified by RNA sequencing. Pathway analysis was carried out to determine deregulated cellular functions. A cohort of gastric cancer patients undergoing gastrectomy was recruited, and nested polymerase chain reaction was performed to detect the presence of *F. nucleatum* in resected cancer tissues. Statistical analysis was performed to determine whether *F. nucleatum* colonization

CORPG6G0072 and No. CORPG6G0073 to Hsieh YY.

**Institutional review board statement:** All data acquisition and use of clinical specimens in this study were performed in accordance with the Declaration of Helsinki. Resected gastric cancer specimens were obtained from Tissue Bank, Department of Medical Research, Chang Gung Memorial Hospital at Chiayi. All the patients participating in this study were informed about the study and signed a written informed consent.

**Conflict-of-interest statement:** The authors declare that they have no competing interests.

**Data sharing statement:** Participants gave informed consent for data sharing.



**Specialty type:** Gastroenterology and hepatology

**Country/Territory of origin:** Taiwan

**Peer-review report's scientific quality classification**

Grade A (Excellent): 0
Grade B (Very good): B
Grade C (Good): C
Grade D (Fair): 0
Grade E (Poor): 0

**Received:** April 11, 2021
**Peer-review started:** April 11, 2021
**First decision:** May 24, 2021
**Revised:** May 31, 2021
**Accepted:** September 14, 2021

affects patient survival.

***RESULTS***

RNA sequencing and subsequent pathway analysis revealed a drastic interferon response induced by a high colonization load. This response peaked within 24 h and subsided after 72 h of incubation. In contrast, deregulation of actin and its regulators was observed during prolonged incubation under a low colonization load, likely altering the mobility of gastric cancer cells. According to the clinical specimen analysis, approximately one-third of the gastric cancer patients were positive for *F. nucleatum*, and statistical analysis indicated that the risk for colonization increases in late-stage cancer patients. Survival analysis demonstrated that *F. nucleatum* colonization was associated with poorer outcomes among patients also positive for *Helicobacter pylori* (*H. pylori*).

***CONCLUSION***

*F. nucleatum* colonization leads to deregulation of actin dynamics and likely changes cancer cell mobility. Cohort analysis demonstrated that *F. nucleatum* colonization leads to poorer prognosis in *H. pylori*-positive patients with late-stage gastric cancer. Hence, combined colonization of *F. nucleatum* and *H. pylori* is a predictive biomarker for poorer survival in late-stage gastric cancer patients treated with gastrectomy.

**Key Words:** *Fusobacterium nucleatum*; *Helicobacter pylori*; Gastric cancer; Survival; Interferon; Mobility



**Core Tip:** *Fusobacterium nucleatum* (*F. nucleatum*) is frequently enriched in the gastric cancer-associated microbiota. Here, we showed that *F. nucleatum* solicits a rapid but transient interferon response from gastric cancer cells. In addition, *F. nucleatum* leads to deregulation of the genes functioning in regulation of actin filament dynamics, likely changing cell mobility. In a patient cohort receiving gastrectomy, combined infection of *F. nucleatum* and *Helicobacter pylori* infection incurs poorer survival, indicating these two pathogens act synergistically to promote invasiveness of gastric cancer. Our finding suggests that *F. nucleatum* is a biomarker as treatment outcome predicator.

**Citation:** Hsieh YY, Tung SY, Pan HY, Chang TS, Wei KL, Chen WM, Deng YF, Lu CK, Lai YH, Wu CS, Li C. *Fusobacterium nucleatum* colonization is associated with decreased survival of *helicobacter pylori*-positive gastric cancer patients. *World J Gastroenterol* 2021; 27(42): 7311-7323
**URL:** https://www.wjgnet.com/1007-9327/full/v27/i42/7311.htm
**DOI:** https://dx.doi.org/10.3748/wjg.v27.i42.7311

## INTRODUCTION

Disruption of the normal gastrointestinal flora is often associated with pathogenic conditions. In general, the extreme acidity and thick protective mucosa of the gastric environment limit the complexity and abundance of the microbiota and prevent direct gastric epithelium colonization by pathogenic microbes[1]. However, *Helicobacter pylori* (*H. pylori*), which is well recognized as a risk factor for gastric cancer, is able to penetrate the mucosa layer and establish long-term colonization[2]. Indeed, virulence factors produced by this bacterium facilitate the transformation of gastric mucosal cells and lead to a drastic increase in the risk of gastric cancer[2,3].

Although it is well established that *H. pylori* is more frequently found in gastric cancer patients than in noncancer controls[4], recent microbiota profiling studies have revealed that the abundance of *H. pylori* in the gastric microbiota is frequently decreased in gastric cancer patients compared with that in noncancer patients[5,6]. Our hypothesis to account for the decrease in *H. pylori* abundance in gastric cancer is microbial succession: Once colonization occurs, *H. pylori* creates a niche microenvir-

**Article in press:** September 14, 2021
**Published online:** November 14, 2021

**P-Reviewer:** Krzyżek P
**S-Editor:** Wu YXJ
**L-Editor:** Filipodia
**P-Editor:** Liu JH

onment on the gastric epithelium that facilitates the colonization of secondary settler bacteria. Accordingly, the predominance of *H. pylori* in the microenvironment can be replaced by other bacteria after a prolonged colonization period. It is possible that the secondary gastric microbes also participate in promoting the development of gastric cancer[7].

Advanced sequencing technology has enabled profiling of the microbiota without the need to isolate pure cultures, and in a previous study employing this experimental approach, we identified *Fusobacterium nucleatum* (*F. nucleatum*) as being enriched in the gastric cancer-associated microbiota. An increased presence of *F. nucleatum* in the colorectal cancer-associated microbiota has also been reported[8,9]. *F. nucleatum* colonization correlates with high microsatellite instability, disruption of the mismatch repair mechanism, and poor prognosis[10]. The genomic instability that is observed is likely mediated by the metabolites produced by *F. nucleatum*. One such metabolite is hydrogen sulfide, which has been shown to generate reactive oxygen species, induce DNA damage, and cause single-nucleotide mutations[11]. Hence, it is possible that *F. nucleatum* promotes oncogenesis by acting as a DNA-damaging agent. In fact, *F. nucleatum* has been shown to promote the growth and metastasis of colorectal cancer [12,13], and the level of *F. nucleatum* in the colorectal cancer-associated microbiota correlates with poor patient prognosis[14,15]. Therefore, *F. nucleatum* may be used as a prognostic biomarker for colorectal cancer.

Detection of *Fusobacterium* DNA using polymerase chain reaction (PCR) facilitates screening of colorectal cancer by increasing the sensitivity of the standard fecal immunochemical test[15]. Although the role of *F. nucleatum* in colorectal cancer has been intensively studied, it remains unclear whether this bacterium exerts a similar oncogenic effect on the gastric epithelium. In this study, a nested PCR-based method was developed to detect the presence of *F. nucleatum* in resected gastric cancer tissue specimens. Based on statistical analysis, we found that the risk of *F. nucleatum* colonization is greatly increased in patients with late-stage gastric cancer. Moreover, *F. nucleatum* colonization was associated with a poor prognosis in *H. pylori*-positive patients. Our findings suggest that invasion of *F. nucleatum* into the gastric cancer-associated microenvironment promotes gastric cancer aggressiveness and subsequently leads to poorer prognosis.

## MATERIALS AND METHODS

### *Coculturing of F. nucleatum with gastric cancer cells*

*F. nucleatum* strain ATCC25586[16] was obtained from Bioresource Collection and Research Center (Hsinchu, Taiwan). The bacteria were cultured on EG culture medium containing 2.5 g Lab-Lemco powder, 10 g proticasepeptone, 5 g yeast extract, 4 g glucose, 0.5 g starch, 0.2 g L-cystine, 0.5 g L-cysteine HCl, 4 g $Na_2HPO_4$, 15 g Bacto-Agar, and 50 mL defibrinated horse blood per liter under anaerobic conditions using a BD GasPak system (Thermo Fisher Scientific, Waltham, MA, United States). The bacteria were scrapped from a plate and resuspended in Dulbecco's Modified Eagle's Medium. The number of cells per millimeter in the resuspended medium was determined using light microscopy. The correlation between the observed cell number and colony forming units was determined by reculturing the bacteria after serial dilutions. The gastric cancer cell line 008L-C2 used in this study was originally isolated from resected gastric cancer tissue. The cells were cultured in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum. *F. nucleatum* strain ATCC25586 was cultured with 008L-C2 gastric cancer cells under anaerobic conditions at 37 °C and 5% $CO_2$ and harvested when the coculture experiment was performed. In coculture experiments, the initial multiplicity of infection (MOI) was 10 and 100. After 0, 24, and 72 hours of coculture with *F. nucleatum*, the cells were collected and washed twice with phosphate-buffered saline. Total RNA was extracted from the washed cells using TRIzol reagent (Thermo Fisher Scientific) according to the manufacturer's protocol, and RNA expression analysis was conducted using next-generation sequencing.

### *RNA sequencing*

The integrity and concentration of purified RNA samples were determined using capillary electrophoresis with a TapeStation 2200 instrument (Agilent, Santa Clara, CA, United States) and fluorometric analysis (Qubit fluorometer; Invitrogen, Waltham, MA, United States). Libraries for RNA sequencing analysis were prepared using a SureSelect strand-specific mRNA library preparation kit (Agilent); the manufacturer's

protocol was closely followed. The libraries were pooled and sequenced using a NextSeq 550 sequencer. Quality filtering, mapping, annotation, and calculation of gene expression levels were performed using CLC Genomic Workbench v.12.0.3 (Qiagen, Redwood City, CA, United States). The RNA level is expressed as transcripts per million. The sequencing data were deposited in the Sequence Read Archive, National Center for Biotechnology Information, United States. The BioProject ID is PRJNA-630089. The BioSample accession numbers are SAMN14823957, SAMN14823958, SAMN14823959, SAMN14823960, and SAMN14823961.

### Gastric cancer specimens and DNA extraction

Resected cancer tissues from patients undergoing gastrectomy were obtained from Chiayi Chang Gung Memorial Hospital Tissue Bank. The acquisition and use of clinical specimens in this study were carried out in accordance with the Declaration of Helsinki. This study was approved by the Institutional Review Board of Chiayi Chang Gung Memorial Hospital (Institutional Review Board approval No. 201700169A3, No. 201700172A3, and No. 201700173A3). Frozen biopsies were briefly rinsed in phosphate-buffered saline to remove extra mucus. The rinsed specimens were immersed overnight in RNAlater reagent (Thermo Fisher Scientific) and stored at -80 °C. The biopsies were pulverized in TRI reagent (Thermo Fisher Scientific) and centrifuged to remove undissolved debris. Total DNA, including both cellular and microbial DNA, was extracted according to the manufacturer's protocol, and the concentration of DNA was determined by fluorometric quantification.

### Detection of H. pylori and F. nucleatum

The tissue specimens were examined for the presence of *H. pylori* using a standard rapid urease test. The presence of *F. nucleatum* in the specimens was determined by nested PCR detection of the *NusG* gene. The forward and reverse primer sequences used for the first-stage PCR were 5′-TGTTAGAGGAAAGCCCAAGAAG and 5′-CTTCTTCCATAGGAATAGGGTCAG, respectively. The initial amplification cycle (denaturation) was as follows: 94 °C for 5 min; followed by 36 cycles at 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s and a final extension step at 72 °C for 5 min. The product of the first-stage amplification was directly used in the second-stage PCR. The forward and reverse primer sequences of the second-stage PCR were 5′-GCTTGAAATGGAAGCTACAAGAG and 5′-GGTCAGAACCAACTCCTACAAA, respectively. The second-stage PCR amplification cycle parameter was identical to that of the first-stage PCR. The sequence of the PCR product was determined by dideoxynucleotide sequencing to confirm that the amplified product is the *F. nucleatum NusG* target sequence. The PCR product was examined by nondenaturing 6% polyacrylamide gel electrophoresis and documented. Statistical analysis was carried out using SAS/STAT statistical analysis software v. 9.4 (College Station, TX, United States). Survival probability was calculated with Kaplan-Meier analysis.

## RESULTS

In our previous metagenomic analysis, we discovered that *F. nucleatum* colonizes and becomes enriched in the gastric cancer-associated microbiota[17]. Among the 11 cancer biopsies collected in that study, four showed *F. nucleatum* colonization, suggesting that *F. nucleatum* is a frequent cocolonizer among gastric cancer patients in the southwestern region of Taiwan. Moreover, clinical data confirmed that *F. nucleatum* colonization is frequently observed in gastric cancer patients. Nevertheless, the pathogenic effects of *F. nucleatum* have not been investigated in gastric cancer cells. Thus, to explore the role of *F. nucleatum* in gastric cancer, we examined gastric cancer cell growth using an *in vitro* coculture system using the gastric cancer cell line 008L-C2, derived from resected gastric cancer tissue. During the incubation period, an increase in *F. nucleatum* abundance was observed under a microscope, though we did not determine the precise doubling time of *F. nucleatum*.

RNA sequencing analysis revealed that the presence of *F. nucleatum* is associated with dose- and time-dependent changes in the gene expression profile of cancer cells. After 24 h of coculture with *F. nucleatum* at a low MOI, only a limited number of marginally expressed genes (transcripts per million < 1) exhibited more than four-fold changes (Figure 1). In contrast, the expression level of a specific set of genes was strongly upregulated at a high MOI (100) (Figure 1). After 72 h of coculture, low-MOI treatment led to a significantly higher number of genes with more than four-fold increases in expression (Figure 1). However, the number of strongly upregulated genes

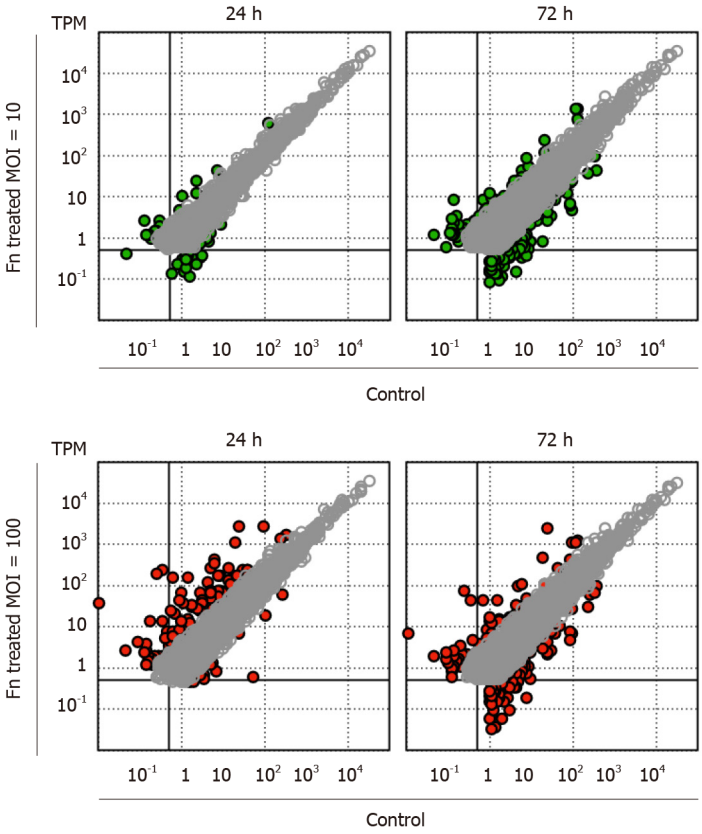


**Figure 1 Changes in the gene expression profile of 008L-C2 cancer cells cocultured with *Fusobacterium nucleatum*.** *Fusobacterium nucleatum* was added to the cell culture at multiplicity of infection (MOI) 10 and 100. After 24 h and 72 h of incubation, the cells were rinsed extensively to remove unattached bacteria. RNA was collected and analyzed using RNA sequencing. The cells collected at time 0 were used as control. Sequencing reads were trimmed and mapped to hg19 using CLC Genomic Workbench v.12.0.3. The colored dots (green for MOI = 10 and red for MOI = 100) are genes showing more than four-fold change at the respective incubation time. Genes with transcripts per million < 1 in all datasets and less than a four-fold change are not shown.

in high MOI-treated cells decreased after longer incubation, and there was an increased number of genes with more than four-fold decreased expression (Figure 1). Our results indicate a rapid and strong cellular response to a large amount of *F. nucleatum*. Additionally, *F. nucleatum* caused a prolonged change in gastric cancer cells.

Ontological analysis indicated that interferon and its response genes as well as inflammatory cytokines are immediately activated by high MOI treatment with *F. nucleatum*. The activated genes, including MX1, MX2, interferon induced protein (IFI)35, IFIT1, IFIT2, IFIT3, IFI44, IFI44L, IFITM1, IFITM3, IFIH1, and IFI6[18], are well-known interferon response genes. Furthermore, tripartite motif-containing 14[19,20], interferon -stimulated gene 15[21], ubiquitin specific peptidase 18[22], 2'-5'-oligoadenylate synthetase (OAS)1, OAS2, and OAS3[23,24] were found to be involved in interferon-dependent antiviral activity (Figure 2A and B). In addition to interferon response genes, simultaneous increases in interleukin (IL)6, IL8, IL32, C-X-C motif ligand (CXCL)1, CXCL2, and CXCL6 expression were observed in the high-MOI-treated cells after 24 h of exposure (Figure 2C). In contrast to the short-term activation profile observed for interleukins and chemokines, C-C ligand 2 remained highly expressed even after 72 h of exposure (Figure 2A). Not only genes involved in the regulation of immunological functions but also those related to cell mobility and adhesion were dysregulated by *F. nucleatum* colonization. For instance, epithelial-stromal interaction 1[25,26] and intercellular adhesion molecule 1[27], both of which are involved in cell-matrix interaction, were upregulated at 24 h and returned to basal levels after 72 h of incubation (Figure 2B). Other cytoskeleton and cell adhesion genes were activated in a distinct pattern, and actin alpha 2; actin, alpha cardiac muscle precursor 1; actin gamma 2, smooth muscle; calponin 1, endothelin 1, and cell migration inducing hyaluronan binding protein were continuously stimulated by *F. nucleatum* (Figure 2C). Increased expression of these genes was time dependent but not dose (MOI) dependent, indicating that chronic colonization with a small number of *F. nucleatum* bacteria may lead to long-term strong effects on cytoskeletal dynamics and cell mobility.

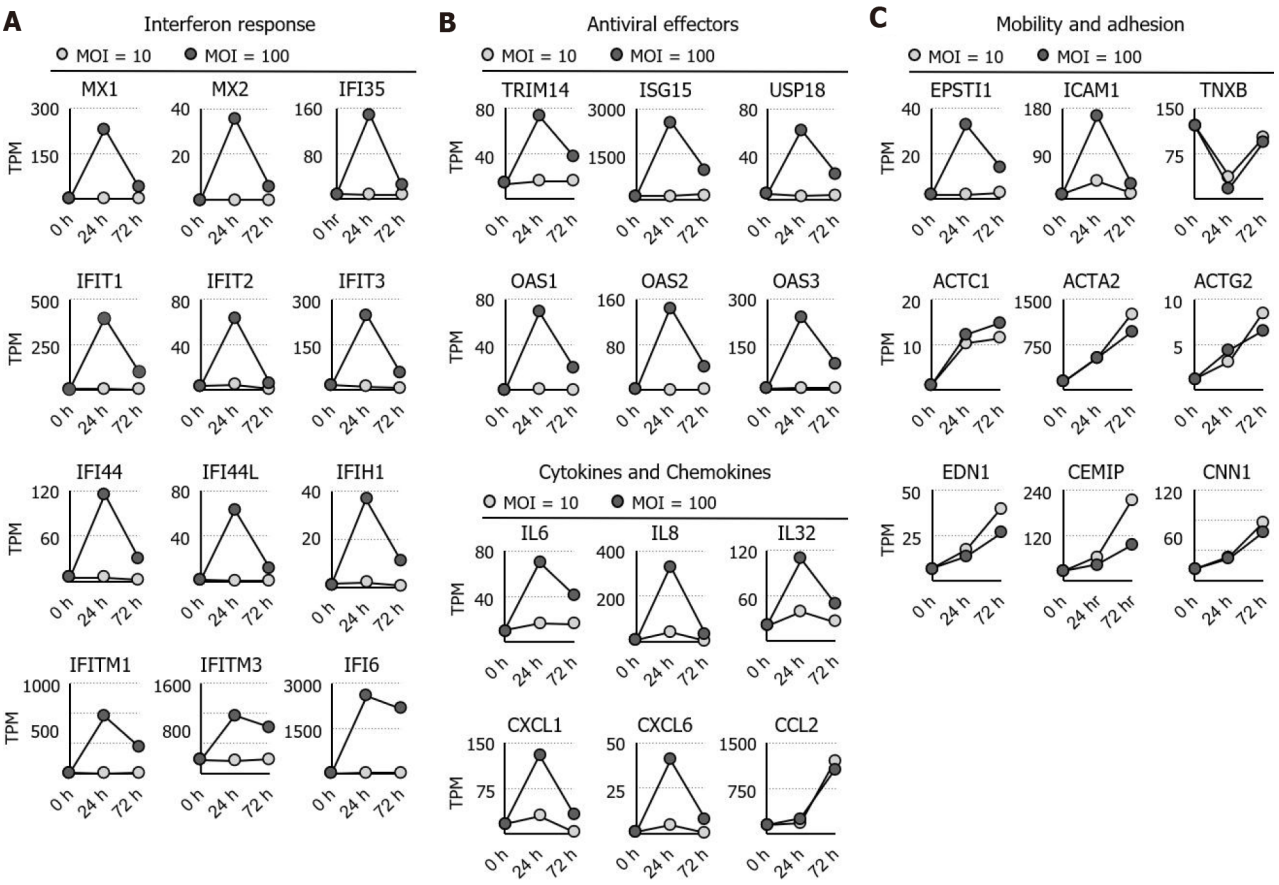


**Figure 2 Identification and ontological analysis of the RNA sequencing dataset.** A: The expression dynamics of the interferon response, cytokines, and chemokines genes are shown; B: The expression dynamic of the antiviral genes is shown; C: The expression dynamics of actin, its regulators, and genes involved in cell adhesion are shown. MOI: Multiplicity of infection; TPM: Transcripts per million.

Overall, our data show that coculturing gastric cancer cells with *F. nucleatum* results in a short-term immune response and continuous dysregulation of actin-related signaling *in vitro*. The change in actin cytoskeleton dynamics was associated with an increase in cell mobility, thereby promoting the invasiveness and metastasis of gastric cancer. Therefore, we investigated whether *F. nucleatum* colonization influences the survival of gastric cancer patients after gastrectomy. Gastric cancer progression is marked by tumor spread and distant metastasis, which are associated with increased cancer cell mobility. To test our hypothesis, we examined resected gastric cancer tissues from patients who underwent gastrectomy. The survival rate of these patients was 67%, which is higher than the 37% survival rate reported by Cancer Registry Annual Report, 2018, Taiwan. The clinical and pathological characteristics of the patient cohort are presented in Table 1.

To determine the presence of *F. nucleatum* in the specimens, we employed a nested PCR method with increased detection sensitivity and specificity. In a previous study utilizing conventional PCR, *F. nucleatum* was found at relatively low frequencies in gastric cancer specimens[28]. Our results obtained by metagenomic profiling indicated that the *F. nucleatum* frequency is much higher. The discrepancy could account for the higher frequency of *F. nucleatum* in the tested cohort, suggesting that the observed phenomenon is specific for this region of Taiwan. Nonetheless, the previously observed low frequency might be associated with methodological limitations resulting in the detection of lower amounts of *F. nucleatum*. This is a likely scenario, considering that resected cancer tissues may contain less surface mucosa. Nested PCR allowed for higher *F. nucleatum* detection sensitivity.

The detection target in this study was the highly conserved *NusG* gene of *F. nucleatum*[28,29]. The sizes of the first- and second-stage PCR products were expected to be 175 bp and 124 bp, respectively. The PCR product was resolved by acrylamide electrophoresis, and identity was confirmed by dideoxynucleotide sequencing. To validate the nested PCR protocol, we tested DNA specimens from our previous study. The method allowed us to identify the majority of *F. nucleatum*-positive specimens, except for those with an exceptionally low bacterial load. The positive identification

**Table 1 The clinicopathalogical characteristics of the study cohort**

| Sex | Age | Stage | TNM classification | Tumor size | *H. pylori* | *F. nucleatum* |
|---|---|---|---|---|---|---|
| M | 70 | IA | pT1aN0 | 1.2 cm × 0.5 cm | Positive | Positive |
| F | 78 | IA | pT1bN0 | 7 cm × 5 cm | Positive | Negative |
| F | 84 | IA | pT1aN0Mx | 2 cm × 2 cm | Positive | Negative |
| M | 73 | IA | pT1aN0Mx | 0.5 cm × 0.3 cm | Positive | Negative |
| M | 73 | IA | pT1aN0 | 1.5 cm × 0.3 cm | Positive | Negative |
| M | 73 | IA | pT1aN0MX | 6 cm × 6 cm | Negative | Negative |
| M | 64 | IA | pT1bN0MX | 9.5 cm × 1.5 cm | Negative | Negative |
| M | 67 | IA | pT1bN0 | 2.5 cm × 2 cm | Positive | Negative |
| M | 66 | IA | pT1bN0 | 0.8 cm × 0.5 cm | Positive | Negative |
| M | 65 | IA | pT1bN0 | 3 cm × 2.7 cm | Positive | Negative |
| F | 68 | IB | pT2N0Mx | 6 cm × 4 cm | Negative | Positive |
| M | 73 | IB | pT2N0Mx | 1.5 cm × 1.5 cm | Positive | Positive |
| M | 60 | IB | pT2N0 | 4.5 cm × 4 cm | Positive | Positive |
| F | 68 | IB | pT2N0 | 3 cm × 1.8 cm | Positive | Negative |
| F | 67 | IB | pT2N0 | 1.5 cm × 1.0 cm | Negative | Negative |
| M | 71 | IB | pT1bN1MX | 2 cm × 1.8 cm | Positive | Negative |
| M | 69 | IB | pT2N0 | 2.2 cm × 2.1 cm | Positive | Negative |
| F | 86 | IIA | pT3N0Mx | 5 cm × 4.5 cm | Positive | Positive |
| F | 87 | IIA | pT3N0Mx | 7.5 cm × 7.0 cm | Negative | Positive |
| M | 68 | IIA | pT3N0 | 4 cm × 3.5 cm | Positive | Positive |
| M | 58 | IIA | pT2N1 | 7 cm × 6 cm | Positive | Negative |
| M | 50 | IIA | pT2N1MX | 3.5 cm × 3.5 cm | Negative | Negative |
| M | 71 | IIA | pT3N0 | 1 cm × 1 cm | Positive | Negative |
| F | 69 | IIB | pT3N1 | 1.2 cm × 1.8 cm | Positive | Positive |
| M | 61 | IIB | pT4aN0 | 2 cm × 1.8 cm | Positive | Positive |
| M | 74 | IIB | pT2N2 | 4.5 cm × 2.0 cm | Positive | Negative |
| M | 64 | IIB | pT4aN0 | 2.6 cm × 2.0 cm | Positive | Negative |
| M | 75 | IIB | pT2N2 | 4.2 cm × 4.0 cm | Negative | Negative |
| M | 76 | IIIA | pT3N2MX | 5 cm × 4.5 cm | Positive | Positive |
| M | 79 | IIIA | pT3N2Mx | 3.5 cm × 3.5 cm | Negative | Negative |
| M | 51 | IIIA | pT4N1 | 8 cm × 6 cm | Positive | Negative |
| M | 63 | IIIA | pT3N2 | 3.2 cm × 2.2 cm | Negative | Negative |
| M | 87 | IIIB | pT3N3b | 6.5 cm × 5 cm | Positive | Positive |
| F | 64 | IIIB | pT3N3 | 7 cm × 5 cm | Positive | Negative |
| F | 65 | IIIB | pT4aN2 | 12 cm × 10 cm | Negative | Negative |
| F | 78 | IIIB | pT3N3b | 3 cm × 2 cm | Positive | Negative |
| M | 59 | IIIB | pT4bN1Mx | 8.5 cm × 7.5 cm | Positive | Negative |
| M | 58 | IIIB | pT3N3aMX | 3 cm × 3 cm | Negative | Negative |
| M | 87 | IIIB | pT4aN2 | 3 cm × 2 cm | Positive | Negative |
| M | 83 | IIIB | pT4aN2 | 6 cm × 5 cm | Negative | Negative |
| M | 69 | IIIB | pT4aN2 | 6 cm × 5 cm | Positive | Negative |

| | | | | | | |
|---|---|---|---|---|---|---|
| M | 65 | IIIB | pT3N3b | 5.5 cm × 4 cm | Positive | Negative |
| F | 73 | IIIC | pT4N3a | 4.5 cm × 4 cm | Positive | Positive |
| F | 74 | IIIC | pT4a N3b | 2.0 cm × 2.0 cm | Positive | Positive |
| M | 57 | IIIC | pT4bN2Mx | 4 cm × 4 cm | Positive | Positive |
| M | 67 | IIIC | pT4aN3b | 8 cm × 7.5 cm | Positive | Positive |
| F | 53 | IIIC | pT4bN3a | 6 cm × 5 cm | Negative | Negative |
| F | 72 | IIIC | pT4aN3a | 7 cm × 2 cm | Negative | Negative |
| F | 79 | IIIC | pT4aN3a | 4.5 cm × 2.5 cm | Negative | Negative |
| M | 52 | IIIC | pT4bN3aMX | 8 cm × 6 cm | Positive | Negative |
| M | 76 | IIIC | pT4aN3a | 2.0 cm × 1.8 cm | Positive | Negative |
| M | 71 | IIIC | pT4aN3a | 4.8 cm × 4.5 cm | Positive | Negative |
| F | 47 | IV | pT4aN3aM1 | 4.5 cm × 4.5 cm | Positive | Positive |
| M | 47 | IV | pT4bN3aM1 | 6 cm × 3 cm | Negative | Positive |
| M | 69 | IV | pT4N3M1 | 5 cm × 4.5 cm | Positive | Positive |
| M | 73 | IV | pT3N3bM1 | 12 cm × 10.5 cm | Positive | Positive |
| M | 75 | IV | pT1bN1M1 | 2.2 cm × 2.0 cm | Positive | Negative |
| M | 66 | IV | pT4aN3bM1b | 7 cm × 4 cm | Positive | Negative |
| M | 75 | IV | pT4bN3bM1 | 3 cm × 3 cm | Positive | Negative |
| M | 70 | IV | pT3N3aM1 | 9 cm × 8 cm × 2.5 cm | Negative | Negative |

M: Male; F: Female; TNM: Tumor, Node, Metastasis; *H. pylori*: *Helicobacter pylori*; *F. nucleatum*: *Fusobacterium nucleatum*.

rate was 90%, with no false-positives. Thus, we used nested PCR to assess the presence of *F. nucleatum* in the resected gastric cancer tissues used in this study (Figure 3A). The results showed 19 of the 60 examined specimens to be positive for *F. nucleatum*. The other 41 specimens were negative or showed only marginal PCR product levels, all of which were defined as *F. nucleatum* negative. Accordingly, the proportion of *F. nucleatum*-positive patients was approximately one-third of the cohort. The results are consistent with our previous study examining upper endoscopic-collected gastric biopsies.

The risk of *F. nucleatum* colonization was analyzed against clinical characteristics and cancer stages (Figure 3B), indicating that older age (≥ 75) is not associated with the risk of *F. nucleatum* colonization ($P$ value = 0.636). Although *H. pylori* colonization appears to be associated with a higher risk of *F. nucleatum* colonization, no statistical significance was obtained ($P$ value = 0.208). The risk of *F. nucleatum* colonization was increased in late-stage gastric cancer patients, suggesting that the microenvironment during cancer progression is more suitable for colonization and growth of *F. nucleatum* ($P$ value = 0.012). Interestingly, we found that the risk of *F. nucleatum* colonization was significantly decreased in male patients ($P$ value = 0.02), which may be associated with the lifestyle or hormonal status of the patients and requires confirmation in larger and/or independent gastric cancer patient cohorts.

We then analyzed the impact of *F. nucleatum* on the survival of gastric cancer patients. Although results showed that *F. nucleatum*-positive patients had a lower survival rate, this was not significant (Figure 4A). Stage I cancer patients with a nearly 100% 5-year survival rate were then excluded from the survival analysis, but significance was still not reached while poorer survival outcomes were maintained (Figure 4A). As noted above, patients who undergo gastrectomy have good treatment outcomes, and it is likely that oncogenic factors, including pathogenic gastric microbiota, are reduced after surgery. Despite the lack of statistical significance, our analysis shows that *F. nucleatum* colonization may adversely impact treatment outcome.

Approximately one-third of the patients in our cohort tested positive for *H. pylori* colonization, and our previous study demonstrated that *F. nucleatum* is likely a secondary settler of the gastric microbiota after *H. pylori* colonizes the gastric epithelium. Consecutive or simultaneous colonization of *H. pylori* and *F. nucleatum*

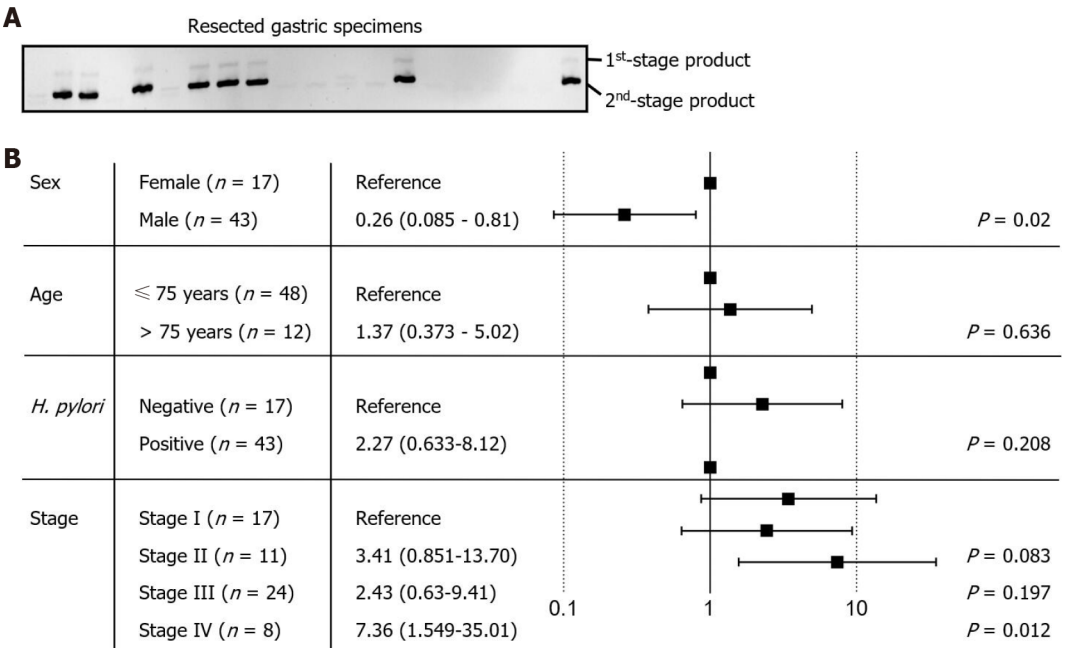


**Figure 3 Identification of *Fusobacterium nucleatum* in the resected gastric cancer tissues.** A: *Fusobacterium nucleatum* (*F. nucleatum*) was detected by amplification of the conserved *NusG* gene sequence using nested polymerase chain reaction method. The end point product was analyzed using 6% nondenaturing polyacrylamide gel electrophoresis; B: The relative risk of *F. nucleatum* colonization was analyzed against the sex, age, status of *Helicobacter pylori* colonization, and cancer stage. Data analysis is shown as Forest plot.

might have synergistic effects and promote cancer progression. To test this hypothesis, we analyzed the impact of *F. nucleatum* on the survival of *H. pylori*-positive patients. Our data demonstrate that patients positive for both *H. pylori* and *F. nucleatum* had a poorer survival outcome than those who were positive for *H. pylori* alone (Figure 4B). Similar results were observed when stage I cancer patients were excluded from the analysis (Figure 4B). Therefore, the presence of *F. nucleatum* colonization negatively impacts gastric cancer prognosis. Our analysis confirms that *F. nucleatum* colonization is frequent in patients with advanced-stage gastric cancer, with an unfavorable effect on patient survival.

## DISCUSSION

*F. nucleatum* is an opportunistic pathogen mainly residing in the oral cavity[30]. Although it is likely that oral-resident microbes are regularly passed to the stomach through dietary intake, they are unable to colonize the rest of the gastric microenvironment under normal physiological conditions. However, *H. pylori* colonization and the growth of carcinoma cells create a suitable microenvironment[31,32] and likely allow *F. nucleatum* invasion. Our previous study, which utilized 16S metagenomic analysis, showed that *F. nucleatum* can be present in the gastric cancer-associated microbiota[17]. In the present study, we employed nested PCR to determine the existence of *F. nucleatum* in resected gastric cancer tissues. The results confirmed our previous finding, showing *F. nucleatum* colonization in approximately one-third of gastric cancer patients in southwestern Taiwan. Whether *F. nucleatum* colonization is common remains to be investigated.

Analysis of our *in vitro* coculture experiment indicated that *F. nucleatum* evokes two distinct cellular responses. Based on dosage dependence and expression pattern, these two responses are likely activated through independent signaling pathways. One is an immediate response that peaked at 24 h to 48 h of incubation and declined to a near-unstimulated level after 72 h. This immediate response was induced only by a high amount of *F. nucleatum* and marked by activation of interferon response genes, antiviral genes, cytokines, and chemokines. Other inflammation-inducing pathogens, such as *H. pylori*, may collaborate with *F. nucleatum* to sustain expression of these genes. The second response involved activation of actin and genes that regulate cell mobility. High expression of these gene products has multiple effects in promoting cancer progression, especially in increasing cell mobility and promoting distant

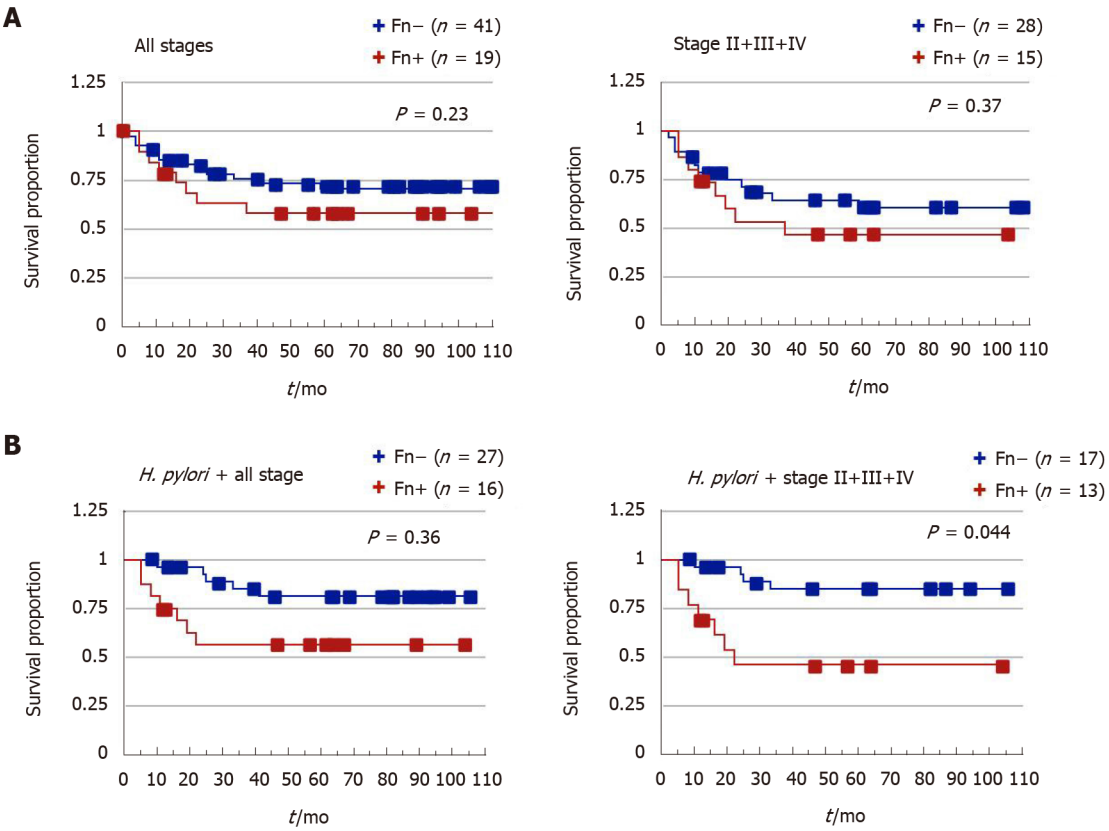


**Figure 4 Analysis of *Fusobacterium nucleatum* colonization in gastric cancer patients.** A: The study cohort was divided into *Helicobacter pylori* (*H. pylori*)-positive and negative groups according to the result of nested-polymerase chain reaction analysis. Survival probability was calculated using Kaplan-Meier analysis in all or stage II, III, and IV cancer patients with at least 1-year follow-up; B: Survival probability was calculated in stages II, III, and IV cancer patients.

metastasis[33-34]. Overall, our experiment demonstrates that colonization by *F. nucleatum* activates specific signaling pathways and promotes aggressiveness in gastric cancer cells.

After determining the prevalence of *F. nucleatum* colonization in gastric cancer patients, we evaluated its clinical impact. All enrolled patients underwent gastrectomy, in which the main cancerous lesions were completely removed. Gastrectomy also results in extraction of cancer-associated microbiota, minimizing the negative impact of pathogenic microbes. The average 5-year survival in our study cohort was approximately 60%, indicating the effectiveness of treatment. Notably, survival analysis showed that *F. nucleatum* colonization negatively impacts the survival outcome of *H. pylori*-positive patients. It is possible that *F. nucleatum* sequentially and/or synergistically cooperates with *H. pylori* to promote gastric cancer progression. Our analysis indicates a complex interaction between multiple pathogens and gastric cancer cells. Moreover, our data suggest that *F. nucleatum* colonization may serve as a prognostic biomarker for *H. pylori*-positive patients.

Our experimental findings show that *F. nucleatum* colonization in the gastric microbiota is a common event in gastric cancer patients. The risk of colonization appears to increase toward the later stages of gastric cancer progression. Cocolonization of *F. nucleatum* with *H. pylori* results in poorer survival than that observed for patients with *H. pylori* colonization alone. As the majority of gastric microbiota components are presumably removed during gastrectomy, preliminary exposure to *F. nucleatum* may exert a long-term impact on the aggressiveness of cancer cells. Our findings indicate that *F. nucleatum* may precondition and promote gastric cancer progression.

## CONCLUSION

In this study, we found that *F. nucleatum* is able to alter actin filament dynamics to promote cell mobility. Additionally, the prevalence of *F. nucleatum* colonization in gastric cancer tissues is much higher than previously thought. Importantly, cocolon-

ization of *F. nucleatum* with *H. pylori* was found to lead to reduced survival in gastric cancer patients. Our study suggests that *F. nucleatum* increases the aggressiveness of gastric cancer and negatively impacts the prognosis of gastric cancer patients.

## ARTICLE HIGHLIGHTS

### Research background

Our previous research identified *Fusobacterium nucleatum* (*F. nucleatum*) as an opportunistic pathogen frequently found in the gastric cancer-associated microbiota. *F. nucleatum* has been demonstrated to promote carcinogenesis and metastasis of colorectal cancer. However, the role of *F. nucleatum* in gastric cancer remains unclear.

### Research motivation

It is our goal to determine the impact of *F. nucleatum* colonization to progression of gastric cancer.

### Research objectives

The objective of current study is to identify the impact of *F. nucleatum* to the cellular function of gastric cancers and to the prognosis of gastric cancer patients.

### Research methods

*F. nucleatum*-induced expression change of a patient-derived gastric cancer cell line was profiled by RNA sequencing and ontological analysis. The presence of *F. nucleatum* in patients' tumor tissue was determined by nested polymerase chain reaction. Statistical analysis of *F. nucleatum* colonization status was performed to determine the correlation with clinical characterization and patients' survival.

### Research results

*F. nucleatum* induces a drastic but temporary interferon response and prolonged deregulation of actin and its regulators from gastric cancer cells. A survey of clinical specimens showed that approximately one-third of gastric cancer patients are positive for *F. nucleatum*. Survival analysis showed that the combined colonization of *Helicobacter pylori* (*H. pylori*) and *F. nucleatum* leads to poorer survival of late-stage patients.

### Research conclusions

The actin filament dynamic change caused by *F. nucleatum* colonization likely promotes cell mobility and cancer metastasis. This observation is correlated with the finding that *F. nucleatum* colonization leads to poor survival of *H. pylori*-positive late-stage patients.

### Research perspectives

*F. nucleatum* colonization leads to poorer survival of gastrectomy-received patients. Our findings indicate the importance of tumor-associated microbiota to the progression of gastric cancer.

## REFERENCES


1 **Shah MA**. Gastric cancer: The gastric microbiota - bacterial diversity and implications. *Nat Rev Gastroenterol Hepatol* 2017; **14**: 692-693 [PMID: 29042691 DOI: 10.1038/nrgastro.2017.140]

2 **Holleczek B**, Schöttker B, Brenner H. Helicobacter pylori infection, chronic atrophic gastritis and risk of stomach and esophagus cancer: Results from the prospective population-based ESTHER cohort study. *Int J Cancer* 2020; **146**: 2773-2783 [PMID: 31376284 DOI: 10.1002/ijc.32610]

3 **Yong X**, Tang B, Li BS, Xie R, Hu CJ, Luo G, Qin Y, Dong H, Yang SM. Helicobacter pylori virulence factor CagA promotes tumorigenesis of gastric cancer *via* multiple signaling pathways. *Cell Commun Signal* 2015; **13**: 30 [PMID: 26160167 DOI: 10.1186/s12964-015-0111-0]

4 **Uemura N**, Okamoto S, Yamamoto S, Matsumura N, Yamaguchi S, Yamakido M, Taniyama K, Sasaki N, Schlemper RJ. Helicobacter pylori infection and the development of gastric cancer. *N Engl J Med* 2001; **345**: 784-789 [PMID: 11556297 DOI: 10.1056/NEJMoa001999]

5 **Ferreira RM**, Pereira-Marques J, Pinto-Ribeiro I, Costa JL, Carneiro F, Machado JC, Figueiredo C. Gastric microbial community profiling reveals a dysbiotic cancer-associated microbiota. *Gut* 2018; **67**: 226-236 [PMID: 29102920 DOI: 10.1136/gutjnl-2017-314205]

6 **Liu X**, Shao L, Liu X, Ji F, Mei Y, Cheng Y, Liu F, Yan C, Li L, Ling Z. Alterations of gastric mucosal microbiota across different stomach microhabitats in a cohort of 276 patients with gastric cancer. *EBioMedicine* 2019; **40**: 336-348 [PMID: 30584008 DOI: 10.1016/j.ebiom.2018.12.034]

7 **Espinoza JL**, Matsumoto A, Tanaka H, Matsumura I. Gastric microbiota: An emerging player in Helicobacter pylori-induced gastric malignancies. *Cancer Lett* 2018; **414**: 147-152 [PMID: 29138097 DOI: 10.1016/j.canlet.2017.11.009]

8 **Mima K**, Nishihara R, Qian ZR, Cao Y, Sukawa Y, Nowak JA, Yang J, Dou R, Masugi Y, Song M, Kostic AD, Giannakis M, Bullman S, Milner DA, Baba H, Giovannucci EL, Garraway LA, Freeman GJ, Dranoff G, Garrett WS, Huttenhower C, Meyerson M, Meyerhardt JA, Chan AT, Fuchs CS, Ogino S. Fusobacterium nucleatum in colorectal carcinoma tissue and patient prognosis. *Gut* 2016; **65**: 1973-1980 [PMID: 26311717 DOI: 10.1136/gutjnl-2015-310101]

9 **Castellarin M**, Warren RL, Freeman JD, Dreolini L, Krzywinski M, Strauss J, Barnes R, Watson P, Allen-Vercoe E, Moore RA, Holt RA. Fusobacterium nucleatum infection is prevalent in human colorectal carcinoma. *Genome Res* 2012; **22**: 299-306 [PMID: 22009989 DOI: 10.1101/gr.126516.111]

10 **Oh HJ**, Kim JH, Bae JM, Kim HJ, Cho NY, Kang GH. Prognostic Impact of Fusobacterium nucleatum Depends on Combined Tumor Location and Microsatellite Instability Status in Stage II/III Colorectal Cancers Treated with Adjuvant Chemotherapy. *J Pathol Transl Med* 2019; **53**: 40-49 [PMID: 30586952 DOI: 10.4132/jptm.2018.11.29]

11 **Yoshida Y**, Suwabe K, Nagano K, Kezuka Y, Kato H, Yoshimura F. Identification and enzymic analysis of a novel protein associated with production of hydrogen sulfide and L-serine from L-cysteine in Fusobacterium nucleatum subsp. nucleatum ATCC 25586. *Microbiology (Reading)* 2011; **157**: 2164-2171 [PMID: 21493682 DOI: 10.1099/mic.0.048934-0]

12 **Chen Y**, Peng Y, Yu J, Chen T, Wu Y, Shi L, Li Q, Wu J, Fu X. Invasive Fusobacterium nucleatum activates beta-catenin signaling in colorectal cancer *via* a TLR4/P-PAK1 cascade. *Oncotarget* 2017; **8**: 31802-31814 [PMID: 28423670 DOI: 10.18632/oncotarget.15992]

13 **Rubinstein MR**, Baik JE, Lagana SM, Han RP, Raab WJ, Sahoo D, Dalerba P, Wang TC, Han YW. *Fusobacterium nucleatum* promotes colorectal cancer by inducing Wnt/β-catenin modulator Annexin A1. *EMBO Rep* 2019; **20** [PMID: 30833345 DOI: 10.15252/embr.201847638]

14 **Chen Y**, Lu Y, Ke Y, Li Y. Prognostic impact of the Fusobacterium nucleatum status in colorectal cancers. *Medicine (Baltimore)* 2019; **98**: e17221 [PMID: 31574832 DOI: 10.1097/MD.0000000000017221]

15 **Wong SH**, Kwong TNY, Chow TC, Luk AKC, Dai RZW, Nakatsu G, Lam TYT, Zhang L, Wu JCY, Chan FKL, Ng SSM, Wong MCS, Ng SC, Wu WKK, Yu J, Sung JJY. Quantitation of faecal *Fusobacterium* improves faecal immunochemical test in detecting advanced colorectal neoplasia. *Gut* 2017; **66**: 1441-1448 [PMID: 27797940 DOI: 10.1136/gutjnl-2016-312766]

16 **Kapatral V**, Anderson I, Ivanova N, Reznik G, Los T, Lykidis A, Bhattacharyya A, Bartman A, Gardner W, Grechkin G, Zhu L, Vasieva O, Chu L, Kogan Y, Chaga O, Goltsman E, Bernal A, Larsen N, D'Souza M, Walunas T, Pusch G, Haselkorn R, Fonstein M, Kyrpides N, Overbeek R. Genome sequence and analysis of the oral bacterium Fusobacterium nucleatum strain ATCC 25586. *J Bacteriol* 2002; **184**: 2005-2018 [PMID: 11889109 DOI: 10.1128/JB.184.7.2005-2018.2002]

17 **Hsieh YY**, Tung SY, Pan HY, Yen CW, Xu HW, Lin YJ, Deng YF, Hsu WT, Wu CS, Li C. Increased Abundance of Clostridium and Fusobacterium in Gastric Microbiota of Patients with Gastric Cancer in Taiwan. *Sci Rep* 2018; **8**: 158 [PMID: 29317709 DOI: 10.1038/s41598-017-18596-0]

18 **Schoggins JW**. Interferon-Stimulated Genes: What Do They All Do? *Annu Rev Virol* 2019; **6**: 567-584 [PMID: 31283436 DOI: 10.1146/annurev-virology-092818-015756]

19 **Chen M**, Meng Q, Qin Y, Liang P, Tan P, He L, Zhou Y, Chen Y, Huang J, Wang RF, Cui J. TRIM14 Inhibits cGAS Degradation Mediated by Selective Autophagy Receptor p62 to Promote Innate Immune Responses. *Mol Cell* 2016; **64**: 105-119 [PMID: 27666593 DOI: 10.1016/j.molcel.2016.08.025]

20 **Tan P**, He L, Cui J, Qian C, Cao X, Lin M, Zhu Q, Li Y, Xing C, Yu X, Wang HY, Wang RF. Assembly of the WHIP-TRIM14-PPP6C Mitochondrial Complex Promotes RIG-I-Mediated Antiviral Signaling. *Mol Cell* 2017; **68**: 293-307.e5 [PMID: 29053956 DOI: 10.1016/j.molcel.2017.09.035]

21 **Zhao C**, Collins MN, Hsiang TY, Krug RM. Interferon-induced ISG15 pathway: an ongoing virus-host battle. *Trends Microbiol* 2013; **21**: 181-186 [PMID: 23414970 DOI: 10.1016/j.tim.2013.01.005]

22 **Basters A**, Knobeloch KP, Fritz G. USP18 - a multifunctional component in the interferon response. *Biosci Rep* 2018; **38** [PMID: 30126853 DOI: 10.1042/BSR20180250]

23 **Hornung V**, Hartmann R, Ablasser A, Hopfner KP. OAS proteins and cGAS: unifying concepts in sensing and responding to cytosolic nucleic acids. *Nat Rev Immunol* 2014; **14**: 521-528 [PMID: 25033909 DOI: 10.1038/nri3719]

24 **Wang Y**, Holleufer A, Gad HH, Hartmann R. Length dependent activation of OAS proteins by dsRNA. *Cytokine* 2020; **126**: 154867 [PMID: 31629990 DOI: 10.1016/j.cyto.2019.154867]

25 **de Neergaard M**, Kim J, Villadsen R, Fridriksdottir AJ, Rank F, Timmermans-Wielenga V, Langerød A, Børresen-Dale AL, Petersen OW, Rønnov-Jessen L. Epithelial-stromal interaction 1 (EPSTI1) substitutes for peritumoral fibroblasts in the tumor microenvironment. *Am J Pathol* 2010; **176**: 1229-1240 [PMID: 20133812 DOI: 10.2353/ajpath.2010.090648]

26 **Nielsen HL**, Rønnov-Jessen L, Villadsen R, Petersen OW. Identification of EPSTI1, a novel gene induced by epithelial-stromal interaction in human breast cancer. *Genomics* 2002; **79**: 703-710

[PMID: 11991720 DOI: 10.1006/geno.2002.6755]

27 **Chen S**, Pan S, Wu H, Zhou J, Huang Y, Wang S, Liu A. ICAM1 Regulates the Development of Gastric Cancer and May Be a Potential Biomarker for the Early Diagnosis and Prognosis of Gastric Cancer. *Cancer Manag Res* 2020; **12**: 1523-1534 [PMID: 32184657 DOI: 10.2147/CMAR.S237443]

28 **Huang S**, Yang Z, Zou D, Dong D, Liu A, Liu W, Huang L. Rapid detection of nusG and fadA in Fusobacterium nucleatum by loop-mediated isothermal amplification. *J Med Microbiol* 2016; **65**: 760-769 [PMID: 27339262 DOI: 10.1099/jmm.0.000300]

29 **Yamamura K**, Baba Y, Miyake K, Nakamura K, Shigaki H, Mima K, Kurashige J, Ishimoto T, Iwatsuki M, Sakamoto Y, Yamashita Y, Yoshida N, Watanabe M, Baba H. *Fusobacterium nucleatum* in gastroenterological cancer: Evaluation of measurement methods using quantitative polymerase chain reaction and a literature review. *Oncol Lett* 2017; **14**: 6373-6378 [PMID: 29151903 DOI: 10.3892/ol.2017.7001]

30 **Brennan CA**, Garrett WS. Fusobacterium nucleatum - symbiont, opportunist and oncobacterium. *Nat Rev Microbiol* 2019; **17**: 156-166 [PMID: 30546113 DOI: 10.1038/s41579-018-0129-6]

31 **Bravo D**, Hoare A, Soto C, Valenzuela MA, Quest AF. *Helicobacter pylori* in human health and disease: Mechanisms for local gastric and systemic effects. *World J Gastroenterol* 2018; **24**: 3071-3089 [PMID: 30065554 DOI: 10.3748/wjg.v24.i28.3071]

32 **Coker OO**, Dai Z, Nie Y, Zhao G, Cao L, Nakatsu G, Wu WK, Wong SH, Chen Z, Sung JJY, Yu J. Mucosal microbiome dysbiosis in gastric carcinogenesis. *Gut* 2018; **67**: 1024-1032 [PMID: 28765474 DOI: 10.1136/gutjnl-2017-314281]

33 **Izdebska M**, Zielińska W, Grzanka D, Gagat M. The Role of Actin Dynamics and Actin-Binding Proteins Expression in Epithelial-to-Mesenchymal Transition and Its Association with Cancer Progression and Evaluation of Possible Therapeutic Targets. *Biomed Res Int* 2018; **2018**: 4578373 [PMID: 29581975 DOI: 10.1155/2018/4578373]

34 **Ohtaki S**, Wanibuchi M, Kataoka-Sasaki Y, Sasaki M, Oka S, Noshiro S, Akiyama Y, Mikami T, Mikuni N, Kocsis JD, Honmou O. ACTC1 as an invasion and prognosis marker in glioma. *J Neurosurg* 2017; **126**: 467-475 [PMID: 27081897 DOI: 10.3171/2016.1.JNS152075]

World Journal of Gastroenterology

Submit a Manuscript: https://www.f6publishing.com

*World J Gastroenterol* 2021 November 14; 27(42): 7324-7339

DOI: 10.3748/wjg.v27.i42.7324

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

ORIGINAL ARTICLE

Basic Study

# Detailing the ultrastructure's increase of prion protein in pancreatic adenocarcinoma

Matteo Bianchini, Maria Anita Giambelluca, Maria Concetta Scavuzzo, Gregorio Di Franco, Simone Guadagni, Matteo Palmeri, Niccolò Furbetta, Desirée Gianardi, Niccola Funel, Claudio Ricci, Raffaele Gaeta, Luca Emanuele Pollina, Alfredo Falcone, Caterina Vivaldi, Giulio Di Candio, Francesca Biagioni, Carla Letizia Busceti, Luca Morelli, Francesco Fornai

**ORCID number:** Matteo Bianchini 0000-0002-2403-0621; Maria Anita Giambelluca 0000-0002-8715-9465; Maria Concetta Scavuzzo 0000-0002-4183-9393; Gregorio Di Franco 0000-0003-2156-369X; Simone Guadagni 0000-0001-9072-8166; Matteo Palmeri 0000-0002-9343-278X; Niccolò Furbetta 0000-0002-7135-2666; Desirée Gianardi 0000-0002-6238-9176; Niccola Funel 0000-0002-5028-2363; Claudio Ricci 0000-0002-0110-8836; Raffaele Gaeta 0000-0001-5220-5997; Luca Emanuele Pollina 0000-0001-7163-8050; Alfredo Falcone 0000-0001-5840-2529; Caterina Vivaldi 0000-0001-9307-2326; Giulio Di Candio 0000-0002-5221-2756; Francesca Biagioni 0000-0003-3566-4889; Carla Letizia Busceti 0000-0002-1417-1893; Luca Morelli 0000-0002-7742-9556; Francesco Fornai 0000-0002-3883-5084.

**Author contributions:** Bianchini M and Giambelluca MA contributed equally to this work; Morelli L and Fornai F contributed equally to this work; Bianchini M, Giambelluca MA, Scavuzzo MC, Morelli L and Fornai F studied the conception and design; Bianchini M, Giambelluca MA, Scavuzzo MC, Di Franco G, Guadagni S, Palmeri M, Furbetta N, Gianardi D, Funel N, Ricci C, Gaeta R, Pollina LE,

**Matteo Bianchini, Gregorio Di Franco, Simone Guadagni, Matteo Palmeri, Niccolò Furbetta, Desirée Gianardi, Giulio Di Candio, Luca Morelli,** General Surgery Unit, Department of Translational Research and New Technologies in Medicine and Surgery, University of Pisa, Pisa 56124, Italy

**Maria Anita Giambelluca, Maria Concetta Scavuzzo, Francesco Fornai,** Human Anatomy, Department of Translational Research and New Technologies in Medicine and Surgery, University of Pisa, Pisa 56124, Italy

**Niccola Funel, Claudio Ricci, Raffaele Gaeta, Luca Emanuele Pollina,** Division of Surgical Pathology, Department of Surgical, Medical, Molecular Pathology and Critical Area, University of Pisa, Pisa 56124, Italy

**Alfredo Falcone, Caterina Vivaldi,** Division of Medical Oncology, Department of Translational Research and New Technologies in Medicine and Surgery, University of Pisa, Pisa 56124, Italy

**Francesca Biagioni, Carla Letizia Busceti, Francesco Fornai,** IRCCS Neuromed, Istituto Neurologico Mediterraneo, Pozzilli 86077, Italy

**Luca Morelli,** EndoCAS (Center for Computer Assisted Surgery), University of Pisa, Pisa 56124, Italy

**Corresponding author:** Luca Morelli, MD, Professor, General Surgery Unit, Department of Translational Research and New Technologies in Medicine and Surgery, University of Pisa, Via Paradisa 2, Pisa 56124, Italy. luca.morelli@unipi.it

## Abstract

### *BACKGROUND*

Recent evidences have shown a relationship between prion protein (PrPc) expression and pancreatic ductal adenocarcinoma (PDAC). Indeed, PrPc could be one of the markers explaining the aggressiveness of this tumor. However, studies investigating the specific compartmentalization of increased PrPc expression within PDAC cells are lacking, as well as a correlation between ultrastructural evidence, ultrastructural morphometry of PrPc protein and clinical data. These

Falcone A, Vivaldi C and Di Candio G contributed to data acquisition; Morelli L and Fornai F are responsible for data analysis and interpretation, and revised the manuscript critically; Bianchini M, Giambelluca MA, Scavuzzo MC, Di Franco G, Guadagni S, Palmeri M, Furbetta N, Gianardi D, Funel N, Pollina LE, Falcone A, Vivaldi C, Di Candio C, Biagioni F and Busceti CL drafted the manuscript; all authors approved the final manuscript.

**Supported by** Tizzi Foundation and Arpa Foundation ( www.fondazionearpa.it).

**Institutional review board statement:** The study was approved by Ethics committee of "Area Vasta Nord Ovest (CEAVNO)".

**Informed consent statement:** All patients signed an informed consent to authorize the scientific use of the collected data.

**Conflict-of-interest statement:** The authors declare that they do not have any conflict of interest.

**Data sharing statement:** No additional data available.



**Specialty type:** Oncology

**Country/Territory of origin:** Italy

**Peer-review report's scientific quality classification**

Grade A (Excellent): 0

Grade B (Very good): B, B

data, as well as the quantitative stoichiometry of this protein detected by immuno-gold, provide a significant advancement in understanding the biology of disease and the outcome of surgical resection.

***AIM***

To analyze quantitative stoichiometry and compartmentalization of PrPc in PDAC cells and to correlate its presence with prognostic data

***METHODS***

Between June 2018 and December 2020, samples from pancreatic tissues of 45 patients treated with pancreatic resection for a preoperative suspicion of PDAC at our Institution were collected. When the frozen section excluded a PDAC diagnosis, or the nodules were too small for adequate sampling, patients were ruled out from the present study. Western blotting was used to detect, quantify and compare the expression of PrPc in PDAC and control tissues, such as those of non-affected neighboring pancreatic tissue of the same patient. To quantify the increase of PrPc and to detect the subcellular compartmentalization of PrPc within PDAC cells, immuno-gold stoichiometry within specific cell compartments was analyzed with electron microscopy. Finally, an analysis of quantitative PrPc expression according to prognostic data, such as cancer stage, recurrence of the disease at 12 mo after surgery and recurrence during adjuvant chemotherapy was made.

***RESULTS***

The amount of PrPc within specimen from 38 out of 45 patients was determined by semi-quantitative analysis by using Western blotting, which indicates that PrPc increases almost three-fold in tumor pancreatic tissue compared with healthy pancreatic regions [242.41 ± 28.36 optical density (OD) *vs* 95 ± 17.40 OD, $P < 0.0001$]. Quantitative morphometry carried out by using immuno-gold detection at transmission electron microscopy confirms an increased PrPc expression in PDAC ductal cells of all patients and allows to detect a specific compartmentalization of PrPc within tumor cells. In particular, the number of immuno-gold particles of PrPc was significantly higher in PDAC cells respect to controls, when considering the whole cell (19.8 ± 0.79 particles *vs* 9.44 ± 0.45, $P < 0.0001$). Remarkably, considering PDAC cells, the increase of PrPc was higher in the nucleus than cytosol of tumor cells, which indicates a shift in PrPc compartmentalization within tumor cells. In fact, the increase of immuno-gold within nuclear compartment exceeds at large the augment of PrPc which was detected in the cytosol (nucleus: 12.88 ± 0.59 particles *vs* 5.12 ± 0.32, $P < 0.0001$; cytosol: 7.74. ± 0.44 particles *vs* 4.3 ± 0.24, $P < 0.0001$).

***RESULTS***

In order to analyze the prognostic impact of PrPc, we found a correlation between PrPc expression and cancer stage according to pathology results, with a significantly higher expression of PrPc for advanced stages. Moreover, 24 patients with a mean follow-up of 16.8 mo were considered. Immuno-blot analysis revealed a significantly higher expression of PrPc in patients with disease recurrence at 12 mo after radical surgery (360.71 ± 69.01 OD *vs* 170.23 ± 23.06 OD, $P = 0.023$), also in the subgroup of patients treated with adjuvant CT (368.36 ± 79.26 OD in the recurrence group *vs* 162.86 ± 24.16 OD, $P = 0.028$), which indicates a correlation with a higher chemo-resistance.

***CONCLUSION***

Expression of PrPc is significantly higher in PDAC cells compared with control, with the protein mainly placed in the nucleus. Preliminary clinical data confirm the correlation with a poorer prognosis.

**Key Words:** Pancreatic ductal adenocarcinoma; Prion protein; Western blotting; Electron microscopy; Cellular compartmentalization; Neuroinvasion

Grade C (Good): C
Grade D (Fair): 0
Grade E (Poor): 0

**Received:** April 27, 2021
**Peer-review started:** April 27, 2021
**First decision:** June 13, 2021
**Revised:** June 14, 2021
**Accepted:** October 25, 2021
**Article in press:** October 25, 2021
**Published online:** November 14, 2021

**P-Reviewer:** Şurlin VM, Yamanaka K
**S-Editor:** Wu YXJ
**L-Editor:** A
**P-Editor:** Wu YXJ

**Core Tip:** Pancreatic ductal adenocarcinoma (PDAC) is an extremely lethal cancer and we are far away from understanding its biology. Recent *in vitro* evidence hypothesizes some role of cellular prion protein (PrPc) in PDAC carcinogenesis. We found that this protein is over-expressed *in vivo* within PDAC tissue of surgically resected patients, here we quantify the stoichiometric increase and provide evidence for a shift towards a nuclear compartmentalization. Such a protein amount is associated with poorer post-surgical prognosis and neuro-invasion. Thus, PrPc might be a key in the puzzled evidence of PDAC biology leading to better comprehend and cure such an aggressive disorder.

**Citation:** Bianchini M, Giambelluca MA, Scavuzzo MC, Di Franco G, Guadagni S, Palmeri M, Furbetta N, Gianardi D, Funel N, Ricci C, Gaeta R, Pollina LE, Falcone A, Vivaldi C, Di Candio G, Biagioni F, Busceti CL, Morelli L, Fornai F. Detailing the ultrastructure's increase of prion protein in pancreatic adenocarcinoma. *World J Gastroenterol* 2021; 27(42): 7324-7339
**URL:** https://www.wjgnet.com/1007-9327/full/v27/i42/7324.htm
**DOI:** https://dx.doi.org/10.3748/wjg.v27.i42.7324

## INTRODUCTION

Pancreatic cancer is currently the fourth most frequent cause of death among cancers with a prevalence, which is increasing, mostly in western countries, where it is estimated to become the second prevalent cause of death from cancer by 2030[1]. The overall median survival of patients with pancreatic ductal adenocarcinoma (PDAC) is 6 mo and the 5-year survival rate is less than 10%[2,3].

Surgical resection is still the only approach with a curative intent, but it is feasible for less than 20% of patients at diagnosis, while 80% of cases are considered too advanced for surgery, and this is based on regional infiltration or distant metastasis [4]. Even in surgically eligible cases, the 5-years survival rate is extremely low, since patients experience early local or distant relapse. The aggressiveness of the disease is mainly related to the extensive local infiltration and to the early lymphatic and hematogenous spread, which rely on the high propensity of these cells to produce neuro-invasion.

In this scenario, an in-depth knowledge of the biology of the disease is fundamental. The comprehension of the mechanisms involved in tumorigenesis is needed, in order to discover early diagnostic tools, and novel therapeutic strategies to improve patients' prognosis. When comparing PDAC with other tumors, the biology of the disease is scarcely investigated, and it remains largely unknown.

Recent evidence demonstrates that cellular Prion Protein (PrPc) is overexpressed either *in vitro* and *in vivo* in PDAC cells and that it interacts with several pathways, enhancing cellular growth, tumoral proliferation and invasion[5-7].

Still, the occurrence of increased PrPc was described so far by using quite general approaches, in the absence of subcellular localization of the protein and without a quantitative, stoichiometric count of protein particles within cancer cells. Thus, we felt it mandatory to provide a quantitative measurement of the increase in PrPc, by using ultrastructural stoichiometry, which is more reliable in protein analytical detection compared with immunohistochemistry. Again, understanding cell compartmentalization of PrPc, and whether this is shifted within PDAC cells is key to establish its potential role in the biology of disease. This is expected to improve the knowledge about the intrinsic role of PrPc in PDAC pathogenesis. So far, studies focusing on these aspects based on an *in vivo* approach are lacking. Again, it is fundamental to strengthen whether a higher expression of PrPc in PDAC cells does associate with specific clinical phenotypes of PDAC. Therefore, the aim of this study is to quantify the increase in PrPc and analyze the subcellular localization of PrPc in PDAC cells from surgically resected patients. These findings are correlated with clinical data, in order to dissect a potential role of PrPc as a biological marker to predict disease severity.

## MATERIALS AND METHODS

### *Patients and specimens*

Samples from tumors of patients surgically treated with pancreatic resections at our Institution were collected between January 2018 and December 2020. Written informed consent was obtained from patients to use their surgical specimens and clinical pathological data for research purposes.

All patients had a preoperative suspicion of PDAC. Preoperative evaluation included medical history, physical, laboratory and radiological examinations, computed tomography (CT) and magnetic resonance imaging, often with magnetic resonance cholangiopancreatography. In addition, abdominal ultrasound with and without contrast, endoscopic ultrasonography (EUS), and fine-needle aspiration (FNA) during EUS were also performed in selected patients. Preoperative data included age and gender.

All the specimens were frozen intraoperatively and further sliced and scored for histology. Tissue from pancreatic nodules other than adenocarcinomas were ruled out from the present study.

Similarly, we could not proceed when tumor specimens were too small. When PDAC diagnosis was confirmed, the pathologist took specimens from the pancreatic tumor and from normal pancreatic tissue. From each specimen (either from controls or tumor tissues) a part was fixed and kept in glutaraldehyde and paraformaldehyde for electron microscopy analysis, while the other one was rapidly frozen and kept at -80°C for storage until for western blotting analyses (SDS-PAGE immunoblotting) to be carried out.

Histological data included: (1) Histological phenotype of the tumor; (2) Grade of differentiation; (3) Tumor size; (4) Number of harvested lymph nodes; (5) Number of metastatic lymph nodes; and (6) Occurrence of angio-invasion and peri-neural infiltration.

Patients were staged according to the T and N definitions proposed for the AJCC 8$^{th}$ edition[8]. Proposed T-stage definitions are the following: T1 ≤ 2 cm maximal diameter, T2 > 2 ≤ 4 cm maximal diameter, T3 > 4 cm maximal diameter, T4 = locally unresectable. Extra-pancreatic extension was not included in T-stage definitions. The N-staging included the following: N0 = node negative, N1 = 1-3 nodes positive for metastatic disease, N2 ≥ 4 nodes positive for metastatic disease.

Pathology, post-operative disease outcome and oncologic follow-up were prospectively retrieved and organized in a specific database. For our clinical analysis patients with at least 12 mo from surgical resection were considered. The degree of PrPc expression in PDAC was reported and compared also on the basis of cancer stage according to AJCC 8$^{th}$ edition.

### *SDS-PAGE immunoblotting*

Pancreatic tissue to be immunoblotted was homogenized in ice-cold lysis buffer containing 50 mmol/L Tris-HCl (pH = 7.5), 150 mmol/L NaCl, 5 mmol/L EDTA, 1% SDS, 0.1% IGEPAL (NP40) and Complete Protease Inhibitor Cocktail Tablet (leupeptin, pepstatin, aprotinin) (Santa Cruz Biotechnology, Dallas, TX, United States). Then tissue was sonicated, and homogenates were centrifuged at 5000 x g for 5 min. An aliquot of supernatant was used to determine the protein concentration by a protein assay kit (Sigma-Aldrich, St. Louis, MO).

Samples (25 μg) were separated on 4%-20% sodium dodecyl sulfate-polyacrylamide gel. Following electrophoresis, proteins were transferred to a nitrocellulose membrane (Biorad; Milano, Italy). The membrane was then immersed in blocking solution containing PBS with 0.05% Tween-20 (PBS-T) and 5% not fat dried milk (Sigma) for 1 h at room temperature. Then the membrane was incubated overnight at 4°C with primary antibody anti-protein PrPc (1:2000, Abcam, Cambridge, United Kingdom) diluted in PBS-T containing 2% BSA (Sigma). The blots were washed three times with PBS-T and incubated for 1 h with goat anti-rabbit horseradish peroxidase-labelled secondary antibody (1:2000; KPL, Maryland, United States) diluted in PBS-T containing 2% not fat dried milk (Sigma).

The bands were visualized with enhanced chemiluminescence reagents (Immuno-Star HRP Substrate; Bio-RadLaboratories) and image analysis was carried out by ChemiDoc System (Bio-RadLaboratories). β-Actin was used as an internal standard for semi-quantitative protein measurement, so-called "house-keeping protein". Densitometric analysis was performed with ImageJ software and the unit of measure was the optical density (OD). Western blotting of PDAC tissues were compared with control tissues.

### Semi-thin sections

When preparing embedded pancreas tissue blocks for electron microscopy analysis, firstly we carried out semi-thin sections in order to better focus on those areas of the tissue where ductal and parenchymal area could be evidenced. Each semi-thin section owning a thickness range of 3-6 μm was cut at ultra-microtome. After cutting, slices were picked up with an iron loop 1 cm long and 2 mm thick. By using the loop, the slice was moved into a drop of distilled water lying on a glass slide and it was then placed on a hot plate at approximately 60°C to be dried. Then, 1 or 2 drops of a toluidine blue staining solution were added on the semi-thin slice. When the edge of the staining drop switched the color from blue to metallic gold, the slide was removed quickly from hot plate to be rinsed with distilled water. Finally, these slides were mounted by using the mounting medium DPX to be analyzed by a Nikon Eclipse 80i light microscope, which was connected to the NIS Elements software for image analysis (Nikon, Tokyo, Japan).

### Electron microscopy

For electron microscopy small fragments of normal and tumoral pancreatic tissue were fixed in 0.1% glutaraldehyde and 2 % paraformaldehyde in phosphate buffer pH = 7.4 for 90 min, creating a fixing solution minimally covering antigen epitope, while fairly preserving tissue architecture.

After washing for 10 min in buffer, samples were post-fixed in 1% OsO4 buffered solution for 1 h at 4°C. Then samples were dehydrated in a series of increasing ethanol concentration (50%, 70%, 90% 95%, 100%) followed by propylene oxide for 20 min. Afterward, samples were embedded in a mixture of Epon-Araldite and propylene oxide (ratio of 1:1 overnight at room temperature) and finally they were embedded in pure Epon-Araldite resin for 72 h at 60°C. Ultra-thin sections were stained with uranyl acetate and lead citrate and examined with Jeol Jem 100SX transmission electron microscope (TEM) (Jeol, Tokyo, Japan) at an acceleration voltage of 80 kV.

### Post–embedding immunocytochemistry

Post-embedding procedure was carried out on ultrathin sections collected on nickel grids. Grids were washed in PBS and incubated in a blocking solution containing 10% goat serum and 0.2% saponin for 20 min, at room temperature, then they were incubated with a primary antibody solution containing anti-rabbit Prion protein PrPc (Abcam, diluted 1:50), with 0.2% saponin and 1% goat serum in a humidified chamber, overnight, at 4°C. After washing in PBS, grids were incubated with secondary anti-rabbit antibodies conjugated with gold particles (20 nm mean diameter, BB International Crumlin, United Kingdom) which were diluted 1:40 in PBS containing 0.2 % saponin and 1% goat antiserum for 1 h, at room temperature. Slices used as methodological controls were incubated with secondary antibody only.

After washing in PBS, grids were incubated on droplet of 1% glutaraldehyde for 3 min; an additional extensive washing of grids with distilled water was carried out to remove an excess of salt traces. Sections were stained with uranyl acetate and lead citrate and examined at TEM.

From each experimental group (Controls and PDAC) 20 grids were observed each one containing a mean of 5 cells: In particular we selected the region in which the cellular ducts were present. In order to measure the distribution of the immuno-gold particles, first we counted the total number of gold particles within each cell, then their numbers in nucleus and in cytosol. TEM analysis was performed at a magnification of 6000-8000x which allowed the concomitant visualization of immuno-gold particles and all cell organelles, using higher magnification when it was necessary to better visualize the immuno-gold particles and lower magnification when an ensemble view of the whole ultrastructure was requested for our analysis. The expression of PrPc was revealed by counting the immuno-gold particles in whole cells, nucleus and cytosol both in control and PDAC groups.

### Statistical analysis

Continuous variables with normal distribution are expressed as the mean ± SD of the mean and they were compared by using Student's *t* test. A *P* value equal or lower than 0.05 was arbitrarily considered as not due to biological variability ($H_0$ hypothesis rejected). The software used for such a statistical analysis was SPSS (Statistical Production and Service Solution for Windows, SPSS Inc., Chicago, IL, United States), version 23.

## RESULTS

### Patients

Surgical specimens of 45 patients were collected. 7 of them were ruled out because of the small dimension of the tumor that prevented an adequate sampling or because the diagnosis of PDAC was excluded at frozen section.

The samples from 38 PDAC patients which were included in the analysis, 19 (50%) were from male, while 19 (50%) from female. The mean age was 72.7 ± 7.9 years (range 52-87). Histological exam confirmed the presence of PDAC in all these 38 cases. The grading of the pancreatic tumor was "moderately differentiated" in 35/38 cases (92.1%), "poorly differentiated" in 3/38 (7.9%). The mean tumor size was 3.2 ± 1.1 cm (range 1.5-6.5 cm). The mean harvested lymph nodes were 34.2 ± 15 (range 14-79). Metastatic lymph-nodes occurred in 30/38 cases (78.9%) with a number of metastatic lymph-nodes of 4.5 ± 4.9 (range 1-23). The presence of angio-invasion was reported in 5/38 cases (13%), while the presence of peri-neural infiltration was reported in 33/38 cases (86.8%). Three cancer stage groups were identified according to pTNM, stage I ($n$ = 8, 21.1%), stage II ($n$ = 14, 36.8%) and stage III ($n$ = 16, 42.1%). These data are shown in Table 1.

### Expression of PrPc in pancreatic tissue at western blotting

PrPc was markedly expressed in tumor pancreatic tissues, while the expression in non-cancer tissues was scarce, with a significant difference (242.41 ± 28.36 OD *vs* 95 ± 17.40 OD, $P < 0.0001$) (Figure 1).

### Semi-thin sections

When observing representative semi-thin sections at different magnification (Figure 2) the difference between control and PDAC pancreas was strikingly evident, mostly at the level of ductal tissue. In detail, the pancreas from control at low magnification possesses a quite homogeneous staining at toluidine blue, where ductal areas are simply evident as empty roundish areas surrounded by cell staining as much as those in the neighboring parenchyma (Figure 2A). This was further evidenced at higher magnification showing a pale toluidine staining of ductal cells (Figure 2B). Conversely, in the pancreas affected by PDAC, low magnification showed a highly non-homogeneous tissue where ductal regions were markedly stained. Also, neighboring areas possess a scattered toluidine staining (Figure 2C). At high magnification, the ductal cells from PDAC are overwhelmed and they tend to occlude the ductal lumen with multiple cell layers with an abnormal shape and abundant cell protrusions (Figure 2D). This ductal tissue was the topographical reference to proceed with electron microscopy analysis.

### Electron microscopy

The ultrastructure of pancreatic healthy cells owns normal architecture with well-preserved cell compartments and well-defined membranes. The zymogen granules maintain their integrity, which suggests fair biochemical activity (Figure 3A). Conversely, PDAC cells (Figure 3B) possess severe cell pathology, which is concomitant with a marked derangement of vacuolar compartment and damaged organelles.

### Expression of PrPc immuno-gold particles within pancreatic tissue

Immuno-cytochemistry shows increased PrPc expression in PDAC cells of all patients. In particular the number of immuno-gold particles was significantly higher in PDAC cells compared with controls (Figure 4) either considering the whole cell (19.8 ± 0.79 particles *vs* 9.44 ± 0.45, $P < 0.0001$) (Figure 5A), or the nuclear compartment (12.88 ± 0.59 particles *vs* 5.12 ± 0.32, $P < 0.0001$) (Figure 5C) or the cytosol (7.74. ± 0.44 particles and 4.3 ± 0.24, $P < 0.0001$, respectively) (Figure 5B).

Within PDAC cells, PrPc was more abundant in the nucleus compared with the cytosol (12.88 ± 0.59 particles *vs* 7.74. ± 0.44, $P < 0.0001$) (Figure 5D). Conversely, in normal cells no difference was noticeable for the placement of PrPc within nucleus and cytosol (5.12 ± 0.32 particles and 4.3 ± 0.24, $P > 0.05$, respectively).

### Clinical data

When correlating within PDAC group the amount of PrPc expression with specific cancer stages, a significantly higher expression of PrPc for advanced stages was detected. In particular, PrPc expression at Western Blotting was 161.69 ± 63.92 OD in stage I, 173.25 ± 76.5 OD in stage II and 346.86 ± 55.26 OD in stage III ($P = 0.0042$).

**Table 1 Patients and postoperative data**

| | |
|---|---|
| Number of patients, *n* | 38 |
| Pancreatic ductal adenocarcinoma, *n* (%) | 38 (100) |
| Mean tumor dimension, cm | 3.2 1.1 (1.5-6.5) |
| Mean harvest lymph nodes, *n* | 34.2 15 (14-79) |
| Mean metastatic lymph nodes, *n* | 4.5 4.9 (1-23) |
| Angioinvasion, *n* (%) | 5 (13) |
| Perineural infiltration, *n* (%) | 33 (86.8) |
| T status, *n* (%) | |
| T1 | 2 (8.7) |
| T2 | 14 (60.9) |
| T3 | 7 (30.4) |
| T status, *n* (%) | |
| T1 | 3 (7.9) |
| T2 | 24 (63.2) |
| T3 | 11 (28.9) |
| N status, *n* (%) | |
| N0 | 7 (18.4) |
| N1 | 15 (39.5) |
| N2 | 16 (42.1) |
| Stage, *n* (%) | |
| I | 8 (21.1) |
| II | 14 (36.8) |
| III | 16 (42.1) |
| Patients with available follow-up, *n* | 24 |
| Patients with disease recurrence at 12 months, *n* (%) | 13 (54.1) |
| Patients treated with adjuvant CT, *n* (%) | 21 (87.5) |
| Recurrence during CT, *n* (%) | 11 (52.4) |

CT: Chemotherapy.

When considering clinical data retrieved from our follow-up, patients surgically treated in the last 12 mo were ruled out from correlation with disease prognosis. Overall, 27 patients were considered, of which 3 were ruled out for missing information.

For the remaining 24 patients, the mean follow-up was 16.8 mo (range 5.6-34.4 mo). Median overall survival and disease-free survival were 15.9 mo and 11.2 mo, respectively. The 12-mo recurrence rate was 54.1% ($n$ = 13). Comparing patients with relapse at 1 year with those without evidence of the disease, the difference in PrPc expression at immuno-blotting was statistically significant between the two groups (360.71 ± 69.01 OD *vs* 170.23 ± 23.06 OD, $P$ = 0.023) (Figure 6A).

Moreover, 21 patients out of 24 received adjuvant chemotherapy (CT). Of these, 10/21 (47.6%) were without evidence of disease relapse at 12 mo, while 11/21 (52.4%) experienced a relapse of the disease. Detailing our analysis of PrPc expression correlated with disease recurrence a significant higher PrPc expression was detected for patients who experienced a relapse, despite the administration of adjuvant CT compared with those receiving CT without evidence of disease at follow-up (368.36 ± 79.26 OD *vs* 162.86 ± 24.16 OD, respectively, $P$ = 0.028) (Figure 6B).

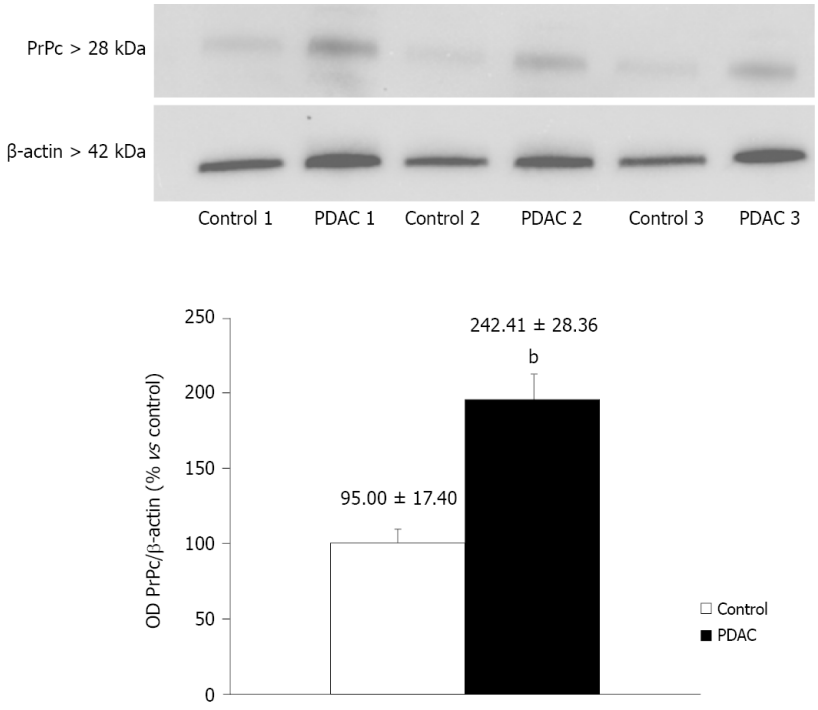


**Figure 1 Immuno-blots for prion protein and the house keeping protein b-actin in control tissues and pancreatic ductal adenocarcinoma tissues.** $^{b}P < 0.0001$. Comparisons between two groups were made by using Student *t*-test. Values are given as the mean ± SD.

## DISCUSSION

Cellular prion protein is a cell surface glycoprotein, firstly discovered as the normal isoform of the scrapie prion protein ($PrP^{Sc}$), the infectious agent of Transmissible Spongiform Encephalopathies. Despite the normal isoform is a highly conserved glycoprotein present in all vertebrates, which indicates some intrinsic and fundamental roles in cell homeostasis, studies about the physiology of PrPc have long been overlooked.

Only recently some authors focused on the role of PrPc and many paths have been opened[11]. PrPc was proposed to protect neurons against cell death and oxidative stress[12], to control copper metabolism[13], to regulate cell cycle[14], synaptic transmission[15], cell adhesion[16], and activation of the immuno-system[17]. It is remarkable that PrPc plays a role in pluripotency and differentiation of embryonic stem cells[18], cell proliferation and differentiation[19-24] and muscle cell regeneration [25].

In detail, the discovery of a role of this protein in regulating the cell cycle led some authors to focus on studying a relationship between PrPc and carcinogenesis. It is now well established that PrPc may sustain cancer cell proliferation in various types of cancers[26]: Gastric[27], colon cancer[28-30], as well as glioblastoma[31-33]. Overall, the contribution of PrPc to cancer cell proliferation fully fits with a gain of its physiological function in normal cells, where it controls the activation of several effectors associated with cell growth[24,34]. A second field of investigation focuses on the correlation between PrPc and chemo-resistance. High PrPc expression levels are indeed associated with increased resistance to various types of agents in glioblastoma [35], gastric[27,36-38], breast[39-41], and colon cancer[29,42,43]. According to several studies this effect could be related to the activation of several pathways that lead to over expression of MDR1 (multidrug-resistance protein 1)[44]. For instance it was recently found that PrPc confers doxorubicin resistance to breast cancer cells[45].

PrPc also promotes cancer cells invasion/migration. In fact, elevated PrPc levels confer enhanced invasive properties to glioblastoma[46], gastric[47], breast[40,48], colon[49], lung[50] and melanoma[51] cell lines.

Finally, PrPc directly induces proliferation and confers resistance to apoptosis in cancer stem cells[18], by dysregulating their interactions with surrounding environment and thus causing cancer stem cells proliferation[52]. As with proliferation, the contribution of PrPc to cancer stem cells self-renewal may be envisioned as a diversion of its physiological role into normal stem cell maintenance[18].

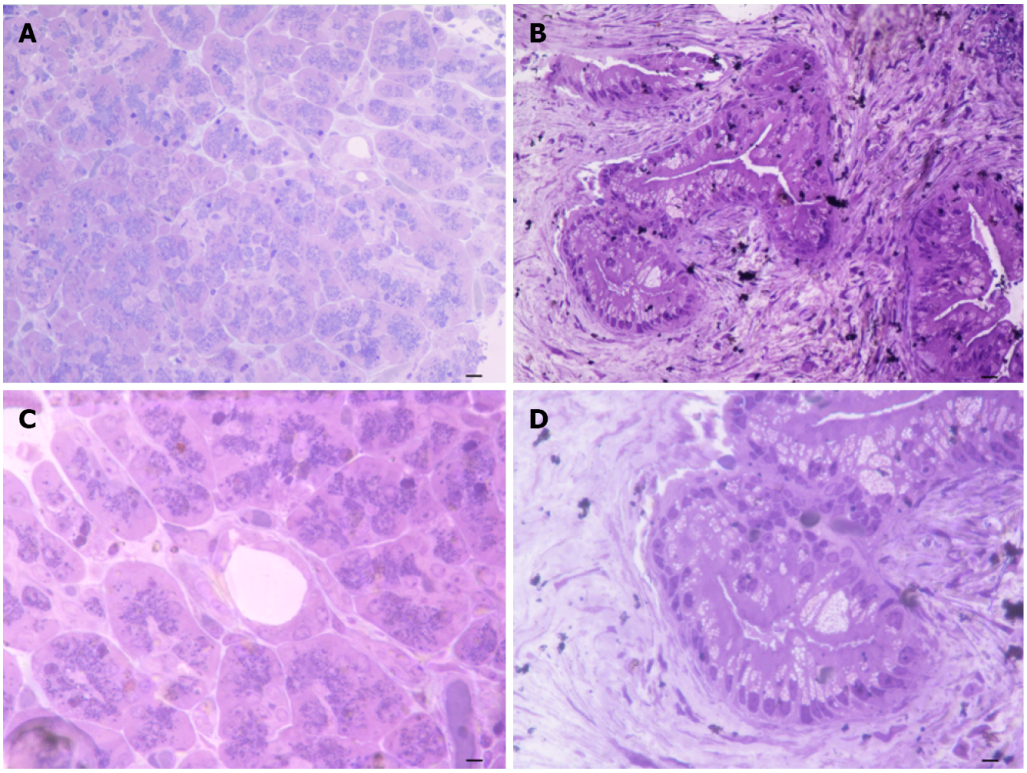


**Figure 2 Semi-thin sections of control and pancreatic ductal adenocarcinoma pancreas.** A: Pancreas from control tissue at low magnification (magnification: 20 ×, scale bar: 12.5 μm). The ductal areas are evident as empty roundish areas surrounded by cell staining as much as those in the neighboring parenchyma; B: The normal pancreatic tissue characteristics are more evident at higher magnification, where there is a pale toluidine staining of ductal cells (magnification: 40 ×, scale bar: 6.25 μm); C: In pancreatic ductal adenocarcinoma (PDAC) tissue a highly non-homogeneous tissue is present and ductal regions are markedly stained (magnification: 20 ×, scale bar: 12.5 μm); D: PDAC tissue at higher magnification (magnification: 40 ×, scale bar: 6.25 μm). The ductal cells are overwhelmed and they tend to occlude the ductal lumen.

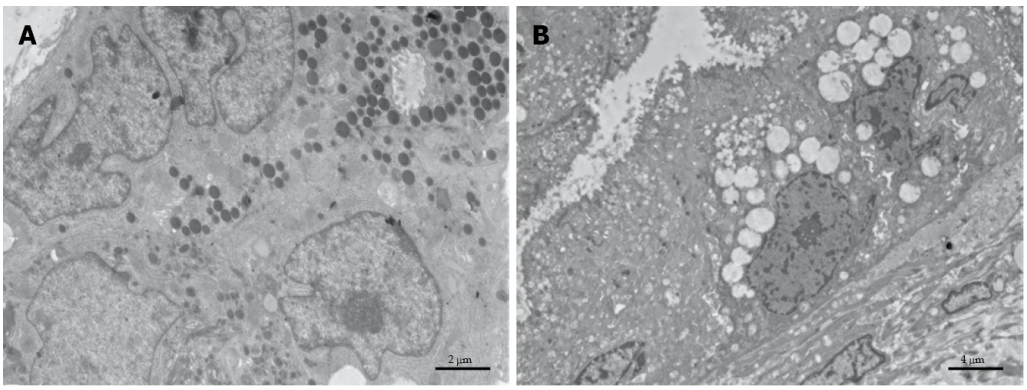


**Figure 3 Ultrastructural organization.** A: Pancreatic normal tissue. In pancreatic healthy tissue the cells surrounding ductal systems are well preserved. The cellular organelles, nuclei and secretory zymogen granules have normal architecture (magnification: 2000 ×, scale bar: 2 μm); B: Pancreatic normal tissue. The cells surrounding duct have increasing loss of cellular architecture (magnification: 1000 ×, scale bar: 4 μm).

Collectively, the involvement of PrPc in various aspects of cancer progression may be viewed as directly related to its physiological role in normal cells. Prion-mediated changes may represent initiating events that promote the emergence of the hallmarks of cancer, including self-sufficiency in growth signals, insensitivity to antigrowth signals, tissue invasion and metastasis, limitless replicative potential and inhibition of apoptosis[53]. Moreover, over-expression of PrPc has been found to be related to a higher chemoresistance[54]. This is likely to have clinical implications: from a therapeutic perspective, reducing PrPc expression may be beneficial, as it was documented for glioblastoma[55] or colon cancer[56]. Besides, alternative opportunities may ensue from a better knowledge of the signals upregulating PrPc expression in cancer cells.

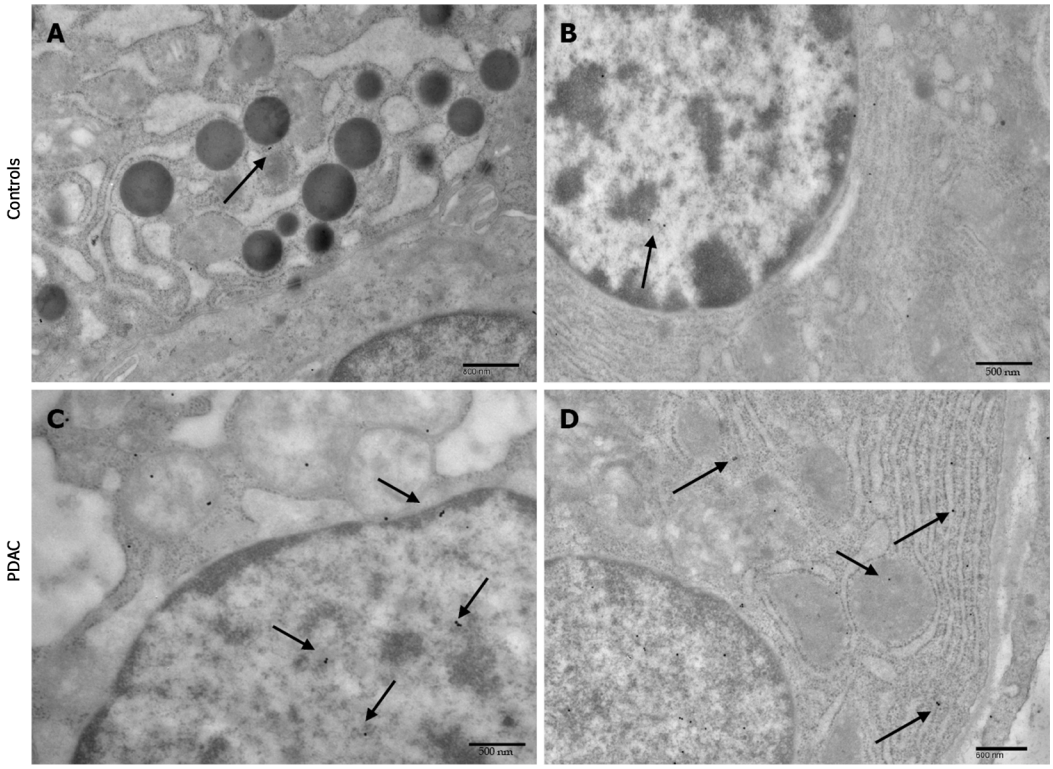


**Figure 4 Prion protein immunocytochemistry in controls and pancreatic ductal adenocarcinoma.** A: Prion protein immunocytochemistry in controls. Few particles of gold prion protein (PrPc) are evident (arrows) in the cytosol (magnification: 6000 ×, scale bar: 800 nm); B: Few particles of gold PrPc are evident (arrows) in the nucleus (magnification: 8000 ×, scale bar: 500 nm); C: Prion protein immunocytochemistry in pancreatic ductal adenocarcinoma. The arrows highlight some of the PrPc immuno-gold particles in the nucleus (magnification: 8000 ×, scale bar: 500 nm); D: The arrows highlight some of the PrPc immuno-gold particles in the cytosol (magnification: 7000 ×, scale bar: 600 nm).

In particular, looking to PDAC, a deeper comprehension of its role may add a significant piece of evidence in the puzzle of this highly aggressive pathology, with a potentially relevant clinical implication. The present study indicates a higher expression of PrPc in PDAC tissues *in vivo*, and provides the first evidence of the cellular localization of this protein. A higher amount of PrPc specifically within PDAC cells is now confirmed in a wider pool of patients compared with our recent *in vivo* study[7].

Moreover, the present study provides a stoichiometric measurement of the protein *is situ* within PDAC cells and indicates a shift in its sub-cellular placement in PDAC. Such a latter novelty was provided by ultrastructural morphometry, and immuno-gold staining under transmission electron microscopy. For instance, such an approach allowed to detect a misplacement of PrPc towards the nuclear compartment, which is in line with its strong effects on cancer-related gene expression. In particular, in PDAC cells the number of PrPc immuno-gold particles was two-fold higher compared with controls, although such an increase is more relevant in the cell nucleus, where it rises up to three-fold of the amount measured in controls. When comparing PrPc compartmentalization in PDAC cells and normal ductal pancreatic cells, the nuclear concentration of PrPc is 1.65-fold higher compared with cytosol, while in the normal cells there is no significant difference between nucleus and cytosol.

From one hand, this may be due to a marked ongoing over-expression of PrPc gene (PRNP) in PDAC cells. In fact, the first hint of a link between PrPc and pancreatic cancer dates back to the early 2000s, when PRNP was identified as one of the 30 genes mostly expressed in pancreatic cancer cell lines when compared with normal cells[57]. On the other hand, such a preferential nuclear compartmentalization may disclose a specific role of PrPc in the biology of PDAC. So far, the evidences from PDAC cell cultures show that PrPc act as a cell surface glycoprotein to activate specific intracellular pathways and signaling that bring to a proliferative effect[5]. However, the detection of a peculiar and prominent concentration in the nucleus suggests an involvement of PrPc in regulating directly gene expression, acting as a nucleo-junctional interplay in order to modulate the transcriptional activity of different pathways involved in carcinogenesis. In fact, PrPc could have a role in signaling complexes that contribute to a regulation of proliferation and cell-to-cell adhesion.

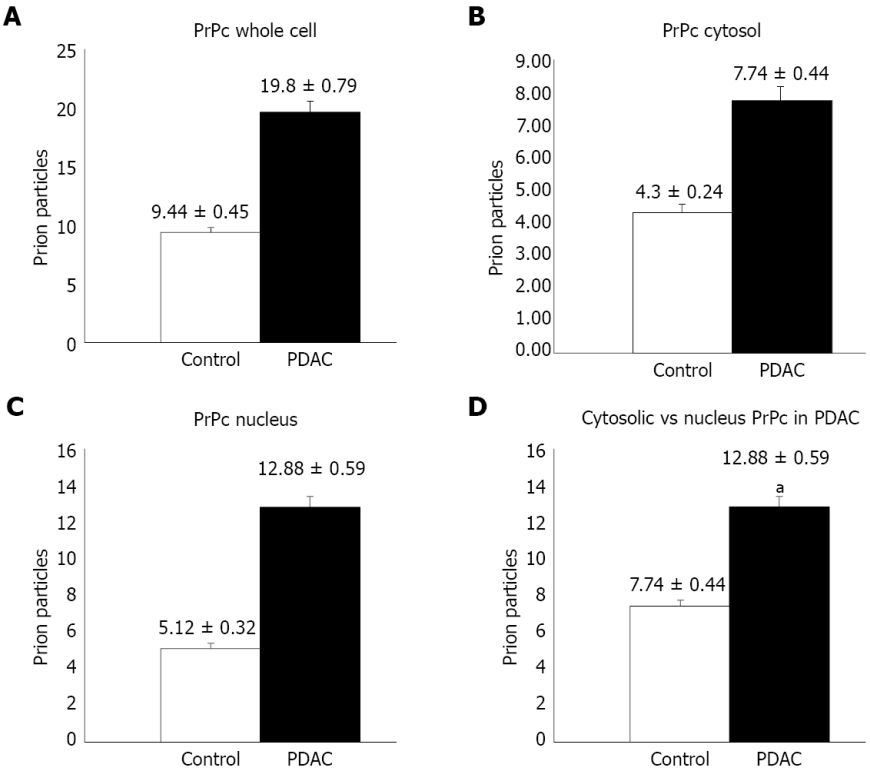


**Figure 5 Prion protein immuno-gold particles count.** A: Prion protein immuno-gold particles count in whole cells (controls *vs* pancreatic ductal adenocarcinoma); B: Prion protein immuno-gold particles count in cytosol (controls *vs* pancreatic ductal adenocarcinoma); C and D: Prion protein immuno-gold particles count in nuclear compartment. A significative difference in prion protein (PrPc) immunogold particles is observed between pancreatic ductal adenocarcinoma (PDAC) and control tissues (C); Even when analyzing the location only in PDAC cells, the nuclear compartment has a significantly higher PrPc concentration (D). [a]*P* < 0.0001. Comparisons between two groups were made by using Student *t*-test. Values are given as the mean ± SD.

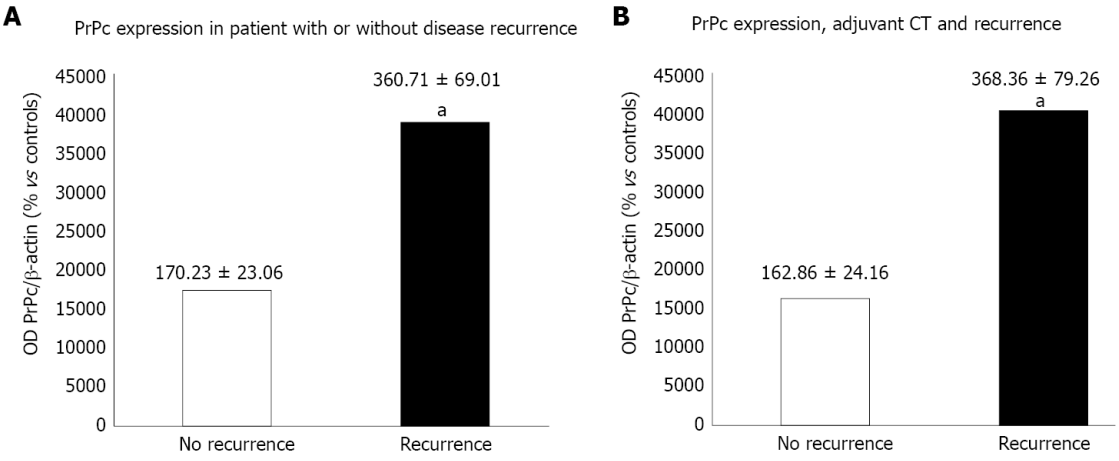


**Figure 6 Prion protein expression.** Comparisons between two groups were made by using Student *t*-test. Values are given as the mean ± SD. A: Prion protein expression according to the presence of disease recurrence after surgery. The histogram compares the expression of prion protein with western blot in specimen from patients surgically resected for pancreatic ductal adenocarcinoma with no evidence of disease at 12-mo follow-up (*n* = 11) with those from patients with disease recurrence (*n* = 13). [a]*P* = 0.023. B: Prion protein expression according to the presence of disease recurrence after surgery in patients treated with adjuvant chemotherapy. The histogram compares the expression of prion protein with western blot in specimen from patients surgically resected for pancreatic ductal adenocarcinoma with no evidence of disease at 12 mo and treated with adjuvant chemotherapy (CT) (*n* = 10) with those from patients treated with adjuvant CT with disease recurrence (*n* = 11). [a]*P* = 0.028.

Recently an association has been found in enterocytes between nuclear PrPc and Wnt and Hippo pathways, which are modulated by cell contacts and are de-regulated at high frequency in many human cancers. In this way, PrPc should be considered as an actor in oncogenic processes through its role in the dynamics of cell-to-cell junctions because its nuclear localization could lead to modulate transcriptional activity of Wnt and Hippo effectors, some of the pathways clearly involved in carcinogenesis[58].

The relevance of these findings in clinical setting is supported by the evidence of a more aggressive behavior of PDAC depending on the amount of PrPc expression. As already demonstrated in our previous study[7], the expression of PrPc correlates with predicted patients' prognosis based on cancer stage according to pathology results. These data are encouraging, since they are confirmed also in a wider group of patients. Moreover, by further collecting early clinical data, we were able to validate such a hypothesis. In fact, in the subgroup of patients with a relapse of the disease after a surgical R0 resection, the expression of PrPc was significantly higher compared with those patients without evidence of relapse.

Moreover, these data indicate a relationship between PrPc and chemo-resistance, since the relapse during adjuvant CT was significantly higher in patients according to their expression of PrPc at western blot.

This is the first project that investigates the sub-cellular nuclear compartmentalization of increased PrPc expression with electron microscopy *in vivo* in patients with PDAC treated with surgery. Our findings could open new research avenues: in fact, being PrPc markedly expressed in the nuclear compartment, studies should investigate whether this protein is involved in specific activation of some unknown nuclear pathways that can lead to a higher cancer aggressiveness and de-differentiation. Similarly, the correlation of PrPc expression with a chemo-resistant phenotype could be used not only for prognostic purposes. For instance, patients may be stratified for prognosis according to PrPc expression in the resected specimen. This may involve also the development of novel therapeutic outcomes. In fact, specific therapeutic agents designed to target PrPc metabolism should be able to reduce a pathological cell growth and to revert chemo-resistance.

## CONCLUSION

The study confirms our previous data, highlighting *in vivo* in patients PDAC tissue the role of PrPc in PDAC aggressiveness. The evidence of a peculiar nuclear compartmentalization of this protein in the cellular nuclei of PDAC cells is in line with *in vitro* data from literature showing over-expression of PRNP gene in PDAC cells, and suggests the presence of some, still unknown, molecular pathways triggered by PrPc in the nuclear compartment. This extends the influence of PrPc beyond its role as cell-membrane glycoprotein. Finally, PrPc expression seems to be associated with a greater risk of relapse after radical surgery and in shifting the cancer towards a phenotype with a higher chemo-resistance. These data provide a step forward in the comprehension of PDAC biology, confirming that PrPc is likely to represent a marker of disease severity and a determinant of PDAC biology. Further studies are needed to validate these results and to investigate the molecular mechanism of PrPc in PDAC pathogenesis and its potential clinical application.

## ARTICLE HIGHLIGHTS

### *Research background*

Pancreatic ductal adenocarcinoma (PDAC) is one of the most lethal human cancers, but its molecular basis is still poorly understood. Among the several new targets for the comprehension of its biology, cellular Prion protein (PrPc) deserves particular mention, since it seems to be involved also in tumorigenesis.

### *Research motivation*

Recent evidences have shown a relationship between PrPc expression and PDAC, with a possible role of this protein in the molecular basis of PDAC aggressiveness itself.

### *Research objectives*

The present study aimed to further analyze the occurrence of PrPc within PDAC tissues, by investigating the specific compartmentalization of PrPc within PDAC cells, which is a fundamental aspect in order to provide a significant advancement in understanding the biology of disease. Moreover, we aimed to correlate the presence of PrPc with clinical data in order to find an association with patients' prognosis.

### Research methods

Samples from pancreatic tissues of 45 patients treated with pancreatic resection PDAC at a single institution were collected. Immune-gold stoichiometry within specific cell compartments was analyzed with electron microscopy in order to elucidate the subcellular compartmentalization of PrPc within PDAC cells. Western blotting was used to detect, quantify and compare the expression of PrPc in PDAC and control tissues, such as those of non-affected neighboring pancreatic tissue of the same patient. Data from western blot analysis where also used to perform a correlation with prognostic data from patients' follow-up.

### Research results

Immune-electron microscopy highlighted an increased PrPc expression in PDAC ductal cells of all patients and allowed to detect a peculiar compartmentalization of PrPc within tumor cells, with a specific increase of PrPc in the nucleus. Furthermore, semi-quantitative analysis by using Western blotting, showed that PrPc increased almost three-fold in tumor pancreatic tissue compared with healthy pancreatic regions, with a significantly higher expression of PrPc in patients with disease recurrence at 12 mo after radical surgery, also in the subgroup of patients treated with adjuvant CT, thus revealing a possible higher chemoresistance.

### Research conclusions

Our study provides evidence for a correlation between PrPc expression in PDAC and a worse biological behavior, with a higher recurrence rate and chemo-resistance. Moreover, it provides for the first-time the evidence of a peculiar subcellular compartmentalization of PrPc itself within PDAC cells.

### Research perspectives

PrPc could be a molecular marker associated to PDAC aggressiveness and ominous prognosis. Its nuclear compartmentalization suggests the activation of specific, but still unknown, molecular pathways involved in the biology of the disease and further studies in this sense are necessary, since specific therapeutic agents designed to target PrPc metabolism should be able to reduce a pathological cell growth and to revert chemo-resistance.

## ACKNOWLEDGEMENTS

The authors thank King SB for the Language Editing.

## REFERENCES

1 **Rahib L**, Smith BD, Aizenberg R, Rosenzweig AB, Fleshman JM, Matrisian LM. Projecting cancer incidence and deaths to 2030: The unexpected burden of thyroid, liver, and pancreas cancers in the united states. *Cancer Res* 2014 [DOI: 10.1158/0008-5472.CAN-14-0155]

2 **Warshaw AL**, Fernández-del Castillo C. Pancreatic carcinoma. *N Engl J Med* 1992; **326**: 455-465 [PMID: 1732772 DOI: 10.1056/NEJM199202133260706]

3 **Hidalgo M**. Pancreatic cancer. *N Engl J Med* 2010; **362**: 1605-1617 [PMID: 20427809 DOI: 10.1056/NEJMra0901557]

4 **Weledji EP**, Enoworock G, Mokake M, Sinju M. How Grim is Pancreatic Cancer? *Oncol Rev* 2016; **10**: 294 [PMID: 27471581 DOI: 10.4081/oncol.2016.294]

5 **Wang Y**, Yu S, Huang D, Cui M, Hu H, Zhang L, Wang W, Parameswaran N, Jackson M, Osborne B, Bedogni B, Li C, Sy MS, Xin W, Zhou L. Cellular Prion Protein Mediates Pancreatic Cancer Cell Survival and Invasion through Association with and Enhanced Signaling of Notch1. *Am J Pathol* 2016; **186**: 2945-2956 [PMID: 27639164 DOI: 10.1016/j.ajpath.2016.07.010]Available]

6 **Sy MS**, Li C, Yu S, Xin W. The fatal attraction between pro-prion and filamin A: prion as a marker in human cancers. *Biomark Med* 2010; **4**: 453-464 [PMID: 20550479 DOI: 10.2217/bmm.10.14]Available]

7 **Bianchini M**, Giambelluca MA, Scavuzzo MC, Di Franco G, Guadagni S, Palmeri M, Furbetta N, Gianardi D, Funel N, Pollina LE, Di Candio G, Fornai F, Morelli L. The occurrence of prion protein in surgically resected pancreatic adenocarcinoma. *Pancreatology* 2020; **20**: 1218-1225 [PMID: 32828686 DOI: 10.1016/j.pan.2020.08.004]

8 **Amin MB**, Edge SB; American Joint Committee on Cancer. AJCC cancer staging manual [Internet]. [cited 17 September 2019]. Available from: https://www.springer.com/gp/book/9783319406176

9 **Funel N**, Giovannetti E, Del Chiaro M, Mey V, Pollina LE, Nannizzi S, Boggi U, Ricciardi S, Del Tacca M, Bevilacqua G, Mosca F, Danesi R, Campani D. Laser microdissection and primary cell cultures improve pharmacogenetic analysis in pancreatic adenocarcinoma. *Lab Invest* 2008; **88**: 773-784 [PMID: 18490900 DOI: 10.1038/Labinvest.2008.40]

10 **Ricci C**, Mota C, Moscato S, D'Alessandro D, Ugel S, Sartoris S, Bronte V, Boggi U, Campani D, Funel N, Moroni L, Danti S. Interfacing polymeric scaffolds with primary pancreatic ductal adenocarcinoma cells to develop 3D cancer models. *Biomatter* 2014; **4**: e955386 [PMID: 25482337 DOI: 10.4161/21592527.2014.955386]

11 **Manni G**, Lewis V, Senesi M, Spagnolli G, Fallarino F, Collins SJ, Mouillet-Richard S, Biasini E. The cellular prion protein beyond prion diseases. *Swiss Med Wkly* 2020; **150**: w20222 [PMID: 32330284 DOI: 10.4414/smw.2020.20222]

12 **McLennan NF**, Brennan PM, McNeill A, Davies I, Fotheringham A, Rennison KA, Ritchie D, Brannan F, Head MW, Ironside JW, Williams A, Bell JE. Prion protein accumulation and neuroprotection in hypoxic brain damage. *Am J Pathol* 2004; **165**: 227-235 [PMID: 15215178 DOI: 10.1016/S0002-9440(10)63291-9]

13 **Brown DR**, Schulz-Schaeffer WJ, Schmidt B, Kretzschmar HA. Prion protein-deficient cells show altered response to oxidative stress due to decreased SOD-1 activity. *Exp Neurol* 1997; **146**: 104-112 [PMID: 9225743 DOI: 10.1006/exnr.1997.6505]

14 **Liang J**, Pan Y, Zhang D, Guo C, Shi Y, Wang J, Chen Y, Wang X, Liu J, Guo X, Chen Z, Qiao T, Fan D. Cellular prion protein promotes proliferation and G1/S transition of human gastric cancer cells SGC7901 and AGS. *FASEB J* 2007; **21**: 2247-2256 [PMID: 17409275 DOI: 10.1096/fj.06-7799com]

15 **Bremer J**, Baumann F, Tiberi C, Wessig C, Fischer H, Schwarz P, Steele AD, Toyka K V, Nave K-A, Weis J, Aguzzi A. Axonal prion protein is required for peripheral myelin maintenance. *Nat Neurosci* **13**: 310-318 [DOI: 10.1038/nn.2483]

16 **Málaga-Trillo E**, Solis GP, Schrock Y, Geiss C, Luncz L, Thomanetz V, Stuermer CA. Regulation of embryonic cell adhesion by the prion protein. *PLoS Biol* 2009; **7**: e55 [PMID: 19278297 DOI: 10.1371/journal.pbio.1000055]

17 **Mabbott N**, Turner M. Prions and the blood and immune systems. *Haematologica* 2005; **90**: 542-548 [PMID: 15820951]

18 **Martin-Lannerée S**, Hirsch TZ, Hernandez-Rapp J, Halliez S, Vilotte JL, Launay JM, Mouillet-Richard S. PrP(C) from stem cells to cancer. *Front Cell Dev Biol* 2014; **2**: 55 [PMID: 25364760 DOI: 10.3389/fcell.2014.00055]

19 **Steele AD**, Emsley JG, Ozdinler PH, Lindquist S, Macklis JD. Prion protein (PrPc) positively regulates neural precursor proliferation during developmental and adult mammalian neurogenesis. *Proc Natl Acad Sci U S A* 2006; **103**: 3416-3421 [PMID: 16492732 DOI: 10.1073/pnas.0511290103]

20 **Zhang CC**, Steele AD, Lindquist S, Lodish HF. Prion protein is expressed on long-term repopulating hematopoietic stem cells and is important for their self-renewal. *Proc Natl Acad Sci U S A* 2006; **103**: 2184-2189 [PMID: 16467153 DOI: 10.1073/pnas.0510577103]

21 **Lima FR**, Arantes CP, Muras AG, Nomizo R, Brentani RR, Martins VR. Cellular prion protein expression in astrocytes modulates neuronal survival and differentiation. *J Neurochem* 2007; **103**: 2164-2176 [PMID: 17868300 DOI: 10.1111/j.1471-4159.2007.04904.x]

22 **Lee YJ**, Baskakov I V. Treatment with normal prion protein delays differentiation and helps to maintain high proliferation activity in human embryonic stem cells. *J Neurochem***114**: 362-373 [DOI: 10.1111/j.1471-4159.2010.06601.x]

23 **Panigaj M**, Glier H, Wildova M, Holada K. Expression of prion protein in mouse erythroid progenitors and differentiating murine erythroleukemia cells. *PLoS One* 2011; **6**: e24599 [PMID: 21912705 DOI: 10.1371/journal.pone.0024599]

24 **Santos TG**, Silva IR, Costa-Silva B, Lepique AP, Martins VR, Lopes MH. Enhanced neural progenitor/stem cells self-renewal *via* the interaction of stress-inducible protein 1 with the prion protein. *Stem Cells* 2011; **29**: 1126-1136 [PMID: 21608082 DOI: 10.1002/stem.664]

25 **Stella R**, Massimino ML, Sandri M, Sorgato MC, Bertoli A. Cellular prion protein promotes regeneration of adult muscle tissue. *Mol Cell Biol* 2010; **30**: 4864-4876 [PMID: 20679477 DOI: 10.1128/MCB.01040-09]

26 **Ryskalin L**, Biagioni F, Busceti CL, Giambelluca MA, Morelli L, Frati A, Fornai F. The role of cellular prion protein in promoting stemness and differentiation in cancer. *Cancers (Basel)* 2021 [DOI: 10.3390/cancers13020170]

27 **Liang J**, Luo G, Ning X, Shi Y, Zhai H, Sun S, Jin H, Liu Z, Zhang F, Lu Y, Zhao Y, Chen X, Zhang H, Guo X, Wu K, Fan D. Differential expression of calcium-related genes in gastric cancer cells transfected with cellular prion protein. *Biochem Cell Biol* 2007; **85**: 375-383 [PMID: 17612632 DOI: 10.1139/O07-052]

28 **Le Corre D**, Ghazi A, Balogoun R, Pilati C, Aparicio T, Martin-Lannerée S, Marisa L, Djouadi F, Poindessous V, Crozet C, Emile JF, Mulot C, Le Malicot K, Boige V, Blons H, de Reynies A, Taieb J, Ghiringhelli F, Bennouna J, Launay JM, Laurent-Puig P, Mouillet-Richard S. The cellular prion protein controls the mesenchymal-like molecular subtype and predicts disease outcome in colorectal cancer. *EBioMedicine* 2019; **46**: 94-104 [PMID: 31377347 DOI: 10.1016/j.ebiom.2019.07.036]

29 **Chieng CK**, Say YH. Cellular prion protein contributes to LS 174T colon cancer cell carcinogenesis by increasing invasiveness and resistance against doxorubicin-induced apoptosis. *Tumour Biol* 2015; **36**: 8107-8120 [PMID: 25983001 DOI: 10.1007/s13277-015-3530-z]

30 **Li QQ**, Sun YP, Ruan CP, Xu XY, Ge JH, He J, Xu ZD, Wang Q, Gao WC. Cellular prion protein

promotes glucose uptake through the Fyn-HIF-2α-Glut1 pathway to support colorectal cancer cell survival. *Cancer Sci* 2011; **102**: 400-406 [PMID: 21265952 DOI: 10.1111/j.1349-7006.2010.01811.x]

31 **Ryskalin L**, Busceti CL, Biagioni F, Limanaqi F, Familiari P, Frati A, Fornai F. Prion protein in glioblastoma multiforme. *Int J Mol Sci* 2019 [DOI: 10.3390/ijms20205107]

32 **Lopes MH**, Santos TG, Rodrigues BR, Queiroz-Hazarbassanov N, Cunha IW, Wasilewska-Sampaio AP, Costa-Silva B, Marchi FA, Bleggi-Torres LF, Sanematsu PI, Suzuki SH, Oba-Shinjo SM, Marie SK, Toulmin E, Hill AF, Martins VR. Disruption of prion protein-HOP engagement impairs glioblastoma growth and cognitive decline and improves overall survival. *Oncogene* 2015; **34**: 3305-3314 [PMID: 25151961 DOI: 10.1038/onc.2014.261]

33 **Corsaro A**, Bajetto A, Thellung S, Begani G, Villa V, Nizzari M, Pattarozzi A, Solari A, Gatti M, Pagano A, Würth R, Daga A, Barbieri F, Florio T. Cellular prion protein controls stem cell-like properties of human glioblastoma tumor-initiating cells. *Oncotarget* 2016; **7**: 38638-38657 [PMID: 27229535 DOI: 10.18632/oncotarget.9575]

34 **Halliez S**, Martin-Lannerée S, Passet B, Hernandez-Rapp J, Castille J, Urien C, Chat S, Laude H, Vilotte JL, Mouillet-Richard S, Béringue V. Prion protein localizes at the ciliary base during neural and cardiovascular development, and its depletion affects α-tubulin post-translational modifications. *Sci Rep* 2015; **5**: 17146 [PMID: 26679898 DOI: 10.1038/srep17146]

35 **Zhuang D**, Liu Y, Mao Y, Gao L, Zhang H, Luan S, Huang F, Li Q. TMZ-induced PrPc/par-4 interaction promotes the survival of human glioma cells. *Int J Cancer* 2012; **130**: 309-318 [PMID: 21328340 DOI: 10.1002/ijc.25985]

36 **Zhao Y**, You H, Liu F, An H, Shi Y, Yu Q, Fan D. Differentially expressed gene profiles between multidrug resistant gastric adenocarcinoma cells and their parental cells. *Cancer Lett* 2002; **185**: 211-218 [PMID: 12169395 DOI: 10.1016/S0304-3835(02)00264-1]

37 **Du J**, Pan Y, Shi Y, Guo C, Jin X, Sun L, Liu N, Qiao T, Fan D. Overexpression and significance of prion protein in gastric cancer and multidrug-resistant gastric carcinoma cell line SGC7901/ADR. *Int J Cancer* 2005; **113**: 213-220 [PMID: 15386405 DOI: 10.1002/ijc.20570]

38 **Luo G**, Wang W, Wu Q, Lu Y, Su T, Gu N, Li K, Wang J, Du R, Zhao X, Li X, Fan R, Zhang H, Nie Y, Zhou X, Shi Y, Liang J, Wang X, Fan D. MGr1-Antigen/37 kDa laminin receptor precursor promotes cellular prion protein induced multi-drug-resistance of gastric cancer. *Oncotarget* 2017 [DOI: 10.18632/oncotarget.17795]

39 **Diarra-Mehrpour M**, Arrabal S, Jalil A, Pinson X, Gaudin C, Piétu G, Pitaval A, Ripoche H, Eloit M, Dormont D, Chouaib S. Prion protein prevents human breast carcinoma cell line from tumor necrosis factor alpha-induced cell death. *Cancer Res* 2004; **64**: 719-727 [PMID: 14744790]

40 **Cheng Y**, Tao L, Xu J, Li Q, Yu J, Jin Y, Chen Q, Xu Z, Zou Q, Liu X. CD44/cellular prion protein interact in multidrug resistant breast cancer cells and correlate with responses to neoadjuvant chemotherapy in breast cancer patients. *Mol Carcinog* 2014; **53**: 686-697 [PMID: 23681900 DOI: 10.1002/mc.22021]

41 **Meslin F**, Conforti R, Mazouni C, Morel N, Tomasic G, Drusch F, Yacoub M, Sabourin JC, Grassi J, Delaloge S, Mathieu MC, Chouaib S, Andre F, Mehrpour M. Efficacy of adjuvant chemotherapy according to Prion protein expression in patients with estrogen receptor-negative breast cancer. *Ann Oncol* 2007; **18**: 1793-1798 [PMID: 17872899 DOI: 10.1093/annonc/mdm406]

42 **Atkinson CJ**, Kawamata F, Liu C, Ham S, Győrffy B, Munn AL, Wei MQ, Möller A, Whitehall V, Wiegmans AP. EGFR and Prion protein promote signaling *via* FOXO3a-KLF5 resulting in clinical resistance to platinum agents in colorectal cancer. *Mol Oncol* 2019; **13**: 725-737 [PMID: 30478887 DOI: 10.1002/1878-0261.12411]

43 **Park JY**, Jeong JK, Lee JH, Moon JH, Kim SW, Lee YJ, Park SY. Induction of cellular prion protein (PrPc) under hypoxia inhibits apoptosis caused by TRAIL treatment. *Oncotarget* 2015; **6**: 5342-5353 [PMID: 25742790 DOI: 10.18632/oncotarget.3028]

44 **Liang J**, Ge F, Guo C, Luo G, Wang X, Han G, Zhang D, Wang J, Li K, Pan Y, Yao L, Yin Z, Guo X, Wu K, Ding J, Fan D. Inhibition of PI3K/Akt partially leads to the inhibition of PrP(C)-induced drug resistance in gastric cancer cells. *FEBS J* 2009; **276**: 685-694 [PMID: 19143835 DOI: 10.1111/j.1742-4658.2008.06816.x]

45 **Wiegmans AP**, Saunus JM, Ham S, Lobb R, Kutasovic JR, Dalley AJ, Miranda M, Atkinson C, Foliaki ST, Ferguson K, Niland C, Johnstone CN, Lewis V, Collins SJ, Lakhani SR, Al-Ejeh F, Möller A. Secreted cellular prion protein binds doxorubicin and correlates with anthracycline resistance in breast cancer. *JCI Insight* 2019; **5** [PMID: 30830863 DOI: 10.1172/jci.insight.124092]

46 **Iglesia RP**, Prado MB, Cruz L, Martins VR, Santos TG, Lopes MH. Engagement of cellular prion protein with the co-chaperone Hsp70/90 organizing protein regulates the proliferation of glioblastoma stem-like cells. *Stem Cell Res Ther* 2017; **8**: 76 [PMID: 28412969 DOI: 10.1186/s13287-017-0518-1]

47 **Pan Y**, Zhao L, Liang J, Liu J, Shi Y, Liu N, Zhang G, Jin H, Gao J, Xie H, Wang J, Liu Z, Fan D. Cellular prion protein promotes invasion and metastasis of gastric cancer. *FASEB J* 2006; **20**: 1886-1888 [PMID: 16877520 DOI: 10.1096/fj.06-6138fje]

48 **Gil M**, Kim YK, Kim KE, Kim W, Park CS, Lee KJ. Cellular prion protein regulates invasion and migration of breast cancer cells through MMP-9 activity. *Biochem Biophys Res Commun* 2016; **470**: 213-219 [PMID: 26778001 DOI: 10.1016/j.bbrc.2016.01.038]

49 **de Lacerda TC**, Costa-Silva B, Giudice FS, Dias MV, de Oliveira GP, Teixeira BL, Dos Santos TG, Martins VR. Prion protein binding to HOP modulates the migration and invasion of colorectal cancer cells. *Clin Exp Metastasis* 2016; **33**: 441-451 [PMID: 27112151 DOI: 10.1007/s10585-016-9788-8]

50 **Lin SC**, Lin CH, Shih NC, Liu HL, Wang WC, Lin KY, Liu ZY, Tseng YJ, Chang HK, Lin YC, Yeh

YC, Minato H, Fujii T, Wu YC, Chen MY, Chou TY. Cellular prion protein transcriptionally regulated by NFIL3 enhances lung cancer cell lamellipodium formation and migration through JNK signaling. *Oncogene* 2020; **39**: 385-398 [PMID: 31477838 DOI: 10.1038/s41388-019-0994-0]

51 **Li C**, Yu S, Nakamura F, Pentikäinen OT, Singh N, Yin S, Xin W, Sy MS. Pro-prion binds filamin A, facilitating its interaction with integrin beta1, and contributes to melanomagenesis. *J Biol Chem* 2010; **285**: 30328-30339 [PMID: 20650901 DOI: 10.1074/jbc.M110.147413]

52 **Rezza A**, Sennett R, Rendl M. Adult Stem Cell Niches [Internet]. In: Current topics in developmental biology. 2014 [cited 2019 Sep 17]. page 333–372 Available from: http://www.ncbi.nlm.nih.gov/pubmed/24439812 [PMID: 24439812]

53 **Hanahan D**, Weinberg RA. Hallmarks of cancer: the next generation. *Cell* 2011; **144**: 646-674 [PMID: 21376230 DOI: 10.1016/j.cell.2011.02.013]

54 **Mehrpour M**, Codogno P. Prion protein: From physiology to cancer biology. *Cancer Lett* 2010; **290**: 1-23 [PMID: 19674833 DOI: 10.1016/j.canlet.2009.07.009]

55 **Barbieri G**, Palumbo S, Gabrusiewicz K, Azzalin A, Marchesi N, Spedito A, Biggiogera M, Sbalchiero E, Mazzini G, Miracco C, Pirtoli L, Kaminska B, Comincini S. Silencing of cellular prion protein (PrPC) expression by DNA-antisense oligonucleotides induces autophagy-dependent cell death in glioma cells. *Autophagy* 2011; **7**: 840-853 [PMID: 21478678 DOI: 10.4161/auto.7.8.15615]

56 **Du L**, Rao G, Wang H, Li B, Tian W, Cui J, He L, Laffin B, Tian X, Hao C, Liu H, Sun X, Zhu Y, Tang DG, Mehrpour M, Lu Y, Chen Q. CD44-positive cancer stem cells expressing cellular prion protein contribute to metastatic capacity in colorectal cancer. *Cancer Res* 2013; **73**: 2682-2694 [PMID: 23418321 DOI: 10.1158/0008-5472.CAN-12-3759]

57 **Han H**, Bearss DJ, Browne LW, Calaluce R, Nagle RB, Von Hoff DD. Identification of differentially expressed genes in pancreatic cancer cells using cDNA microarray. *Cancer Res* 2002; **62**: 2890-2896 [PMID: 12019169]

58 **Rousset M**, Leturque A, Thenet S. The nucleo-junctional interplay of the cellular prion protein: A new partner in cancer-related signaling pathways? *Prion* 2016; **10**: 143-152 [PMID: 27216988 DOI: 10.1080/19336896.2016.1163457]

World Journal of Gastroenterology

Submit a Manuscript: https://www.f6publishing.com

*World J Gastroenterol* 2021 November 14; 27(42): 7340-7349

DOI: 10.3748/wjg.v27.i42.7340

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

ORIGINAL ARTICLE

Basic Study

# Gut microbiome composition can predict the response to nivolumab in advanced hepatocellular carcinoma patients

Min-Woo Chung, Moon-Ju Kim, Eun Jeong Won, Yu Jeong Lee, Yong-Woon Yun, Sung Bum Cho, Young-Eun Joo, Jun-Eul Hwang, Woo Kyun Bae, Ik-Joo Chung, Myung Geun Shin, Jong Hee Shin

**ORCID number:** Min-Woo Chung 0000-0003-3144-1450; Moon-Ju Kim 0000-0002-8018-6903; Eun Jeong Won 0000-0002-8750-4257; Yu Jeong Lee 0000-0003-1282-4210; Yong-Woon Yun 0000-0001-6715-1768; Sung Bum Cho 0000-0001-9816-3446; Young-Eun Joo 0000-0003-0422-2439; Jun-Eul Hwang 0000-0002-3768-4149; Woo Kyun Bae 0000-0003-0267-1943; Ik-Joo Chung 0000-0003-0479-8067; Myung Geun Shin 0000-0002-0372-9185; Jong Hee Shin 0000-0001-9593-476X.

**Author contributions:** Won EJ conceived of the study, participated in the study design and data analysis, and was responsible for writing and submission of the final manuscript; Kim MJ and Lee YJ carried out the experimental studies; Chung MW, Cho SB, Joo Y, Yun YW and Kim MJ analyzed and interpreted the data; Yun YW interpreted the statistical data; Hwang JE, Bae WK, Chung IJ, Shin MG and Shin JH were responsible for patient care and sample acquisition; All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published.

**Supported by** the National Research Foundation of Korea

**Min-Woo Chung, Sung Bum Cho, Young-Eun Joo, Jun-Eul Hwang, Woo Kyun Bae, Ik-Joo Chung,** Department of Internal Medicine, Chonnam National University Hospital and College of Medicine, Hwasun 58128, Jeollanam-do, South Korea

**Moon-Ju Kim, Eun Jeong Won, Yu Jeong Lee,** Department of Parasitology and Tropical Medicine, Chonnam National University Medical School, Hwasun 58128, Jeollanam-do, South Korea

**Eun Jeong Won, Myung Geun Shin,** Department of Laboratory Medicine, Chonnam National University Hwasun Hospital, Hwasun 58128, Jeollanam-do, South Korea

**Yong-Woon Yun,** Department of Preventive Medicine, Chonnam National University Medical School, Hwasun-eup, Hwasun 58128, Jeollanam-do, South Korea

**Jong Hee Shin,** Department of Laboratory Medicine, Chonnam National University Hospital, Gwangju 61469, South Korea

**Corresponding author:** Eun Jeong Won, MD, PhD, Professor, Department of Parasitology and Tropical Medicine, Chonnam National University Medical School, 322, Seoyang-ro, Hwasun-eup, Hwasun 58128, Jeollanam-do, South Korea. parasite.woni@jnu.ac.kr

## Abstract

### *BACKGROUND*

Immunotherapy has revolutionized the clinical outcomes of intractable cancer patients. Little is known about the intestinal nonpathogenic bacterial composition of hepatocellular carcinoma (HCC) patients treated by immunotherapy.

### *AIM*

To determine whether there is a correlation between gut bacterial composition and prognosis in HCC patients.

### *METHODS*

From September 2019 to March 2020, we prospectively collected fecal samples and examined the gut microbiome of 8 advanced HCC patients treated with nivolumab as a second- or third-line systemic treatment. Fecal samples were collected before the start of immunotherapy. Fecal samples of patients with progression during treatment were collected at the time of progression, and fecal

(NRF) Grant funded by the Ministry of Education, Science, and Technology, No. NRF-2019R1C1C1004605, No. NRF-2019M3E5D1A02067953, and No. NRF-2021M3E5D1A0201518021.

**Institutional review board statement:** This study was carried out in accordance with all relevant institutional guidelines. The Ethics Committee of Chonnam National University Hwasun Hospital approved this study (CNUHH-2019-134) and written informed consent was obtained from all subjects.

**Conflict-of-interest statement:** All authors declare having no conflicts of interest.

**Data sharing statement:** No additional data are available.



**Specialty type:** Gastroenterology and hepatology

**Country/Territory of origin:** South Korea

**Peer-review report's scientific quality classification**

Grade A (Excellent): 0
Grade B (Very good): 0
Grade C (Good): C
Grade D (Fair): 0
Grade E (Poor): 0

**Received:** May 20, 2021
**Peer-review started:** May 20, 2021
**First decision:** June 13, 2021
**Revised:** June 18, 2021
**Accepted:** November 3, 2021

samples of patients who showed good response to nivolumab were collected after 5-7 mo as follow-up. Metagenomic data from 16S ribosomal RNA sequencing were analyzed using CLC Genomics Workbench. Microbiome data were analyzed according to therapeutic response.

***RESULTS***

All 8 patients were male, of which 6 had underlying chronic hepatitis B. A higher Shannon index was found in the responders than in the non-responders after nivolumab therapy ($P$ = 0.036). The unweighted beta diversity analysis also showed that the overall bacterial community structure and phylogenetic diversity were clearly distinguished according to therapeutic response. There was no significant difference in the diversity or composition of the patient gut microbiome according to the immunotherapy used. Several taxa specific to therapeutic response were designated as follows: *Dialister pneumosintes, Escherichia coli, Lactobacillus reteri, Streptococcus mutans, Enterococcus faecium, Streptococcus gordonii, Veillonella atypica, Granulicatella* sp., and *Trchuris trichiura* for the non-responders; *Citrobacter freundii, Azospirillum* sp. and *Enterococcus durans* for the responders. Of note, a skewed *Firmicutes/Bacteroidetes* ratio and a low *Prevotella/Bacteroides* ratio can serve as predictive markers of non-response, whereas the presence of *Akkermansia* species predicts a good response.

***CONCLUSION***

The current presumptive study suggests a potential role for the gut microbiome as a prognostic marker for the response to nivolumab in treatment of HCC patients.

**Key Words:** Microbiome; Nivolumab; *Firmicutes/Bacteroidetes* ratio; *Prevotella/Bacteroides* ratio; Hepatocellular carcinoma; Prognosis



**Core Tip:** Immune check point inhibitors are known to be an effective treatment option for advanced hepatocellular carcinoma (HCC), not only for second-line, but also as a first-line treatment. However, there are few predictive or prognostic markers for which patient group will have a good treatment response to immunotherapy or systemic therapy for HCC until now. Our study shows that non-responders to nivolumab in HCC patients have dysbiotic fecal composition, whereas a high *Prevotella/Bacteroides* ratio can predict a better response to nivolumab, highlighting a potential role for the gut microbiome as a prognostic marker for the response to nivolumab therapy.

**Citation:** Chung MW, Kim MJ, Won EJ, Lee YJ, Yun YW, Cho SB, Joo YE, Hwang JE, Bae WK, Chung IJ, Shin MG, Shin JH. Gut microbiome composition can predict the response to nivolumab in advanced hepatocellular carcinoma patients. *World J Gastroenterol* 2021; 27(42): 7340-7349
**URL:** https://www.wjgnet.com/1007-9327/full/v27/i42/7340.htm
**DOI:** https://dx.doi.org/10.3748/wjg.v27.i42.7340

## INTRODUCTION

Hepatocellular carcinoma (HCC) is the most frequent primary malignancy of the liver, and it is now the second leading cause of cancer death worldwide[1]. Curative HCC treatment is only feasible in the early stages and involves local ablative procedures, surgical resection, or liver transplantation. For patients not amenable to curative therapy and in those with metastatic disease, other systemic treatments, such as sorafenib, are available. Beyond the limits of standard therapies, immunotherapy has been introduced for the oncological treatment of various solid malignancies. Nivolumab, as the first programmed cell death protein-1 (PD-1) agent, has shown great promise in treatment of various cancers, including melanoma and squamous cell carcinoma of the head and neck[2,3]. Recently, it was approved as the second-line agent for advanced HCC patients who have experienced sorafenib failure[4,5]. In line

**Article in press:** November 3, 2021
**Published online:** November 14, 2021

**P-Reviewer:** Tun HM
**S-Editor:** Gong ZM
**L-Editor:** A
**P-Editor:** Gong ZM

with this growing clinical relevance, stratification of patients receiving nivolumab therapy is now required to predict the prognosis and tumor aggressiveness.

There is mounting evidence that the gut microbiota can influence and modulate host immune responses; thus, microbiome profiling has been revealed as a predictor for response to immunotherapy among different groups and in different countries[6-8]. Moreover, some immunostimulatory bacterial species, including *Akkermansia muciniphila*[6], *Bifidobacterium longum*[7] and *Bacteroidetes fragilis*[8], were reported to elicit systemic immune responses and to reprogram the tumor microenvironment in mouse tumors treated with anti–cytotoxic T-lymphocyte-associated-4 and/or anti–PD-1 antibodies. The human gut microbiota has been shown to be associated with clinical responses to anti-PD-1/programmed death ligand 1 (PD-L1) immunotherapy in melanoma, non-small cell lung cancer, and renal cell carcinoma. However, the association between gut microbiota and response to nivolumab therapy is still not clear.

This study aimed to elucidate the impact of the gut microbiota on the prediction of prognosis in advanced HCC patients receiving nivolumab immunotherapy.

## MATERIALS AND METHODS

### Study design and patients

A total of 8 patients who received nivolumab (from September 2019 to August 2020) as second- or third-line treatment after sorafenib failure were included in the study. Tumor response was assessed using the Response Evaluation Criteria in Solid Tumors version 1.1[9]. The study was approved by the Chonnam National University Hwasun Hospital Institutional Review Board (CNUHH-2019-134). All patients signed a written informed consent form based on the principles of the Declaration of Helsinki. Patients were classified based on disease status (absence or progression at 12 mo after initiation of nivolumab therapy) and overall response (complete response, partial response, or stable disease for > 6 mo). Non-responders were those showing disease progression or stable disease for < 6 mo as well as those who died.

### Collection of fecal samples and microbiome analysis

Fecal samples were collected prospectively, according to International Human Microbiome Standards guidelines (SOP_03_V1) at two time points — before the first nivolumab injection (< 1 mo, T0) and at the 3-mo follow-up (T1). The samples were immediately transferred on ice to our clinic and immediately stored at -80 °C until sample processing. Fecal genomic DNA was extracted as previously described[6-8]. DNA was extracted using the Cica Geneus® DNA Prep Kit (Kanto Chemical, Tokyo, Japan) following the manufacturer's instructions. The fecal microbiome was assessed by sequencing various regions (V3-V4) of the 16S ribosomal RNA bacterial gene at months 0 and 2. Briefly, polymerase chain reaction amplification was performed using 16S universal primers targeting the V3-V4 region of the bacterial 16S ribosomal gene. The joint pair length was set to encompass 467 base pair amplicons using the 2 × 300 paired-end MiSeq kit (Illumina, San Diego, CA, United States). For each sample, a sequencing library was generated by adding sequencing adapters. Detection of the sequencing fragments was performed using MiSeq technology by Macrogen (Seoul, South Korea).

### Metagenomic and network analysis

The targeted metagenomic sequences from the microbiota were analyzed using the bioinformatics pipeline established by Vaiomer (Labège, France) using the FROGS guidelines. Low-depth samples (less than 9000 sequences per sample) were removed from the analysis. Sequences were trimmed and merged and then clustered into operational taxonomic units (OTUs) using CLC Genomics Workbench v. 10.1.1 and CLC Microbial Genomics Module v. 2.5 (Qiagen, Hilden, Germany). Taxonomic assignment of these sequences was carried based on the National Center for Biotechnology Information taxonomy database, with an OTU cutoff of 3%. The most abundant sequences were considered representative of each cluster and assigned to a taxonomy level based CLC Microbial Genomics default values.

Alpha diversity metrics (richness and Shannon's index) were calculated using the phyloseq R package based on rarefied OTU counts[10]. The beta diversity index was defined as the difference between the total number of species across the two groups and the number of species common to both[11]. Exploratory analysis of beta-diversity (between-sample diversity) was performed based on the Bray-Curtis measure of

dissimilarity as a principal coordinate analysis.

For the hierarchical cluster analysis, Bray-Curtis metrics and complete linkage clustering were implemented. At deeper taxonomic levels (from the phylum to genus level), we performed linear discriminant analysis (LDA) effect size analysis based on the non-parametric factorial Kruskal-Wallis sum rank test, to detect bacterial taxa with significantly different abundance between responders and non-responders. Then, LDA effect size was used to estimate the size effect of each differentially abundant taxon based on the criteria of LDA ≥ 3.0 and $P < 0.05$. Volcano plots showed the estimated log 2-fold difference in OTU abundance between responders and non-responders.

### Statistical analysis

Alpha diversity metrics were compared by Mann-Whitney tests; for comparisons involving more than two groups, Bonferroni's correction was applied. The LDA was performed, and the volcano plots generated, at http://huttenhower.sph.harvard.edu/galaxy/. Two-tailed student's *t* test and the $\chi^2$ test were used to test for differences in the phenotypical characteristics of the microbiome using GraphPad Prism software (GraphPad Software Inc., San Diego, CA, United States).

## RESULTS

### Patients and sample

A single-site correlative study design was used to investigate the effects of gut microbiota on the efficacy of nivolumab in 8 adult HCC patients (Supplementary Table 1). All 8 patients received nivolumab as second- or third-line treatment after sorafenib failure. The median age was 62.5 (interquartile range: 58.0-66.25) years overall, and was not significantly different between the groups. All patients were male. Six patients (75%) were chronically infected with hepatitis B virus (HBV), and four patients (50%) had alcohol-related liver disease. All patients were Barcelona Clinic Liver Cancer stage C and stage IV by the modified Union for International Cancer Control stage system. While all 3 patients of the non-responder group had vascular invasion, only 1 patient (20%) had vascular invasion in the responder group. Presence of biliary invasion was 66.7% (2/3) in the non-responder group and 20% (1/5) in the responder group. The percentage of patients with an alpha-fetoprotein score ≥ 400 ng/mL at baseline was 66.7% (2/3) and 40% (2/5), each. Most patients (87.5%) were Child-Pugh class A, and only 1 patient in the good response group was Child-Pugh class B. All patients received three or more prior therapies before nivolumab.

### Metagenome analysis

A total of 2027154 good-quality reads with a mean length of 301 base pairs were generated. A higher Shannon index was found in the responders when compared to the non-responders after nivolumab therapy ($P = 0.036$), reflecting a significantly higher species richness in the former group (Figure 1A and B). In contrast, there was no significant difference in alpha diversity within the same patients according to the nivolumab therapy (Figure 1C). The unweighted beta diversity analysis also showed that the overall bacterial community structure and phylogenetic diversity were similar between T0 and T1, but the responders and non-responders were clearly distinguished (Figure 2).

To identify the bacterial taxa associated with a good prognosis, different taxonomical levels were compared using LDA, and 36 and 4 species were found to be differentially abundant in the responders and non-responders, respectively (Figure 3). Of the bacterial species, *Ruminococcus gnavus* was abundant in the non-responders, while several bacterial taxa, including *Clostridia, Prevotella 9*, *Rikenellaceae, Alistipes,* the *Christensenellaceae R-7* group, *Dialister, Muribaculaceae, Desulfovibrionales, Deltaproteobacteria,* the *Eubacterium coprostanoligenes* group, *Acidaminococcaceae,* the *Lachnospiraceae NK4A136* group, *Roseburia, Mitsuokella, Ruminiclostridium 9, Marinifilaceae, Lachnospiraceae,* and *Ruminococcaceae* groups other than *Ruminococcus gnavus* were highly enriched in the responders. A volcano plot also showed several bacterial species specifically associated with the prognosis of nivolumab therapy in the HCC patients. *Dialister pneumosintes, Escherichia coli, Lactobacillus reteri, Streptococcus mutans, Enterococcus faecium, Streptococcus gordonii, Veillonella atypica, Granulicatella* sp., and *Trchuris trichiura* were specific to the non-responders, while *Citrobacter freundii, Azospirillum* sp., and *Enterococcus durans* were specific to the responders.

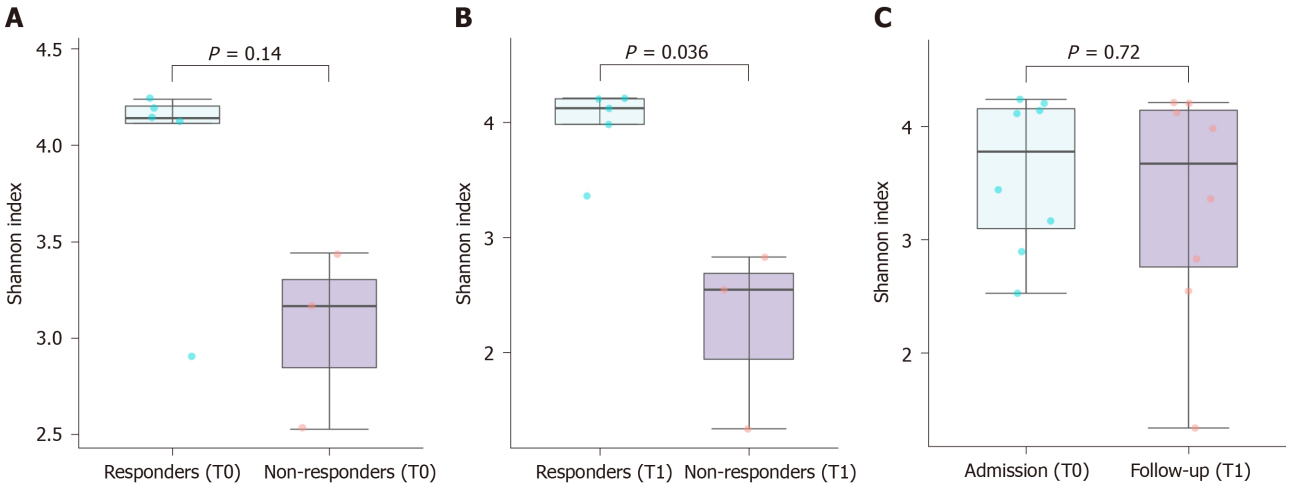


**Figure 1 Alpha diversity indices (Shannon index) of hepatocellular carcinoma patients based on treatment and prognosis.** A: The Shannon index of the non-responders on admission (T0) was not significantly lower than that of the responders ($P$ = 0.14); B: The Shannon index of the non-responders was significantly lower than that of the responders after nivolumab therapy (T1); C: Nivolumab therapy did not alter the Shannon index. HCC: Hepatocellular carcinoma.

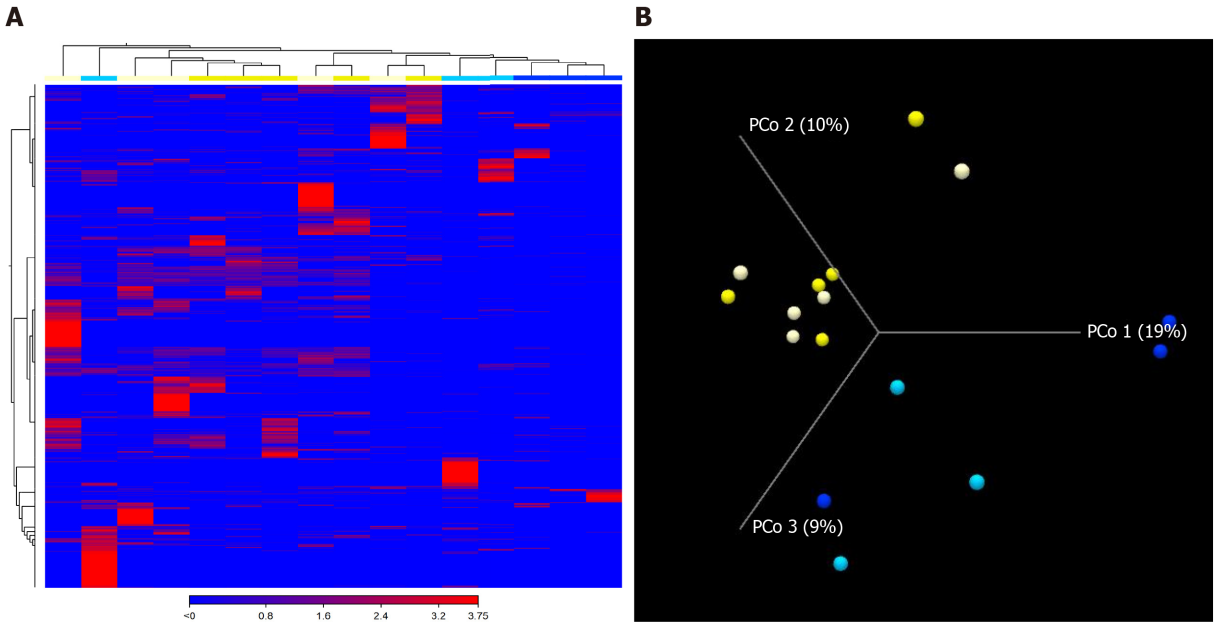


**Figure 2 Composition of the gut microbiome in hepatocellular carcinoma patients is associated with the response to nivolumab.** A: Heatmap showing the abundance of operational taxonomic units in responders (yellow) and non-responders (blue). The original comprehensive figure, including the names of bacterial taxa, is presented as Supplemental Figure 1; B: Unweighted beta diversity analysis showed that the overall bacterial community structure and phylogenetic diversity at T0 (light yellow and light blue) and T1 (yellow and blue) were similar; distinct clusters were not observed for responders (yellow) and non-responders (blue). Statistical values obtained by the PERMANOVA test are presented in Supplemental Table 2.

The gut microbiota composition in HCC patients was also described at the phylum and genus levels (Figure 4). At the phylum level, a skewed *Firmicutes/Bacteroidetes* ratio (< 0.5 or > 1.5) was more frequently found in the non-responders than in the responders (66.7% *vs* 10%, $P < 0.05$). The mean ratio of *Prevotella* species to *Bacteroides* species (P/B ratio) was significantly higher in the responders than in the non-responders (22.99 *vs* 2.312, $P = 0.024$). In addition, the presence of *Akkermansia* species was observed in two responders only, indicating that this could be a useful prognostic marker of the response to nivolumab therapy in advanced HCC patients.

## DISCUSSION

A number of clinical trials investigating the therapeutic potential of manipulation of

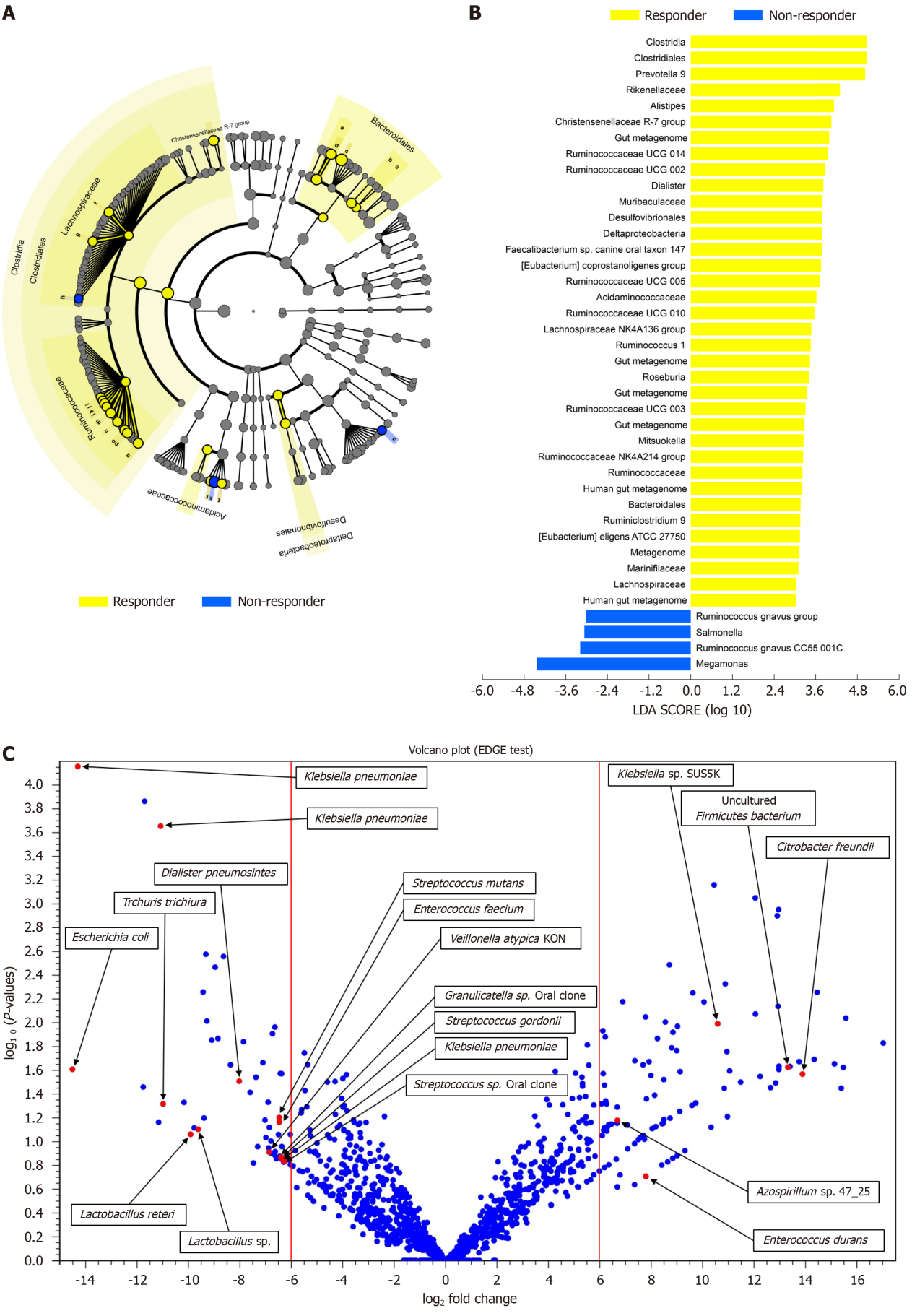


**Figure 3 Specific bacterial taxa are associated with the prognosis of nivolumab therapy in hepatocellular carcinoma patients according to linear discriminant analysis effect size analysis.** A: Taxonomic cladogram based on linear discriminant analysis (LDA) using effect size (LEfSe) showing

differences in fecal taxa. Dot size is proportional to the abundance of the taxon. Letters correspond to the following taxa: (a) *Marinifilaceae*, (b) *Muribaculaceae*, (c) *Prevotella 9*, (d) *Alistipes*, (e) *Rikenellaceae*, (f) the *Lachnospiraceae NK4A136* group, (g) *Roseburia*, (h) the *[Ruminococcus]gnavus* group, (i) *Ruminiclostridium 9*, (j) the *Ruminococcaceae NK4A214* group, (k) *Ruminococcaceae UCG-002*, (l) *Ruminococcaceae UCG-003*, (m) *Ruminococcaceae UCG-005*, (n) *Ruminococcaceae UCG-010*, (o) *Ruminococcaceae UCG-014*, (p) *Ruminococcus 1*, the (q) *[Eubacterium]coprostanoligenes* group, (r) *Dialister*, (s) *Megamonas*, (t) *Mitsuokella*, and (u) *Salmonella*; B: LDA scores for the differentially abundant taxa in the fecal microbiome of responders (yellow) and non-responders (blue). The length denotes the effect size for a taxon. $P = 0.05$ for the Kruskal-Wallis test; LDA score > 3; C: Volcano plot showing several bacterial taxa specifically related to the prognosis of nivolumab therapy in hepatocellular carcinoma patients. LDA: Linear discriminant analysis.

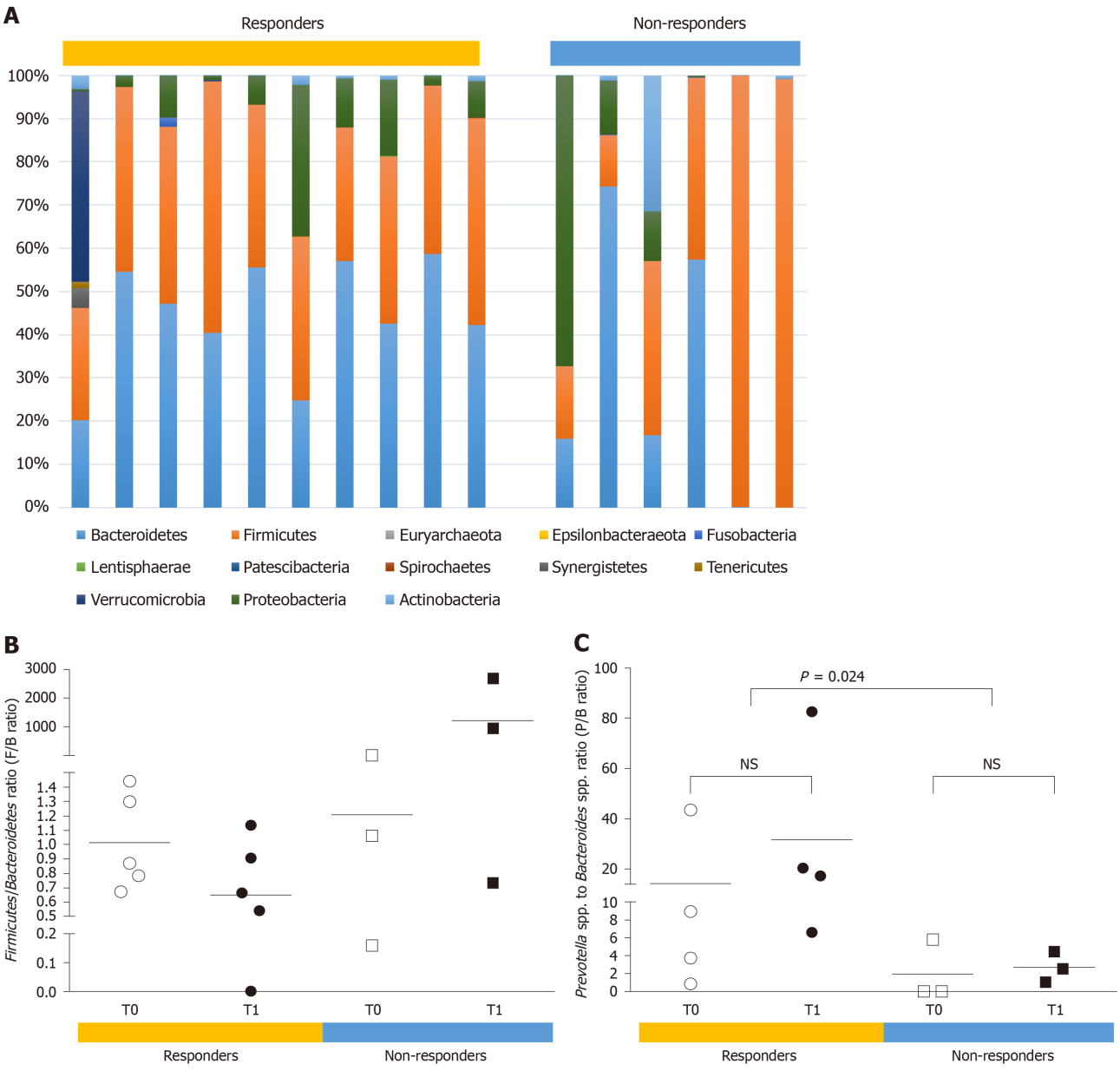


**Figure 4 Potential prognostic markers of the response to nivolumab therapy in hepatocellular carcinoma patients.** A and B: At the phylum level, a skewed *Firmicutes/Bacteroidetes* ratio (< 0.5 or > 1.5) was more frequent in the non-responders than the responders (66.7% *vs* 10.0%, $P = 0.018$); C: At the genus level, the ratio of *Prevotella* species to *Bacteroides* species (P/B ratio) was a prognostic marker of the response to nivolumab in hepatocellular carcinoma patients. The responders showed a significantly higher mean P/B ratio than the non-responders (22.99 *vs* 2.312, $P = 0.024$). NS: Statistically non-specific.

the gut microbiota in non-HCC cancer patients has already begun[6-8,12]. Regarding HCC, however, only two Chinese studies have been reported to date[13,14]. Reduced alpha diversity was found in the non-responders in those studies, in line with our data. In addition, we found that several bacterial taxa, such as *Dialister pneumosintes, Escherichia coli, Lactobacillus reteri, Streptococcus mutans, Enterococcus faecium, Streptococcus gordonii, Veillonella atypica, Granulicatella* sp., and *Trchuris trichiura*, were specific to non-responders, while *Citrobacter freundii, Azospirillum* sp., and *Enterococcus durans*

were specific to responders. The gut bacteria associated with the therapeutic response did not overlap with those in previous studies[13,14]. This might be partly due to differences in the techniques used to analyze samples, the reference databases, and geographical or racial/ethnic differences. While this study has not proven the clinical efficacy and benefit of our approach, it is feasible that it could reduce the therapeutic failure rate of nivolumab. Furthermore, it is not clear whether the aforementioned findings regarding the role of the gut microbiome in antitumor immune responses in animal models and patients with other tumor types also apply to patients with HCC.

Beyond dysbiosis, we suggested additional potential prognostic markers of the response to nivolumab therapy in HCC patients. We found that in all the responders, the *Firmicutes/Bacteroidetes* ratio ranged from 0.54 to 1.44 (mean of 0.88), whereas 66.7% of non-responders exhibited a highly skewed ratio (< 0.5 or > 1.5). Previous studies have shown elevated *Firmicutes/Bacteroidetes* ratio in the gut microbiota to be associated with obesity and older age[15], suggesting that it could be the result of dysbiosis arising from adaptation of individual microbial communities to long-term metabolic dysfunction[16]. Until recently, few studies have discussed the association between the *Firmicutes/Bacteroidetes* ratio and gastrointestinal cancer. Yu *et al*[17] reported that, in a rat model, precancerous lesions of gastric cancer had the highest *Firmicutes/Bacteroidetes* ratio. A reduced *Firmicutes/Bacteroidetes* ratio was also found to be related to the progression of liver diseases (liver cirrhosis and primary liver cancer) [18]. Notably, our data strongly support the notion that a highly skewed *Firmicutes/Bacteroidetes* ratio can be used to predict a lack of response to nivolumab therapy. This could facilitate the selection of appropriate immunotherapies for advanced HCC patients.

Furthermore, we found that a high P/B ratio was a better prognostic marker of the response to nivolumab therapy outcome in HCC patients. Overall, we found that responders had a significantly higher P/B ratio than non-responders. This observation is partly supported by a recent study reporting an elevated P/B ratio in fecal samples obtained from advanced-stage gastrointestinal cancer patients receiving anti-PD-1/PD-L1 and an improved response to anti-PD-1/PD-L1 treatment[19]. In line with previous studies, we also found that the presence of *Akkermansia* species can serve as a good prognostic marker of the response to nivolumab therapy in advanced HCC patients[5,13]. Although we did not analyze the gut microbiome of non-HCC patients, but previous studies have shown that patients with HCC and underlying cirrhosis typically present with profound dysbiosis. Thus, it is tempting to speculate that underlying dysbiosis could contribute to immunotherapy failure in some patients and that gut microbiome modulation may have even more profound effects in HCC than other tumors.

In this study, all 5 responders and 1 of 3 non-responders were infected by HBV. However, this may reflect the relatively higher proportion of HBV-related HCC in the Korean population in general, rather than the positive impact of HBV infection on nivolumab outcome. The association of HBV in nivolumab treatment response is not clear yet. For further consideration, in the CheckMate 040 data, the efficacy and safety of nivolumab treatment in sorafenib-experienced patients with advanced HCC were comparable between the HBV-infected group and the non-infected group[5].

A major limitation of this study is the small size of its cohort, which did not provide sufficient statistical power. This preliminary data should be interpreted with caution and further studies enrolling larger numbers of subjects may, thus, reveal additional microbial patterns. In addition, translation of a prognosis-associated microbial signature may not be straightforward, and thus several inherent variabilities between individuals within each cohort should be also considered as potential confounding factors. Nevertheless, our data highlights the promising possibility that a feasible approach may be to combine several microbial features for prediction of nivolumab treatment.

## CONCLUSION

The current study suggests that non-responders to nivolumab have dysbiotic fecal composition. Moreover, a high P/B ratio predicted a better response to nivolumab in HCC patients, albeit that cohort was relatively small. Further studies should help define the role of these bacteria and their potential as novel biomarkers. New insights into the pathophysiological relevance of intestinal dysbiosis in the prognosis of HCC may lead to innovative therapeutic solutions, such as supplementation with probiotics, to prevent primary resistance to therapy.

## ARTICLE HIGHLIGHTS

### *Research background*

Systemic chemotherapy for hepatocellular carcinoma (HCC) has an important role, and immunotherapy in HCC is challenging.

### *Research motivation*

Predictive or prognostic markers for systemic treatment for HCC have not been elucidated.

### *Research objectives*

To investigate the correlation between gut microbiome and treatment response in advanced HCC.

### *Research methods*

Patients who were treated with nivolumab for HCC were identified from one tertiary hospital in South Korea from September 2019 to August 2020. Metagenomic data from 16S ribosomal RNA sequencing were analyzed according to therapeutic response.

### *Research results*

A higher Shannon index was found in the responders than in the non-responders after nivolumab therapy ($P$ = 0.036). The unweighted beta diversity analysis also showed that the overall bacterial community structure and phylogenetic diversity were clearly distinguished according to therapeutic response. A skewed *Firmicutes/Bacteroidetes* ratio and a low *Prevotella/Bacteroides* ratio can serve as predictive markers of non-response, whereas the presence of *Akkermansia* species predicts a good response.

### *Research conclusions*

Gut microbiome has a potential role as a prognostic marker for the response to nivolumab in the treatment of HCC patients.

### *Research perspectives*

Microbiome study before and/or follow up of treatment with immunotherapy for HCC patients could be a prognostic marker, and it can be a criterion for selecting which systemic treatment should be used for each patient as part of precision medicine.

## ACKNOWLEDGEMENTS

We would like to thank all participants in this study.

## REFERENCES

1 **Wallace MC**, Preen D, Jeffrey GP, Adams LA. The evolving epidemiology of hepatocellular carcinoma: a global perspective. *Expert Rev Gastroenterol Hepatol* 2015; **9**: 765-779 [PMID: 25827821 DOI: 10.1586/17474124.2015.1028363]

2 **Larkin J**, Chiarion-Sileni V, Gonzalez R, Grob JJ, Cowey CL, Lao CD, Schadendorf D, Dummer R, Smylie M, Rutkowski P, Ferrucci PF, Hill A, Wagstaff J, Carlino MS, Haanen JB, Maio M, Marquez-Rodas I, McArthur GA, Ascierto PA, Long GV, Callahan MK, Postow MA, Grossmann K, Sznol M, Dreno B, Bastholt L, Yang A, Rollin LM, Horak C, Hodi FS, Wolchok JD. Combined Nivolumab and Ipilimumab or Monotherapy in Untreated Melanoma. *N Engl J Med* 2015; **373**: 23-34 [PMID: 26027431 DOI: 10.1056/NEJMoa1504030]

3 **Harrington KJ**, Ferris RL, Blumenschein G Jr, Colevas AD, Fayette J, Licitra L, Kasper S, Even C, Vokes EE, Worden F, Saba NF, Kiyota N, Haddad R, Tahara M, Grünwald V, Shaw JW, Monga M, Lynch M, Taylor F, DeRosa M, Morrissey L, Cocks K, Gillison ML, Guigay J. Nivolumab *versus* standard, single-agent therapy of investigator's choice in recurrent or metastatic squamous cell carcinoma of the head and neck (CheckMate 141): health-related quality-of-life results from a randomised, phase 3 trial. *Lancet Oncol* 2017; **18**: 1104-1115 [PMID: 28651929 DOI: 10.1016/S1470-2045(17)30421-7]

4 **El-Khoueiry AB**, Sangro B, Yau T, Crocenzi TS, Kudo M, Hsu C, Kim TY, Choo SP, Trojan J, Welling TH Rd, Meyer T, Kang YK, Yeo W, Chopra A, Anderson J, Dela Cruz C, Lang L, Neely J, Tang H, Dastani HB, Melero I. Nivolumab in patients with advanced hepatocellular carcinoma

(CheckMate 040): an open-label, non-comparative, phase 1/2 dose escalation and expansion trial. *Lancet* 2017; **389**: 2492-2502 [PMID: 28434648 DOI: 10.1016/S0140-6736(17)31046-2]

5 **Yau T**, Hsu C, Kim TY, Choo SP, Kang YK, Hou MM, Numata K, Yeo W, Chopra A, Ikeda M, Kuromatsu R, Moriguchi M, Chao Y, Zhao H, Anderson J, Cruz CD, Kudo M. Nivolumab in advanced hepatocellular carcinoma: Sorafenib-experienced Asian cohort analysis. *J Hepatol* 2019; **71**: 543-552 [PMID: 31176752 DOI: 10.1016/j.jhep.2019.05.014]

6 **Routy B**, Le Chatelier E, Derosa L, Duong CPM, Alou MT, Daillère R, Fluckiger A, Messaoudene M, Rauber C, Roberti MP, Fidelle M, Flament C, Poirier-Colame V, Opolon P, Klein C, Iribarren K, Mondragón L, Jacquelot N, Qu B, Ferrere G, Clémenson C, Mezquita L, Masip JR, Naltet C, Brosseau S, Kaderbhai C, Richard C, Rizvi H, Levenez F, Galleron N, Quinquis B, Pons N, Ryffel B, Minard-Colin V, Gonin P, Soria JC, Deutsch E, Loriot Y, Ghiringhelli F, Zalcman G, Goldwasser F, Escudier B, Hellmann MD, Eggermont A, Raoult D, Albiges L, Kroemer G, Zitvogel L. Gut microbiome influences efficacy of PD-1-based immunotherapy against epithelial tumors. *Science* 2018; **359**: 91-97 [PMID: 29097494 DOI: 10.1126/science.aan3706]

7 **Sivan A**, Corrales L, Hubert N, Williams JB, Aquino-Michaels K, Earley ZM, Benyamin FW, Lei YM, Jabri B, Alegre ML, Chang EB, Gajewski TF. Commensal Bifidobacterium promotes antitumor immunity and facilitates anti-PD-L1 efficacy. *Science* 2015; **350**: 1084-1089 [PMID: 26541606 DOI: 10.1126/science.aac4255]

8 **Vétizou M**, Pitt JM, Daillère R, Lepage P, Waldschmitt N, Flament C, Rusakiewicz S, Routy B, Roberti MP, Duong CP, Poirier-Colame V, Roux A, Becharef S, Formenti S, Golden E, Cording S, Eberl G, Schlitzer A, Ginhoux F, Mani S, Yamazaki T, Jacquelot N, Enot DP, Bérard M, Nigou J, Opolon P, Eggermont A, Woerther PL, Chachaty E, Chaput N, Robert C, Mateus C, Kroemer G, Raoult D, Boneca IG, Carbonnel F, Chamaillard M, Zitvogel L. Anticancer immunotherapy by CTLA-4 blockade relies on the gut microbiota. *Science* 2015; **350**: 1079-1084 [PMID: 26541610 DOI: 10.1126/science.aad1329]

9 **Eisenhauer EA**, Therasse P, Bogaerts J, Schwartz LH, Sargent D, Ford R, Dancey J, Arbuck S, Gwyther S, Mooney M, Rubinstein L, Shankar L, Dodd L, Kaplan R, Lacombe D, Verweij J. New response evaluation criteria in solid tumours: revised RECIST guideline (version 1.1). *Eur J Cancer* 2009; **45**: 228-247 [PMID: 19097774 DOI: 10.1016/j.ejca.2008.10.026]

10 **Magurran AE**. An index of diversity. In: Measuring Biological Diversity. *Afr J Aquat Sci* 2004; **29**: 285-286 [DOI: 10.2989/16085910409503825]

11 **Veech JA**, Summerville KS, Crist TO, Gering JC. The additive partitioning of species diversity: recent revival of an old idea. *Oikos* 2002; **99**: 93-99 [DOI: 10.1034/j.1600-0706.2002.]

12 **Gopalakrishnan V**, Helmink BA, Spencer CN, Reuben A, Wargo JA. The Influence of the Gut Microbiome on Cancer, Immunity, and Cancer Immunotherapy. *Cancer Cell* 2018; **33**: 570-580 [PMID: 29634945 DOI: 10.1016/j.ccell.2018.03.015]

13 **Zheng Y**, Wang T, Tu X, Huang Y, Zhang H, Tan D, Jiang W, Cai S, Zhao P, Song R, Li P, Qin N, Fang W. Gut microbiome affects the response to anti-PD-1 immunotherapy in patients with hepatocellular carcinoma. *J Immunother Cancer* 2019; **7**: 193 [PMID: 31337439 DOI: 10.1186/s40425-019-0650-9]

14 **Li L**, Ye J. Characterization of gut microbiota in patients with primary hepatocellular carcinoma received immune checkpoint inhibitors: A Chinese population-based study. *Medicine (Baltimore)* 2020; **99**: e21788 [PMID: 32925716 DOI: 10.1097/MD.0000000000021788]

15 **Grigor'eva IN**. Gallstone Disease, Obesity and the Firmicutes/Bacteroidetes Ratio as a Possible Biomarker of Gut Dysbiosis. *J Pers Med* 2020; **11** [PMID: 33375615 DOI: 10.3390/jpm11010013]

16 **Payne AN**, Chassard C, Zimmermann M, Müller P, Stinca S, Lacroix C. The metabolic activity of gut microbiota in obese children is increased compared with normal-weight children and exhibits more exhaustive substrate utilization. *Nutr Diabetes* 2011; **1**: e12 [PMID: 23154580 DOI: 10.1038/nutd.2011.8]

17 **Yu C**, Su Z, Li Y, Liu K, Chu F, Liu T, Chen R, Ding X. Dysbiosis of gut microbiota is associated with gastric carcinogenesis in rats. *Biomed Pharmacother* 2020; **126**: 110036 [PMID: 32172061 DOI: 10.1016/j.biopha.2020.110036]

18 **Zhang L**, Wu YN, Chen T, Ren CH, Li X, Liu GX. Relationship between intestinal microbial dysbiosis and primary liver cancer. *Hepatobiliary Pancreat Dis Int* 2019; **18**: 149-157 [PMID: 30661942 DOI: 10.1016/j.hbpd.2019.01.002]

19 **Peng Z**, Cheng S, Kou Y, Wang Z, Jin R, Hu H, Zhang X, Gong JF, Li J, Lu M, Wang X, Zhou J, Lu Z, Zhang Q, Tzeng DTW, Bi D, Tan Y, Shen L. The Gut Microbiome Is Associated with Clinical Response to Anti-PD-1/PD-L1 Immunotherapy in Gastrointestinal Cancer. *Cancer Immunol Res* 2020; **8**: 1251-1261 [PMID: 32855157 DOI: 10.1158/2326-6066.CIR-19-1014]

World Journal of Gastroenterology

Submit a Manuscript: https://www.f6publishing.com

*World J Gastroenterol* 2021 November 14; 27(42): 7350-7361

DOI: 10.3748/wjg.v27.i42.7350

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

ORIGINAL ARTICLE

Retrospective Cohort Study

# Presentation, patterns and predictive value of baseline liver tests on outcomes in COVID-19 patients without chronic liver disease

David Bernstein, Nitzan Roth, Angela Kim, Marcia Epstein, David Hirschwerk, Charlotte L Kvasnovsky, Sanjaya K Satapathy

**ORCID number:** David Bernstein 0000-0001-7826-2981; Nitzan Roth 0000-0003-2079-2917; Angela Kim 0000-0001-7159-2427; Marcia Epstein 0000-0002-2966-0075; David Hirschwerk 0000-0002-9748-0316; Charlotte L Kvasnovsky 0000-0002-6649-1784; Sanjaya K Satapathy 0000-0003-0153-2829.

**Author contributions:** Bernstein D, Roth N, and Satapathy SK developed the concept for the manuscript; Bernstein D wrote the manuscript; Kvasnovsky CL performed the statistical analysis; Kim A, Epstein M, and Hirschwerk D reviewed the literature; Bernstein D, Satapathy S, Roth N, Kim A, Epstein M, Hirschwerk D, and Kvasnovsky CL reviewed and edited the manuscript.

**Supported by** National Institute on Aging of the National Institute of Health, No. R24AG06419; National Library of Medicine of the National Institutes of Health, No. R01LM012836.

**Institutional review board statement:** This study protocol was approved by the Northwell Health Institutional Review Board.

**Informed consent statement:** Informed consent statement was waived.

**David Bernstein, Nitzan Roth, Sanjaya K Satapathy,** Department of Medicine/Hepatology, Northwell Health, Zucker School of Medicine at Hofstra/Northwell, Manhasset, NY 11030, United States

**Angela Kim, Marcia Epstein, David Hirschwerk,** Division of Infectious Diseases, Department of Medicine Northwell Health, Zucker School of Medicine at Hofstra/Northwell, Manhasset, NY 11030, United States

**Charlotte L Kvasnovsky,** Cohen Children's Medical Center, Zucker School of Medicine at Hofstra/Northwell, New Hyde Park, NY 11042, United States

**Corresponding author:** David Bernstein, FAASLD, MACG, MD, Professor, Department of Medicine/Hepatology, Northwell Health, Zucker School of Medicine at Hofstra/Northwell, 400 Community Drive, Manhasset, NY 11030, United States. dbernste@northwell.edu

## Abstract

### *BACKGROUND*

Coronavirus disease 2019 (COVID-19) infection is known to cause abnormal hepatic enzymes. The long term consequences of such elevations are uncertain.

### *AIM*

To assessed the prevalence and prognostic value of initial liver enzymes in a large cohort of COVID-19 patients.

### *METHODS*

We reviewed electronic medical records of 10614 COVID-19 patients without known chronic liver disease who were admitted to our health system from March 1, 2020, to April 30, 2020. We analyzed baseline demographics and liver chemistries. The primary outcome was in-hospital mortality, and the secondary outcome was a composite of in-hospital mortality or need for mechanical ventilation.

### *RESULTS*

Subjects with abnormal liver tests had increased risks of mortality and composite outcome when compared to patients with normal measurements on unadjusted analysis and after adjustment for demographic factors.

**Conflict-of-interest statement:** The authors declare no conflicts of interest related to the content of the manuscript.

**Data sharing statement:** The data that support the findings of this study are available on request from COVID19@northwell.edu. The data are not publicly available due to restrictions as it could compromise the privacy of research participants.

**STROBE statement:** The authors have read the STROBE Statement—checklist of items, and the manuscript was prepared and revised according to the STROBE Statement—checklist of items.



**Specialty type:** Gastroenterology and hepatology

**Country/Territory of origin:** United States

**Peer-review report's scientific quality classification**

Grade A (Excellent): 0
Grade B (Very good): B
Grade C (Good): C
Grade D (Fair): 0
Grade E (Poor): 0

**Received:** January 30, 2021
**Peer-review started:** January 30, 2021
**First decision:** June 4, 2021
**Revised:** July 12, 2021
**Accepted:** October 24, 2021
**Article in press:** October 24, 2021
**Published online:** November 14, 2021

*CONCLUSION*

In our diverse patient population, liver enzyme abnormalities are associated with increased mortality and the need for mechanical ventilation in subjects without chronic liver disease. Cholestasis patients are at the greatest risk for poor outcomes.

**Key Words:** COVID-19; Liver enzymes; Outcomes; Predictors



**Core Tip:** We believe that our paper is an important contribution to the literature for the following reasons: (1) The cohort size is the largest to date; (2) We show the importance of initial liver tests in predicting outcomes; (3) In this large cohort, the finding of initial cholestatic pattern of injury being most predictive of poor outcome has not yet been described; and (4) This is a cohort from a large urban health system in the United States (New York) whose subject demographics reflect more the population seen in the United States. The other publications listed below are more uniform populations not representative of what our practitioners see in daily practice

**Citation:** Bernstein D, Roth N, Kim A, Epstein M, Hirschwerk D, Kvasnovsky CL, Satapathy SK. Presentation, patterns and predictive value of baseline liver tests on outcomes in COVID-19 patients without chronic liver disease. *World J Gastroenterol* 2021; 27(42): 7350-7361
**URL:** https://www.wjgnet.com/1007-9327/full/v27/i42/7350.htm
**DOI:** https://dx.doi.org/10.3748/wjg.v27.i42.7350

## INTRODUCTION

The outbreak of coronavirus disease 2019 (COVID-19) caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), first reported in Wuhan, China[1], was declared a global health emergency in January 2020 by the World Health Organization (WHO)[2].

Liver enzymes have been reported to be elevated in 15%-76% of patients with COVID-19 infection[3-14]. While most reported liver enzyme abnormalities have been mild, severe acute hepatitis and severe cholestasis has been reported secondary to COVID-19 infection[15,16]. The degree of enzyme elevation and patterns of liver enzymes can indicate patients' outcomes such as the need for mechanical ventilation and in-hospital mortality[3,12-17] but are not well described in a large, multi-ethnic cohort. Our study reports the results of our analysis of these factors in the largest cohort to date of hospitalized adults with COVID-19 infection without chronic liver disease.

## MATERIALS AND METHODS

### *Study population and data collection*

We obtained the medical records and compiled data from our electronic medical record on all patients with documented COVID-19 infection who were admitted to 12 hospitals in New York City, Long Island, and Westchester County, New York, within the Northwell Health system from the period of March 1, 2020, to April 30, 2020. A confirmed case was defined as a positive reverse transcriptase polymerase chain reaction for SARS-CoV-2 on a specimen obtained through nasopharyngeal swabbing, including if an initial test result was negative but repeat testing was positive. We collected the following demographic information: Age, sex, race, ethnicity, presence of co-morbid conditions, and body mass index (BMI). Race and ethnicity data were collected by self-report in pre-specified fixed categories. Baseline laboratory testing was defined as the first measurement available within 24 h of presentation. For this study, we excluded children under 18 years of age, patients missing a baseline value for serum alanine aminotransferase (ALT), and patients with known chronic liver

**P-Reviewer:** Hasan M, Lukito AA
**S-Editor:** Fan JR
**L-Editor:** A
**P-Editor:** Fan JR

disease. Exclusion for chronic liver disease was based on initial identification of this group of patients using ICD-10 codes and followed by manual chart review to confirm a diagnosis of chronic liver disease.

This study was supported by the Northwell Health COVID-19 Research Consortium and was approved by the Institutional Review Board for the Feinstein Institutes of Medical Research at Northwell Health as minimal-risk research using data collected for routine clinical practice, with the requirement for informed consent waived.

### Statistical analysis

Liver enzymes, namely, serum levels of aspartate aminotransferase (AST), ALT, alkaline phosphatase, and total bilirubin levels were stratified into 4 groups: (1) Within normal limits; (2) Greater than the upper limit of normal (ULN) to less than or equal to 4 times the ULN; (3) Greater than 4 times the ULN to less than or equal to 10 times the ULN; and (4) Greater than 10 times the ULN. As *per* the American Association for the Study of Liver Diseases expert consensus recommendations, we defined the ULN for ALT as 25 U/L for women and 35 U/L for men[18]. The ULNs of other liver chemistries were defined using standard definitions and based on our health system laboratory's ULNs, which were: 40 U/L for AST, 125 U/L for alkaline phosphatase, and 1.2 mg/dL for total bilirubin. We classified patients who had more severe liver enzyme abnormalities into three patterns of liver injury: Hepatocellular (defined as ALT > 3 × ULN and alkaline phosphatase ≤ 2 × ULN), cholestatic (defined as alkaline phosphatase > 2 × ULN and ALT ≤ 3 × ULN), or mixed (defined as ALT > 3 × ULN and alkaline phosphatase > 2 × ULN)[7].

The primary outcome of interest was in-hospital mortality. The secondary outcome was a composite outcome of the need for mechanical ventilation or in-hospital mortality.

We summarized each continuous variable using its median and interquartile range (IQR). Categorical variables were summarized using counts and percentages. Comparisons between groups were assessed using Wilcoxon rank sum tests, chi-squared tests, and Fisher exact tests as appropriate. For survival analyses, we censored patients as alive without the event of interest on their date of hospital discharge or at 28 d of follow-up, whichever was earlier. Due to the low numbers of patients with elevations in alkaline phosphatase or bilirubin, we analyzed survival based on normal *vs* elevated measurements rather than the previously defined four categories. We analyzed survival using Kaplan-Meier survival curves using log-rank tests and estimated hazard ratios (HRs) and 95% confidence intervals (CIs) using univariate and multivariate Cox proportional hazards models. Multivariate models were adjusted for age, sex, race, ethnicity, BMI (< 30 kg/m$^2$ *vs* ≥ 30 kg/m$^2$), and presence of co-morbid conditions (hypertension, diabetes mellitus, coronary artery disease, chronic obstructive pulmonary disease, heart failure, chronic kidney disease, end-stage renal disease, and malignancy). We checked the proportional hazards assumption for each variable included in our Cox regression models using graphical assessment of the Kaplan-Meier survival curves and log[-log(survival)] *vs* log(time) graphs to look for parallel curves and by ensuring that Schoenfeld residuals were independent of time. Because the variables of age, sex, and race violated the proportional hazards assumption, we included interaction terms with time for those variables in our multivariate models. We used two-sided tests with alpha = 0.05. Statistical analyses were performed using SAS version 9.4 (SAS Institute, Cary, NC).

## RESULTS

There were 11265 patients with COVID-19 hospitalized from March 1, 2020, to April 30, 2020. After excluding 106 children who were under 18 years old, 300 patients without baseline measurements of AST and ALT, and 245 patients with chronic liver disease, our study population included 10614 patients. Median length of hospital stay was 6 d (range: 0-58 d, IQR: 3-11 d). Baseline characteristics of the study population are described in Table 1. The median age was 65 years (range: 18-107 years, IQR: 54-77 years). The majority of patients were male (59%), white (38%), and non-Hispanic (21%). The most common comorbidities were hypertension (58%), obesity (39%), and diabetes (36%).

More than half of patients had elevations in AST (59%) and ALT (54%) on presentation, whereas alkaline phosphatase and bilirubin levels were elevated for only 13% and 5% of patients, respectively (Table 1). Most transaminase elevations were in group 2. 0.8% had ALT > 10 × ULN. 76% had AST higher than ALT on presentation.

**Table 1 Baseline characteristics of the study population (*n* = 10614)**

| Demographics | |
|---|---|
| Sex | |
| Male | 6243 (58.8%) |
| Female | 4371 (41.2%) |
| Race | |
| White | 4037 (38.0%) |
| Black | 2248 (21.2%) |
| Asian | 914 (8.6%) |
| Other/multiracial | 2942 (27.7%) |
| Unknown | 473 (4.5%) |
| Ethnicity | |
| Non-Hispanic or Latino or other/unknown | 8338 (78.6%) |
| Hispanic or Latino | 2276 (21.4%) |
| Age (yr) | 65 (54-77) |
| Body mass index (kg/m$^2$)[1] | 28.3 (24.9-32.6) |
| < 30 | 5120 (61.4%) |
| ≥ 30 | 3220 (38.6%) |
| **Presence of co-morbid conditions** | |
| Hypertension | 6204 (58.5%) |
| Diabetes mellitus | 3764 (35.5%) |
| Coronary artery disease | 1328 (12.5%) |
| Heart failure | 832 (7.8%) |
| Malignancy | 791 (7.5%) |
| Chronic obstructive pulmonary disease | 608 (5.7%) |
| Chronic kidney disease (stage I-IV) | 391 (3.7%) |
| End-stage renal disease | 434 (4.1%) |
| **Liver chemistries (within 24 h)** | |
| AST (IU/L)[1] | 46 (31-72) |
| Normal | 4377 (41.3%) |
| > 1 to ≤ 4 × ULN | 5713 (53.8%) |
| > 4 to ≤ 10 × ULN | 437 (4.1%) |
| > 10 × ULN | 85 (0.8%) |
| ALT (IU/L) | 33 (21-56) |
| Normal | 4883 (46.0%) |
| > 1 to ≤ 4 × ULN | 5159 (48.6%) |
| > 4 to ≤ 10 × ULN | 484 (4.6%) |
| > 10 × ULN | 88(0.8%) |
| Alkaline phosphatase (IU/L)[1] | 75 (59-98) |
| Normal | 9242 (87.1%) |
| > 1 to ≤ 4 × ULN | 1333 (12.6%) |
| > 4 to ≤ 10 × ULN | 35(0.3%) |
| > 10 × ULN | 2 (0.02%) |

| | |
|---|---|
| Bilirubin (mg/dL)[1] | 0.5 (0.4-0.7) |
| Normal | 10119 (95.4%) |
| > 1 to ≤ 4 × ULN | 486 (4.6%) |
| > 4 to ≤ 10 × ULN | 5 (0.05%) |
| > 10 × ULN | 3 (0.03%) |

[1]Missing data for some patients: Body mass index ($n$ = 2274), alkaline phosphatase ($n$ = 2), aspartate aminotransferase ($n$ = 2), and bilirubin ($n$ = 1).
Data summarized as $n$ (%) for categorical variables and median (interquartile range) for continuous variables. AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; ULN: Upper limit of normal.

1160 patients (10.95) had severe hepatic enzyme elevations. Of these, 936 (8.9%) had a hepatocellular pattern, 133 (1.3%) a cholestatic pattern, and 91 (0.9%) a mixed pattern. The prevalence of hepatic test elevations differed based on sex, ethnicity, race, and presence of comorbidities (Table 2) Males were more likely to have elevations in AST (64.8% *vs* 50.1%, $P < 0.001$) and bilirubin (5.9% *vs* 2.8%, $P < 0.001$) but less likely to have elevations in alkaline phosphatase (12.0% *vs* 14.2%, $P = 0.001$) than females. There was no difference in ALT levels between males and females ($P = 0.34$).

In Kaplan-Meier survival analyses, any elevated liver chemistry was associated with increased mortality (Figure 1) or need for mechanical ventilation (Figure 2).

Having an elevated AST was associated with a higher risk of in-hospital mortality (unadjusted HR 1.34, 95%CI: 1.22-1.47, $P < 0.001$; adjusted HR 1.67, 95%CI: 1.49-1.86, $P < 0.001$) and in-hospital mortality or need for mechanical ventilation (unadjusted HR 1.34, 95%CI: 1.18-1.52, $P < 0.001$; adjusted HR 1.77, 95%CI: 1.51-2.08, $P < 0.001$). Increasing severity of AST abnormalities was associated with incrementally poor survival and need for mechanical ventilation (Table 3), and the trend remained significant after adjustment for age, sex, race, ethnicity, and the presence of co-morbid conditions. Compared to patients with normal AST, patients with AST > 10 × ULN were three times as likely to have in-hospital mortality (adjusted HR 2.64, 95%CI: 1.73-4.04, $P < 0.001$) and three times as likely to have in-hospital mortality or need for mechanical ventilation (adjusted HR 3.38, 95%CI: 1.78-6.40, $P < 0.001$).

In unadjusted models, having an elevated ALT appeared to be associated with a lower risk of in-hospital mortality (unadjusted HR 0.85, 95%CI: 0.78-0.92, $P < 0.001$) and the composite outcome (unadjusted HR 0.73, 95%CI: 0.65-0.83, $P < 0.001$). However, after adjusting for the confounding factors of age, sex, race, ethnicity and the presence of co-morbid conditions, an elevated ALT was associated with a 1.2 times higher risk of in-hospital mortality (adjusted HR 1.21, 95%CI: 1.09-1.34, $P < 0.001$) and the composite outcome (adjusted HR 1.21, 95%CI: 1.04-1.41, $P = 0.02$). Elevation in alkaline phosphatase was associated with a similar modestly increased risk of in-hospital mortality (adjusted HR 1.29, 95%CI: 1.12-1.48, $P < 0.001$) and in the composite outcome (adjusted HR 1.39, 95%CI: 1.12-1.72, $P = 0.003$). The associations between elevated bilirubin levels and outcomes became attenuated and lost significance after adjustment for confounding factors (Table 3).

The pattern of liver enzyme abnormalities affected outcomes. Patients with initial AST higher than ALT had a higher risk of in-patient mortality (adjusted HR 1.72, 95%CI: 1.46-2.02, $P < 0.001$) and the composite outcome of in-patient mortality or need for mechanical ventilation (adjusted HR: 1.84, 95%CI: 1.41-2.40, $P < 0.001$), compared to patients with AST less than or equal to ALT. Compared to patients with normal or non-severe elevations in ALT and alkaline phosphatase (with ALT < 3 × ULN and alkaline phosphatase ≤ 2 × ULN), patients with a severe cholestatic liver injury had a higher risk of in-patient mortality (adjusted HR 1.57, 95%CI: 1.07-2.28, $P = 0.02$) and the composite outcome of in-patient mortality or need for mechanical ventilation (adjusted HR 2.05, 95%CI: 1.17-3.58, $P = 0.01$). A hepatocellular or mixed pattern of liver injury was not associated with outcomes in adjusted models.

## DISCUSSION

Our study shows that initial, abnormal liver enzymes are associated with poor outcomes in patients with COVID-19 infection in a diverse, multi-ethnic patient population. More than half of our patients upon presentation to the hospital had abnormal levels of serum aminotransferases, and a small proportion had elevations of

**Table 2 Correlations between baseline liver chemistry elevations and demographic characteristics of the study population**

| | Elevated ALP | *P* value | Elevated AST | *P* value | Elevated ALT | *P* value | Elevated Bilirubin | *P* value |
|---|---|---|---|---|---|---|---|---|
| Sex | | 0.001 | | < 0.001 | | 0.34 | | < 0.001 |
| Female | 619 (14.2%) | | 2191 (50.1%) | | 2336 (53.4%) | | 124 (2.8%) | |
| Male | 751 (12.0%) | | 4044 (64.8%) | | 3395 (54.4%) | | 370 (5.9%) | |
| Race | | < 0.001 | | < 0.001 | | < 0.001 | | 0.51 |
| Asian | 134 (14.7%) | | 613 (67.1%) | | 533 (58.3%) | | 43 (4.7%) | |
| Black | 228 (10.1%) | | 1332 (59.3%) | | 1146 (51.0%) | | 117 (5.2%) | |
| Other/multiracial | 468 (15.9%) | | 1845 (62.7%) | | 1836 (62.4%) | | 139 (4.7%) | |
| Unknown | 74 (15.6%) | | 300 (63.4%) | | 304 (64.3%) | | 17 (3.6%) | |
| White | 466 (11.6%) | | 2145 (53.2%) | | 1912 (47.4%) | | 178 (4.4%) | |
| Ethnicity | | < 0.001 | | < 0.001 | | < 0.001 | | 0.51 |
| Hispanic or Latino | 398 (17.5%) | | 1410 (62.0%) | | 1450 (63.7%) | | 97 (4.3%) | |
| Non-Hispanic or Latino | 864 (11.3%) | | 4384 (57.3%) | | 3852 (50.4%) | | 361 (4.7%) | |
| Unknown or other | 108 (15.7%) | | 441 (64.0%) | | 429 (62.3%) | | 36 (5.2%) | |
| BMI (kg/m2) | | < 0.001 | | 0.08 | | < 0.001 | | < 0.001 |
| < 30 | 730 (14.3%) | | 2996 (58.5%) | | 2694 (52.6%) | | 269 (5.3%) | |
| ≥ 30 | 336 (10.5%) | | 1946 (60.4%) | | 1885 (58.5%) | | 111 (3.5%) | |
| Diabetes mellitus | | 0.03 | | < 0.001 | | < 0.001 | | < 0.001 |
| No | 920 (13.4%) | | 1158 (60.7%) | | 3897 (56.9%) | | 356 (5.2%) | |
| Yes | 450 (12.0%) | | 2077 (55.2%) | | 1834 (48.7%) | | 138 (3.7%) | |
| Hypertension | | < 0.001 | | < 0.001 | | < 0.001 | | 0.36 |
| No | 642 (14.6%) | | 2683 (60.9%) | | 2649 (60.1%) | | 215 (4.9%) | |
| Yes | 728 (11.7%) | | 3552 (57.3%) | | 3082 (49.7%) | | 279 (4.5%) | |
| Coronary artery disease | | 0.69 | | 0.006 | | < 0.001 | | 0.09 |
| No | 1194 (12.9%) | | 5501 (59.3%) | | 5184 (55.8%) | | 420 (4.5%) | |
| Yes | 176 (13.3%) | | 734 (55.3%) | | 547 (41.2%) | | 74 (5.6%) | |
| Congestive heart failure | | < 0.001 | | < 0.001 | | < 0.001 | | < 0.001 |
| No | 1223 (12.5%) | | 5811 (59.4%) | | 5419 (55.4%) | | 432 (4.4%) | |
| Yes | 147 (17.7%) | | 424 (51.0%) | | 312 (37.5%) | | 62 (7.5%) | |
| COPD | | 0.24 | | < 0.001 | | < 0.001 | | 0.46 |
| No | 1301 (13.0%) | | 5939 (59.4%) | | 5504 (55.0%) | | 462 (4.6%) | |
| Yes | 69 (11.4%) | | 296 (48.7%) | | 227 (37.3%) | | 32 (5.3%) | |
| CKD (stage I-IV) | | 0.82 | | 0.001 | | < 0.001 | | 0.59 |
| No | 1321 (12.9%) | | 6036 (59.1%) | | 5582 (54.6%) | | 478 (4.7%) | |
| Yes | 49 (12.5%) | | 199 (50.9%) | | 149 (38.1%) | | 16 (4.1%) | |
| End-stage renal disease | | < 0.001 | | < 0.001 | | < 0.001 | | 0.09 |
| No | 1265 (12.4%) | | 6044 (59.4%) | | 5621 (55.2%) | | 481 (4.7%) | |
| Yes | 105 (24.2%) | | 191 (44.0%) | | 110 (25.4%) | | 13 (3.0%) | |
| Malignancy | | 0.58 | | < 0.001 | | < 0.001 | | 0.049 |
| No | 1273 (13.0%) | | 5827 (59.3%) | | 5380 (54.8%) | | 446 (4.5%) | |
| Yes | 97 (12.3%) | | 408 (51.7%) | | 351 (44.4%) | | 48 (6.1%) | |

ALP: Alkaline phosphatase; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; BMI: Body mass index; CKD: Chronic kidney disease; COPD: Chronic obstructive pulmonary disease.

**Table 3 Associations between liver chemistries and outcomes**

| | In-hospital mortality | | | | In-hospital mortality or need for mechanical ventilation | | | |
|---|---|---|---|---|---|---|---|---|
| | Unadjusted analysis-HR (95%CI) | *P* value | Adjusted analysis[1]-HR (95%CI) | *P* value | Unadjusted analysis-HR (95%CI) | *P* value | Adjusted analysis[1]-HR (95%CI) | *P* value |
| AST | | | | | | | | |
| Normal | Ref. | | Ref. | | Ref. | | Ref. | |
| > 1 to ≤ 4 × ULN | 1.29 (1.17-1.42) | < 0.001 | 1.65 (1.47-1.85) | < 0.001 | 1.29 (1.13-1.46) | < 0.001 | 1.73 (1.47-2.04) | < 0.001 |
| > 4 to ≤ 10 × ULN | 1.66 (1.38-1.99) | < 0.001 | 1.69 (1.33-2.14) | < 0.001 | 1.67 (1.27-2.20) | < 0.001 | 2.09 (1.42-3.07) | < 0.001 |
| > 10 × ULN | 3.50 (2.56-4.78) | < 0.001 | 2.64 (1.73-4.04) | < 0.001 | 3.89 (2.48-6.10) | < 0.001 | 3.38 (1.78-6.40) | < 0.001 |
| ALT | | | | | | | | |
| Normal | Ref. | | Ref. | | Ref. | | Ref. | |
| > 1 to ≤ 4 × ULN | 0.84 (0.77-0.91) | < 0.001 | 1.21 (1.09-1.34) | < 0.001 | 0.72 (0.63-0.81) | < 0.001 | 1.20 (1.02-1.40) | 0.02 |
| > 4 to ≤ 10 × ULN | 0.88 (0.71-1.09) | 0.24 | 1.22 (0.93-1.61) | 0.15 | 0.79 (0.57-1.09) | 0.15 | 1.40 (0.87-2.26) | 0.16 |
| > 10 × ULN | 1.71 (1.20-2.45) | 0.003 | 1.34 (0.81-2.22) | 0.26 | 1.42 (0.80-2.51) | 0.23 | 1.36 (0.58-3.19) | 0.48 |
| Alkaline phosphatase | | | | | | | | |
| Normal | Ref. | | Ref. | | Ref. | | Ref. | |
| Elevated | 1.27 (1.14-1.43) | < 0.001 | 1.29 (1.12-1.48) | < 0.001 | 1.19 (1.01-1.42) | 0.04 | 1.39 (1.12-1.72) | 0.003 |
| Bilirubin | | | | | | | | |
| Normal | Ref. | | Ref. | | Ref. | | Ref. | |
| Elevated | 1.55 (1.32-1.82) | < 0.001 | 1.12 (0.92-1.37) | 0.27 | 1.69 (1.34-2.13) | < 0.001 | 0.96 (0.71-1.30) | 0.79 |
| AST:ALT ratio | | | | | | | | |
| ≤ 1 | Ref. | | Ref. | | Ref. | | Ref. | |
| > 1 | 2.08 (1.82-2.37) | < 0.001 | 1.72 (1.46-2.02) | < 0.001 | 2.61 (2.13-3.19) | < 0.001 | 1.84 (1.41-2.40) | < 0.001 |
| Pattern of liver injury[2] | | | | | | | | |
| Normal or non-severe | Ref. | | Ref. | | Ref. | | Ref. | |
| Hepatocellular | 0.93 (0.80-1.09) | 0.39 | 0.90 (0.74-1.11) | 0.33 | 0.84 (0.66-1.07) | 0.15 | 1.18 (0.85-1.65) | 0.33 |
| Mixed | 1.14 (0.74-1.75) | 0.56 | 1.06 (0.65-1.73) | 0.82 | 1.00 (0.52-1.93) | 0.99 | 1.02 (0.44-2.37) | 0.96 |
| Cholestatic | 1.17 (0.85-1.61) | 0.33 | 1.57 (1.07-2.28) | 0.02 | 1.25 (0.81-1.93) | 0.31 | 2.05 (1.17-3.58) | 0.01 |

[1]Adjusted for age (time-varying), sex (time-varying), race (time-varying), ethnicity, and comorbidities (obesity, hypertension, diabetes, coronary artery disease, chronic kidney disease, end-stage renal disease, chronic obstructive pulmonary disease, heart failure, and cancer).
[2]Defined as: Normal or non-severe [alanine aminotransferase (ALT) ≤ 3 × ULN and alkaline phosphatase ≤ 2 × upper limit of normal (ULN)], hepatocellular (ALT > 3 × ULN and alkaline phosphatase ≤ 2 × ULN), mixed (ALT > 3 × ULN and alkaline phosphatase > 2 × ULN), and cholestatic (ALT ≤ 3 × ULN and alkaline phosphatase > 2 × ULN).
AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; CI: Confidence interval; HR: Hazard ratio; ULN: Upper limit of normal.

bilirubin and alkaline phosphatase. The predominant pattern of liver injury was hepatocellular. Of those who presented with abnormal transaminases, the overwhelming majority presented with enzymes one-four times the ULN and had an AST greater than ALT. Elevations in either ALT, AST, alkaline phosphatase or bilirubin were associated with increased mortality or need for mechanical ventilation.

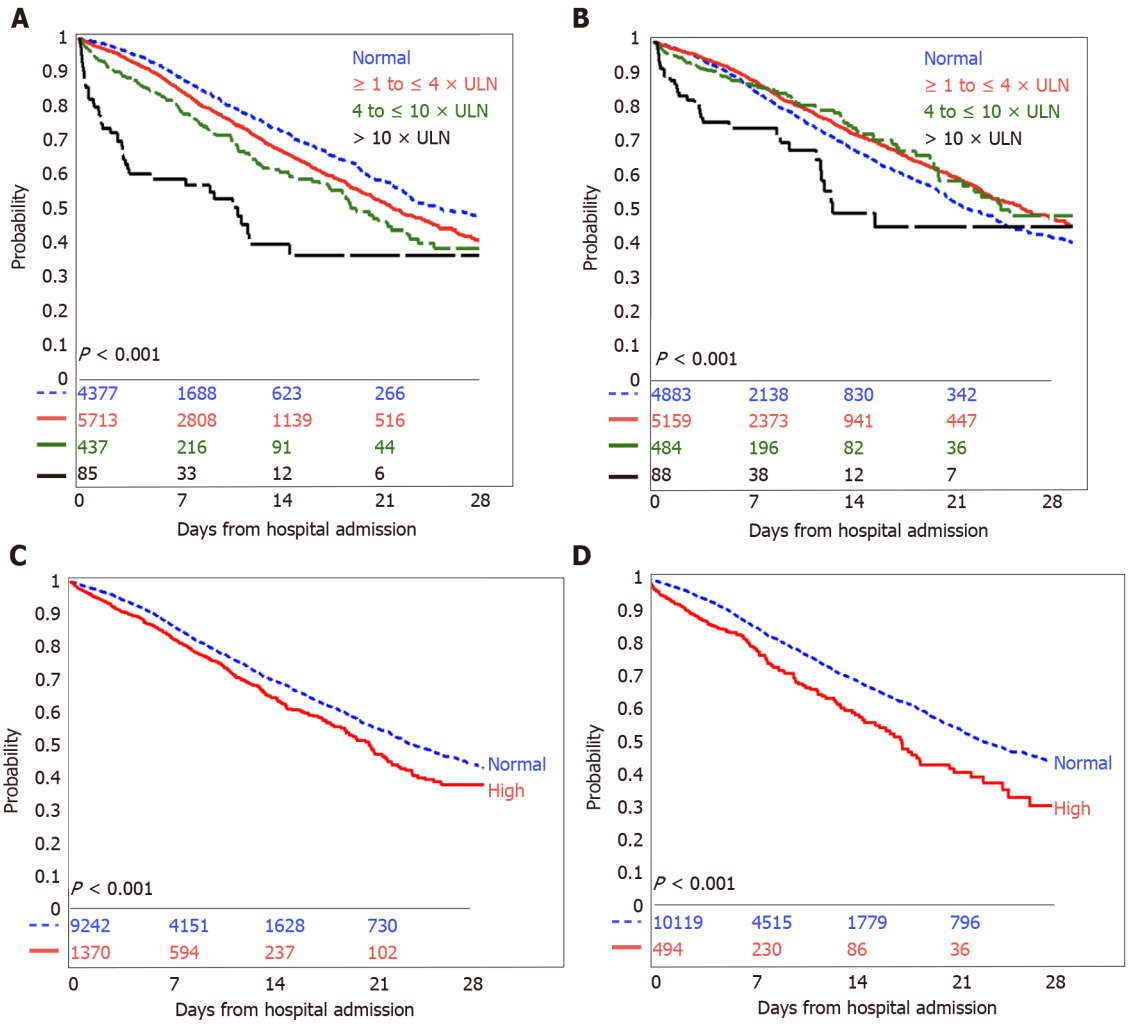


**Figure 1 Kaplan-Meier curves for in-hospital mortality based on liver chemistries.** A: Aspartate aminotransferase; B: Alanine aminotransferase; C: alkaline phosphatase; D: Bilirubin. ULN: Upper limit of normal.

Initial AST elevations appear to be the more significant predictor of mortality and the need for mechanical ventilation than elevations in ALT, alkaline phosphatase and total bilirubin with these risks increasing as AST elevations were more severe, even when adjusted for baseline demographics and the presence of co-morbidities. The mechanism behind this is unclear and still needs to be determined. One study noted AST-dominant aminotransferase elevation is common in COVID-19, can mirror disease severity, and appears to reflect true hepatic injury[12]. In this largest cohort of patients reported so far, we have demonstrated that AST predominant hepatic injury is common and associated with survival outcomes.

It has been postulated that abnormal liver enzymes in COVID-19 infection may be related to a direct cytotoxicity from active viral replication of SARS-CoV-2 in the liver, immune-mediated liver damage secondary to the systemic inflammatory response syndrome, hypoxic changes induced by respiratory failure, vascular changes due to COVID-19 induced thrombotic disease, endothelitis, right heart failure or drug induced liver injury[6,7,19-21]. SARS-CoV-2 virions have been detected in portal vein vessel lumens and endothelial cells by in situ hybridization[21]. SARS-CoV-2 enters host cells through the cell receptor, angiotensin converting enzyme 2 (ACE2). Cells with high ACE2 levels are associated with more severe COVID disease. ACE2 is highly expressed in the lung, cholangiocytes and hepatic vessel endothelial cells with some but less expression in hepatocytes. The high ACE2 expression in cholangiocytes may explain the severe cholestatic disease seen in the subset of patients presenting with this liver enzyme pattern[16,21]. Hypoxia is associated with increased expression of ACE2 receptors in hepatocytes and cholangiocytes[21]. While our study is unable to differentiate between a direct viral effect or an immune-induced response, we show that patients with COVID-19 infection have a high prevalence of abnormal liver chemistries prior to the administration of any COVID-19 specific medications.

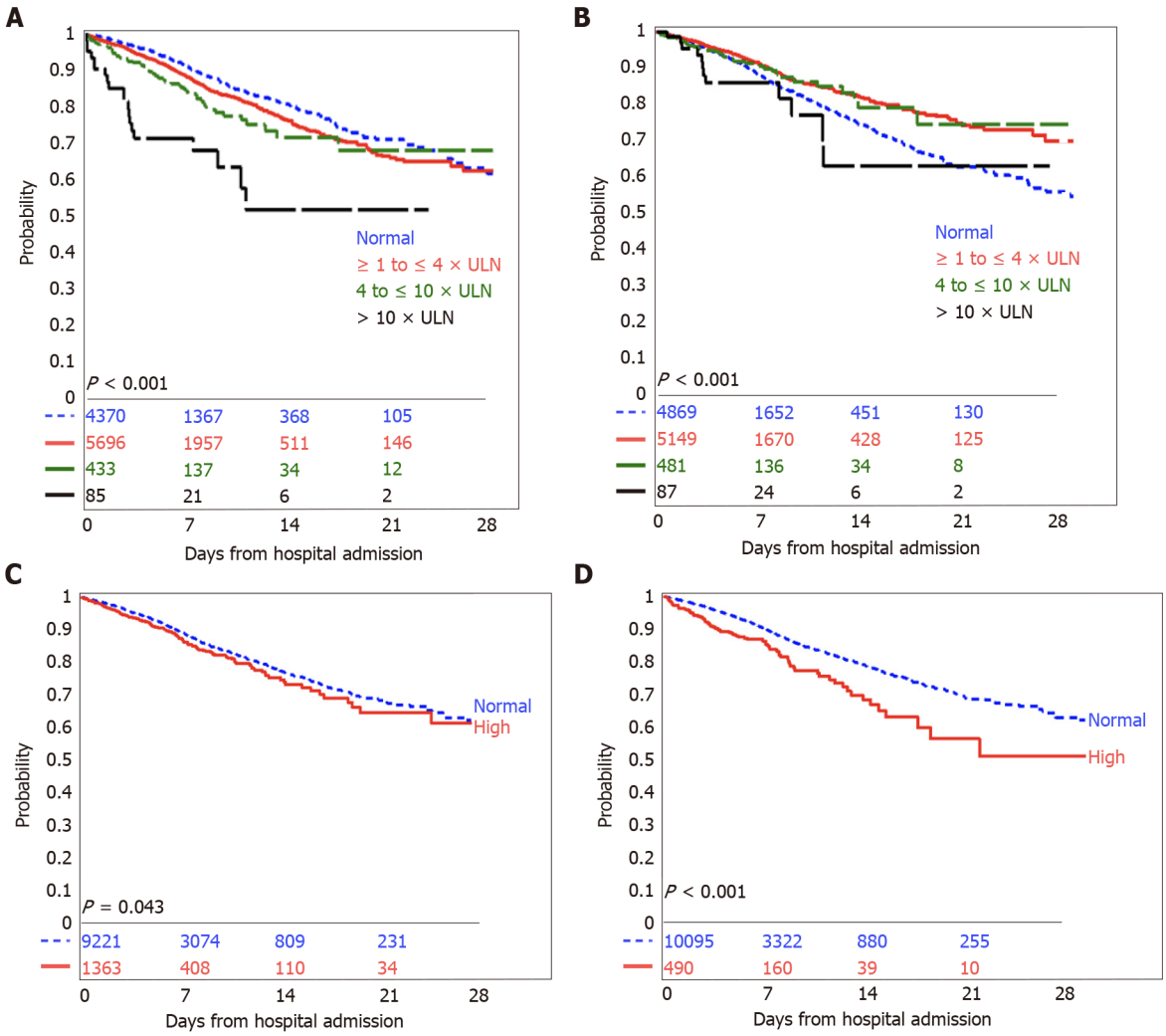


**Figure 2 Kaplan-Meier curves for in-hospital mortality or need for mechanical ventilation based on liver chemistries.** A: Aspartate aminotransferase; B: Alanine aminotransferase; C: Alkaline phosphatase; D: Bilirubin. ULN: Upper limit of normal.

Previous published smaller studies have reported that the pattern of liver injury, either hepatocellular or mixed hepatocellular-cholestatic, appears to be more common in people with severe COVID-19[6-8,14,22]. Based upon our strict definition of these patterns, we found that the hepatocellular pattern of injury on presentation was the most common type of injury noted and that this pattern was not associated with outcomes in an adjusted model. While an initial presentation of severe cholestasis was uncommon, it was associated with a higher risk of in-patient mortality or the need for mechanical ventilation. This is an important finding and should alert caregivers of the risk of decreased survival with this specific pattern of presentation.

Our study does have several limitations. All data was collected from the electronic health record database, as the large size of the cohort precluded a manual review of all cases. Baseline laboratory tests collected were defined as within 24 h of presentation to the emergency department, but this definition does not take into account the length of patient symptoms prior to presentation. Our study specifically evaluated initial laboratory data prior to the initiation of any multimodal treatments such as patient positioning, supplemental oxygen and medical therapies, both standard of care and experimental as our sites had access to several clinical trials medications. While initiation of therapies during hospital admission may have influenced overall outcomes, it is beyond the scope of this paper to assess the effects of individual therapies on outcomes. The absence of data on patients who remain hospitalized at the final study date may have biased the survival outcomes of the study.

Our study specifically excluded patients with known chronic liver disease. This was done through review of ICD-10 diagnostic codes followed by manual chart review as needed for confirmation. Despite this methodology, it remains a possibility that our cohort did include patients with undiagnosed non-alcoholic fatty liver disease, as the

prevalence of obesity and diabetes in our population was 39% and 36%, respectively, and both of these co-morbidities are associated with this condition. Diabetic patients in our study had significantly lower prevalence of AST and ALT elevations than non-diabetics. In contrast, compared to non-obese patients, obese patients had a higher prevalence of ALT elevations but not the other measured liver chemistries. These findings may make it unclear whether the presence of undiagnosed non-alcoholic fatty liver disease affects the risk of liver injury, survival or the need for mechanical ventilation in patients infected with COVID-19 and further study is warranted.

## CONCLUSION

In this largest cohort of hospitalized COVID-19 patients reported so far, we have shown an increased mortality and the need for mechanical ventilation is associated with hepatic test elevations, and in particular those with cholestasis, these findings should be taken into consideration during the initial evaluation of COVID-19 patients, both in the in-patient and out-patient setting and patients with significantly elevated liver enzymes should be prioritized for future treatments as these treatments become available.

## ARTICLE HIGHLIGHTS

### *Research background*

Liver enzyme abnormalities are commonly seen in coronavirus disease 2019 (COVID-19) infection. We assessed the prevalence and prognostic value of the initial liver enzymes in patients admitted to hospital with COVID-10 infection.

### *Research motivation*

At the time of the writing of this manuscript, our health system had data on 10614 individual patients admitted with COVID-10 infection. We wanted to assess the prevalence of liver enzyme abnormalities in these patients and determine if any particular enzyme pattern would predict prognosis.

### *Research objectives*

Determine the prevalence of abnormal liver enzymes in patients admitted to the hospital with COVID-19 infection. Determine the prognostic value of initial liver enzymes on mortality and/or the need for mechanical ventilation. Determine if any particular abnormal liver enzyme pattern was most predictive of poor outcome in COVID-19 infection.

### *Research methods*

Review of electronic medical records of 10614 patients admitted to the hospital with COVID-19 infection.

### *Research results*

Elevated liver enzymes are common upon initial hospital presentation of COVID-19 infection.

### *Research conclusions*

Increased mortality and the need for mechanical ventilation is associated with elevated hepatic enzymes in COVID-19 patients without chronic liver disease.

### *Research perspectives*

This is an important study which highlights the importance of initial liver enzyme patterns in predicting outcomes. Health care workers should be aware of these findings to better triage COVID-19 patients.

## REFERENCES

1 **Huang C**, Wang Y, Li X, Ren L, Zhao J, Hu Y, Zhang L, Fan G, Xu J, Gu X, Cheng Z, Yu T, Xia J,

Wei Y, Wu W, Xie X, Yin W, Li H, Liu M, Xiao Y, Gao H, Guo L, Xie J, Wang G, Jiang R, Gao Z, Jin Q, Wang J, Cao B. Clinical features of patients infected with 2019 novel coronavirus in Wuhan, China. *Lancet* 2020; **395**: 497-506 [PMID: 31986264 DOI: 10.1016/S0140-6736(20)30183-5]

2 **World Health Organization**. Coronavirus disease (COVID-19) outbreak. Geneva: World Health Organization; 2020. [cited 8 October 2020]. Available from: https://www.who.int/emergencies/diseases/novel-coronavirus-2019

3 **Lei F**, Liu YM, Zhou F, Qin JJ, Zhang P, Zhu L, Zhang XJ, Cai J, Lin L, Ouyang S, Wang X, Yang C, Cheng X, Liu W, Li H, Xie J, Wu B, Luo H, Xiao F, Chen J, Tao L, Cheng G, She ZG, Zhou J, Wang H, Lin J, Luo P, Fu S, Ye P, Xiao B, Mao W, Liu L, Yan Y, Chen G, Huang X, Zhang BH, Yuan Y. Longitudinal Association Between Markers of Liver Injury and Mortality in COVID-19 in China. *Hepatology* 2020; **72**: 389-398 [PMID: 32359177 DOI: 10.1002/hep.31301]

4 **Guan WJ**, Ni ZY, Hu Y, Liang WH, Ou CQ, He JX, Liu L, Shan H, Lei CL, Hui DSC, Du B, Li LJ, Zeng G, Yuen KY, Chen RC, Tang CL, Wang T, Chen PY, Xiang J, Li SY, Wang JL, Liang ZJ, Peng YX, Wei L, Liu Y, Hu YH, Peng P, Wang JM, Liu JY, Chen Z, Li G, Zheng ZJ, Qiu SQ, Luo J, Ye CJ, Zhu SY, Zhong NS; China Medical Treatment Expert Group for Covid-19. Clinical Characteristics of Coronavirus Disease 2019 in China. *N Engl J Med* 2020; **382**: 1708-1720 [PMID: 32109013 DOI: 10.1056/NEJMoa2002032]

5 **Wang C**, Horby PW, Hayden FG, Gao GF. A novel coronavirus outbreak of global health concern. *Lancet* 2020; **395**: 470-473 [PMID: 31986257 DOI: 10.1016/S0140-6736(20)30185-9]

6 **Xu L**, Liu J, Lu M, Yang D, Zheng X. Liver injury during highly pathogenic human coronavirus infections. *Liver Int* 2020; **40**: 998-1004 [PMID: 32170806 DOI: 10.1111/liv.14435]

7 **Cai Q**, Huang D, Yu H, Zhu Z, Xia Z, Su Y, Li Z, Zhou G, Gou J, Qu J, Sun Y, Liu Y, He Q, Chen J, Liu L, Xu L. COVID-19: Abnormal liver function tests. *J Hepatol* 2020; **73**: 566-574 [PMID: 32298767 DOI: 10.1016/j.jhep.2020.04.006]

8 **Zhang C**, Shi L, Wang FS. Liver injury in COVID-19: management and challenges. *Lancet Gastroenterol Hepatol* 2020; **5**: 428-430 [PMID: 32145190 DOI: 10.1016/S2468-1253(20)30057-1]

9 **Wang D**, Hu B, Hu C, Zhu F, Liu X, Zhang J, Wang B, Xiang H, Cheng Z, Xiong Y, Zhao Y, Li Y, Wang X, Peng Z. Clinical Characteristics of 138 Hospitalized Patients With 2019 Novel Coronavirus-Infected Pneumonia in Wuhan, China. *JAMA* 2020; **323**: 1061-1069 [PMID: 32031570 DOI: 10.1001/jama.2020.1585]

10 **Goyal P**, Choi JJ, Pinheiro LC, Schenck EJ, Chen R, Jabri A, Satlin MJ, Campion TR Jr, Nahid M, Ringel JB, Hoffman KL, Alshak MN, Li HA, Wehmeyer GT, Rajan M, Reshetnyak E, Hupert N, Horn EM, Martinez FJ, Gulick RM, Safford MM. Clinical Characteristics of Covid-19 in New York City. *N Engl J Med* 2020; **382**: 2372-2374 [PMID: 32302078 DOI: 10.1056/NEJMc2010419]

11 **Richardson S**, Hirsch JS, Narasimhan M, Crawford JM, McGinn T, Davidson KW; the Northwell COVID-19 Research Consortium, Barnaby DP, Becker LB, Chelico JD, Cohen SL, Cookingham J, Coppa K, Diefenbach MA, Dominello AJ, Duer-Hefele J, Falzon L, Gitlin J, Hajizadeh N, Harvin TG, Hirschwerk DA, Kim EJ, Kozel ZM, Marrast LM, Mogavero JN, Osorio GA, Qiu M, Zanos TP. Presenting Characteristics, Comorbidities, and Outcomes Among 5700 Patients Hospitalized With COVID-19 in the New York City Area. *JAMA* 2020; **323**: 2052-2059 [PMID: 32320003 DOI: 10.1001/jama.2020.6775]

12 **Bloom PP**, Meyerowitz EA, Reinus Z, Daidone M, Gustafson J, Kim AY, Schaefer E, Chung RT. Liver Biochemistries in Hospitalized Patients With COVID-19. *Hepatology* 2021; **73**: 890-900 [PMID: 32415860 DOI: 10.1002/hep.31326]

13 **Hajifathalian K**, Krisko T, Mehta A, Kumar S, Schwartz R, Fortune B, Sharaiha RZ; WCM-GI research group*. Gastrointestinal and Hepatic Manifestations of 2019 Novel Coronavirus Disease in a Large Cohort of Infected Patients From New York: Clinical Implications. *Gastroenterology* 2020; **159**: 1137-1140.e2 [PMID: 32389667 DOI: 10.1053/j.gastro.2020.05.010]

14 **Hundt MA**, Deng Y, Ciarleglio MM, Nathanson MH, Lim JK. Abnormal Liver Tests in COVID-19: A Retrospective Observational Cohort Study of 1,827 Patients in a Major U.S. Hospital Network. *Hepatology* 2020; **72**: 1169-1176 [PMID: 32725890 DOI: 10.1002/hep.31487]

15 **Wander P**, Epstein M, Bernstein D. COVID-19 Presenting as Acute Hepatitis. *Am J Gastroenterol* 2020; **115**: 941-942 [PMID: 32301760 DOI: 10.14309/ajg.0000000000000660]

16 **Roth NC**, Kim A, Vitkovski T, Xia J, Ramirez G, Bernstein D, Crawford JM. Post-COVID-19 Cholangiopathy: A Novel Entity. *Am J Gastroenterol* 2021; **116**: 1077-1082 [PMID: 33464757 DOI: 10.14309/ajg.0000000000001154]

17 **Goel H**, Harmouch F, Garg K, Saraiya P, Daly T, Kumar A, Hippen JT. The liver in COVID-19: prevalence, patterns, predictors, and impact on outcomes of liver test abnormalities. *Eur J Gastroenterol Hepatol* 2020 [PMID: 33369962 DOI: 10.1097/MEG.0000000000002021]

18 **Kim JH**. 2018 Korean Association for the Study of the Liver (KASL) Clinical Practice Guidelines of Chronic Hepatitis B: What's Different? *Korean J Gastroenterol* 2019; **73**: 132-140 [PMID: 31013556 DOI: 10.4166/kjg.2019.73.3.132]

19 **Lee IC**, Huo TI, Huang YH. Gastrointestinal and liver manifestations in patients with COVID-19. *J Chin Med Assoc* 2020; **83**: 521-523 [PMID: 32243269 DOI: 10.1097/JCMA.0000000000000319]

20 **Jothimani D**, Venugopal R, Abedin MF, Kaliamoorthy I, Rela M. COVID-19 and the liver. *J Hepatol* 2020; **73**: 1231-1240 [PMID: 32553666 DOI: 10.1016/j.jhep.2020.06.006]

21 **Nardo AD**, Schneeweiss-Gleixner M, Bakail M, Dixon ED, Lax SF, Trauner M. Pathophysiological mechanisms of liver injury in COVID-19. *Liver Int* 2021; **41**: 20-32 [PMID: 33190346 DOI: 10.1111/liv.14730]

22 **Piano S**, Dalbeni A, Vettore E, Benfaremo D, Mattioli M, Gambino CG, Framba V, Cerruti L, Mantovani A, Martini A, Luchetti MM, Serra R, Cattelan A, Vettor R, Angeli P; COVID-LIVER study group. Abnormal liver function tests predict transfer to intensive care unit and death in COVID-19. *Liver Int* 2020; **40**: 2394-2406 [PMID: 32526083 DOI: 10.1111/liv.14565]

World Journal of Gastroenterology

Submit a Manuscript: https://www.f6publishing.com

*World J Gastroenterol* 2021 November 14; 27(42): 7362-7375

DOI: 10.3748/wjg.v27.i42.7362

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

ORIGINAL ARTICLE

Retrospective Cohort Study

# Survival and outcomes for co-infection of chronic hepatitis C with and without cirrhosis and COVID-19: A multicenter retrospective study

Shimaa Afify, Basem Eysa, Fatma Abdel Hamid, Omnia M Abo-Elazm, Mohamed A Edris, Rabab Maher, Ahmed Abdelhalim, Muhammad Mostafa Abdel Ghaffar, Dalia A Omran, Hend Ibrahim Shousha

**ORCID number:** Shimaa Afify 0000-0001-5937-4240; Basem Eysa 0000-0003-4806-0443; Fatma Abdel Hamid 0000-0002-0072-4135; Omnia M Abo-Elazm 0000-0002-0506-9459; Mohamed A Edris 0000-0003-1244-0890; Rabab Maher 0000-0002-8728-2156; Ahmed Abdelhalim 0000-0001-9152-4886; Muhammad Mostafa Abdel Ghaffar 0000-0003-2768-3465; Dalia A Omran 0000-0002-5513-6955; Hend Ibrahim Shousha 0000-0002-0928-4738.

**Author contributions:** Afify S and Omran DA were the guarantors and designed the study; Afify S, Maher R, Eysa B, Abdel Ghaffar MM, Abdelhalim A and Edris MA participated in the acquisition of the data; Abo-Elazm OM participated in the analysis, and interpretation of the data; Shousha HI and Hamid FA drafted the initial manuscript; Afify S, Maher R, Omran DA, and Eysa B revised the article critically for important intellectual content.

**Institutional review board statement:** This study was approved by the research ethics committee of the General Organization for Teaching Hospitals and Institutes(number ITH00123) 22July 2020).

**Shimaa Afify, Basem Eysa, Mohamed A Edris, Ahmed Abdelhalim,** Department of Gastroenterology, National Hepatology and Tropical Medicine Research Institute, Cairo 20222, Egypt

**Fatma Abdel Hamid,** Department of Endemic Medicine, Faculty of Medicine, Fayoum University, El-Fayoum 13524, Egypt

**Omnia M Abo-Elazm,** Department of Biostatistics and Cancer Epidemiology, National Cancer Institute, Cairo 20222, Egypt

**Rabab Maher,** Department of Gastroenterology, Students Hospital, Cairo University, Giza 12111, Egypt

**Muhammad Mostafa Abdel Ghaffar,** The General Organization for Teaching Hospitals and Institutes, Cairo 11562, Egypt

**Dalia A Omran, Hend Ibrahim Shousha,** Department of Endemic Medicine, Faculty of Medicine, Cairo University, Cairo 11562, Egypt

**Corresponding author:** Shimaa Afify, MD, MSc, PhD, Consultant Physician-Scientist, Department of Gastroenterology, National Hepatology and Tropical Medicine Research Institute, 10 Fom Elkhalig, Kasr Alainy St., Cairo 20222, Egypt. drshima202@yahoo.com

## Abstract

### *BACKGROUND*

Chronic liver disease, particularly cirrhosis, is associated with worse outcomes in patients infected with coronavirus disease 2019 (COVID-19).

### *AIM*

To assess outcomes of COVID-19 infection among patients with pre-existing hepatitis C with or without liver cirrhosis.

### *METHODS*

This multicenter, retrospective cohort study included all cases of confirmed co-infection of severe acute respiratory syndrome coronavirus 2 and chronic hepatitis

**Informed consent statement:** All study subjects gave written informed consent before study inclusion.

**Conflict-of-interest statement:** The authors declare that there are no conflicts of interest to report.

**Data sharing statement:** No additional data are available.

**STROBE statement:** The authors have read the STROBE Statement checklist of items, and the manuscript was prepared and revised according to the STROBE Statement—checklist of items.



**Specialty type:** Gastroenterology and hepatology

**Country/Territory of origin:** Egypt

**Peer-review report's scientific quality classification**

Grade A (Excellent): 0
Grade B (Very good): B, B
Grade C (Good): 0
Grade D (Fair): 0
Grade E (Poor): 0

**Received:** March 21, 2021
**Peer-review started:** March 21, 2021
**First decision:** April 29, 2021
**Revised:** May 12, 2021
**Accepted:** October 24, 2021
**Article in press:** October 24, 2021
**Published online:** November 14, 2021

**P-Reviewer:** Beraldo RF, Gupta T
**S-Editor:** Ma YJ
**L-Editor:** A

C with or without liver cirrhosis who were admitted to six hospitals (Al-Sahel Hospital, Al-Matareya Hospital, Al-Ahrar Hospital, Ahmed Maher Teaching Hospital, Al-Gomhoreya Hospital, and the National Hepatology and Tropical Medicine Research Institute) affiliated with the General Organization for Teaching Hospitals and Institutes in Egypt. Patients were recruited from May 1, 2020, to July 31, 2020. Demographic, laboratory, imaging features, and outcomes were collected. Multivariate regression analysis was performed to detect factors affecting mortality.

*RESULTS*

This retrospective cohort study included 125 patients with chronic hepatitis C and COVID-19 co-infection, of which 64 (51.20%) had liver cirrhosis and 40 (32.00%) died. Fever, cough, dyspnea, and fatigue were the most frequent symptoms in patients with liver cirrhosis. Cough, sore throat, fatigue, myalgia, and diarrhea were significantly more common in patients with liver cirrhosis than in non-cirrhotic patients. There was no difference between patients with and without cirrhosis regarding comorbidities. Fifteen patients (23.40%) with liver cirrhosis presented with hepatic encephalopathy. Patients with liver cirrhosis were more likely than non-cirrhotic patients to have combined ground-glass opacities and consolidations in CT chest scans: 28 (43.75%) *vs* 4 (6.55%), respectively ($P$ value < 0.001). These patients also were more likely to have severe COVID-19 infection, compared to patients without liver cirrhosis: 29 (45.31%) *vs* 11 (18.04%), respectively ($P$ value < 0.003). Mortality was higher in patients with liver cirrhosis, compared to those with no cirrhosis: 33 (51.56%) *vs* 9 (14.75%), respectively ($P$ value < 0.001). All patients in Child-Pugh class A recovered and were discharged. Cirrhotic mortality occurred among decompensated patients only. A multivariate regression analysis revealed the following independent factors affecting mortality: Male gender (OR 7.17, 95%CI: 2.19–23.51; $P$ value = 0.001), diabetes mellitus (OR 4.03, 95%CI: 1.49–10.91; $P$ value = 0.006), and liver cirrhosis (OR 1.103, 95%CI: 1.037–1.282; $P$ value < 0.0001). We found no differences in liver function, COVID-19 disease severity, or outcomes between patients who previously received direct-acting antiviral therapy (and achieved sustained virological response) and patients who did not receive this therapy.

*CONCLUSION*

Patients with liver cirrhosis are susceptible to higher severity and mortality if infected with COVID-19. Male gender, diabetes mellitus, and liver cirrhosis are independent factors associated with increased mortality risk.

**Key Words:** COVID-19; Egypt; Outcome; Liver cirrhosis; Chronic hepatitis C



**Core Tip:** Chronic liver disease, particularly cirrhosis, is associated with worse outcomes in patients infected with coronavirus disease 2019 (COVID-19). This study examined the impact of COVID-19 infection on patients with chronic hepatitis C during the first COVID-19 peak in Egypt. This retrospective cohort study was performed in six Egyptian hospitals. We found that cirrhotic patients had higher rates of pneumonia, severe COVID-19, and mortality. Cirrhotic mortality was observed among decompensated patients only. Male gender, diabetes mellitus, and liver cirrhosis were independent factors associated with increased mortality risk in Egyptian patients with COVID-19 and chronic hepatitis C.

**Citation:** Afify S, Eysa B, Hamid FA, Abo-Elazm OM, Edris MA, Maher R, Abdelhalim A, Abdel Ghaffar MM, Omran DA, Shousha HI. Survival and outcomes for co-infection of chronic hepatitis C with and without cirrhosis and COVID-19: A multicenter retrospective study. *World J Gastroenterol* 2021; 27(42): 7362-7375
**URL:** https://www.wjgnet.com/1007-9327/full/v27/i42/7362.htm
**DOI:** https://dx.doi.org/10.3748/wjg.v27.i42.7362

**P-Editor:** Ma YJ

## INTRODUCTION

On March 11, 2020, WHO declared severe acute respiratory syndrome coronavirus 2 (SARS-COV-2), also known as coronavirus disease 2019 (COVID-19), as the sixth pandemic of the 21st century[1]. As of January 2021, the virus has caused more than 85 million confirmed infections and about 2 million deaths worldwide[2]. In Egypt, about 140000 confirmed infections and about 8000 deaths were recorded by the Egyptian Ministry of Health and Population as of January 2021[3]. Lung lesions can cause major damage in those infected with COVID-19, and liver injury also has been reported[4]. Studies conducted in Wuhan early in the epidemic outbreak found that up to 50% of infected patients had abnormal liver enzymes. Zhang *et al*[5] showed that SARS-CoV-2 infection occurs in 2%–11% of patients with pre-existing liver conditions.

COVID-19's adverse effects on the liver could be explained by both direct cytopathic injury and indirect effects of the virus[4]. It is well-established that SARS-CoV-2 uses angiotensin-converting enzyme receptors to gain entry into cells. These receptors are much more abundant in cholangiocytes (59.70%) than in hepatocytes (2.60%)[6]. The bile duct epithelium also plays an important role in regeneration after injury and immune response. Thus, the indirect effects may be due to exposure to multiple insults. For example, in severe cases admitted to intensive care, hemodynamic instability can lead to hypoperfusion and ischemic liver injury. Pneumonitis-associated hypoxia can lower mean arterial pressure, causing a synergistic effect contributing to this ischemic insult[4]. Another contributing factor could be toxic effects of medications (*e.g.*, steroids, non-steroid anti-inflammatory drugs, antibiotics, anticoagulants, antivirals), which are associated predominantly with hepatocellular rather than cholestatic liver injury[6,7]. Finally, immune dysregulation can lead to systemic inflammatory response syndrome, or cytokine storm, which is the release of inflammatory mediators (*e.g.*, interleukins IL-6 and IL-1, TNF- α, and interferon) that can cause and exacerbate liver injury[8].

Hepatitis C virus remains the most common etiology of overt and occult chronic liver diseases, liver cirrhosis, and risk of hepatocellular carcinoma in Egypt[9]. Chronic liver disease is associated with immune dysregulation and multiple system involvement (*e.g.*, cardiomyopathy, hepatopulmonary syndrome, and coagulopathy)[9]. Patients in Child-Pugh classes B and C and those with higher MELD scores have much higher mortality rates than patients in class A or with lower MELD scores[10]. Importantly, the cause of death in most of these patients is respiratory failure rather than acute on top of chronic liver failure[10].

Transaminitis, which is abnormal levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST), is the most frequent and direct cause of disease severity in patients without cirrhosis[4]. Decompensation with worsening liver symptoms (*e.g.*, ascites, spontaneous bacterial peritonitis, hepatic encephalopathy, and variceal hemorrhage) has been reported in patients with chronic liver disease and is associated with a high risk of death. Interestingly, decompensation occurs without respiratory symptoms[11]. This study aimed to demonstrate the impact of COVID-19 infection on patients with pre-existing hepatitis C with or without liver cirrhosis during the first COVID-19 peak in Egypt.

## MATERIALS AND METHODS

### *Study design and patient selection*

This multicenter, retrospective cohort study included all cases of confirmed co-infection of SARS-CoV-2 and chronic hepatitis C with or without liver cirrhosis who were admitted to six hospitals (Al-Sahel Hospital, Al-Matareya Hospital, Al-Ahrar Hospital, Ahmed Maher Teaching Hospital, Al-Gomhoreya Hospital, and the National Hepatology and Tropical Medicine Research Institute) in the General Organization for Teaching Hospitals and Institutes in Egypt. Patients were recruited from May 1, 2020, to July 31, 2020. The diagnosis of COVID-19 was based on a positive RT-PCR from nasopharyngeal swabs. Patients with negative RT-PCR results or those who did not undergo the swab were excluded.

### *Ethics statement*

The authors assert that all procedures contributing to this work complied with the ethical standards of the relevant national and institutional committees on human experimentation and with the Helsinki Declaration of 1975, as revised in 2008. All procedures involving human subjects and patients were approved by the research

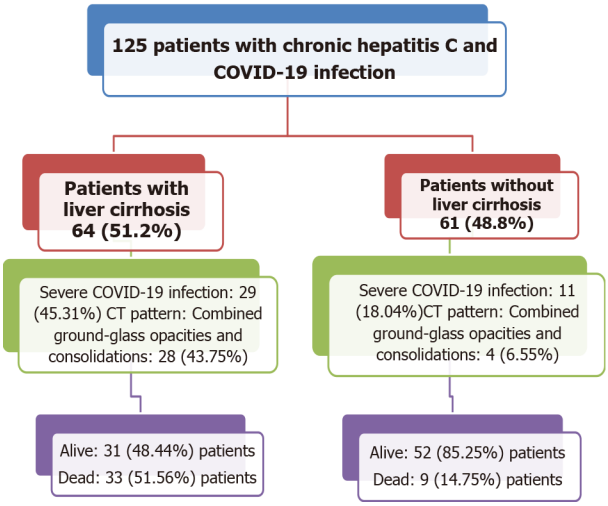


**Figure 1 Flow chart of the study cohort**.

ethics committee of the General Organization for Teaching Hospitals and Institutes. Written informed consent was obtained from all patients.

### *Methods*

Baseline demographic data collected included age, gender, cigarette smoking status, and comorbidities. Other information recorded included general respiratory and gastrointestinal symptoms, chest CT scans, and laboratory results (complete blood count, liver and renal function, coagulation, D-dimer, ferritin, and C-reactive protein). We also included treatments administered to the patients and COVID-19 disease classification and outcome. COVID-19 severity was categorized as mild, moderate, or severe, according to the management protocol of the Egyptian Ministry of Health and Population[12]. Mild cases were symptomatic with lymphopenia or leucopenia and no radiological lung affection by pneumonia. Moderate cases were symptomatic with radiological features of pneumonia with or without leucopenia and lymphopenia. Severe and critical cases included any of the following: respiratory rate > 30 per minute; $SaO_2$ < 92 in room air; $PaO_2/FiO_2$ ratio < 300; chest radiology showing > 50% lung affection or progressive lung affection within 24 to 48 h; or critically ill at $SaO_2$ < 92, respiratory rate > 30 per minute, or $PaO_2/FiO_2$ ratio < 200 despite oxygen therapy. Severe and critical cases were indicated for intensive care unit (ICU) admission. Treatments were applied according to the protocol[12].

### *Statistical analysis*

Data were analyzed using SPSS version 25 (SPSS Inc., Chicago, IL, United States). Numerical data are expressed as mean and standard deviation or median and range, as appropriate. Qualitative data are expressed as frequency and percentage. The Chi-square test (Fisher's exact test) was used to examine the relation between qualitative variables. For quantitative data, Student's *t*-test or the Mann-Whitney test (non-parametric *t*-test) was used to compare groups, as appropriate. A $P$ value ≤ 0.05 was considered significant. To study possible associations between selected variables (gender, liver cirrhosis, and diabetes mellitus) and mortality, we fitted multiple logistic regression models. The results are expressed as the odds ratio (OR) with 95% confidence interval (CI).

## RESULTS

This study included 125 patients infected with chronic hepatitis C virus and COVID-19 (Figure 1). Of those, 64 (51.2%) patients had liver cirrhosis: 25 (39.06%) were classified as Child-Pugh class A, 22 (34.38%) as class B, and 17 (26.56%) as class C. Patient residences included five Egyptian governorates: Cairo, Giza, Al-Behera, Al-Qalubya, and Al-Menofeya. Over half (61.2%) were older than 60 years, and most (68.8%) were men. Table 1 presents the baseline demographic features. Regarding COVID-19 symptoms, five (4.0%) patients were asymptomatic. The most common symptoms

**Table 1 Baseline demographic features of patients (*n* = 125 patients)**

| Item | *n* (%) |
|---|---|
| **Egyptian governorate** | |
| Cairo | 78 (62.4) |
| Al-Menofeya | 17 (13.6) |
| Giza | 14 (11.2) |
| Al-Qalubya | 9 (7.2) |
| Al-Behera | 7 (5.6) |
| **Age (yr)** | |
| 20 to 30 | 1 (0.8) |
| 30 to 40 | 6 (4.8) |
| 40 to 50 | 10 (8.1) |
| 50 to 60 | 31 (25.0) |
| 60 to 70 | 35 (28.2) |
| 70 to 80 | 35 (28.2) |
| 80 to 90 | 6 (4.8) |
| **Gender** | |
| Male | 86 (68.8) |
| Female | 39 (31.2) |
| **Cigarette smoking** | 10 (8.0) |
| **History of contact with COVID-19 case** | 32 (25.6) |
| **Diabetes mellitus** | 52 (41.6) |
| **Hypertension** | 52 (41.6) |
| **Direct-acting antiviral therapy treatment** | |
| Not previously treated | 108 (86.4) |
| Sustained virological response | 17 (13.6) |
| **COPD** | 1 (0.8) |
| **Coronary artery disease** | 12 (9.6) |
| **Acute kidney injury** | 1 (0.8) |
| **Chronic renal insufficiency** | 8 (6.4) |
| **Heart failure** | 2 (1.6) |
| **Bronchial asthma** | 1 (0.8) |
| **CT pattern** | 1 (0.8) |
| Consolidations and ground-glass opacities | 31 (24.8) |
| Ground-glass opacities | 94 (75.2) |
| **Lesion distribution on CT** | |
| Bilateral | 122 (97.6) |
| Unilateral | 3 (2.4) |
| **COVID-19 case severity** | |
| Moderate | 86 (68.8) |
| Severe | 39 (31.2) |
| **Admission zone** | |
| ICU | 24 (19.2) |

| | |
|---|---|
| Intermediate care | 6 (4.8) |
| Ward | 95 (76.0) |

COVID-19: Coronavirus disease 2019; COPD: Chronic obstructive pulmonary disease; CT: Computed tomography; ICU: Intensive care unit.

were dyspnea (80 patients, 64.0%), fever (78 patients, 62.4%), and cough (55 patients, 44.0%), and five (4.0%) had diarrhea. Table 2 summarizes the patient symptoms. Table 3 summarizes the baseline laboratory results and treatment protocols among the studied patients.

We compared the characteristics of patients with and without liver cirrhosis. Fever, cough, dyspnea, and fatigue were the most frequent symptoms in patients with liver cirrhosis. Cough, sore throat, fatigue, myalgia, and diarrhea were significantly more common in patients with liver cirrhosis than non-cirrhotic patients. There was no difference between patients with and without cirrhosis regarding comorbidities. Fifteen (23.4%) patients with liver cirrhosis presented with hepatic encephalopathy (Table 4).

Patients with liver cirrhosis were more likely to show combined ground-glass opacities and consolidations in their chest CT scans: 28 (43.75%) *vs* 4 (6.55%), respectively ($P$ value < 0.001). These patients also were more likely to present with severe COVID-19 infection: 29 (45.31%) *vs* 11 (18.04%), respectively (P-value 0.003), compared to patients without liver cirrhosis. Mortality was higher in patients with liver cirrhosis: 33 (51.56%) *vs* 9 (14.75%), respectively ($P$ value < 0.001) (Table 5). All patients classified as Child-Pugh class A recovered and were discharged, whereas 18 (45%) mortalities occurred among patients considered class B and 15 (37.5%) among those considered class C.

A multivariate logistic regression revealed that male patients were more likely than female patients to die, after adjusting for liver cirrhosis and diabetes mellitus status (OR 7.166, 95%CI: 2.185–23.506; $P$ value 0.001). Mortality was four times more likely among patients with diabetes mellitus, after adjusting for other factors in the model (OR 4.029, 95%CI: 1.488–10.906; $P$ value 0.006). Patients with liver cirrhosis also were more likely to die (OR 1.103, 95%CI: 1.037–1.282; $P$ value 0.0001), compared to those without cirrhosis (Table 6).

Within our cohort, 17 (13.6%) patients received direct-acting antiviral therapy (DAA) and achieved sustained virological response before acquiring COVID-19 infection. Among them, 10 (15.6%) patients had liver cirrhosis and seven (11.5%) did not. Three (17.6%) DAA recipients had severe COVID-19 disease, and the rest had moderate COVID-19 disease on admission. Regarding liver function, COVID-19 disease severity, and outcome, we found no difference between patients who previously received DAA and those who did not (Table 7).

## DISCUSSION

Little research has assessed outcomes of patients co-infected with chronic hepatitis C virus and COVID-19, and existing studies include either a small number of patients or report data from patients with chronic liver disease and multiple underlying etiologies (viral and non-viral). This is the first Egyptian study reporting the outcome of SARS-CoV-2 infection in patients with isolated chronic hepatitis C as the etiology of their underlying chronic liver disease. We found a substantially increased incidence (23.43%) of hepatic encephalopathy in our cirrhotic patients infected with COVID-19. The 1-year cumulative incidence of hepatic encephalopathy in liver cirrhosis ranges from 0% to 21%[13]. We also found that the severity of COVID-19 symptoms and mortality rates were significantly higher in patients with liver cirrhosis. All patients assigned to Child-Pugh class A recovered and were discharged, but mortalities occurred among patients assigned to Child-Pugh class B or class C on admission. Male gender, diabetes mellitus, and liver cirrhosis were independent factors affecting mortality in our cohort.

In our study, fever (67.2%), cough (57.8%), dyspnea (56.3%), and fatigue (32.8%) were the most frequent symptoms in patients with liver cirrhosis, followed by diarrhea (7.8%). Among patients with cirrhosis, 6.3% were asymptomatic. Iavarone *et al*[14] studied 50 patients with liver cirrhosis with hepatitis C, hepatitis B, non-viral (*e.g.*, alcoholic), or multiple etiologies and reported fever in 64%, fatigue in 60%, dyspnea in

**Table 2 Symptoms of the studied patients**

| Symptom | *n* (%) |
|---|---|
| Dyspnea | 80 (64.0) |
| Fever | 78 (62.4) |
| Cough | 55 (44.0) |
| Fatigue | 26 (20.8) |
| Disturbed consciousness | 22 (17.6) |
| Sore throat | 15 (12.0) |
| Newly developed hepatic encephalopathy | 15 (12.0) |
| Hepatocellular carcinoma | 15 (12.0) |
| Myalgia | 12 (9.6) |
| Current ascites | 9 (7.2) |
| History of hematemesis from esophageal varices | 9 (7.2) |
| Asymptomatic | 5 (4.0) |
| Diarrhea | 5 (4.0) |
| Arthralgia | 5 (4.0) |
| Anorexia | 4 (3.2) |
| Nausea | 3 (2.4) |
| Vomiting | 3 (2.4) |
| Loss of taste | 2 (1.6) |
| Hemoptysis | 1 (0.8) |
| Abdominal pain | 1 (0.8) |
| Loss of smell | 1 (0.8) |
| Jaundice | 1 (0.8) |
| Rhinorrhea | 1 (0.8) |

42%, and cough in 36%, followed by diarrhea in 10% and no symptoms in 12% of patients. In our study, 15 (23.4%) patients with liver cirrhosis presented with hepatic encephalopathy, compared with 11 (22%) of patients in Iavarone *et al*[14]. For ALT levels in our cohort, 58 (46.4%) were normal, 40 (32%) were elevated 1-2 times above the upper limit of normal, 7 (5.6%) were elevated 2-3 times above the upper limit, 20 (16%) were three times above normal, and 1 patient was five times over the upper limit. Iavarone *et al*[14] define ALT elevation at five times over the upper limit of normal as hepatic flare.

Our results revealed higher mortality among male patients, compared to female patients. Nasiri *et al*[15] similarly reported COVID-19-related mortality to be higher among males, and cohorts from China, Italy, Denmark, and the United States confirmed these findings[16-20]. Underlying sex-related mechanisms could include chromosomal immunological response, lifestyle (alcohol, smoking, and obesity), and comorbidities[19]. We also found that mortality was significantly higher in patients with liver cirrhosis, particularly those with decompensated cirrhosis. Boettler *et al*[21] reported that chronic viral hepatitis did not seem to raise the risk of a severe COVID-19 in a study by Guan *et al*[22], which included patients in China with chronic hepatitis B only. Shalimar *et al*[23] found no difference in mortality among patients with and without cirrhosis, but their sample size was small. An Italian study by Mangia *et al*[24] found that cirrhosis of metabolic origin, older age, leucopenia, and lymphopenia were risk factors for mortality. They also suggested that the very low prevalence of SARS-CoV-2 infection in patients with chronic hepatitis C infection could play a protective role against SARS-CoV-2 infection.

A meta-analysis by Váncsa *et al*[25], which included mainly studies of chronic hepatitis B in China, reported that liver failure and platelet count could predict in-hospital mortality with high specificity and lactate dehydrogenase with moderate

**Table 3 Baseline laboratory results and treatments of the studied 125 patients**

| | mean ± SD/*n* (%) |
|---|---|
| **Pulse rate** | 92 ± 17 |
| **Temperature** | 37 ± 1 |
| **Respiratory rate** | 23 ± 5 |
| **Oxygen saturation** | 93 ± 5 |
| **Hemoglobin (gm/dL)** | 12 ± 2 |
| **Platelet count (× 1000/cmm)** | 183 ± 107 |
| **Total leucocyte count (× 1000/cmm)** | 10 ± 8 |
| **Neutrophil : Lymphocyte ratio** | 1 ± 0 |
| **INR** | 1.16 ± 0.29 |
| **PTT** | 33.9 ± 13.2 |
| **Serum creatinine (mg/dL)** | 1.39 ± 1.43 |
| **Serum sodium (mEq/L)** | 137 ± 9 |
| **Serum potassium (mEq/L)** | 4.18 ± 0.77 |
| **Total bilirubin (mg/dL)** | 3 ± 5 |
| **Direct bilirubin (mg/dL)** | 2.1 ± 3.8 |
| **Serum albumin (g/dL)** | 3.2 ± 0.7 |
| **Alanine Transaminase (U/L)** | 58 ± 53 |
| **Aspartate Transaminase (U/L)** | 92 ± 123 |
| **Alkaline phosphatase (U/L)** | 262 ± 226 |
| **Serum ferritin (ng/mL)** | 667 ± 483 |
| **D-dimer (mg/mL)** | 1560 ± 2503 |
| **Fibrinogen (mg/dL) × 100 if presented in g/L** | 77 |
| **Azithromycin** | 72 (57.6) |
| **Paracetamol** | 45 (36.0) |
| **Supplementary vitamin C** | 85 (68.0) |
| **Zinc** | 69 (55.2) |
| **Colchicine** | 2 (1.6) |
| **Lactoferrin** | 18 (14.4) |
| **Other antibiotics** | 71 (56.8) |
| **Steroids** | 38 (30.4) |
| **Hydroxyl-chloroquine** | 29 (23.2) |
| **Low-molecular weight heparin** | 56 (44.8) |
| **Warfarin** | 3 (2.4) |

PTT: Partial thromboplastin time; INR: International normalized ratio.

specificity. Singh *et al*[26] found that patients with cirrhosis had a higher relative risk of mortality and a higher risk of hospitalization, compared to patients without liver disease. Another study by Galiero *et al*[27] described outcomes in 35 patients with liver cirrhosis, though they did not compare cirrhotic *vs* non-cirrhotic patients, they found that male sex, chronic liver disease, and malignancies were independent factors of poor prognosis in hospitalized patients with COVID-19. They also reported that patients with advanced chronic liver disease had worse clinical conditions compared to patients with no liver disease[27].

**Table 4 Baseline features and symptoms of patients with and without liver cirrhosis**

| Variable | | No liver cirrhosis61 (48.8%) | Liver cirrhosis 64 (51.2%) | *P* value |
|---|---|---|---|---|
| **Gender** | Male | 42 (68.9%) | 44 (68.8%) | 0.57 |
| | Female | 19 (31.1%) | 20 (31.3%) | |
| **Age (yr)** | 20 to 30 | 0 (0.0%) | 1 (1.6%) | 0.004 |
| | 30 to 40 | 3 (4.9%) | 3 (4.8%) | |
| | 40 to 50 | 0 (0.0%) | 10 (15.9%) | |
| | 50 to 60 | 19 (31.1%) | 12 (19.0%) | |
| | 60 to 70 | 14 (23.0%) | 21 (33.3%) | |
| | 70 to 80 | 21 (34.4%) | 14 (22.2%) | |
| | 80 to 90 | 4 (6.6%) | 2 (3.2%) | |
| **Cigarette smoking** | | 2 (3.2%) | 8 (11.5%) | 0.06 |
| **Diabetes mellitus** | | 24 (39.3%) | 28 (43.8%) | 0.71 |
| **Hypertension** | | 23 (37.7%) | 29 (45.3%) | 0.46 |
| **COPD** | | 0 (0.0%) | 1 (1.6%) | 1 |
| **Coronary artery disease** | | 5 (8.2%) | 7(10.9%) | 0.76 |
| **Chronic renal insufficiency** | | 4 (6.6%) | 4 (6.3%) | 1 |
| **Heart failure** | | 0 | 2 (3.1%) | 0.49 |
| **Hepatocellular carcinoma** | | 3 (4.9%) | 12 (18.8%) | 0.026 |
| **Esophageal varices** | | 0 (0.0%) | 9 (14.1%) | 0.003 |
| **Asymptomatic** | | 1 (1.6%) | 4 (6.3%) | 0.36 |
| **Fever** | | 35 (57.4%) | 43 (67.2%) | 0.27 |
| **Cough** | | 18 (29.5% | 37 (57.8%) | 0.002 |
| **Dyspnea** | | 44 (72.1%) | 36 (56.3%) | 0.09 |
| **Sore throat** | | 2 (3.3%) | 13 (20.3%) | 0.005 |
| **Hemoptysis** | | 0 (0.0%) | 1 (1.6%) | 1 |
| **Fatigue** | | 5 (8.2%) | 21 (32.8%) | 0.001 |
| **Anorexia** | | 1 (1.6%) | 3 (4.7%) | 0.62 |
| **Diarrhea** | | 0 (0.0%) | 5 (7.8%) | 0.05 |
| **Nausea** | | 1 (1.6%) | 2 (3.1%) | 1 |
| **Vomiting** | | 1 (1.6%) | 2 (3.1%) | 1 |
| **Abdominal pain** | | 1 (1.6%) | 0 (0.0%) | 0.48 |
| **Arthralgia** | | 1 (1.6%) | 4 (6.3%) | 0.36 |
| **Myalgia** | | 1 (1.6%) | 11 (17.2%) | 0.004 |
| **Loss of taste** | | 0 (0.0%) | 2 (3.1%) | 0.49 |
| **Loss of smell** | | 0 (0.0%) | 1 (1.6%) | 1 |
| **Disturbed consciousness** | | 3 (4.9%) | 19 (29.7%) | 0.001 |
| **Hepatic encephalopathy** | | 0 (0.0%) | 15 (23.4%) | 0.033 |
| **Direct-acting antiviral therapy with sustained virological response before COVID-19 infection** | | 7 (11.5%) | 10 (15.6%) | 0.61 |

COPD: Chronic obstructive pulmonary disease; COVID-19: Coronavirus disease 2019.

In a retrospective multicenter study from 16 hospitals in China that included 21

**Table 5 Comparison between patients with and without liver cirrhosis**

| | | No liver cirrhosis 61 (48.8%) | Liver cirrhosis 64 (51.2%) | *P* value |
|---|---|---|---|---|
| **Hemoglobin (mg/dL)** | | 12 ± 2 | 12 ± 2 | 0.85 |
| **Platelet count (× 1000/cmm)** | | 193 ± 107 | 171 ± 107 | 0.36 |
| **TLC (× 1000/cmm)** | | 12 ± 10 | 9 ± 5 | 0.31 |
| **Serum creatinine (mg/dL)** | | 1.58 ± 1.89 | 1.21 ± 0.76 | 0.59 |
| **Total bilirubin (mg/dL)** | | 3 ± 4 | 3 ± 5 | 0.79 |
| **Serum albumin (g/dL)** | | 3.3 ± 0.7 | 3.1 ± 0.6 | 0.36 |
| **Alanine transaminase (U/L)** | | 67 ± 67 | 50 ± 40 | 0.21 |
| **Aspartate transaminase (U/L)** | | 60 ± 37 | 101 ± 137 | 0.89 |
| **CT pattern** | Consolidations and ground-glass opacities | 4 (6.55%) | 28 (43.75%) | < 0.001 |
| | Ground-glass opacities | 57 (93.45%) | 36 (56.25%) | |
| **Lesion distribution on CT** | Bilateral | 58 (95.1%) | 64 (100.0%) | 0.11 |
| | Unilateral | 3 (4.9%) | 0 | |
| **COVID-19 severity** | Moderate | 50 (81.96%) | 35 (54.69%) | 0.003 |
| | Severe | 11 (18.04%) | 29 (45.31%) | |
| **Azithromycin** | | 33 (54.1%) | 39 (60.9%) | 0.47 |
| **Paracetamol** | | 16 (26.2%) | 29 (45.3%) | 0.04 |
| **Supplementary vitamin C** | | 37 (60.7%) | 48 (75.0%) | 0.12 |
| **Supplementary zinc** | | 33 (54.1%) | 36 (56.3%) | 0.85 |
| **Lactoferrin** | | 3 (4.9%) | 15 (23.4%) | 0.004 |
| **Other antibiotics** | | 28 (45.9%) | 43 (67.2%) | 0.019 |
| **Anticoagulants** | LMWH | 28 (45.9%) | 28 (43.8%) | 1 |
| | Warfarin | 1 (1.6%) | 2 (3.1%) | 1 |
| **Steroids** | | 16 (26.2%) | 22 (34.4%) | 0.339 |
| **Alive (discharged)** | | 52 (85.25%) | 31 (48.44%) | < 0.001 |
| **Died at the hospital** | | 9 (14.75%) | 33 (51.56%) | |

TLC: Thin layer chromatography; CT: Computed tomography; COVID-19: Coronavirus disease 2019.

**Table 6 Multivariate regression analysis of factors affecting mortality**

| | B | S.E. | *P* value | Odds ratio | 95%CI | |
|---|---|---|---|---|---|---|
| | | | | | Lower | Upper |
| Male | 1.969 | 0.606 | 0.001 | 7.166 | 2.185 | 23.506 |
| Diabetes mellitus | 1.393 | 0.508 | 0.006 | 4.029 | 1.488 | 10.906 |
| Liver cirrhosis | 2.274 | 0.515 | 0.0001 | 1.103 | 1.037 | 1.282 |
| Constant | -1.879 | 0.604 | 0.002 | 0.153 | | |

patients with COVID-19 and hepatitis B virus-related liver cirrhosis (Child-Pugh classes A, B, and C in 16, 3, and 2 cases, respectively), mortalities occurred in patients assigned to class A (3; 60.0%) and C (2; 40.0%)[10]. In another multinational cohort study of 745 patients from 29 countries, Marjot *et al*[28] found that liver cirrhosis was present in 386 patients: 171 (44%) in Child-Pugh class A, 124 (32%) in class B, and 91 (24%) in class C. Mortality rates significantly increased with worsening scores: 33 (19%) in class A, 44 (35%) in class B, and 46 (51%) in class C died. Age, Child-Pugh

**Table 7 Comparison between patients with COVID-19 who received or did not receive previous direct-acting antiviral therapy**

| | | Did not receive previous DAA (*n* = 108) | Received previous DAA (*n* = 17) | *P* value |
|---|---|---|---|---|
| **COVID-19 severity** | **Moderate** | 72 (66.7%) | 14 (82.4%) | 0.26 |
| | **Severe** | 36 (33.3%) | 3 (17.6%) | |
| **Admission zone** | **ICU** | 22 (20.4%) | 2 (11.8%) | 0.52 |
| | **Intermediate care** | 6 (5.6%) | 0 (0.0%) | |
| | **Ward** | 80 (74.1%) | 15 (88.2%) | |
| **Vital status** | **Alive** | 73 (67.6%) | 12 (70.6%) | 1 |
| | **Dead** | 35 (32.4%) | 5 (29.4%) | |
| **Total bilirubin (mg/dL)** | | 1.00 (1-20) | 1.30 (1-6) | 0.84 |
| **Direct bilirubin (mg/dL)** | | 0.9 (0.2-15.6) | 0.9 (0.1-2.7) | 0.93 |
| **Serum albumin (g/dL)** | | 3.1 (1.7-5) | 3.15 (2.4-3.8) | 0.45 |
| **Alanine transaminase (U/L)** | | 41.5 (13-324) | 49 (18-175) | 0.54 |
| **Aspartate transaminase (U/L)** | | 46.5 (10- 549) | 53 (16-415) | 0.42 |

DAA: Direct-acting antiviral; COVID-19: coronavirus disease 2019; ICU: Intensive care unit.

class, and alcoholic liver disease were the independent factors affecting mortality in their study, and hepatic encephalopathy occurred in 104 (27%) of their patients[28]. Another study from 13 Asian countries studied 43 patients with liver cirrhosis and reported worsening liver disease and increased hepatic complications in patients with COVID-19 infection ($P$ value < 0.05); they found that a baseline Child-Pugh score ≥ 9 was associated with higher mortality (area under the ROC 0.94, hazard ratio 19.2, 95%CI: 2.3–163.3; $P$ value < 0.001). The independent factors affecting mortality in their study were increased serum bilirubin and AST/ALT ratio[29].

The exact mechanism causing poor outcomes among patients with COVID-19 and preexisting liver disease remains unknown; however, interactions between local liver injury and systemic disturbances seem likely culprits, especially in patients with cirrhosis. Patients with liver cirrhosis are theoretically more susceptible to poor outcomes from COVID-19-related liver injury. Moreover, patients with advanced liver disease exhibit immune deficiency and systemic inflammation, as reflected by activated circulating immune cells and increased serum levels of pro-inflammatory cytokines. These factors can predispose this population to cytokine storms[26]. Furthermore, some cirrhotic patients may have an underlying hepato-pulmonary syndrome, portopulmonary hypertension, or hydrothorax, which can increase the risk of respiratory failure, as indicated in a study by Oyelade *et al*[30].

Sun *et al*[31] suggested SARS-CoV-2-related direct cytotoxicity (*i.e.*, severe inflammatory response leading to immune-mediated liver damage), hypoxic hepatitis due to anoxia (particularly in patients with severe COVID-19), drug□induced liver injury (especially related to the use of antiviral agents such as lopinavir, ritonavir, remdesivir, chloroquine, tocilizumab, umifenovir, and traditional Chinese preparations), and reactivation of pre-existing chronic liver disease as possible mechanisms of hepatic injury. They also found that liver biopsy specimens from deceased patients with severe COVID-19 showed moderate microvascular steatosis and mild lobular and portal activity, indicating that liver injury could have been caused by either SARS-CoV-2 infection or drug-induced liver injury[31].

A limitation to our study is its retrospective nature and the limited number of patients. It remains unknown whether liver injuries and mortalities among patients with chronic hepatitis C and COVID-19 co-infection are due to patients' pre-existing chronic conditions or the impact of SARS-CoV-2 infection. We found that patients with decompensated hepatitis C-related cirrhosis were at higher risk of COVID-19-related mortality. Hepatic encephalopathy could be the presentation of underlying SARS-CoV-2 infection in patients with liver cirrhosis. The underlying etiology of chronic liver disease also could impact COVID-19 disease course and outcome. Further studies comparing outcomes and prognostic factors in patients with isolated underlying etiologies of chronic liver disease are therefore encouraged, rather than combining them into one group, which could obscure relevant risk factors for disease severity and

outcomes.

## CONCLUSION

In Egypt, among patients with chronic hepatitis C who also were infected with COVID-19, those with cirrhosis had higher rates of pneumonia, severe COVID-19, and mortality, though cirrhotic mortality occurred among decompensated patients only. Male gender, diabetes mellitus, and liver cirrhosis were the independent factors affecting mortality in these patients.

## ARTICLE HIGHLIGHTS

### *Research background*

Coronavirus disease 2019 (COVID-19) disease severity and outcomes are affected by pre-existing chronic liver disease, particularly cirrhosis. Patients with decompensated liver cirrhosis (Child-Pugh classes B and C) are severely affected, with higher mortality rates than patients with compensated disease.

### *Research motivation*

Comprehensive research on the outcome of COVID-19 in patients with isolated etiology of pre-existing chronic liver disease is needed to understand the clinical presentations and outcomes.

### *Research objectives*

This study aimed to demonstrate the impact of COVID-19 factors affecting mortality among patients with pre-existing hepatitis C with or without liver cirrhosis during the first peak of the pandemic in Egypt.

### *Research methods*

This multicenter retrospective cohort study included 125 patients with COVID-19 at six quarantine hospitals in Egypt from May 1, 2020, to July 31, 2020. Clinical, laboratory features, COVID-19 severity, and outcomes were recorded. A regression analysis was performed to detect factors affecting mortality.

### *Research results*

Fever, cough, dyspnea, and fatigue were the most frequent symptoms in patients with liver cirrhosis. Cough, sore throat, fatigue, myalgia, and diarrhea were significantly more common in patients with liver cirrhosis than in non-cirrhotic patients. Fifteen (23.4%) patients with liver cirrhosis presented with hepatic encephalopathy. Patients with liver cirrhosis were more likely to exhibit combined ground-glass opacities and consolidations in their chest CT scans and more likely to present with severe COVID-19 infection, compared to patients without liver cirrhosis. Mortality was higher among patients with liver cirrhosis: 33 (51.56%) *vs* 9 (14.75%), respectively ($P$ value < 0.001. All patients in Child-Pugh class A recovered and were discharged, and mortalities occurred among patients in Child-Pugh classes B and C. A multivariate logistic regression revealed that male gender, diabetes mellitus, and liver cirrhosis were independent factors affecting mortality. Regarding liver function, COVID-19 disease severity, and outcomes, we found no difference between patients who previously received direct acting antiviral therapy (and achieved sustained virological response) and patients who did not receive such therapy.

### *Research conclusions*

Patients with decompensated hepatitis C virus-related liver cirrhosis are at higher risk of severe COVID-19 disease and mortality. Male gender, diabetes mellitus, and liver cirrhosis are the independent factors affecting mortality.

### *Research perspectives*

Male gender, diabetes mellitus, and liver cirrhosis significantly increased mortality in patients with COVID-19 and isolated hepatitis C virus-related chronic liver disease. Previous achievement of sustained virological response after direct acting antiviral therapy for chronic hepatitis C does not impact COVID-19 disease severity, outcome,

or the results of liver function tests.

## REFERENCES


1 **World Health Organization**. [cited March 22, 2020] Available from: https://www.who.int/dg/speeches/detail/who-director-general-s-opening-remarks-at-the-media-briefing-on-covid-19-11-march-2020

2 **WorldOmeter**. World / Countries / Egypt. Last updated: September 28, 2021. Available from: https://www.worldometers.info/coronavirus/country/egypt/

3 **Anirvan P**, Bharali P, Gogoi M, Thuluvath PJ, Singh SP, Satapathy SK. Liver injury in COVID-19: The hepatic aspect of the respiratory syndrome - what we know so far. *World J Hepatol* 2020; **12**: 1182-1197 [PMID: 33442447 DOI: 10.4254/wjh.v12.i12.1182]

4 **Huang C**, Wang Y, Li X, Ren L, Zhao J, Hu Y, Zhang L, Fan G, Xu J, Gu X, Cheng Z, Yu T, Xia J, Wei Y, Wu W, Xie X, Yin W, Li H, Liu M, Xiao Y, Gao H, Guo L, Xie J, Wang G, Jiang R, Gao Z, Jin Q, Wang J, Cao B. Clinical features of patients infected with 2019 novel coronavirus in Wuhan, China. *Lancet* 2020; **395**: 497-506 [PMID: 31986264 DOI: 10.1016/S0140-6736(20)30183-5]

5 **Zhang C**, Shi L, Wang FS. Liver injury in COVID-19: management and challenges. *Lancet Gastroenterol Hepatol* 2020; **5**: 428-430 [PMID: 32145190 DOI: 10.1016/S2468-1253(20)30057-1]

6 **Kumar P**, Sharma M, Kulkarni A, Rao PN. Pathogenesis of Liver Injury in Coronavirus Disease 2019. *J Clin Exp Hepatol* 2020; **10**: 641-642 [PMID: 32837092 DOI: 10.1016/j.jceh.2020.05.006]

7 **Cabibbo G**, Rizzo GEM, Stornello C, Craxì A. SARS-CoV-2 infection in patients with a normal or abnormal liver. *J Viral Hepat* 2021; **28**: 4-11 [PMID: 33190321 DOI: 10.1111/jvh.13440]

8 **Mantovani A**, Beatrice G, Dalbeni A. Coronavirus disease 2019 and prevalence of chronic liver disease: A meta-analysis. *Liver Int* 2020; **40**: 1316-1320 [PMID: 32329563 DOI: 10.1111/liv.14465]

9 **Strickland GT**, Elhefni H, Salman T, Waked I, Abdel-Hamid M, Mikhail NN, Esmat G, Fix A. Role of hepatitis C infection in chronic liver disease in Egypt. *Am J Trop Med Hyg* 2002; **67**: 436-442 [PMID: 12452500 DOI: 10.4269/ajtmh.2002.67.436]

10 **Qi X**, Liu Y, Wang J, Fallowfield JA, Li X, Shi J, Pan H, Zou S, Zhang H, Chen Z, Li F, Luo Y, Mei M, Liu H, Wang Z, Li J, Yang H, Xiang H, Liu T, Zheng MH, Liu C, Huang Y, Xu D, Kang N, He Q, Gu Y, Zhang G, Shao C, Liu D, Zhang L, Kawada N, Jiang Z, Wang F, Xiong B, Takehara T, Rockey DC; COVID-Cirrhosis-CHESS Group. Clinical course and risk factors for mortality of COVID-19 patients with pre-existing cirrhosis: a multicentre cohort study. *Gut* 2021; **70**: 433-436 [PMID: 32434831 DOI: 10.1136/gutjnl-2020-321666]

11 **Moon AM**, Webb GJ, Aloman C, Armstrong MJ, Cargill T, Dhanasekaran R, Genescà J, Gill US, James TW, Jones PD, Marshall A, Mells G, Perumalswami PV, Qi X, Su F, Ufere NN, Barnes E, Barritt AS, Marjot T. High mortality rates for SARS-CoV-2 infection in patients with pre-existing chronic liver disease and cirrhosis: Preliminary results from an international registry. *J Hepatol* 2020; **73**: 705-708 [PMID: 32446714 DOI: 10.1016/j.jhep.2020.05.013]

12 **Ministry of Health and Population**, Egypt. Management protocol for COVID-19 Patients. Version 1.4 / 30th May 2020. Available from: https://www.elwatannews.com/data/iframe/pdf/17175200761591035127.pdf

13 **Elsaid MI**, Rustgi VK. Epidemiology of Hepatic Encephalopathy. *Clin Liver Dis* 2020; **24**: 157-174 [PMID: 32245524 DOI: 10.1016/j.cld.2020.01.001]

14 **Iavarone M**, D'Ambrosio R, Soria A, Triolo M, Pugliese N, Del Poggio P, Perricone G, Massironi S, Spinetti A, Buscarini E, Viganò M, Carriero C, Fagiuoli S, Aghemo A, Belli LS, Lucà M, Pedaci M, Rimondi A, Rumi MG, Invernizzi P, Bonfanti P, Lampertico P. High rates of 30-day mortality in patients with cirrhosis and COVID-19. *J Hepatol* 2020; **73**: 1063-1071 [PMID: 32526252 DOI: 10.1016/j.jhep.2020.06.001]

15 **Galbadage T**, Peterson BM, Awada J, Buck AS, Ramirez DA, Wilson J, Gunasekera RS. Systematic Review and Meta-Analysis of Sex-Specific COVID-19 Clinical Outcomes. *Front Med (Lausanne)* 2020; **7**: 348 [PMID: 32671082 DOI: 10.3389/fmed.2020.00459]

16 **Richardson S**, Hirsch JS, Narasimhan M, Crawford JM, McGinn T, Davidson KW; the Northwell COVID-19 Research Consortium, Barnaby DP, Becker LB, Chelico JD, Cohen SL, Cookingham J, Coppa K, Diefenbach MA, Dominello AJ, Duer-Hefele J, Falzon L, Gitlin J, Hajizadeh N, Harvin TG, Hirschwerk DA, Kim EJ, Kozel ZM, Marrast LM, Mogavero JN, Osorio GA, Qiu M, Zanos TP. Presenting Characteristics, Comorbidities, and Outcomes Among 5700 Patients Hospitalized With COVID-19 in the New York City Area. *JAMA* 2020; **323**: 2052-2059 [PMID: 32320003 DOI: 10.1001/jama.2020.6775]

17 **Onder G**, Rezza G, Brusaferro S. Case-Fatality Rate and Characteristics of Patients Dying in Relation to COVID-19 in Italy. *JAMA* 2020; **323**: 1775-1776 [PMID: 32203977 DOI: 10.1001/jama.2020.4683]

18 **Petrilli CM**, Jones SA, Yang J, Rajagopalan H, O'Donnell L, Chernyak Y, Tobin KA, Cerfolio RJ, Francois F, Horwitz LI. Factors associated with hospital admission and critical illness among 5279 people with coronavirus disease 2019 in New York City: prospective cohort study. *BMJ* 2020; **369**: m1966 [PMID: 32444366 DOI: 10.1136/bmj.m1966]

19 **Kragholm K**, Andersen MP, Gerds TA, Butt JH, Østergaard L, Polcwiartek C, Phelps M, Andersson C, Gislason GH, Torp-Pedersen C, Køber L, Schou M, Fosbøl EL. Association between male sex and

outcomes of Coronavirus Disease 2019 (Covid-19) - a Danish nationwide, register-based study. *Clin Infect Dis* 2020 [PMID: 32634827 DOI: 10.1093/cid/ciaa924]

20 **Chen T**, Wu D, Chen H, Yan W, Yang D, Chen G, Ma K, Xu D, Yu H, Wang H, Wang T, Guo W, Chen J, Ding C, Zhang X, Huang J, Han M, Li S, Luo X, Zhao J, Ning Q. Clinical characteristics of 113 deceased patients with coronavirus disease 2019: retrospective study. *BMJ* 2020; **368**: m1091 [PMID: 32217556 DOI: 10.1136/bmj.m1091]

21 **Boettler T**, Newsome PN, Mondelli MU, Maticic M, Cordero E, Cornberg M, Berg T. Care of patients with liver disease during the COVID-19 pandemic: EASL-ESCMID position paper. *JHEP Rep* 2020; **2**: 100113 [PMID: 32289115 DOI: 10.1016/j.jhepr.2020.100113]

22 **Guan WJ**, Ni ZY, Hu Y, Liang WH, Ou CQ, He JX, Liu L, Shan H, Lei CL, Hui DSC, Du B, Li LJ, Zeng G, Yuen KY, Chen RC, Tang CL, Wang T, Chen PY, Xiang J, Li SY, Wang JL, Liang ZJ, Peng YX, Wei L, Liu Y, Hu YH, Peng P, Wang JM, Liu JY, Chen Z, Li G, Zheng ZJ, Qiu SQ, Luo J, Ye CJ, Zhu SY, Zhong NS; China Medical Treatment Expert Group for Covid-19. Clinical Characteristics of Coronavirus Disease 2019 in China. *N Engl J Med* 2020; **382**: 1708-1720 [PMID: 32109013 DOI: 10.1056/NEJMoa2002032]

23 **Shalimar**, Elhence A, Vaishnav M, Kumar R, Pathak P, Soni KD, Aggarwal R, Soneja M, Jorwal P, Kumar A, Khanna P, Singh AK, Biswas A, Nischal N, Dar L, Choudhary A, Rangarajan K, Mohan A, Acharya P, Nayak B, Gunjan D, Saraya A, Mahapatra S, Makharia G, Trikha A, Garg P. Poor outcomes in patients with cirrhosis and Corona Virus Disease-19. *Indian J Gastroenterol* 2020; **39**: 285-291 [PMID: 32803716 DOI: 10.1007/s12664-020-01074-3]

24 **Mangia A**, Cenderello G, Verucchi G, Ciancio A, Fontana A, Piazzolla V, Minerva N, Squillante MM, Copetti M. Is positivity for hepatitis C virus antibody predictive of lower risk of death in COVID-19 patients with cirrhosis? *World J Clin Cases* 2020; **8**: 5831-5834 [PMID: 33344581 DOI: 10.12998/wjcc.v8.i22.5831]

25 **Váncsa S**, Hegyi PJ, Zádori N, Szakó L, Vörhendi N, Ocskay K, Földi M, Dembrovszky F, Dömötör ZR, Jánosi K, Rakonczay Z Jr, Hartmann P, Horváth T, Erőss B, Kiss S, Szakács Z, Németh D, Hegyi P, Pár G. Pre-existing Liver Diseases and On-Admission Liver-Related Laboratory Tests in COVID-19: A Prognostic Accuracy Meta-Analysis With Systematic Review. *Front Med (Lausanne)* 2020; **7**: 572115 [PMID: 33282888 DOI: 10.3389/fmed.2020.572115]

26 **Singh S**, Khan A. Clinical Characteristics and Outcomes of Coronavirus Disease 2019 Among Patients With Preexisting Liver Disease in the United States: A Multicenter Research Network Study. *Gastroenterology* 2020; **159**: 768-771.e3 [PMID: 32376408 DOI: 10.1053/j.gastro.2020.04.064]

27 **Galiero R**, Pafundi PC, Simeon V, Rinaldi L, Perrella A, Vetrano E, Caturano A, Alfano M, Beccia D, Nevola R, Marfella R, Sardu C, Coppola C, Scarano F, Maggi P, De Lucia Sposito P, Vocciante L, Rescigno C, Sbreglia C, Fraganza F, Parrella R, Romano A, Calabria G, Polverino B, Pagano A, Bologna C, Amitrano M, Esposito V, Coppola N, Maturo N, Adinolfi LE, Chiodini P, Sasso FC; COVOCA Study Group. Impact of chronic liver disease upon admission on COVID-19 in-hospital mortality: Findings from COVOCA study. *PLoS One* 2020; **15**: e0243700 [PMID: 33301529 DOI: 10.1371/journal.pone.0243700]

28 **Marjot T**, Moon AM, Cook JA, Abd-Elsalam S, Aloman C, Armstrong MJ, Pose E, Brenner EJ, Cargill T, Catana MA, Dhanasekaran R, Eshraghian A, García-Juárez I, Gill US, Jones PD, Kennedy J, Marshall A, Matthews C, Mells G, Mercer C, Perumalswami PV, Avitabile E, Qi X, Su F, Ufere NN, Wong YJ, Zheng MH, Barnes E, Barritt AS 4th, Webb GJ. Outcomes following SARS-CoV-2 infection in patients with chronic liver disease: An international registry study. *J Hepatol* 2021; **74**: 567-577 [PMID: 33035628 DOI: 10.1016/j.jhep.2020.09.024]

29 **Sarin SK**, Choudhury A, Lau GK, Zheng MH, Ji D, Abd-Elsalam S, Hwang J, Qi X, Cua IH, Suh JI, Park JG, Putcharoen O, Kaewdech A, Piratvisuth T, Treeprasertsuk S, Park S, Wejnaruemarn S, Payawal DA, Baatarkhuu O, Ahn SH, Yeo CD, Alonzo UR, Chinbayar T, Loho IM, Yokosuka O, Jafri W, Tan S, Soo LI, Tanwandee T, Gani R, Anand L, Esmail ES, Khalaf M, Alam S, Lin CY, Chuang WL, Soin AS, Garg HK, Kalista K, Batsukh B, Purnomo HD, Dara VP, Rathi P, Al Mahtab M, Shukla A, Sharma MK, Omata M; APASL COVID Task Force, APASL COVID Liver Injury Spectrum Study (APCOLIS Study-NCT 04345640). Pre-existing liver disease is associated with poor outcome in patients with SARS CoV2 infection; The APCOLIS Study (APASL COVID-19 Liver Injury Spectrum Study). *Hepatol Int* 2020; **14**: 690-700 [PMID: 32623632 DOI: 10.1007/s12072-020-10072-8]

30 **Oyelade T**, Alqahtani J, Canciani G. Prognosis of COVID-19 in Patients with Liver and Kidney Diseases: An Early Systematic Review and Meta-Analysis. *Trop Med Infect Dis* 2020; **5** [PMID: 32429038 DOI: 10.3390/tropicalmed5020080]

31 **Sun J**, Aghemo A, Forner A, Valenti L. COVID-19 and liver disease. *Liver Int* 2020; **40**: 1278-1281 [PMID: 32251539 DOI: 10.1111/liv.14470]

World Journal of Gastroenterology

Submit a Manuscript: https://www.f6publishing.com

*World J Gastroenterol* 2021 November 14; 27(42): 7376-7386

DOI: 10.3748/wjg.v27.i42.7376

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

ORIGINAL ARTICLE

Retrospective Study

# Endoscopic ultrasound features of autoimmune pancreatitis: The typical findings and chronic pancreatitis changes

Sheng-Yu Zhang, Yun-Lu Feng, Long Zou, Xi Wu, Tao Guo, Qing-Wei Jiang, Qiang Wang, Ya-Min Lai, Shou-Jiang Tang, Ai-Ming Yang

**ORCID number:** Sheng-Yu Zhang 0000-0001-5321-457X; Yun-Lu Feng 0000-0002-2761-100X; Long Zou 0000-0002-9804-6167; Xi Wu 0000-0003-0007-2410; Tao Guo 0000-0002-1671-090X; Qing-Wei Jiang 0000-0002-3030-9960; Qiang Wang 0000-0003-3879-1349; Ya-Min Lai 0000-0002-7460-0012; Shou-Jiang Tang 0000-0001-9734-4026; Ai-Ming Yang 0000-0002-2907-7904.

**Author contributions:** Zhang SY and Feng YL contributed equally to this work and should be considered as co-first authors; Zhang SY, Wu X, Guo T, Tang SJ and Yang AM designed the study; Zhang SY and Feng YL collected and analyzed the data; Zou L and Lai YM collected the clinical information; Zou L collected the endoscopic images; Wu X, Guo T, Jiang QW and Wang Q collected the images; Zhang SY, Feng YL, Wang Q and Tang SJ analyzed the images; Zhang SY wrote the manuscript; Feng YL and Tang SJ revised the manuscript; Yang AM approved the final version of the manuscript.

**Supported by** Beijing Science and Technology Program, No. Z181100001618013; Peking Union Medical College Education Reform Program, No. 2019zlgc0116.

**Sheng-Yu Zhang, Yun-Lu Feng, Long Zou, Xi Wu, Tao Guo, Qing-Wei Jiang, Qiang Wang, Ya-Min Lai, Ai-Ming Yang,** Department of Gastroenterology, Peking Union Medical College Hospital, Chinese Academy of Medical Science and Peking Union Medical College, Beijing 100730, China

**Shou-Jiang Tang,** Division of Digestive Disease, Department of Medicine, University of Mississippi Medical Center, Jackson, MS 39216, United States

**Corresponding author:** Ai-Ming Yang, MD, Director, Professor, Department of Gastroenterology, Peking Union Medical College Hospital, Chinese Academy of Medical Science and Peking Union Medical College, No. 9 Dongdan Santiao, Dongcheng District, Beijing 100730, China. yangam2020@126.com

## Abstract

### *BACKGROUND*

Few studies have fully described endoscopic ultrasound (EUS) features of newly diagnosed autoimmune pancreatitis (AIP) involving both typical findings and chronic pancreatitis (CP) features. The typical EUS findings are prevalent in the diffuse type AIP but may not be as common for the focal type, and the differences between the diffuse and focal AIP need to be specified.

### *AIM*

To demonstrate the EUS features of newly diagnosed AIP and the difference between diffuse and focal AIP.

### *METHODS*

This retrospective single center study included 285 patients of newly diagnosed type 1 AIP following the international consensus diagnostic criteria, with the EUS procedures accomplished before corticosteroid initiation. We explored the EUS features and compared the typical AIP and CP features between the diffuse and focal AIP cases. The Rosemont criteria were employed for CP features definition and CP change level comparison.

### *RESULTS*

For the typical AIP features, there were significantly more patients in the diffuse group with bile duct wall thickening (158 of 214 cases, 73.4% *vs* 37 of 71 cases, 52.1%, $P = 0.001$) and peripancreatic hypoechoic margin (76 of 214 cases, 35.5% *vs*

**Institutional review board statement:** The study was reviewed and approved by the Ethics Committee of Peking Union Medical College Hospital (approval number S-K1613).

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

**Data sharing statement:** No additional data are available.



**Specialty type:** Gastroenterology and hepatology

**Country/Territory of origin:** China

**Peer-review report's scientific quality classification**

Grade A (Excellent): 0
Grade B (Very good): B, B
Grade C (Good): C
Grade D (Fair): D
Grade E (Poor): 0

**Received:** June 13, 2021
**Peer-review started:** June 13, 2021
**First decision:** July 14, 2021
**Revised:** July 27, 2021
**Accepted:** October 25, 2021
**Article in press:** October 25, 2021
**Published online:** November 14, 2021

**P-Reviewer:** Arcidiacono PG, Emmanuel J, Fujimori N, Nakai Y
**S-Editor:** Ma YJ
**L-Editor:** Filipodia
**P-Editor:** Ma YJ

5 of 71 cases, 7.0%, $P < 0.001$). For the CP features, there were significantly more patients in the focal group with main pancreatic duct dilation (30 of 214 cases, 14.0% *vs* 18 of 71 cases, 25.3%, $P = 0.03$). The cholangitis-like changes were more prevalent in the focal cases with pancreatic head involvement. The CP change level was relatively limited for newly diagnosed AIP cases in both groups.

*CONCLUSION*

This study demonstrated the difference in the typical AIP and CP features between diffuse and focal AIP and indicated the limited CP change level in newly diagnosed AIP.

**Key Words:** Endoscopic ultrasound; Autoimmune pancreatitis; Rosemont criteria; Chronic pancreatitis



**Core Tip:** The endoscopic ultrasound (EUS) features of newly diagnosed autoimmune pancreatitis (AIP) involving both typical findings and chronic pancreatitis (CP) features have rarely been described. The EUS typical features of AIP can help to differentiate diffuse AIP from classic CP and differentiate focal AIP from pancreatic cancer. This study demonstrated the EUS features of newly diagnosed AIP and the difference in the typical AIP features and CP features between the diffuse and focal AIP cases on the basis of the largest number of cases and indicated the relatively limited CP change in newly diagnosed AIP cases.

**Citation:** Zhang SY, Feng YL, Zou L, Wu X, Guo T, Jiang QW, Wang Q, Lai YM, Tang SJ, Yang AM. Endoscopic ultrasound features of autoimmune pancreatitis: The typical findings and chronic pancreatitis changes. *World J Gastroenterol* 2021; 27(42): 7376-7386
**URL:** https://www.wjgnet.com/1007-9327/full/v27/i42/7376.htm
**DOI:** https://dx.doi.org/10.3748/wjg.v27.i42.7376

## INTRODUCTION

In 1995, Yoshida *et al*[1] first proposed the concept of autoimmune pancreatitis (AIP) as a unique disease entity, which can be divided into two subtypes. Type 1 AIP, which is the most common type in Asia, is the pancreatic manifestation of immunoglobulin (Ig)G4-related disease and is often associated with extrapancreatic disorders, especially IgG4-associated cholangitis[2].

AIP can be divided into diffuse type and focal type based on the image findings[3]. With endoscopic ultrasound (EUS) as an effective tool to find parenchymal and main pancreatic ductal (MPD) changes, typical AIP features can be observed for diffuse type AIP, such as diffuse enlargement of the whole pancreas with diffuse hypoechoic parenchyma and hypoechoic peripancreatic margin[4-6]. But the typical EUS findings for AIP may not be as common for the focal type and differences between the diffuse and focal type need to be demonstrated.

As a special form of chronic pancreatitis (CP), almost all CP features could be recognized in AIP cases during EUS examination[4-6]. After recurrent attack or prolonged inflammatory injury from AIP, advanced CP features such as parenchymal calcification/atrophy and MPD calculi will emerge from the diffuse enlarged hypoechoic pancreas[7-9]. So, from newly diagnosed AIP to advanced CP, we need a measure to describe the level of CP change.

Therefore, we conducted this single center retrospective study for a detailed description of the EUS features of newly diagnosed AIP patients and demonstration of the difference between diffuse and focal AIP, and we tried to compare the CP change level in both groups *via* the Rosemont criteria based on all CP features.

## MATERIALS AND METHODS

### Patients

Patients diagnosed with AIP undergoing EUS before the initiation of corticosteroid treatment at the Peking Union Medical College Hospital from January 2012 to August 2018 were included in this retrospective study, and patients who had a history of alcoholism or recent acute pancreatitis onset (within 3 mo) were excluded. All diagnoses was made following the International Consensus Diagnostic Criteria (ICDC; see Supplement Figure)[10]. The study was approved by the Ethics Committee of Peking Union Medical College Hospital (approval number S-K1613).

### EUS feature definitions

All EUS procedures were performed by experienced endoscopists (XW, DSW, TG, QWJ; all with over 5 years EUS experience) with radial or linear echoendoscopes (frequency 5-7.5 MHz; GF-UCT260 or GF-UE260, Olympus, Tokyo, Japan) and ultrasound workstations (EM-2, Olympus, Tokyo, Japan, and Aloka ProSound F75, Hitachi, Tokyo, Japan). EUS images and videos were stored as digital data, and the image analysis was completed by SYZ and YLF in a blinded manner independently, in which LZ selected all image videos for each case from the database, removed the patient's information and diagnosis and showed them to SYZ and YLF. If disagreement existed between the two investigators, the third one (QW) would decide the image interpreting results.

We defined the typical EUS features of AIP, including diffuse enlargement (the pancreas is divided into three parts: head, body, and tail; cases with more than one part of the pancreas enlarged were defined as "diffuse enlargement" and grouped as the "diffuse type"), focal enlargement (cases with less than one part of the pancreas enlarged were defined as "focal enlargement" and grouped as the "focal type"), diffuse hypoechoic area (DHA), focal hypoechoic areas, bile duct wall thickening, extrahepatic bile duct dilation, intrapancreatic bile stenosis, peripancreatic lymphadenopathy, peripancreatic hypoechoic margins, lobular outer margins and peripancreatic vessel involvement (Figure 1).

We used the Rosemont criteria to describe the CP features in AIP[11]. The parenchymal changes included hyperechoic foci (HF), hyperechoic strand (HS), lobularity with/without honeycombing, cystic lesions and calcifications (Figure 2). The MPD changes included MPD stones, duct irregularity, dilation and hyperechoic duct margins (Figure 2).

All definitions are interpreted in detail in Supplementary Table 1.

### The Rosemont criteria application for CP change

We applied the Rosemont criteria to describe the level of CP change in newly diagnosed AIP patients of both diffuse and focal types.

### Statistical analysis

Descriptive analysis was used to describe the clinical and EUS features of AIP patients. Categorical variables were presented as $n$ (%), and continuous variables were presented as the mean ± SD or the median and the interquartile range, depending on the distribution. The distribution of the continuous variables was checked using a visual inspection of the histogram. Statistical analysis was accomplished with SPSS 23.0 (IBM, NY, United States). The differences in EUS features and clinical factors between the diffuse and focal AIP cases were compared using Student's $t$ test/Mann-Whitney $U$ test (for continuous variables, depending on distribution) or $\chi^2$ test (for categorical variables). Multivariate analysis using the binary logistic regression including variables identified to be significant ($P \leq 0.10$) at univariate analysis. A two-tailed $P$ value of less than 0.05 was considered to be statistically significant.

## RESULTS

### Demographics and clinical characteristics

A total of 285 patients were included in this study, with 230 male patients (80.7%) and a median age of 62 [interquartile range 54, 68] years. The mean follow-up duration was 26 [interquartile range 12, 51] mo. All patients were diagnosed with type 1 AIP according to the ICDC criteria. The clinical data (including symptoms and laboratory tests) are shown in Table 1. All items were comparable between the diffuse and focal

**Table 1 Comparison of patients' demographics and clinical manifestations before corticosteroid treatment**

| | All (*n* = 285) | Diffuse type (*n* = 214) | Focal type (*n* = 71) | *P* value |
|---|---|---|---|---|
| Sex | 230 (80.7) | 179 (83.6) | 51 (71.8) | 0.03 |
| Age | 62 (54, 68) | 62 (55, 68) | 59 (53, 68) | 0.12 |
| Follow-up time (mo) | 26 (12, 51) | 28 (13, 51) | 21 (9, 51) | 0.13 |
| Symptoms | | | | |
| Abdominal pain | 73 (26.0) | 51 (23.8) | 22 (31.0) | 0.23 |
| Jaundice | 106 (37.2) | 81 (37.8) | 25 (35.2) | 0.69 |
| Number of involved organs[1] | 1 (1, 2) | 1 (1, 2) | 1 (1, 2) | 0.47 |
| Laboratory tests | | | | |
| ALT (U/L) (9-50) | 47 (18, 133) | 64 (18, 184) | 30 (15, 83) | 0.01 |
| TBil (μmol/L) (5.1-22.2) | 20.6 (12.2, 55.6) | 21.4 (12.9, 68.7) | 14.5 (11.6, 45.2) | 0.08 |
| IgG (mg/dL) (700-1700) | 1590 (1140, 2070) | 1630 (1130, 2140) | 1510 (1130, 1760) | 0.37 |
| IgG4 (mg/dL) (8-140) | 558.0 (280.5, 1270.0) | 605.5 (253.5, 1457.5) | 458.0 (301.0, 1050.0) | 0.26 |
| CA 19-9 (U/L) (0-34.0) | 20.3 (7.9, 61.0) | 23.4 (10.5, 74.1) | 12.6 (6.7, 36.5) | 0.01 |

[1]Involved organs included salivary gland, lacrimal gland, lung, kidney, liver, bile duct, retroperitoneal fibrosis and prostate gland.
Results presented as median (interquartile range) or *n* (%). ALT: Alanine transaminase; CA: Carbohydrate antigen; TBil: Total bilirubin; Ig: Immunoglobulin.

group, except for alanine transaminase and carbohydrate antigen 19-9, which were higher in the diffuse group.

### EUS features and differences

The EUS features of all patients were shown, and the differences between the diffuse and focal types of AIP were compared (Table 2).

There were 214 cases of diffuse type and 71 cases of focal type AIP; among focal cases, more lesions were located in the pancreatic head (50 cases, 70.4%), while less in the body (8 cases, 11.3%) and tail (13 cases, 18.3%).

For the typical AIP features, there were significantly more patients with DHA in the diffuse group (197 of 214 cases, 92.1% *vs* 16 of 71 cases, 22.5%, $P < 0.001$), while there were significantly more patients with focal hypoechoic areas in the focal group (0 of 214 cases, 0% *vs* 59 of 71 cases, 83.1%, $P < 0.001$), which was consistent with the original definitions. For cholangitis-like changes, there were significantly more patients with bile duct wall thickening (158 of 214 cases, 73.4% *vs* 37 of 71 cases, 52.1%, $P = 0.001$) in the diffuse group. For peripancreatic changes, there were significantly more patients with peripancreatic hypoechoic margins (76 of 214 cases, 35.5% *vs* 5 of 71 cases, 7.0%, $P < 0.001$) in the diffuse group.

In the focal group, the cholangitis-like changes (bile duct wall thickening, intrapancreatic bile duct stenosis and extrahepatic bile duct dilation) were more prevalent in cases with pancreatic head involvement compared to those with body or tail involvement ($P < 0.001$, $P = 0.01$ and $P = 0.02$, respectively) (Supplementary Table 2).

For the CP features, parenchymal changes were all comparable in the diffuse and focal group. For MPD changes, there were significantly more patients with MPD dilation in the focal group than in the diffuse group (30 of 214 cases, 14.0% *vs* 18 of 71 cases, 25.3%, $P = 0.03$).

In the logistic regression analysis for the diffuse AIP with the EUS findings, the DHA [odds ratio (OR) = 11.23, 95% confidence index (CI): 3.07–41.03; $P < 0.001$], bile duct wall thickening (OR = 4.44, 95%CI 2.49–7.93; $P < 0.001$) and peripancreatic hypoechoic margin (OR = 4.34, 95%CI: 1.93–9.80; $P < 0.001$) were all predictors of the diffuse AIP (Table 3).

### The Rosemont criteria application for CP change

The Rosemont criteria were applied to describe the CP change level. Only a small portion of patients was diagnosed as "suggestive of CP" (12.1% in the diffuse group *vs* 15.5% in the focal group), and patients with advanced CP change ("consistent with

**Table 2 Comparison of endoscopic ultrasound features between the diffuse and focal types of autoimmune pancreatitis patients**

| EUS findings | All (*n* = 285) | Diffuse type (*n* = 214) | Focal type (*n* = 71) | *P* value |
|---|---|---|---|---|
| **Typical findings** | | | | |
| DHA | 213 (74.7) | 197 (92.1) | 16 (22.5) | < 0.001 |
| FHA | 59 (20.7) | 0 (0) | 59 (83.1) | < 0.001 |
| **Bile duct changes** | | | | |
| Bile duct wall thickening | 195 (68.4) | 158 (73.4) | 37 (52.1) | 0.001 |
| Intrapancreatic bile duct stenosis | 165 (57.9) | 131 (61.2) | 34 (47.9) | 0.05 |
| Extrahepatic bile duct dilation | 122 (42.8) | 97 (45.3) | 25 (35.2) | 0.14 |
| **Peripancreatic changes** | | | | |
| Peripancreatic lymphadenopathy | 89 (31.2) | 72 (33.6) | 17 (23.9) | 0.13 |
| Peripancreatic hypoechoic margin | 81 (28.4) | 76 (35.5) | 5 (7.0) | < 0.001 |
| Lobular outer margin | 40 (14.0) | 34 (15.9) | 6 (8.5) | 0.12 |
| Peripancreatic vessel involvement | 21 (7.4) | 16 (7.5) | 5 (7.0) | 0.90 |
| **Chronic pancreatitis changes** | | | | |
| **Parenchymal changes** | | | | |
| HF | 271 (95.1) | 202 (94.4) | 69 (97.2) | 0.53[1] |
| HS | 174 (61.1) | 131 (61.2) | 43(60.6) | 0.92 |
| Cystic lesion | 18 (6.3) | 14 (6.5) | 4 (5.6) | 1.00[1] |
| Parenchymal calcification | 3 (1.1) | 2 (0.9) | 1 (1.4) | 1.00[1] |
| Lobularity with honeycombing | 26 (9.1) | 19 (8.9) | 7 (9.9) | 0.80 |
| Lobularity without honeycombing | 48 (16.8) | 36 (16.8) | 12 (16.9) | 0.99 |
| **Main pancreatic duct changes** | | | | |
| MPD calculi | 1 (0.4) | 0 (0) | 1 (1.4) | 0.56[1] |
| MPD dilation | 48 (16.8) | 30 (14.0) | 18 (25.3) | 0.03 |
| Diffuse stenosis/irregularity | 29 (10.2) | 20 (9.3) | 9 (12.7) | 0.42 |
| Focal stenosis | 11 (3.9) | 6 (2.8) | 5 (7.0) | 0.15[1] |
| Hyperechoic duct margin | 119 (41.8) | 91 (42.5) | 28 (39.4) | 0.65 |

[1]Fisher's exact test.

Results presented as *n* (%). DHA: Diffuse hypoechoic area; EUS: Endoscopic ultrasound; FHA: Focal hypoechoic area; HF: Hyperechoic foci; HS: Hyperechoic strand; MPD: Main pancreatic duct.

**Table 3 The logistic regression for predictors of diffuse autoimmune pancreatitis**

| Effect | Odds ratio (95%CI) | *P* value |
|---|---|---|
| DHA | 11.23 (3.07, 41.03) | < 0.001 |
| Bile duct wall thickening | 4.44 (2.49, 7.93) | < 0.001 |
| Peripancreatic hypoechoic margin | 4.34 (1.93, 9.80) | < 0.001 |

CI: Confidence index; DHA: Diffuse hypoechoic area.

CP") were even more rare (0.9% *vs* 1.4%) (Table 4). The CP change level was similar for newly diagnosed AIP cases in the diffuse and focal groups.

**Table 4 The Rosemont Criteria for description of chronic pancreatitis change in newly diagnosed autoimmune pancreatitis patients**

| Rosemont criteria | Diffuse type (*n* = 14) | Focal type (*n* = 71) | *P* value |
|---|---|---|---|
| Consistent with CP | 2 (0.9) | 1 (1.4) | 0.45 |
| Suggestive of CP | 26 (12.1) | 11 (15.5) | |
| Indeterminate of CP | 174 (81.3) | 58 (81.7) | |
| Normal | 12 (5.6) | 1 (1.4) | |

Results presented as *n* (%). CP: Chronic pancreatitis.

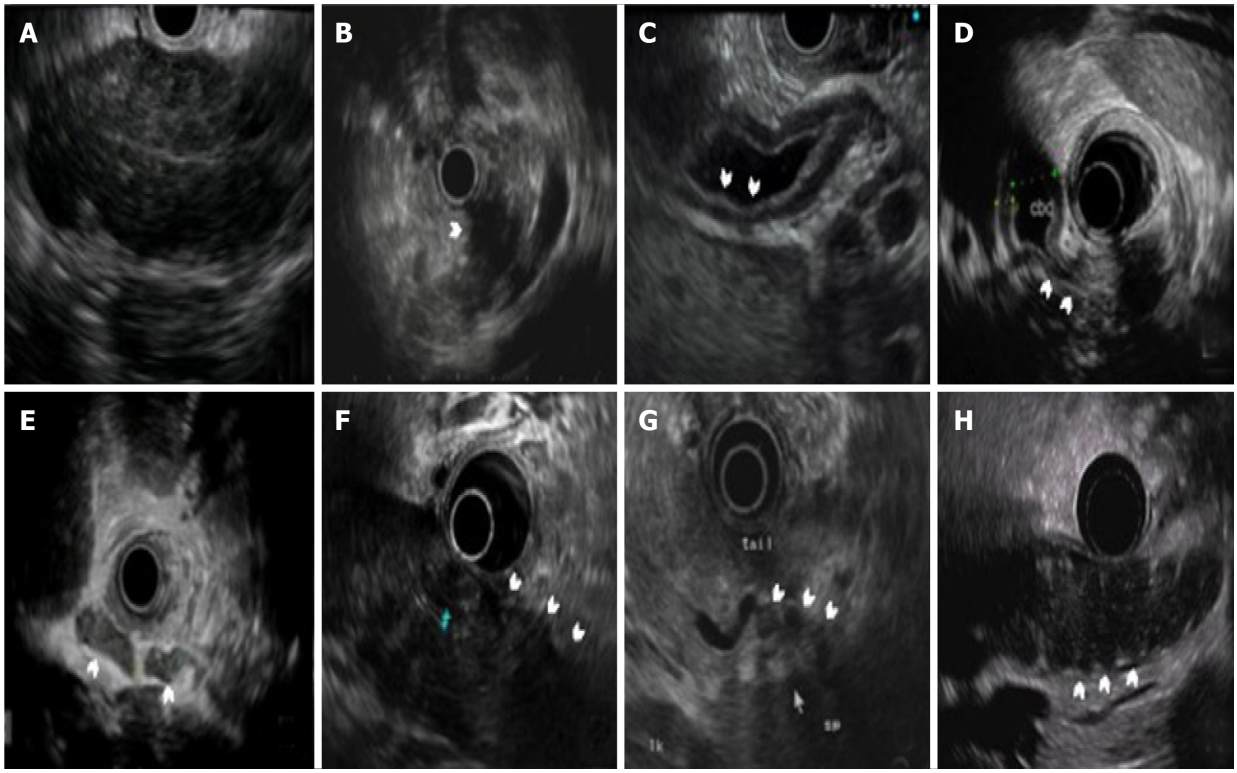


**Figure 1 The typical endoscopic ultrasound findings in autoimmune pancreatitis.** The white arrows show the following: A: Diffuse hypoechoic area of the pancreatic body; B: Focal hypoechoic area of the pancreatic body; C: Thickened wall of the common bile duct; D: Intrapancreatic bile duct stenosis; E: Peripancreatic lymphadenopathy; F: Peripancreatic hypoechoic margin; G: Peripancreatic vessel involvement with stenosis of the splenic vein; H: Lobular outer margin.

## DISCUSSION

Type 1 AIP is a special form of CP that is pathologically characterized by an abundance of lymphoplasmacytic infiltrates, fibrosis and obliterative phlebitis[2]. EUS can detect abnormalities in the parenchyma and pancreatic duct that are possibly not visible by other modalities. To date, this is the largest single center retrospective study demonstrating EUS features in newly diagnosed type 1 AIP patients, which not only describes the typical findings and the CP features of AIP but also figures out the difference between the diffuse and focal type.

We demonstrated the typical EUS features in AIP, including diffuse or focal hypoechoic area, bile duct changes due to IgG4-associated cholangitis and peripancreatic changes. In fact, the typical EUS findings (such as diffuse enlargement of the pancreas, DHA, bile duct wall thickening or stenosis and peripancreatic hypoechoic margin) were still prominent manifestations for AIP patients, which was consistent with previous studies (Supplement Table 3). More patients in the diffuse group showed typical features, especially for bile duct wall thickening and peripancreatic hypoechoic margin due to the profound inflammatory change[6]. In the multivariate regression analysis for the diffuse AIP, we demonstrated the predictors of the diffuse AIP: the DHA, bile duct wall thickening and peripancreatic hypoechoic margin (all $P <$

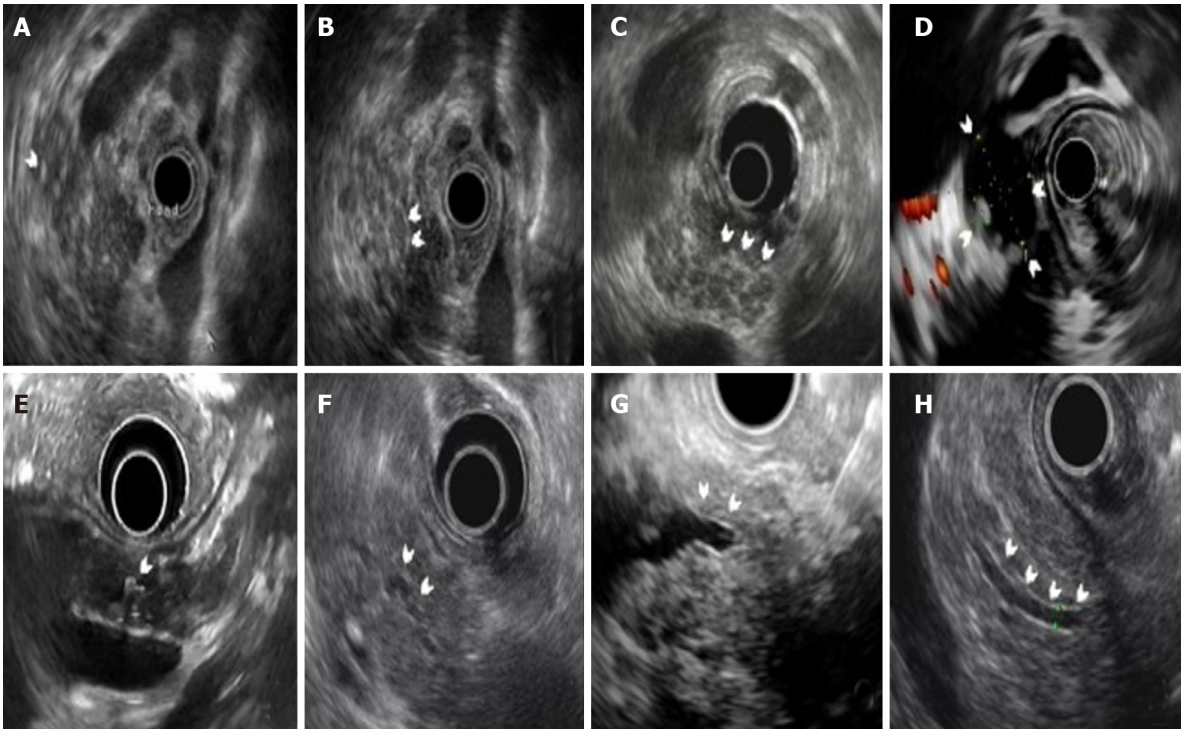


**Figure 2 The pancreatic parenchymal and duct changes of chronic pancreatitis in autoimmune pancreatitis.** The white arrows show the following: A: Multiple hyperechoic foci; B: Hyperechoic strands in pancreatic head; C: Multiple lobularities with honeycombing; D: Cystic lesion connecting to the main pancreatic duct (MPD); E: MPD stone with acoustic shadow; F: Diffuse irregularity change of the MPD; G: Focal stenosis and upstream dilation of the MPD; H: Hyperechoic duct margin.

0.001), which are all typical EUS findings of AIP rather than CP features.

It is also notable that despite hypoechoic parenchyma in the enlarged part of the focal type the rest of the parenchyma also showed hypoechogenicity and hyperechoic foci/strands under EUS, which implied the dynamic change of the parenchyma possibly caused by the spontaneous remission of AIP[12]. But we did not define these cases as "diffuse type" to avoid confusion with the ICDC, which emphasizes the diffuse or focal "enlargement" of the pancreas.

Bile duct changes often happen in AIP, as shown in this study. Bile duct wall thickening (73.4% in the diffuse group, 66.0% in the focal cases with pancreatic head involved and 19.0% in the focal cases without pancreatic head involved) resulting from IgG4-associated cholangitis and intrapancreatic bile duct stenosis (61.2% in the diffuse group, 58.0% in the focal cases with pancreatic head involved and 23.8% in the focal cases without pancreatic head involved) that possibly caused by both bile duct wall thickening and extrinsic pancreatic compression[13] are both more common in the diffuse group (almost all cases have head involvement) and focal AIP cases with pancreatic head involved.

The CP changes of AIP were also fully explored in this study. All parenchymal changes were comparable in the diffuse and focal groups. In previous studies, Farrell *et al*[4] showed that lobularity existed in 7.1% (1/14) of patients[7]. Hoki *et al*[5] demonstrated that the occurrence of HF, HS, lobularity, cystic lesions and calcifications were 32% (8/25), 56% (14/25), 8% (2/25), 16% (4/25) and 16% (4/25), respectively. Okabe *et al*[6] found that HF existed in all patients (32/32), with a lower incidence rate of HS and lobularity (81.3% and 53.1%, respectively). As for MPD changes, there was no difference between the two groups except for MPD dilation, which was more frequently seen in the focal group (14.0% *vs* 25.3%, $P = 0.03$) and seemed to be more prevalent in the focal cases with pancreatic head involved (32.0% in head involved cases *vs* 9.5% in non-head involved cases, $P = 0.05$). Previous studies reported that MPD dilation was present in 12%-37% of AIP patients, which was often located proximally to the AIP affected area where the MPD or surrounding parenchyma was involved, while hyperechoic duct margins and diffuse stenosis/irregularity were present in approximately 12% (3/25) and 40% (10/25) of patients, respectively[8,9] (Supplementary Table 3). The reason for the different incidence rate of parenchymal and MPD changes may be that AIP patients are possibly at different clinical stages (early or advanced) in these studies[14]. After the recurrent

attack or prolonged inflammatory damage, pancreatic duct stones or parenchymal calcifications may be formed, which will make the shape of AIP more similar to advanced CP[7-9].

The focal AIP accompanied by MPD dilation, sometimes also by peripancreatic lymphadenopathy and vessel involvement, is difficult to differentiate from pancreatic carcinoma. There are some EUS features, like bile duct wall thickening and peripancreatic hypoechoic margin that are relatively specific for focal AIP patients[5]. Several noninvasive EUS methods have been developed for differential diagnosis but without satisfactory sensitivity or specificity[15,16]. A prediction model with multiple EUS features could help to differentiate focal AIP from pancreatic carcinoma[17]. The EUS-guided fine-needle aspiration (EUS-FNA) procedure should be considered as the first choice to diagnosis focal AIP or rule out malignancy[18-21]. The diagnostic accuracy of EUS-FNA is between 45%–78%[22]. In this study, 92 initial "non-diagnostic" AIP cases received EUS-FNA procedures, among whom 36 cases (39.1%) got level 1 and 2 histological evidence (Supplementary Figure). All cases receiving EUS-FNA got the final diagnosis of "definite" AIP according to the ICDC.

To date, endoscopic retrograde pancreatography is included as part of the diagnosis of AIP in the Japanese guideline and the ICDC[10,23]. Exploring quantitative and qualitative parenchymal and ductal change, EUS is a reliable method to diagnose CP, which is statistically comparable to the endoscopic retrograde pancreatography and the Cambridge criteria (the gold standard in the past)[24-26]. So, we tried to describe the CP change level of newly diagnosed AIP cases with the CP features in the Rosemont criteria and found that only a small portion of patients was diagnosed as "suggestive of CP" (12.1% in the diffuse group *vs* 15.5% in the focal group), and patients with more advanced CP change ("consistent with CP") were even more rare (0.9% *vs* 1.4%). Therefore, the CP change is relatively limited for most newly diagnosed AIP cases that were probably in the early stage of disease, while advanced CP findings may happen in the long-term recurrent attacks[8,14]. EUS can detect the early parenchymal fibrosis of CP (like HF and HS) in AIP cases, which changes dynamically after corticosteroid therapy[6]. As the tool for accessing the fibrosis degree of the pancreas, the EUS findings of CP may be used for predicting the pancreatic atrophy and diabetes exacerbation, which needs further investigation[27].

This study had limitations. First, this was a single center retrospective study, and all AIP patients included in this study were diagnosed with type 1 AIP. Therefore, selection bias inevitably existed. Second, the EUS-FNA diagnosis accuracy in this study was somehow lower than previously reported (about 39.1% for level 1 and 2 histological evidence), which might be because the fine needle biopsy needles were used sparingly[22] (Supplementary Table 4). The long time period of the study (our center did not have fine needle biopsy needles until 2015) might explain the reason that we did use the fine needle biopsy needles (22G Procore and 20G Procore, COOK, United States) except in a low proportion (12.0% in diffuse AIP patients and 29.9% in focal AIP cases). However, the FNA needles still have the clinical significance of ruling out malignancy in AIP patients[21]. Third, MPD dilation was defined as "> 3 mm in the head, > 2 mm in the body, >1 mm in the tail" in the Rosemont criteria, but in the elderly population of AIP patients, the normal range of MPD diameter might be larger [28]. Therefore, we might have overestimated the incidence rate of MPD dilation. Lastly, the "dilated side branches" of the pancreatic duct in the Rosemont criteria were relatively difficult to evaluate without endoscopic retrograde pancreatography, so they were not included in this study.

## CONCLUSION

In conclusion, this study demonstrated the EUS features of newly diagnosed AIP and the difference in the typical AIP features and CP features between diffuse and focal AIP on the basis of the largest number of cases and indicated the relatively limited CP change in newly diagnosed AIP cases *via* the Rosemont criteria.

## ARTICLE HIGHLIGHTS

### *Research background*

Few studies have fully described the endoscopic ultrasound (EUS) features of newly diagnosed autoimmune pancreatitis (AIP) involving both typical findings and chronic

pancreatitis (CP) features. The typical EUS findings are prevalent in diffuse AIP but may not be as common for the focal type, and the differences between diffuse and focal AIP need to be specified. The EUS typical features of AIP (especially the cholangiopathy-like features) can help to differentiate diffuse AIP from classic CP and differentiate focal AIP from pancreatic cancer.

### Research motivation

This is the largest single center retrospective study demonstrating EUS features in newly diagnosed type 1 AIP patients that not only describes the typical findings and the CP features of AIP but also figures out the difference between the diffuse and focal types.

### Research objectives

The authors conducted this single center retrospective study for a detailed description of the EUS features of newly diagnosed AIP patients and demonstration of the difference between diffuse and focal AIP, and we tried to compare the CP change level in both groups *via* the Rosemont criteria based on all CP features.

### Research methods

This retrospective single center study included 285 patients of newly diagnosed type 1 AIP following the international consensus diagnostic criteria, with the EUS procedures accomplished before corticosteroid initiation. We explored the EUS features and compared the typical AIP and CP features between the diffuse and focal AIP cases. The Rosemont criteria were employed for CP features definition and CP change level comparison.

### Research results

For the typical AIP features, there were significantly more patients in the diffuse group with bile duct wall thickening and peripancreatic hypoechoic margin. In the multivariate regression analysis for diffuse AIP, we demonstrated the predictors of diffuse AIP: the DHA, bile duct wall thickening and peripancreatic hypoechoic margin (all $P < 0.001$), which are all typical EUS findings of AIP rather than CP features. For the CP features, there were significantly more patients in the focal group with main pancreatic duct dilation. The cholangitis-like changes were more prevalent in the focal cases with pancreatic head involvement. The CP change level was relatively limited for newly diagnosed AIP cases in the diffuse and focal groups.

### Research conclusions

This study demonstrated the EUS features of newly diagnosed AIP and the difference in the typical AIP features and CP features between the diffuse and focal AIP cases on the basis of the largest number of cases. It indicated the relatively limited CP change in newly diagnosed AIP cases *via* the Rosemont criteria.

### Research perspectives

EUS can detect the early parenchymal fibrosis of CP in AIP cases, which changes dynamically after corticosteroid therapy. As the tool for accessing the fibrosis degree of the pancreas, the EUS findings of CP may be used for predicting pancreatic atrophy and diabetes exacerbation, which needs further investigation.

## ACKNOWLEDGEMENTS

The authors would like to thank Yue-Lun Zhang, PhD (Central Research Laboratory, Peking Union Medical College Hospital) for his professional assistance in statistical analysis and all the nurses for their expert assistance during the EUS procedures.

## REFERENCES

1 **Yoshida K**, Toki F, Takeuchi T, Watanabe S, Shiratori K, Hayashi N. Chronic pancreatitis caused by an autoimmune abnormality. Proposal of the concept of autoimmune pancreatitis. *Dig Dis Sci* 1995; **40**: 1561-1568 [PMID: 7628283 DOI: 10.1007/BF02285209]

2 **Miyabe K**, Zen Y, Cornell LD, Rajagopalan G, Chowdhary VR, Roberts LR, Chari ST. Gastrointestinal and Extra-Intestinal Manifestations of IgG4-Related Disease. *Gastroenterology* 2018;

**155**: 990-1003.e1 [PMID: 30012334 DOI: 10.1053/j.gastro.2018.06.082]
3 **Kwon JH**, Kim JH, Kim SY, Byun JH, Kim HJ, Lee MG, Lee SS. Differentiating focal autoimmune pancreatitis and pancreatic ductal adenocarcinoma: contrast-enhanced MRI with special emphasis on the arterial phase. *Eur Radiol* 2019; **29**: 5763-5771 [PMID: 31028441 DOI: 10.1007/s00330-019-06200-0]
4 **Farrell JJ**, Garber J, Sahani D, Brugge WR. EUS findings in patients with autoimmune pancreatitis. *Gastrointest Endosc* 2004; **60**: 927-936 [PMID: 15605008 DOI: 10.1016/S0016-5107(04)02230-8]
5 **Hoki N**, Mizuno N, Sawaki A, Tajika M, Takayama R, Shimizu Y, Bhatia V, Yamao K. Diagnosis of autoimmune pancreatitis using endoscopic ultrasonography. *J Gastroenterol* 2009; **44**: 154-159 [PMID: 19214678 DOI: 10.1007/s00535-008-2294-2]
6 **Okabe Y**, Ishida Y, Kaji R, Sugiyama G, Yasumoto M, Naito Y, Toyonaga A, Tsuruta O, Sata M. Endoscopic ultrasonographic study of autoimmune pancreatitis and the effect of steroid therapy. *J Hepatobiliary Pancreat Sci* 2012; **19**: 266-273 [PMID: 21671062 DOI: 10.1007/s00534-011-0392-7]
7 **Takayama M**, Hamano H, Ochi Y, Saegusa H, Komatsu K, Muraki T, Arakura N, Imai Y, Hasebe O, Kawa S. Recurrent attacks of autoimmune pancreatitis result in pancreatic stone formation. *Am J Gastroenterol* 2004; **99**: 932-937 [PMID: 15128363 DOI: 10.1111/j.1572-0241.2004.04162.x]
8 **Kanai K**, Maruyama M, Kameko F, Kawasaki K, Asano J, Oguchi T, Watanabe T, Ito T, Muraki T, Hamano H, Matsumoto A, Arakura N, Kawa S. Autoimmune Pancreatitis Can Transform Into Chronic Features Similar to Advanced Chronic Pancreatitis With Functional Insufficiency Following Severe Calcification. *Pancreas* 2016; **45**: 1189-1195 [PMID: 27400257 DOI: 10.1097/MPA.0000000000000598]
9 **Ito T**, Kawa S, Matsumoto A, Kubota K, Kamisawa T, Okazaki K, Hirano K, Hirooka Y, Uchida K, Masuda A, Ohara H, Shimizu K, Arakura N, Masamune A, Kanno A, Sakagami J, Itoi T, Ito T, Ueki T, Nishino T, Inui K, Mizuno N, Yoshida H, Sugiyama M, Iwasaki E, Irisawa A, Shimosegawa T, Chiba T. Risk Factors for Pancreatic Stone Formation in Type 1 Autoimmune Pancreatitis: A Long-term Japanese Multicenter Analysis of 624 Patients. *Pancreas* 2019; **48**: 49-54 [PMID: 30540679 DOI: 10.1097/MPA.0000000000001210]
10 **Shimosegawa T**, Chari ST, Frulloni L, Kamisawa T, Kawa S, Mino-Kenudson M, Kim MH, Klöppel G, Lerch MM, Löhr M, Notohara K, Okazaki K, Schneider A, Zhang L; International Association of Pancreatology. International consensus diagnostic criteria for autoimmune pancreatitis: guidelines of the International Association of Pancreatology. *Pancreas* 2011; **40**: 352-358 [PMID: 21412117 DOI: 10.1097/MPA.0b013e3182142fd2]
11 **Catalano MF**, Sahai A, Levy M, Romagnuolo J, Wiersema M, Brugge W, Freeman M, Yamao K, Canto M, Hernandez LV. EUS-based criteria for the diagnosis of chronic pancreatitis: the Rosemont classification. *Gastrointest Endosc* 2009; **69**: 1251-1261 [PMID: 19243769 DOI: 10.1016/j.gie.2008.07.043]
12 **Kubota K**, Iida H, Fujisawa T, Yoneda M, Inamori M, Abe Y, Kirikoshi H, Saito S, Ohshiro H, Kakuta Y, Nakajima A. Clinical factors predictive of spontaneous remission or relapse in cases of autoimmune pancreatitis. *Gastrointest Endosc* 2007; **66**: 1142-1151 [PMID: 18061714 DOI: 10.1016/j.gie.2007.06.059]
13 **Hirano K**, Tada M, Isayama H, Yamamoto K, Mizuno S, Yagioka H, Yashima Y, Sasaki T, Kogure H, Togawa O, Arizumi T, Matsubara S, Nakai Y, Sasahira N, Tsujino T, Kawabe T, Omata M. Endoscopic evaluation of factors contributing to intrapancreatic biliary stricture in autoimmune pancreatitis. *Gastrointest Endosc* 2010; **71**: 85-90 [PMID: 19836737 DOI: 10.1016/j.gie.2009.08.008]
14 **Suda K**, Nishimori I, Takase M, Oi I, Ogawa M. Autoimmune pancreatitis can be classified into early and advanced stages. *Pancreas* 2006; **33**: 345-350 [PMID: 17079937 DOI: 10.1097/01.mpa.0000235305.96486.f1]
15 **Cho MK**, Moon SH, Song TJ, Kim RE, Oh DW, Park DH, Lee SS, Seo DW, Lee SK, Kim MH. Contrast-Enhanced Endoscopic Ultrasound for Differentially Diagnosing Autoimmune Pancreatitis and Pancreatic Cancer. *Gut Liver* 2018; **12**: 591-596 [PMID: 29699060 DOI: 10.5009/gnl17391]
16 **Imazu H**, Kanazawa K, Mori N, Ikeda K, Kakutani H, Sumiyama K, Hino S, Ang TL, Omar S, Tajiri H. Novel quantitative perfusion analysis with contrast-enhanced harmonic EUS for differentiation of autoimmune pancreatitis from pancreatic carcinoma. *Scand J Gastroenterol* 2012; **47**: 853-860 [PMID: 22507131 DOI: 10.3109/00365521.2012.679686]
17 **Guo T**, Xu T, Zhang S, Lai Y, Wu X, Wu D, Feng Y, Jiang Q, Wang Q, Qian J, Yang A. The role of EUS in diagnosing focal autoimmune pancreatitis and differentiating it from pancreatic cancer. *Endosc Ultrasound* 2021; **10**: 280-287 [PMID: 34213428 DOI: 10.4103/EUS-D-20-00212]
18 **Kanno A**, Masamune A, Fujishima F, Iwashita T, Kodama Y, Katanuma A, Ohara H, Kitano M, Inoue H, Itoi T, Mizuno N, Miyakawa H, Mikata R, Irisawa A, Sato S, Notohara K, Shimosegawa T. Diagnosis of autoimmune pancreatitis by EUS-guided FNA using a 22-gauge needle: a prospective multicenter study. *Gastrointest Endosc* 2016; **84**: 797-804.e1 [PMID: 27068878 DOI: 10.1016/j.gie.2016.03.1511]
19 **Cao L**, Wang Y, Wang J, Guo Q, Chen Q, Wu X, Tang SJ, Cheng B. The role of EUS-guided fine needle aspiration in autoimmune pancreatitis: a single center prospective study. *Scand J Gastroenterol* 2018; **53**: 1604-1610 [PMID: 30422724 DOI: 10.1080/00365521.2018.1534137]
20 **Kurita A**, Yasukawa S, Zen Y, Yoshimura K, Ogura T, Ozawa E, Okabe Y, Asada M, Nebiki H, Shigekawa M, Ikeura T, Eguchi T, Maruyama H, Ueki T, Itonaga M, Hashimoto S, Shiomi H, Minami R, Hoki N, Takenaka M, Itokawa Y, Uza N, Hashigo S, Yasuda H, Takada R, Kamada H, Kawamoto H, Kawakami H, Moriyama I, Fujita K, Matsumoto H, Hanada K, Takemura T, Yazumi S.

Comparison of a 22-gauge Franseen-tip needle with a 20-gauge forward-bevel needle for the diagnosis of type 1 autoimmune pancreatitis: a prospective, randomized, controlled, multicenter study (COMPAS study). *Gastrointest Endosc* 2020; **91**: 373-381.e2 [PMID: 31654634 DOI: 10.1016/j.gie.2019.10.012]

21 **Sugimoto M**, Takagi T, Suzuki R, Konno N, Asama H, Watanabe K, Nakamura J, Kikuchi H, Waragai Y, Takasumi M, Sato Y, Hikichi T, Ohira H. Endoscopic Ultrasonography-Guided Fine Needle Aspiration Can Be Used to Rule Out Malignancy in Autoimmune Pancreatitis Patients. *J Ultrasound Med* 2017; **36**: 2237-2244 [PMID: 28670760 DOI: 10.1002/jum.14265]

22 **Chhoda A**, Rustagi T. EUS-guided needle biopsy for autoimmune pancreatitis. *Clin J Gastroenterol* 2020; **13**: 669-677 [PMID: 32519311 DOI: 10.1007/s12328-020-01153-0]

23 **Okazaki K**, Kawa S, Kamisawa T, Ito T, Inui K, Irie H, Nishino T, Notohara K, Nishimori I, Tanaka S, Nishiyama T, Suda K, Shiratori K, Tanaka M, Shimosegawa T; Working Committee of the Japan Pancreas Society and the Research Committee for Intractable Pancreatic Disease supported by the Ministry of Health, Labour and Welfare of Japan. Amendment of the Japanese Consensus Guidelines for Autoimmune Pancreatitis, 2013 I. Concept and diagnosis of autoimmune pancreatitis. *J Gastroenterol* 2014; **49**: 567-588 [PMID: 24639057 DOI: 10.1007/s00535-014-0942-2]

24 **Axon AT**, Classen M, Cotton PB, Cremer M, Freeny PC, Lees WR. Pancreatography in chronic pancreatitis: international definitions. *Gut* 1984; **25**: 1107-1112 [PMID: 6479687 DOI: 10.1136/gut.25.10.1107]

25 **Sahai AV**, Zimmerman M, Aabakken L, Tarnasky PR, Cunningham JT, van Velse A, Hawes RH, Hoffman BJ. Prospective assessment of the ability of endoscopic ultrasound to diagnose, exclude, or establish the severity of chronic pancreatitis found by endoscopic retrograde cholangiopancreatography. *Gastrointest Endosc* 1998; **48**: 18-25 [PMID: 9684659 DOI: 10.1016/S0016-5107(98)70123-3]

26 **Stevens T**, Conwell DL, Zuccaro G Jr, Vargo JJ, Dumot JA, Lopez R. Comparison of endoscopic ultrasound and endoscopic retrograde pancreatography for the prediction of pancreatic exocrine insufficiency. *Dig Dis Sci* 2008; **53**: 1146-1151 [PMID: 17934824 DOI: 10.1007/s10620-007-9975-1]

27 **Yamada Y**, Masuda A, Sofue K, Ueshima E, Shiomi H, Sakai A, Kobayashi T, Ikegawa T, Tanaka S, Nakano R, Tanaka T, Kakihara M, Ashina S, Tsujimae M, Yamakawa K, Abe S, Gonda M, Masuda S, Inomata N, Kutsumi H, Itoh T, Murakami T, Kodama Y. Prediction of pancreatic atrophy after steroid therapy using equilibrium-phase contrast computed tomography imaging in autoimmune pancreatitis. *JGH Open* 2020; **4**: 677-683 [PMID: 32782956 DOI: 10.1002/jgh3.12316]

28 **Hastier P**, Buckley MJ, Dumas R, Kuhdorf H, Staccini P, Demarquay JF, Caroli-Bosc FX, Delmont JP. A study of the effect of age on pancreatic duct morphology. *Gastrointest Endosc* 1998; **48**: 53-57 [PMID: 9684665 DOI: 10.1016/S0016-5107(98)70129-4]

World Journal of Gastroenterology

Submit a Manuscript: https://www.f6publishing.com

*World J Gastroenterol* 2021 November 14; 27(42): 7387-7401

DOI: 10.3748/wjg.v27.i42.7387

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

ORIGINAL ARTICLE

**Observational Study**

# Long-term clinical outcomes of lipiodol marking using standard gastroscopy for image-guided radiotherapy of upper gastrointestinal cancers

Kim Hay Be, Richard Khor, Daryl Lim Joon, Ben Starvaggi, Michael Chao, Sweet Ping Ng, Michael Ng, Leonardo Zorron Cheng Tao Pu, Marios Efthymiou, Rhys Vaughan, Sujievvan Chandran

**ORCID number:** Kim Hay Be 0000-0003-0792-3265; Richard Khor 0000-0002-7057-2747; Daryl Lim Joon 0000-0002-1947-9694; Ben Starvaggi 0000-0002-0490-6163; Michael Chao 0000-0002-3487-3746; Sweet Ping Ng 0000-0003-1721-0680; Michael Ng 0000-0003-3539-9237; Leonardo Zorron Cheng Tao Pu 0000-0002-7921-5631; Marios Efthymiou 0000-0003-2569-5163; Rhys Vaughan 0000-0002-4557-1734; Sujievvan Chandran 0000-0002-5015-6287.

**Author contributions:** Be KH, Zorron Cheng Tao Pu L, Khor R, Lim Joon D, Chandran S, Efthymiou M and Vaughan R conceptualized and designed the study; all authors were involved in data collection, analyses, or both; all authors were involved in the interpretation of the results; Be KH, Zorron Cheng Tao Pu L, Khor R and Chandran S drafted the manuscript; Lim Joon D, Ng SP, Efthymiou M and Vaughan R carried the critical revision of the article for important intellectual content; and all authors read and approved the final version of the manuscript.

**Institutional review board statement:** The study was reviewed and approved by the

**Kim Hay Be, Leonardo Zorron Cheng Tao Pu, Marios Efthymiou, Rhys Vaughan, Sujievvan Chandran,** Department of Gastroenterology and Hepatology, Austin Health, Heidelberg 3084, Victoria, Australia

**Richard Khor, Daryl Lim Joon, Ben Starvaggi, Michael Chao, Sweet Ping Ng,** Department of Radiation Oncology, Austin Health, Heidelberg 3084, Victoria, Australia

**Richard Khor, Sweet Ping Ng,** School of Cancer Medicine, La Trobe University, Melbourne 3086, Victoria, Australia

**Michael Chao, Sweet Ping Ng, Marios Efthymiou, Rhys Vaughan, Sujievvan Chandran,** Medicine, Dentistry and Health Sciences, University of Melbourne, Parkville 3010, Victoria, Australia

**Michael Ng,** Genesis Care, East Melbourne 3002, Victoria, Australia

**Corresponding author:** Kim Hay Be, MBBS, Doctor, Department of Gastroenterology and Hepatology, Austin Health, 145 Studley Road, Heidelberg 3084, Victoria, Australia. kim.be@austin.org.au

## Abstract

### *BACKGROUND*

Image-guided radiotherapy (IGRT) has significantly improved the precision in which radiotherapy is delivered in cancer treatment. Typically, IGRT uses bony landmarks and key anatomical structures to locate the tumor. Recent studies have demonstrated the feasibility of peri-tumor fiducials in enabling even more accurate delineation of target and normal tissue. The use of gold coils as fiducials in gastrointestinal tumors has been extensively studied. However, placement requires expertise and specialized endoscopic ultrasound equipment. This article reports the long-term outcomes of using a standard gastroscopy to inject liquid fiducials for the treatment of oesophageal and gastric tumors with IGRT.

### *AIM*

To assess the long-term outcomes of liquid fiducial-guided IGRT in a cohort of oesophageal and gastric cancer patients.

Austin Health Institutional Review Board (Approval No. LNR/18/Austin/254).

**Informed consent statement:** Informed consent was waived.

**Conflict-of-interest statement:** Dr. BE on behalf of all authors has nothing to disclose.

**Data sharing statement:** No additional data are available.

**STROBE statement:** The authors have read the STROBE Statement—checklist of items, and the manuscript was prepared and revised according to the STROBE Statement—checklist of items.



**Specialty type:** Gastroenterology and hepatology

**Country/Territory of origin:** Australia

**Peer-review report's scientific quality classification**

Grade A (Excellent): A
Grade B (Very good): 0
Grade C (Good): C, C
Grade D (Fair): D
Grade E (Poor): 0

**Received:** July 5, 2021
**Peer-review started:** July 5, 2021
**First decision:** July 13, 2021
**Revised:** July 26, 2021
**Accepted:** October 27, 2021
**Article in press:** October 27, 2021
**Published online:** November 14, 2021

**P-Reviewer:** An T, Chien CR, Link

*METHODS*

A retrospective cohort study of consecutive adults with Oesophagogastric cancers referred for liquid fiducial placement before definitive/neo-adjuvant or palliative IGRT between 2013 and 2021 at a tertiary hospital in Melbourne, Australia was conducted. Up to four liquid fiducials were inserted per patient, each injection consisting of 0.2-0.5mL of a 1:1 mixture of iodized oil (Lipiodol; Aspen Pharmacare) and n-butyl 2-cyanoacrylate (Histoacryl®; B. Braun). A 23-gauge injector (Cook Medical) was used for the injection. All procedures were performed by or under the supervision of a gastroenterologist. Liquid fiducial-based IGRT (LF-IGRT) consisted of computer-assisted direct matching of the fiducial region on cone-beam computerised tomography at the time of radiotherapy. Patients received standard-IGRT (S-IGRT) if fiducial visibility was insufficient, consisting of bone match as a surrogate for tumor position. Radiotherapy was delivered to 54Gy in 30 fractions for curative patients and up to 45Gy in 15 fractions for palliative treatments.

*RESULTS*

52 patients were referred for liquid fiducial placement within the study period. A total of 51 patients underwent liquid fiducial implantation. Of these a total of 31 patients received radiotherapy. Among these, the median age was 77.4 years with a range between 57.5 and 88.8, and 64.5% were male. Twenty-seven out of the 31 patients were able to have LF-IGRT while four had S-IGRT. There were no complications after endoscopic implantation of liquid fiducials in our cohort. The cohort overall survival (OS) post-radiotherapy was 19 mo (range 0 to 87 mo). Whilst the progression-free survival (PFS) post-radiotherapy was 13 mo (range 0 to 74 mo). For those treated with curative intent, the median OS was 22.0 mo (range 0 to 87 mo) with a PFS median of 14.0 mo (range 0 to 74 mo). Grade 3 complication rate post-radiotherapy was 29%.

*CONCLUSION*

LF-IGRT is feasible in 87.1% of patients undergoing liquid fiducial placement through standard gastroscopy injection technique. Our cohort has an overall survival of 19 mo and PFS of 13 mo. Further studies are warranted to determine the long-term outcomes of liquid-fiducial based IGRT.

**Key Words:** Image-guided radiotherapy; Lipiodol; Gastroscopy; Gastric cancer; Oesophageal cancer; Fiducial



**Core Tip:** Based on a cohort of 31 patients who had undergone lipiodol fiducial implantation through standard gastroscopy and received radiotherapy, fiducial-based image-guided radiotherapy (IGRT) was possible in 87.1%. Our cohort had an overall survival of 19 mo and progression-free survival of 13 mo. Further studies are warranted to determine the long-term outcomes of liquid-fiducial based IGRT.

**Citation:** Be KH, Khor R, Lim Joon D, Starvaggi B, Chao M, Ng SP, Ng M, Zorron Cheng Tao Pu L, Efthymiou M, Vaughan R, Chandran S. Long-term clinical outcomes of lipiodol marking using standard gastroscopy for image-guided radiotherapy of upper gastrointestinal cancers. *World J Gastroenterol* 2021; 27(42): 7387-7401
**URL:** https://www.wjgnet.com/1007-9327/full/v27/i42/7387.htm
**DOI:** https://dx.doi.org/10.3748/wjg.v27.i42.7387

## INTRODUCTION

In 2020, an estimated 1.69 million cases of oesophageal and gastric cancers were diagnosed worldwide, equating to nearly one in every twelve new diagnoses of cancer. Together esophageal and gastric cancers were responsible for approximately

A
**S-Editor:** Wang JJ
**L-Editor:** A
**P-Editor:** Wang JJ

1.31 million cases of cancer-related mortality[1]. In Australia, the projected age-standardised cancer-related death rates of oesophageal and gastric cancers were 7.8 in 2020, ranking in the top 5 cancer-related death[2]. As with all cancers, patients with earlier-stage tumors display a better prognosis. Unfortunately, most gastrooesophageal cancers are diagnosed at later stages, often warranting chemoradiotherapy with or without surgery[3].

Radiotherapy is one of the main treatment modality in the neoadjuvant and definitive treatment of oesophageal cancers[4-6]. Currently, its use in gastric cancer is largely limited to the post-operatively setting, however more evidence in the neoadjuvant setting is emerging with the near completion of a randomized control trial[7,8]. As such, radiotherapy either as the primary therapy or as an adjunct continues to have an important role in the treatment of patients with oesophageal and gastric cancer[9,10].

A known limitation to the delivery of radiotherapy to patients with upper gastrointestinal malignancies is that early tumors can be difficult to identify on computerized tomography (CT) scans, impairing the delineation of the tumor for radiotherapy treatment design. F-18 Fluorodeoxyglucose positron emission tomography (FDG-PET) can assist in defining the metabolically active tumor volume, but some studies indicate that up to 20% of oesophageal carcinomas are not FDG-PET avid[11]. Furthermore, the mobility of the oesophagus and stomach particularly with respiratory motion and its different physiological states such as fasting and fed states need to be considered during radiotherapy[12,13]. Fiducial placements of markers potentially allow a more accurate definition of the radiotherapy field. The use of fiducial markers to help guide radiotherapy has been demonstrated as feasible in many solid cancer treatments including the prostate, pancreas and hollow viscus of the gastrointestinal tract[12,14,15]. Fiducial-based image-guided radiotherapy (F-IGRT) in the treatment of prostate cancer has been demonstrated to have advantages of optimising radiation dosage to the tumor, whilst minimising radiation exposure to normal tissue[16,17]. Comparatively, the data for the gastrointestinal tract is less robust. A recent meta-analysis, although showing high efficacy and safety of endoscopic ultrasound (EUS)-guided fiducials for pancreatic cancer radiotherapy, highlights that more information is needed regarding the impact of fiducials on long-term clinical outcomes[18]. The insertion of fiducials within the gastrointestinal tract using EUS guidance, although attaining high technical success rates, has substantial limitations with its broad adoption in clinical practice. These include high costs, the necessity of a highly trained endoscopist (who can perform EUS) and a cumbersome solid fiducial loading method[19].

A new type of fiducial was introduced by our group to address these limitations of solid fiducials implantation. Iodized oil-based liquid fiducials are less expensive and can be inserted by an endoscopist who perform gastroscopies. This was described for the first time in our pilot study in 2016[20], in which we demonstrated that similar results could be achieved compared to solid fiducials. Although the data from this study has enabled conclusions on the safety and technical efficacy of the use of liquid fiducials, long-term efficacy data focused on the patients that received LF-IGRT was not available. Hence, this study presents data on long-term follow-up of patients with gastroesophageal cancers who received F-IGRT based on liquid fiducials placed with standard gastroscopy injection technique.

## MATERIALS AND METHODS

### *Ethical approval*

This is a retrospective cohort study of consecutive adults with oesophageal and gastric cancers referred to the endoscopy unit at Austin Health, Melbourne, Australia for liquid fiducial placement before IGRT between January 2013 and January 2021. A database of all patients referred to the endoscopy unit for liquid fiducial placement before IGRT was prospectively maintained. The study was approved after institutional board review (Austin Research Ethics Committee: H2013/04975). Informed consent was waived; patient confidentiality was maintained and protected.

### *Patient selection*

Inclusion criteria were patients with: (1) A management plan discussed in a multi-disciplinary team meeting for radiotherapy for oesophageal (squamous carcinoma or adenocarcinoma) or gastric cancer; and (2) Referred to the endoscopy team for placement of fiducial markers.

Exclusion criteria were patients that did not have liquid fiducials inserted (*e.g.*, deemed as unfit for endoscopic procedure); patients that did not have radiotherapy after placement of fiducials (*e.g.*, declining clinical status) and patients that had surgery before radiotherapy after fiducial placement. In addition, patients who had incomplete treatment and outcome data (*e.g.*, due to loss of follow up) were excluded from our analysis.

### Clinical data collection

Patient clinical data including diagnosis, functional performance status as defined by Eastern Cooperative Oncology Group (ECOG)[21], the American Joint Committee on Cancer (AJCC) 8$^{th}$ edition staging[22], and treatment outcomes were retrospectively collected from patient medical records, endoscopy, radiology, surgical and histopathology reports. The national health database (©Australian Digital Health Agency) was used to assess whether the patient was still alive.

Information for patients treated at other centers was requested from their treating radiation oncologists. When information on the measured primary and secondary outcomes was available, these patients were included in the analysis.

Progression-free survival (PFS) was assessed based on patient disposition at their latest oncological follow-up appointment.

### The endoscopic procedure

The endoscopic procedure aims to insert a total of four fiducials per patient (2 proximal and 2 distal edges of the tumor), each injection consisting of 0.2-0.5mL of a 1:1 mixture of iodized oil (Lipiodol; Aspen Pharmacare) and n-butyl 2-cyanoacrylate (Histoacryl®; B. Braun). If a tumor was obstructing the passage of the gastroscope, only fiducials on the proximal edge were placed. In addition, if a tumor was extending to the level of the cricopharyngeal, it was not technically possible to insert fiducials at the proximal edge and only distal edges were placed. A 23-gauge injector (Cook Medical®) was used for these injections. All procedures were performed by or under the supervision of a gastroenterologists. All procedures were done under sedation, which was performed by an accredited anaesthetist. The endoscope used for all procedures was a standard gastroscope [(GIF-H1180 and H190; Olympus©), Melbourne, Victoria, Australia], and did not require the aid of fluoroscopy. Patients were routinely observed after the procedure for 1 h after which they were discharged if there were no significant adverse events.

### The injection technique

Three 2-mL syringes with a Luer Lock™ that can securely be locked onto the end of the injector needle are required; two are filled with iodized oil and only one will contain the iodised oil/n-butyl 2-cyanoacrylate mixture (1 mL: 1 mL).

Step 1: The 23-gauge injector is primed with the iodised oil only outside the patient.

Step 2: When the endoscopist is ready to inject, the injector is passed down the accessory channel of the gastroscope and further primed with the iodized oil/n-butyl 2-cyanoacrylate mixture, ideally within the stomach.

Step 3: A total of four-point injections (0.2-0.5 mL each) are made into the edges of the tumor. Two injections are placed proximally and another two placed distally into the edges of the tumor, when possible.

Step 4: Once marking is completed, the injector should be flushed with the syringe containing iodised oil only to prevent accidental gluing of the accessory channel, again ideally within the stomach.

Step 5: The gastroscope is then withdrawn with the needle retracted but the injector tip itself is slightly out of the distal tip of the gastroscope.

Step 6: Once the gastroscope is removed from the patient, the injector is cut at the port end so that it can be pulled through from the distal tip.

Step 7: The gastroscope accessory channel is subsequently flushed with water. Images and a video of these steps can be found in a previous publication[20].

### Fiducial IGRT

Following insertion of liquid fiducial markers, the patient underwent CT simulation a minimum of 24 h after insertion. The gross target volume (GTV) was defined using the fiducial markers, endoscopic report and correlative imaging (*e.g.*, diagnostic FDG-PET/CT and CT).

For patients who received definitive treatment, a high dose clinical target volume (CTV) included the GTV plus a 1 cm margin, clipped at anatomic boundaries. A low dose CTV included the GTV plus a 3 cm margin in the cranio-caudal (C-C) axis and 1

cm in other planes (clipped to anatomic boundaries), plus regional lymph nodes at the discretion of the radiation oncologist. A 1 cm planning target volume (PTV) margin was used. The high dose PTV was treated to 54Gy in 30 fractions, and the low dose PTV was assigned 46Gy in 30 fractions. Treatments were planned using the Monaco treatment planning system (Elekta, Stockholm).

For patients who had palliative treatments, the CTV was defined using a 1 cm margin, clipped at anatomical boundaries, with a 1 cm PTV margin. A range of prescription doses were used depending on patient disposition, ranging from 30Gy in 10 fractions to 45Gy in 15 fractions.

Liquid fiducial-based IGRT (LF-IGRT) was performed when the implanted liquid fiducials could be adequately visualized at the time of radiotherapy treatment on cone-beam CT (CBCT). LF-IGRT was performed each fraction using the Elekta XVI software (Elekta Synergy, XVI version 5, Elekta AB, Stockholm, Sweden) online image verification software and an Elekta linear accelerator. A grey value match was performed on an area including the fiducial markers. If liquid fiducials could not be located on the CBCT scan, then patients were treated with S-IGRT. S-IGRT utilized a grey value match on the vertebrae only.

### PFS and complications post radiotherapy

Postprocedural follow-up was assessed through outpatient radiation oncology appointments. Data on patients seen at our center were retrieved from electronic medical records. Patients who had the fiducials implanted at our center but had their follow-up elsewhere had their treating radiation oncologist contacted for information. Data on PFS, late complications from fiducial placement and radiotherapy complications (as per the National Cancer Institute Common Terminology Criteria for Adverse Events v4.0.[23]) were assessed.

### Outcome Measures

**Primary Outcome**: The primary outcome was the overall survival (OS) and PFS of patients who received IGRT after liquid fiducial placement for the treatment of a gastroesophageal tumor. The OS and PFS were referenced to the time of radiotherapy completion. Progression was defined as radiographic or histologic progression (*e.g.*, from recurrence detected on gastroscopy), coded as either local or distant in location.

**Secondary outcomes:** Secondary outcomes included the technical success of liquid fiducial guiding radiotherapy, adverse events rate and subgroup analyses based on radiotherapy treatment (LF-IGRT and S-IGRT), treatment intent (curative and palliative), tumor type (oesophageal and gastric) and oesophageal histology.

**Key definitions:** Technical success was defined as the successful delivery of LF-IGRT after the placement of liquid fiducial(s) through standard gastroscopy technique.

Gastroesophageal junction cancers classified as Siewert types I and II were analyzed as oesophageal cancers in accordance with the 8$^{th}$ edition of the AJCC staging guidelines[22]. Gastroesophageal junction cancers classified as Siewert type III were analyzed as gastric cancers.

Functional performance status was defined by the ECOG performance status[21].

### Statistical analysis

Data are summarized as median and ranges for continuous data, and as frequency and percentages for categorical data. For continuous data, comparisons were done using the Mann-Whitney *U* test or Independent Samples Kruskal-Wallis test based on the normality assumption. For categorical data, Fisher's Exact test and Likelihood Ratio Chi-Squared test were used as per high prevalence of expected cells with a count less than 5. $P$ value of $< 0.05$ was considered significant. Survival rates were estimated using the Kaplan-Meier method. Statistical analyzes were performed with SPSS statistical software (IBM Corp. 2020. IBM SPSS Statistics, Version 26.0. Armonk, NY) and JMP v16.0 (SAS Institute Inc).

## RESULTS

### Population

A total of 52 patients were referred to the endoscopy unit for liquid fiducial placement for IGRT over eight years between January 2013 and January 2021.

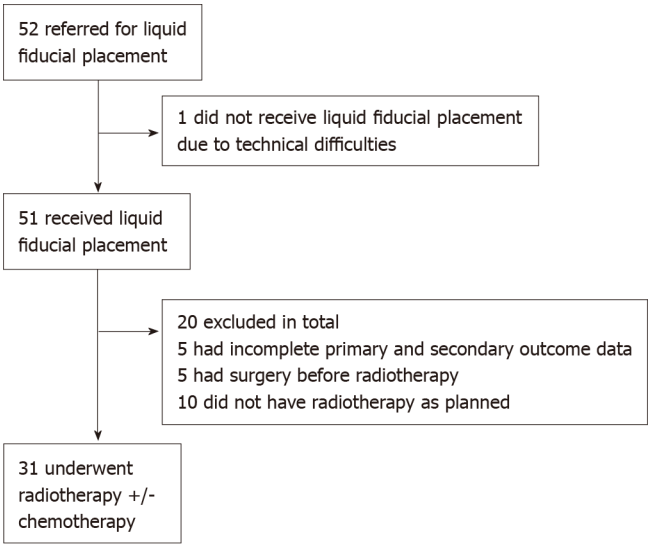


**Figure 1 Study flowchart.**

1 patient did not have liquid fiducial placement after endoscopic assessment as the tumor was obstructive and extended up to the level of the oropharynx and deemed unsafe to proceed. The majority of patients (98.0%) were able to have at least one edge marked with liquid fiducials, with a large proportion (77.4%) having both at distal and proximal edges marked.

51 patients had liquid fiducials inserted during the study period. Of these, 20 were excluded from our analysis. Five underwent radiotherapy at other centers and their clinical data were not available for analysis. Five had surgery prior to radiotherapy and ten did not have radiotherapy as anticipated. For instance, some patients were treated on the TOPGEAR trial and were randomized to no adjuvant therapy[8]. Therefore, data on the use of endoscopically-placed liquid fiducials during radiotherapy was available for 31 patients (Figure 1).

Our cohort of 31 patients had a median age of 77 years (range 57.5 to 88.8). The majority (71.0%) had oesophageal cancers, with a significant subset (72.7%) of these with squamous cell carcinoma (SCC). Only three patients in our cohort had gastroesophageal junction cancer of which two were classified as Siewert type III. Most patients (67.7%) had locally advanced disease without lymph node involvement or metastatic disease. Most of the cohort had an ECOG score of 0 or 1.

Three (9.6%) of patients received neoadjuvant chemoradiotherapy prior to oesophagectomy for oesophageal SCC. A large proportion (38.7%) of our cohort received definitive chemoradiotherapy, whilst a further 25.8% received definitive radiotherapy only. Detailed demographics and treatments are summarized in Table 1.

### *Technical success*

Twenty-seven out of the 31 patients (87.1%) received LF-IGRT. Patients commenced radiotherapy after a median period of 18 d (range 9 to 44) of fiducial placement. The cohort median duration of radiotherapy was 30 d (range 14 to 47). Details on F-IGRT and S-IGRT subgroups are shown in Table 2.

### *OS and PFS*

On the close-out date of 24/06/2021, 54.8% of patients were alive. The cohort OS post-radiotherapy was 19 mo (range 0 to 87 mo). Whilst the PFS post-radiotherapy was 13 mo (range 0 to 74 mo). For those treated with curative intent, the median OS was 22.0 mo (range 0 to 87 mo) with a PFS median of 14.0 mo (range 0 to 74). Nineteen patients were alive at 5 years. The 5-year survival rate for oesophageal cancer was 42.5% and for gastric cancer was 55.6% for this cohort. Kaplan-Meier curves for the overall cohort OS and PFS, treatment intent and type of cancer and histology are described in Figures 2, 3 and 4 respectively. Details on OS, PFS and 5-year survival for each subgroup analysis for the type of radiotherapy treatment (LF-IGRT and S-IGRT), treatment intent (curative and palliative), tumor type (oesophageal and gastric) and oesophageal histology are described in Table 3.

**Table 1 Patient demographics and treatment**

| Variables | | Median/*n* | Range/% |
|---|---|---|---|
| | Age | 77.4 | 57.5-88.0 |
| | Male | 20 | 64.5 |
| Site of cancer | Oesophageal | 21 | 67.7 |
| | GOJ (Siewert I/II) | 1 | 3.2 |
| | GOJ (Siewert III) | 2 | 6.5 |
| | Gastric | 7 | 22.6 |
| Type of cancer | Oesophageal SCC | 16 | 51.6 |
| | Oesophageal adenocarcinoma | 6 | 19.4 |
| | Gastric adenocarcinoma | 9 | 29.0 |
| Endoscopy data | Patients with both proximal and distal fiducial placed | 24 | 77.4 |
| | Patients with solely proximal or distal fiducials placed | 7 | 22.6 |
| ECOG | 0 | 13 | 41.9 |
| | 1 | 11 | 35.5 |
| | 2 | 7 | 22.6 |
| Stages[1] | LN negative without distant metastasis | 21 | 67.7 |
| | LN positive without distant metastasis | 6 | 19.4 |
| | Distant metastasis present | 3 | 9.6 |
| Radiotherapy | Fiducial seen on CBCT | 29 | 93.5 |
| | Fiducial-based IGRT | 27 | 87.1 |
| Treatment intent | Neoadjuvant chemotherapy with oesophagectomy | 3 | 9.7 |
| | Definitive chemoradiotherapy | 12 | 38.7 |
| | Palliative chemoradiotherapy | 3 | 9.7 |
| | Definitive radiotherapy | 8 | 25.8 |
| | Palliative radiotherapy | 5 | 16.1 |

[1]Staging as per AJCC 8th edition. T-stage was not available for all patients as endoscopic ultrasound is not routinely performed at our center.
SCC: Squamous cell carcinoma; ECOG: Eastern Cooperative Oncology Group; GOJ: Gastro-oesophageal junction; LN: Lymph Node; CBCT: Cone-beam computerized tomography; IGRT: Image-guided radiotherapy.

Of note, 12 patients had disease progression during the study period. Seven of these patients had local progression whilst five had distal disease progression.

### *Adverse events*

No early or late adverse events occurred following the insertion of the fiducials as assessed prior to discharge on the day of the procedure and on subsequent radiation oncology follow-ups, respectively. Nine patients experienced grade three adverse events which were odynographia, dysphagia, nausea, dehydration, febrile neutropenia and lung infection during their treatment. No patient experienced grade four or five adverse events. Adverse events from their treatment are summarized in Table 4.

## DISCUSSION

In this study, we describe the OS and PFS of patients with gastroesophageal tumors that underwent LF-IGRT with liquid fiducials inserted through standard gastroscopy injection technique. This report is a follow-up to our initial study which first described this technique[20]. We believe this to be the largest observational cohort study of its kind, adding to the limited body of knowledge on the long-term outcomes of F-IGRT for gastrooesophageal tumors using liquid fiducials.

**Table 2 Patient demographics and treatment details based on fiducial-based image-guided radiotherapy and standard image-guided radiotherapy**

| Variables | | F-IGRT | S-IGRT |
|---|---|---|---|
| | | Median/*n*, range/% | Median/*n*, range/% |
| | Age | 77.4, 57.5-88.0 | 77.3, 64.8-85.4 |
| | Male | 19, 70.4 | 1, 25.0 |
| Site of cancer | Oesophageal | 18, 62.1 | 3, 75.0 |
| | GOJ (Siewert I/II) | 0, 0.0 | 1, 25.0 |
| | GOJ (Siewert III) | 2, 7.4 | 0, 0.0 |
| | Gastric | 7, 25.9 | 0, 0.0 |
| Type of cancer | Oesophageal SCC | 13, 48.1 | 3, 75.0 |
| | Oesophageal adenocarcinoma | 5, 18.5 | 1, 25.0 |
| | Gastric adenocarcinoma | 9, 33.3 | 0, 0.0 |
| Endoscopy data | Patients with both proximal and distal fiducial placed | 22, 81.5 | 2, 50.0 |
| | Patients with solely proximal or distal fiducials placed | 5, 18.5 | 2, 50.0 |
| ECOG | 0 | 11, 40.7 | 2, 50.0 |
| | 1 | 9, 33.3 | 2, 50.0 |
| | 2 | 7, 25.9 | 0, 0.0 |
| Stages[1] | LN negative without distant metastasis | 18, 66.7 | 4, 100.0 |
| | LN positive without distant metastasis | 6, 22.2 | 0, 0.0 |
| | Distant metastasis present | 3, 11.1 | 0, 0.0 |
| Treatment details | Time to treatment (d) | 19.0, 9.0-44 | 14 .0, 9.0-17.0 |
| | Curative intent | 20, 74.1 | 4, 100.0 |
| | Palliative intent | 7, 25.9 | 0, 0.0 |
| | Dose (Grays) | 50, 25.2-55 | 47.7, 41.4-54 |
| | Fraction | 25, 10-30 | 26.5, 23-30 |
| | Duration (d) | 30, 14-47 | 37, 30-39 |
| | Chemotherapy | 14, 51.9 | 4, 100.0 |

[1]Staging as per American Joint Committee on Cancer 8th edition. T-stage was not available for all patients as endoscopic ultrasound is not routinely performed at our center.
F-IGRT: Fiducial-based image-guided radiotherapy; S-IGRT: Standard image-guided radiotherapy; SCC: Squamous cell carcinoma; ECOG: Eastern Cooperative Oncology Group; GOJ: Gastro-oesophageal junction; LN: Lymph Node.

Our cohort consisted mainly of patients with locally advanced oesophageal cancer with SCC. The majority (90.3%) of our cohort received chemoradiotherapy or radiotherapy alone as palliative and definitive treatment. The median OS was 19.0 mo, with the longest OS of 87 mo. Our results, albeit a small cohort, compare favorably to what is available in the literature, the 5-year survival rates for oesophageal and gastric cancers were 42.8% and 55.6%, respectively[24]. In the context of gastric cancer in an inoperable population undergoing chemoradiotherapy, the reported median survival was 25.0 mo[25]. We recognize that this is possibly due to judicious patient selection, or the relatively small numbers of patients included compared with larger randomized trials.

Also, we acknowledge that in Australia access to EUS for endoscopic staging is variable, and at our center is not routinely performed. Thus, limiting our accurate reporting of tumor staging as per AJCC 8th Edition. This further adds to our argument that the use of EUS-guided solid fiducials for marking tumors has limitations of which most can be mitigated with the use of liquid fiducials[19,20]. Additionally, the technical aspects of the injection technique required for placement of liquid fiducials

**Table 3 Subgroup analysis of overall survival, progression-free survival and 5-year survival**

| Subgroups | | OS (mo) | PFS (mo) | 5-yr survival (%) |
|---|---|---|---|---|
| | | Median/*n*, range/% | Median/*n*, range/% | |
| Treatment | F-IGRT (*n* = 27) | 21.0, 0-87 | 12.0, 0-74 | 47.2 |
| | S-IGRT (*n* = 4) | 17.0, 3-49 | 18.0, 2-32 | 0.0 |
| Intent | Curative (*n* = 23) | 22.0, 0-87 | 14.0, 0-74 | 51.5 |
| | Palliative (*n* = 8) | 8.0, 0-79 | 4, 0-13 | 30.0 |
| Location | Oesophageal (*n* = 22) | 18, 0-87 | 13, 0-74 | 42.8 |
| | SCC (*n* = 16) | 20, 1-87 | 14, 1-74 | 62.0 |
| | AC (*n* = 6) | 10, 0-54 | 7, 0-19 | 0.0 |
| | Gastric (*n*=9) | 25, 0-79 | 13, 0-53 | 55.6 |

F-IGRT: Fiducial-based image-guided radiotherapy; S-IGRT: Standard image-guided radiotherapy; SCC: Squamous cell carcinoma; AC: Adenocarcinoma; OS: Overall survival; PFS: Progression-free survival.

**Table 4 Complications**

| | LF-IGRT only | LF-IGRT with chemotherapy | S-IGRT with chemotherapy | Overall |
|---|---|---|---|---|
| Hospitalization | 3 | 4 | 2 | 9 |
| Fatigue (G1/G2/G3) | 1/2/0 | 3/1/0 | 2/0/0 | 6/3/0 |
| Odynophagia G1/G2/G3 | 3/1/1 | 2/4/1 | 0/4/0 | 5/9/2 |
| Dysphagia (G1/G2/G3) | 2/0/0 | 0/1/0 | 0/1/1 | 2/2/1 |
| Oesophageal stricture (G1/G2/G3) | 1/0/0 | 0/0/1 | 0/0/1 | 1/0/2 |
| Nausea (G1/G2/G3) | 0/2/1 | - | - | 0/2/1 |
| Diarrhoea (G1/G2/G3) | - | 1/0/0 | - | 1/0/0 |
| Lung infection (G2, G3) | - | 0/1 | - | 0/1 |
| Dermatitis (G1/G2/G3) | - | 1/0/0 | - | 1/0/0 |
| Febrile neutropenia (G3) | - | 1 | - | 1 |
| Dehydration (G1/G2/G3) | 0/0/1 | - | - | 0/0/1 |

G1: Grade 1 complication; G2: Grade 2 complication; G3: Grade 3 complication; LF-IGRT: Liquid fiducial-based image-guided radiotherapy; S-IGRT: Standard image-guided radiotherapy.

is, in essence, an adaptation of a skillset that most gastroenterologists would already have in the management of gastrooesophageal variceal bleeding[26]. As such, this technique of liquid fiducials can be more easily adopted. Furthermore, our group has previous described the potential cost-saving of liquid fiducials amounting to approximately AU$1150 to AU$1750 per procedure when compared to EUS-guided guided solid fiducials insertion[20]. Since the description of lipiodol as a fiducial for gastroesophageal tumors, similar techniques have been described with similar technical success and safety profiles[27].

The use of F-IGRT has many potential benefits over S-IGRT, including facilitating a higher dose focused on the tumor with a lower dose delivered to cover the submucosal spread, and more accurate treatment delivery (matching to the tumor rather than surrounding bony structures)[17,28]. There are conflicting data regarding the efficacy of increased radiation dose in treating oesophageal cancer[29,30]. While some retrospective studies demonstrated a dose-response, recent randomized control trials failed to find a difference in outcomes[31-33]. The effect of dose escalation in optimizing cure rate may be more evident in those with early-stage SCC where the tumor is radiosensitive and the rate of distant metastasis is low[34]. Higher doses can be associated with increased normal tissue toxicity and hence focusing radiotherapy as much as possible to the tumor area is essential. F-IGRT allows a higher dose to be

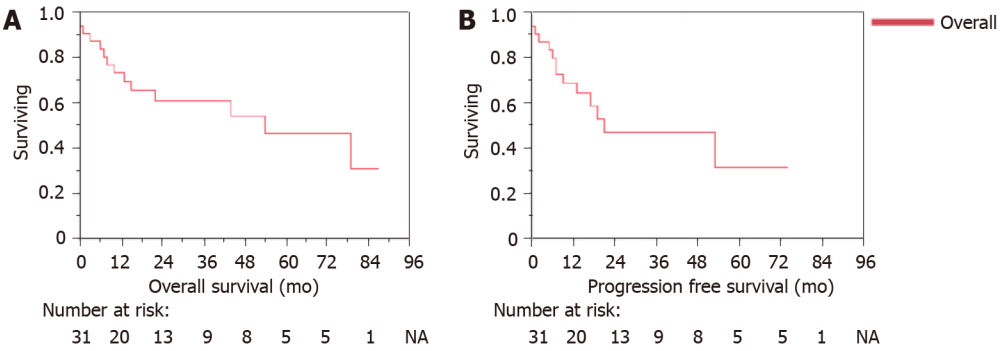


**Figure 2 Kaplan Meier curve–cohort.** A: The Kaplan-Meier curve for the cohort overall survival post-radiotherapy treatment; B: The Kaplan-Meier curve for the cohort progression free survival post-radiotherapy treatment. NA: Not applicable.

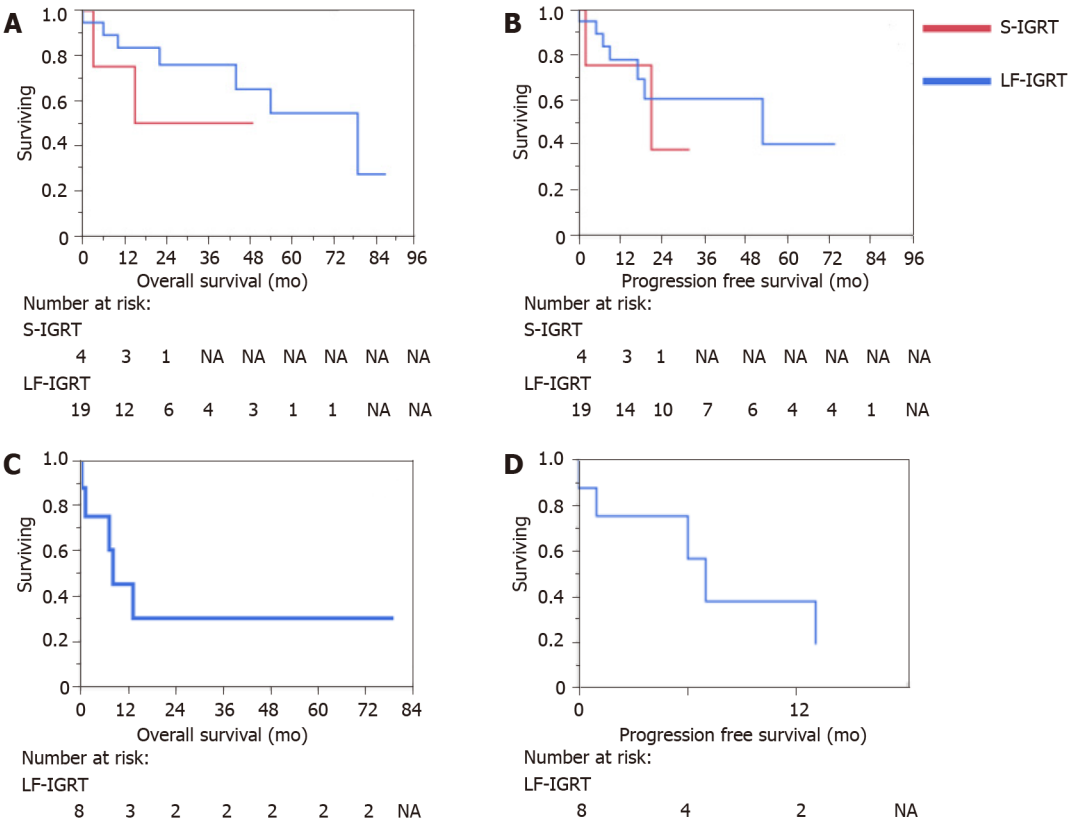


**Figure 3 Kaplan Meier curve-treatment intent.** A: The Kaplan-Meier curve for the overall survival (OS) of patients receiving curative intent standard image-guided radiotherapy (S-IGRT) and fiducial-based IGRT (F-IGRT) treatment; B: The Kaplan-Meier curve for the progression free survival (PFS) of patients receiving curative intent S-IGRT and F-IGRT treatment; C: The Kaplan-Meier curve for the OS of patients receiving palliative intent F-IGRT treatment; D: The Kaplan-Meier curve for the PFS of patients receiving palliative intent F-IGRT treatment, respectively. NA: Not applicable; S-IGRT: Standard image-guided radiotherapy; LF-IGRT: Liquid fiducial-based image-guided radiotherapy.

assigned to the tumor while simultaneously allowing for more confident identification of submucosal spread, and therefore facilitating lower doses to the adjacent esophagus.

The improvement in the delivery of radiotherapy for patients having endoscopically inserted liquid fiducials is illustrated in Figure 5.

In our cohort, 87.1% successfully underwent LF-IGRT after liquid fiducial placement. In two of our patients, the liquid fiducials were not visible at the time of radiation treatment planning. We hypothesis that this could be due to extravasation or diffusion of the submucosal bleb after the procedure. For two patients, the liquid fiducials were visible but were not sufficient for F-IGRT due to the fiducials not being reliably seen. This highlights a difference compared to metallic fiducials, in that liquid fiducials can have variable shapes and distribution. In addition, we consider our definition of technical success to be more clinically relevant relative to previous

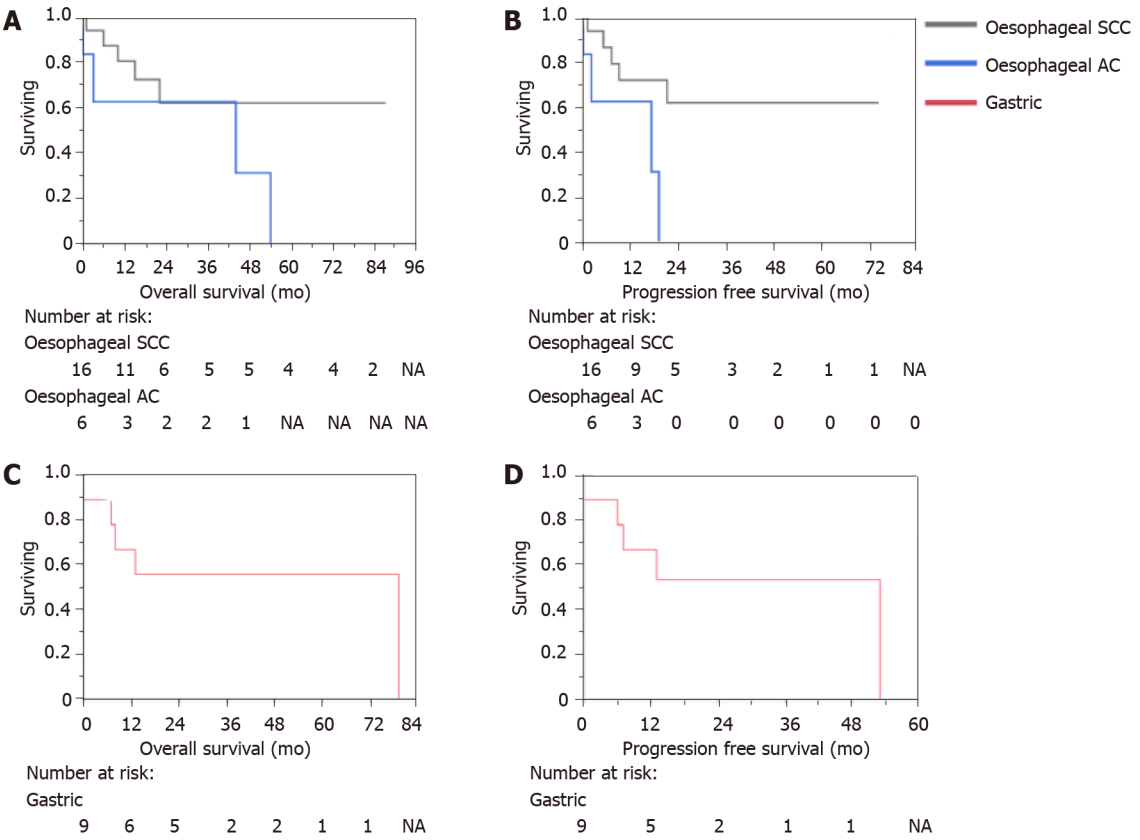


**Figure 4 Kaplan Meier curve-site and histopathology.** A: The Kaplan-Meier curve for the overall survival (OS) of patients with oesophageal squamous cell carcinoma (SCC) and adenocarcinoma (AC); B: The Kaplan-Meier curve for the progression free survival (PFS) of patients with oesophageal SCC and AC; C: The Kaplan-Meier curve for the OS of patients with gastric AC; D: The Kaplan-Meier curve for the PFS of patients with gastric AC. NA: Not applicable; SCC: Squamous cell carcinoma; AC: Adenocarcinoma.

definitions reported in the literature. This would account for our slightly lower success rates. However, applying the same technical definition, our rates of successful placement of liquid fiducials would be 98.1%, compared to 96.3%[18] and 98.0%[35].

Toxicity rates reported in the literature include grade 3 seen in 42% and grade 4 in 7%; mainly hematology, gastrointestinal and mouth ulceration[24]. Our lower complication rate from F-IGRT may be related to advances in treatment or the use of fiducials *per se*. Prospective randomized studies are needed to ascertain the utility of LF-IGRT in reducing complications.

Despite these potential benefits, prospective randomized trials are required as observational studies have failed to show differences in long-term outcomes such as OS for oesophageal cancers using IGRT[36]. Nevertheless, F-IGRT has been deemed by specialists as a promising IGRT modality for the future[37].

The limitations of our study are mainly the small numbers, the heterogeneity of the cohort, the retrospective design and the lack of a direct comparison with S-IGRT. Regarding the certainty of the delivered dose, one other study demonstrated that soft tissue (diaphragm) or bone matching on CBCT resulted in a larger margin to cover the tumor 95% of the time[38]. Direct soft tissue matching of locally advanced tumors would be possible if visible on CBCT but would be not feasible if the tumor is too small to be seen. Secondarily, although we present data on few patients, this is to date the largest cohort of F-IGRT for gastroesophageal tumors utilizing the liquid fiducial technique. The retrospective design and lack of a robust comparison between F- and S-IGRT could not be addressed in the present study but is a promising subject for future research. Furthermore, due to the small number of patients, a multivariate analysis was not performed to address potential bias in this study.

## CONCLUSION

F-IGRT was considered feasible in 87.1% of patients undergoing liquid fiducial placement through standard gastroscopy injection technique. Our cohort had an OS of

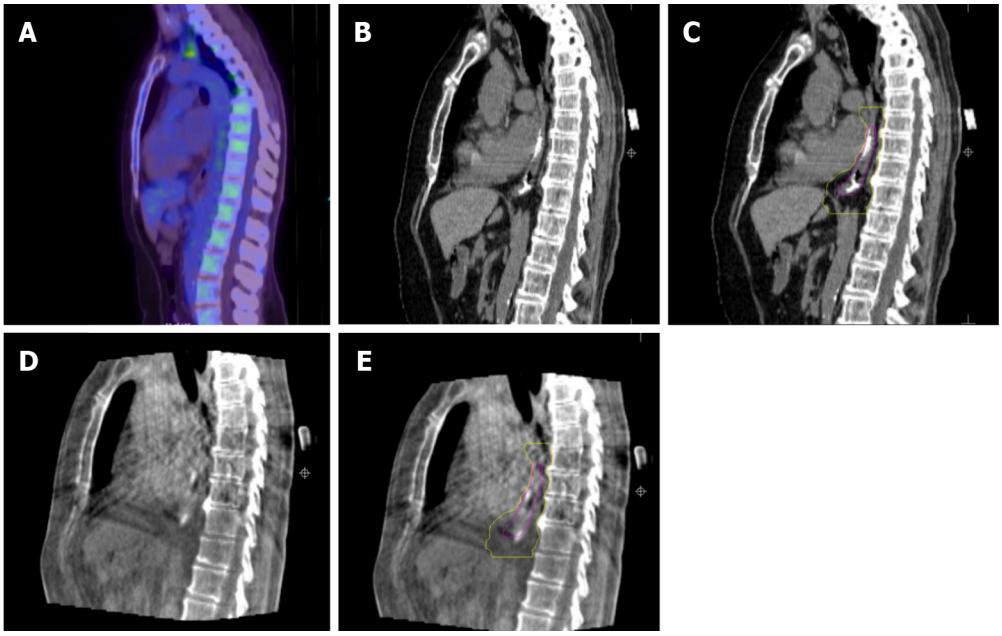


**Figure 5 Endoscopically inserted liquid fiducials and liquid fiducial-based image-guided radiotherapy.** A: An Fluorodeoxyglucose positron emission tomography of a patient with oesophageal cancer which demonstrates no uptake within the lesion; B: Planning computerized tomography (CTs) showing the fiducials without and with the high dose clinical target volume (CTV) (pink); C: Planning CTs showing the fiducials without and with the low dose CTV (yellow); D: Cone-beam CT (CBCT) images that are not labelled; E: CBCT images that are labelled.

19 mo and PFS of 13 mo. Further studies are warranted to determine the long-term outcomes of liquid-fiducial based IGRT.

## ARTICLE HIGHLIGHTS

### *Research background*

Further studies are warranted to determine the long-term outcomes of liquid fiducial-based image-guided radiotherapy (IGRT) in the treatment of oesophagogastric cancers.

### *Research motivation*

Based on a cohort of 31 patients who had undergone lipiodol fiducial implantation through standard gastroscopy and received radiotherapy, fiducial-based IGRT was possible in 87.1%. Our cohort had an overall survival (OS) of 19 mo and progression-free survival (PFS) of 13 mo.

### *Research objectives*

52 patients were referred for liquid fiducial placement within the study period. A total of 51 patients underwent liquid fiducial implantation. Of these a total of 31 patients received radiotherapy. Twenty-seven out of the 31 patients were able to have liquid fiducial-based IGRT (LF-IGRT) while four had standard-IGRT (S-IGRT). There were no complications after endoscopic implantation of liquid fiducials in our cohort. The cohort OS post-radiotherapy was 19 mo (range 0 to 87 mo). Whilst the PFS post-adiotherapy was 13 mo (range 0 to 74 mo).

### *Research methods*

A retrospective cohort study of consecutive adults with oesophagogastric cancers referred for liquid fiducial placement before definitive/neo-adjuvant or palliative IGRT between 2013 and 2021 at a tertiary hospital in Melbourne, Australia was conducted. Up to four liquid fiducials were inserted per patient, each injection consisting of 0.2-0.5mL of a 1:1 mixture of iodized oil (Lipiodol; Aspen Pharmacare) and n-butyl 2-cyanoacrylate (Histoacryl®; B. Braun). A 23-gauge injector (Cook Medical) was used for the injection. All procedures were performed by or under the supervision of a gastroenterologist. LF-IGRT consisted of computer-assisted direct

matching of the fiducial region on cone-beam computerized tomography (CBCT) at the time of radiotherapy. Patients received S-IGRT if fiducial visibility was insufficient, consisting of bone match as a surrogate for tumor position. Radiotherapy was delivered to 54Gy in 30 fractions for curative patients and up to 45Gy in 15 fractions for palliative treatments.

### Research results

To assess the long-term outcomes of liquid fiducial-guided IGRT in a cohort of oesophageal and gastric cancer patients.

### Research conclusions

We believe this to be the largest observational cohort study of its kind, adding to the limited body of knowledge on the long-term outcomes of F-IGRT for gastrooesophageal tumors using liquid fiducials.

### Research perspectives

IGRT has significantly improved the precision in which radiotherapy is delivered in cancer treatment. Typically, IGRT uses bony landmarks and key anatomical structures to locate the tumor. Recent studies have demonstrated the feasibility of peri-tumor fiducials in enabling even more accurate delineation of target and normal tissue. The use of gold coils as fiducials in gastrointestinal tumors has been extensively studied. However, placement requires expertise and specialized endoscopic ultrasound equipment. This article reports the long-term outcomes of using a standard gastroscopy to inject liquid fiducials for the treatment of oesophageal and gastric tumors with IGRT.

## REFERENCES


1 **Sung H**, Ferlay J, Siegel RL, Laversanne M, Soerjomataram I, Jemal A, Bray F. Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries. *CA Cancer J Clin* 2021; **71**: 209-249 [PMID: 33538338 DOI: 10.3322/caac.21660]

2 **Australian Institute of Health and Welfare**. Cancer data in Australia [Internet]. Canberra: Australian Institute of Health and Welfare. [cited 16 October 2021]. Available from: https://www.aihw.gov.au/reports/cancer/cancer-data-in-australia

3 **Arnold M**, Ferlay J, van Berge Henegouwen MI, Soerjomataram I. Global burden of oesophageal and gastric cancer by histology and subsite in 2018. *Gut* 2020; **69**: 1564-1571 [PMID: 32606208 DOI: 10.1136/gutjnl-2020-321600]

4 **Chan KKW**, Saluja R, Delos Santos K, Lien K, Shah K, Cramarossa G, Zhu X, Wong RKS. Neoadjuvant treatments for locally advanced, resectable esophageal cancer: A network meta-analysis. *Int J Cancer* 2018; **143**: 430-437 [PMID: 29441562 DOI: 10.1002/ijc.31312]

5 **Zhu LL**, Yuan L, Wang H, Ye L, Yao GY, Liu C, Sun NN, Li XJ, Zhai SC, Niu LJ, Zhang JB, Ji HL, Li XM. A Meta-Analysis of Concurrent Chemoradiotherapy for Advanced Esophageal Cancer. *PLoS One* 2015; **10**: e0128616 [PMID: 26046353 DOI: 10.1371/journal.pone.0128616]

6 **Noordman BJ**, Verdam MGE, Lagarde SM, Hulshof MCCM, van Hagen P, van Berge Henegouwen MI, Wijnhoven BPL, van Laarhoven HWM, Nieuwenhuijzen GAP, Hospers GAP, Bonenkamp JJ, Cuesta MA, Blaisse RJB, Busch OR, Ten Kate FJW, Creemers GM, Punt CJA, Plukker JTM, Verheul HMW, Spillenaar Bilgen EJ, van Dekken H, van der Sangen MJC, Rozema T, Biermann K, Beukema JC, Piet AHM, van Rij CM, Reinders JG, Tilanus HW, Steyerberg EW, van der Gaast A, Sprangers MAG, van Lanschot JJB. Effect of Neoadjuvant Chemoradiotherapy on Health-Related Quality of Life in Esophageal or Junctional Cancer: Results From the Randomized CROSS Trial. *J Clin Oncol* 2018; **36**: 268-275 [PMID: 29161204 DOI: 10.1200/JCO.2017.73.7718]

7 **Stahl M**, Walz MK, Riera-Knorrenschild J, Stuschke M, Sandermann A, Bitzer M, Wilke H, Budach W. Preoperative chemotherapy *vs* chemoradiotherapy in locally advanced adenocarcinomas of the oesophagogastric junction (POET): Long-term results of a controlled randomised trial. *Eur J Cancer* 2017; **81**: 183-190 [PMID: 28628843 DOI: 10.1016/j.ejca.2017.04.027]

8 **Leong T**, Smithers BM, Haustermans K, Michael M, Gebski V, Miller D, Zalcberg J, Boussioutas A, Findlay M, O'Connell RL, Verghis J, Willis D, Kron T, Crain M, Murray WK, Lordick F, Swallow C, Darling G, Simes J, Wong R. TOPGEAR: A Randomized, Phase III Trial of Perioperative ECF Chemotherapy with or Without Preoperative Chemoradiation for Resectable Gastric Cancer: Interim Results from an International, Intergroup Trial of the AGITG, TROG, EORTC and CCTG. *Ann Surg Oncol* 2017; **24**: 2252-2258 [PMID: 28337660 DOI: 10.1245/s10434-017-5830-6]

9 **Ng SP**, Leong T. Role of Radiation Therapy in Gastric Cancer. *Ann Surg Oncol* 2021; **28**: 4151-4157 [PMID: 33689079 DOI: 10.1245/s10434-021-09639-y]

10 **Yuan M**, Bao Y, Ma Z, Men Y, Wang Y, Hui Z. The Optimal Treatment for Resectable Esophageal Cancer: A Network Meta-Analysis of 6168 Patients. *Front Oncol* 2021; **11**: 628706 [PMID:

33777777 DOI: 10.3389/fonc.2021.628706]
11 **Kato H**, Miyazaki T, Nakajima M, Takita J, Kimura H, Faried A, Sohda M, Fukai Y, Masuda N, Fukuchi M, Manda R, Ojima H, Tsukada K, Kuwano H, Oriuchi N, Endo K. The incremental effect of positron emission tomography on diagnostic accuracy in the initial staging of esophageal carcinoma. *Cancer* 2005; **103**: 148-156 [PMID: 15558794 DOI: 10.1002/cncr.20724]
12 **Hashimoto T**, Shirato H, Kato M, Yamazaki K, Kurauchi N, Morikawa T, Shimizu S, Ahn YC, Akine Y, Miyasaka K. Real-time monitoring of a digestive tract marker to reduce adverse effects of moving organs at risk (OAR) in radiotherapy for thoracic and abdominal tumors. *Int J Radiat Oncol Biol Phys* 2005; **61**: 1559-1564 [PMID: 15817362 DOI: 10.1016/j.ijrobp.2005.01.006]
13 **Abbas H**, Chang B, Chen ZJ. Motion management in gastrointestinal cancers. *J Gastrointest Oncol* 2014; **5**: 223-235 [PMID: 24982771 DOI: 10.3978/j.issn.2078-6891.2014.028]
14 **Pishvaian AC**, Collins B, Gagnon G, Ahlawat S, Haddad NG. EUS-guided fiducial placement for CyberKnife radiotherapy of mediastinal and abdominal malignancies. *Gastrointest Endosc* 2006; **64**: 412-417 [PMID: 16923491 DOI: 10.1016/j.gie.2006.01.048]
15 **Shirato H**, Harada T, Harabayashi T, Hida K, Endo H, Kitamura K, Onimaru R, Yamazaki K, Kurauchi N, Shimizu T, Shinohara N, Matsushita M, Dosaka-Akita H, Miyasaka K. Feasibility of insertion/implantation of 2.0-mm-diameter gold internal fiducial markers for precise setup and real-time tumor tracking in radiotherapy. *Int J Radiat Oncol Biol Phys* 2003; **56**: 240-247 [PMID: 12694845 DOI: 10.1016/s0360-3016(03)00076-2]
16 **Nicosia L**, Sicignano G, Rigo M, Figlia V, Cuccia F, De Simone A, Giaj-Levra N, Mazzola R, Naccarato S, Ricchetti F, Vitale C, Ruggieri R, Alongi F. Daily dosimetric variation between image-guided volumetric modulated arc radiotherapy and MR-guided daily adaptive radiotherapy for prostate cancer stereotactic body radiotherapy. *Acta Oncol* 2021; **60**: 215-221 [PMID: 32945701 DOI: 10.1080/0284186X.2020.1821090]
17 **Takei Y**, Monzen H, Tamura M, Doi H, Nishimura Y. Dose reduction potential of using gold fiducial markers for kilovoltage image-guided radiotherapy. *J Appl Clin Med Phys* 2020; **21**: 151-157 [PMID: 32959957 DOI: 10.1002/acm2.13023]
18 **Patel JB**, Revanur V, Forcione DG, Bechtold ML, Puli SR. Endoscopic ultrasound-guided fiducial marker placement in pancreatic cancer: A systematic review and meta-analysis. *World J Gastrointest Endosc* 2020; **12**: 231-240 [PMID: 32879658 DOI: 10.4253/wjge.v12.i8.231]
19 **Chandran S**, Vaughan R, Efthymiou M, Sia J, Hamilton C. A pilot study of EUS-guided fiducial insertion for the multidisciplinary management of gastric cancer. *Endosc Int Open* 2014; **2**: E153-E159 [PMID: 26134962 DOI: 10.1055/s-0034-1377523]
20 **Chandran S**, Vaughan R, Jacob A, Hamilton C, Joon DL, Lim K, Tog C, Bhatia K, Aly A, Sweeney T, Efthymiou M. A novel endoscopic marker for radiological localization and image-guided radiotherapy in esophageal and gastric cancers (with video). *Gastrointest Endosc* 2016; **83**: 309-317 [PMID: 26239307 DOI: 10.1016/j.gie.2015.06.042]
21 **Oken MM**, Creech RH, Tormey DC, Horton J, Davis TE, McFadden ET, Carbone PP. Toxicity and response criteria of the Eastern Cooperative Oncology Group. *Am J Clin Oncol* 1982; **5**: 649-655 [PMID: 7165009]
22 **Rice TW**, Patil DT, Blackstone EH. 8th edition AJCC/UICC staging of cancers of the esophagus and esophagogastric junction: application to clinical practice. *Ann Cardiothorac Surg* 2017; **6**: 119-130 [PMID: 28447000 DOI: 10.21037/acs.2017.03.14]
23 **National Cancer Institute**. Common terminology criteria for adverse events v4.0 (CTCAE) [Internet]. [cited 16 October 2021]. Available from: https://ctep.cancer.gov/protocoldevelopment/electronic_applications/ctc.htm
24 **Gwynne S**, Hurt C, Evans M, Holden C, Vout L, Crosby T. Definitive chemoradiation for oesophageal cancer--a standard of care in patients with non-metastatic oesophageal cancer. *Clin Oncol (R Coll Radiol)* 2011; **23**: 182-188 [PMID: 21232928 DOI: 10.1016/j.clon.2010.12.001]
25 **Saikawa Y**, Kubota T, Kumagai K, Nakamura R, Kumai K, Shigematsu N, Kubo A, Kitajima M, Kitagawa Y. Phase II study of chemoradiotherapy with S-1 and low-dose cisplatin for inoperable advanced gastric cancer. *Int J Radiat Oncol Biol Phys* 2008; **71**: 173-179 [PMID: 17996385 DOI: 10.1016/j.ijrobp.2007.09.010]
26 **Mahmoudi N**, Whittaker JS. Glueing of fundal varices. *Can J Gastroenterol* 2006; **20**: 691-693 [PMID: 17111049 DOI: 10.1155/2006/753970]
27 **de Blanck SR**, Scherman-Rydhög J, Siemsen M, Christensen M, Baeksgaard L, Irming Jølck R, Specht L, Andresen TL, Persson GF. Feasibility of a novel liquid fiducial marker for use in image guided radiotherapy of oesophageal cancer. *Br J Radiol* 2018; **91**: 20180236 [PMID: 29975152 DOI: 10.1259/bjr.20180236]
28 **Yu S**, Lawrenson L, Wei R, Sehgal V, Hanna N, Kuo J, Daroui P, Ramsinghani N, Al-Ghazi M. The dosimetric impact of image guided radiation therapy by intratumoral fiducial markers. *Pract Radiat Oncol* 2016; **6**: 276-283 [PMID: 26852174 DOI: 10.1016/j.prro.2015.11.007]
29 **Hulshof MCCM**, Geijsen ED, Rozema T, Oppedijk V, Buijsen J, Neelis KJ, Nuyttens JJME, van der Sangen MJC, Jeene PM, Reinders JG, van Berge Henegouwen MI, Thano A, van Hooft JE, van Laarhoven HWM, van der Gaast A. Randomized Study on Dose Escalation in Definitive Chemoradiation for Patients With Locally Advanced Esophageal Cancer (ARTDECO Study). *J Clin Oncol* 2021; **39**: 2816-2824 [PMID: 34101496 DOI: 10.1200/JCO.20.03697]
30 **Xu YJ**, Zhu WG, Liao ZX, Kong Y, Wang WW, Li JC, Huang R, He H, Yang XM, Liu LP, Sun ZW, He HJ, Bao Y, Zeng M, Pu J, Hu WY, Ma J, Jiang H, Liu ZG, Zhuang TT, Tan BX, Du XH, Qiu GQ,

Zhou X, Ji YL, Hu X, Wang J, Ma HL, Zheng X, Huang J, Liu AW, Liang XD, Tao H, Zhou JY, Liu Y, Chen M. [A multicenter randomized prospective study of concurrent chemoradiation with 60 Gy *vs* 50 Gy for inoperable esophageal squamous cell carcinoma]. *Zhonghua Yi Xue Za Zhi* 2020; **100**: 1783-1788 [PMID: 32536123 DOI: 10.3760/cma.j.cn112137-20200303-00574]

31 **Innocente R**, Navarria F, Petri R, Palazzari E, Vecchiato M, Polesel J, Ziccarelli A, Martino A, Ubiali P, Tonin D, Lauretta A, Belluco C, Foltran L, Buonadonna A, Lleshi A, Colombo CB, Barresi L, Gigante M, Franchin G, De Paoli A. Feasibility and Oncological Outcome of Preoperative Chemoradiation With IMRT Dose Intensification for Locally Advanced Esophageal and Gastroesophageal Cancer. *Front Oncol* 2021; **11**: 626275 [PMID: 33680967 DOI: 10.3389/fonc.2021.626275]

32 **Xiao L**, Czito BG, Pang Q, Hui Z, Jing S, Shan B, Wang J. Do Higher Radiation Doses with Concurrent Chemotherapy in the Definitive Treatment of Esophageal Cancer Improve Outcomes? *J Cancer* 2020; **11**: 4605-4613 [PMID: 32489478 DOI: 10.7150/jca.44447]

33 **Li R**, Shinde A, Glaser S, Chao J, Kim J, Karam SD, Goodman K, Chen YJ, Amini A. Analyzing the impact of neoadjuvant radiation dose on pathologic response and survival outcomes in esophageal and gastroesophageal cancers. *J Gastrointest Oncol* 2019; **10**: 712-722 [PMID: 31392052 DOI: 10.21037/jgo.2019.02.18]

34 **Effeney R**, Shaw T, Burmeister BH, Burmeister E, Harvey J, Mai GT, Thomas J, Barbour AP, Smithers BM, Pryor DI. Patterns of Failure Following Dose-escalated Chemoradiotherapy for Fluorodeoxyglucose Positron Emission Tomography Staged Squamous Cell Carcinoma of the Oesophagus. *Clin Oncol (R Coll Radiol)* 2018; **30**: 642-649 [PMID: 30017206 DOI: 10.1016/j.clon.2018.06.011]

35 **Coronel E**, Cazacu IM, Sakuraba A, Luzuriaga Chavez AA, Uberoi A, Geng Y, Tomizawa Y, Saftoiu A, Shin EJ, Taniguchi CM, Koong AC, Herman JM, Bhutani MS. EUS-guided fiducial placement for GI malignancies: a systematic review and meta-analysis. *Gastrointest Endosc* 2019; **89**: 659-670.e18 [PMID: 30445001 DOI: 10.1016/j.gie.2018.10.047]

36 **Kuo YH**, Fang HY, Lin YS, Lein MY, Yang CY, Ho SC, Li CC, Chien CR. Effectiveness of image-guided radiotherapy for locally advanced esophageal squamous cell carcinoma patients treated with definitive concurrent chemoradiotherapy. *Thorac Cancer* 2020; **11**: 113-119 [PMID: 31742897 DOI: 10.1111/1759-7714.13244]

37 **Ng J**, Lee P. The Role of Radiotherapy in Localized Esophageal and Gastric Cancer. *Hematol Oncol Clin North Am* 2017; **31**: 453-468 [PMID: 28501087 DOI: 10.1016/j.hoc.2017.01.005]

38 **Voncken FEM**, Nakhaee S, Stam B, Wiersema L, Vollenbrock SE, van Dieren JM, van Leerdam ME, Sonke JJ, Aleman BMP, Remeijer P. Quantification of Esophageal Tumor Motion and Investigation of Different Image-Guided Correction Strategies. *Pract Radiat Oncol* 2020; **10**: 84-92 [PMID: 31786235 DOI: 10.1016/j.prro.2019.11.012]

Published by **Baishideng Publishing Group Inc**
7041 Koll Center Parkway, Suite 160, Pleasanton, CA 94566, USA
**Telephone:** +1-925-3991568
**E-mail:** bpgoffice@wjgnet.com
**Help Desk:** https://www.f6publishing.com/helpdesk
https://www.wjgnet.com