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FRONTIER

Significance of gut microbiota in alcoholic and non-alcoholic fatty liver diseases

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Abstract

Liver-gut communication is vital in fatty liver diseases, and gut microbes are the key regulators in maintaining liver homeostasis. Chronic alcohol abuse and persistent overnutrition create dysbiosis in gut ecology, which can contribute to fatty liver disease. In this review, we discuss the gut microbial compositional changes that occur in alcoholic and nonalcoholic fatty liver diseases and how this gut microbial dysbiosis and its metabolic products are involved in fatty liver disease pathophysiology. We also summarize the new approaches related to gut microbes that might help in the diagnosis and treatment of fatty liver disease.

Key Words: Fatty liver disease; Alcoholic fatty liver disease; Non-alcoholic fatty liver disease; Gut microbiome; Dysbiosis

Core Tip: In this review, we compare the gut microbial composition in two different fatty liver diseases: Alcoholic fatty liver and nonalcoholic fatty liver. This review enables readers to recognize the gut microbiota compositional differences that occur in these two histopathologically analogous conditions and to explore these gut microbial compositional variations in their research. Additionally, this review will also be helpful in the design of new experiments aiming to develop new diagnostic and/or therapeutic methodologies.

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INTRODUCTION

Significant increases in mortality and morbidity due to chronic fatty liver disease have raised great global health concerns. Alcoholic fatty liver disease (AFLD) and nonalcoholic fatty liver disease (NAFLD) are the most common chronic fatty liver illnesses in the Western world, with prevalences of 6% and 25%, respectively[1], and are the leading causes of liver transplantation[2,3]. Both AFLD and NAFLD start with fat accumulation in the liver, known as benign or simple steatosis, which leads to inflammation identified as steatohepatitis. Advanced disease includes fibrosis and cirrhosis, which can lead to a more severe state, including hepatocellular carcinoma and liver failure, and ultimately can cause death. Only 20% of patients with AFLD and NAFLD develop progressive liver disease[4,5]. In addition to fat accumulation, increased inflammation, and alcohol consumption, other causes, such as an altered gut microbial composition, gut microbial metabolites, or gut barrier function, are associated with the exacerbation of chronic liver disease[6,7].

The recent increase in the understanding of the microbiota and its metabolites has changed the perspectives of various chronic diseases[8]. The human gut microbiota represents a complex ecosystem with various species of microbes that are approximately 1-2 kg in weight in total[9,10]. The gut microbiota maintains homeostasis by interacting with the host and has important functions, including metabolism, digestion, vitamin production, mucosal immune reaction, and the translocation of microbial-associated molecular patterns[11-14]. Importantly, the gut microbiota is known to have a significant role in liver disease progression, but the associated mechanisms are still not fully established.

The liver is the first organ exposed to gut microbial metabolites through portal vein blood. Therefore, the gut microbial community has a vital role in liver homeostasis, and dysbiosis in gut microbial ecology can produce microbial metabolites and components that can have a direct impact on the liver[15-19]. Similarly, the liver also influences gut microbial ecology, particularly in the intestine, through primary bile acids[20-22]. In this way, the liver and gut share a close bidirectional relationship. Interestingly, fecal microbiota transplantation (FMT) studies showed a proof of concept in alcohol-associated and metabolic disease generation and establishment[19,23,24].

Although AFLD and NAFLD have similar histopathological characteristics, they have different etiologies[25]. Thus, gut microbial composition in AFLD and NAFLD could have some commonalities as well as dissimilarities at various classification levels[16,26]. These gut microbial compositional similarities in AFLD and NAFLD could help not only establish common pathophysiological pathways but also increase the chance of finding common treatments. Conversely, gut microbial compositional variations in AFLD and NAFLD could be helpful for the development of specific disease-based signatural gut microbiota profiles. Additionally, these disease-specific gut microbiota profiles could be valuable for the design of gut-microbiota-based therapies such as probiotics[27], synbiotics[28], postbiotics[29] and/or FMT[30] to ameliorate liver disease. These microbial therapeutics also could provide access for developing personalized patient-based treatments to restore liver functions in AFLD and/or NAFLD. AFLD and/or NAFLD-specific gut microbiota profiles could also be useful in the future as diagnostic biomarker tools for the early diagnosis of these diseases.

Considering the importance of the disease-specific gut microbial signature in AFLD and/or NAFLD, herein, we review the gut microbial compositions related to AFLD and/or NAFLD development, especially focusing on the relationship of these compositions with the progression of both diseases, particularly in humans. We also explicitly focus on the microbial signature pertaining to AFLD and/or NAFLD and common microbes in both fatty liver conditions. This review also helps in the understanding of the deep association between the gut microbiota and fatty liver diseases, which can also be considered microbiota-associated fatty liver diseases.

GUT MICROBIAL COMMUNITY EUBIOSIS

The gut microbiota is an endogenous ecosystem that coevolves with the host as a symbiotic organ and regulates the normal physiological functions of the gut, such as food digestion and nutrient absorption, and provides essential micronutrients to the host[31]. The gut microbial ecosystem maintains a balance between the microbial species living inside the gut known as "eubiosis" that is crucial for good health.

Microbial colonization in the gastrointestinal tract starts immediately after birth and is dominated by the *Bifidobacterium* genus, and a decline in this dominance is observed in the first year of life[32]. The infant gut microbiota is changeable, as this microbial colonization is affected by multiple external factors, such as the mode of delivery, medications, nourishment[33,34], age, genetic background, and cultural/geographic influence[32,35,36]. Similarly, breastfed infants have a less diverse gut microbiota than formula-fed infants, which is the best possible explanation for the difference in gut microbial composition between United States infants and non-United States infants, as United States infants have 28 operational taxonomic units dominated by the *Prevotella* genus[32]. As children start consuming solid foods, the gut microbiota becomes more diverse and starts stabilizing[32,35,37,38]. Fecal samples collected from different geographical regions showed that the gut microbiota composition took shape toward an adult-like configuration until 3 years of age[32], after which the gut composition became more persistent[39].

Primarily, the *Firmicutes* and *Bacteroidetes* phyla dominated the adult human gut microbial composition, and *Actinobacteria*, *Proteobacteria* and *Verrucomicrobia* were found in lesser abundance. Fecal metagenomic analysis from 4 different countries identified well-classified robust gut microbial communities, named enterotypes, represented through multiple numbers of 3 genera: *Prevotella*, *Ruminococcus* and *Bacteroides*[40], and this classification of enterotype was independent of nationality, age, body mass index (BMI), and sex. However, this enterotype-based classification remains a topic of debate because external factors such as diet are considered primary regulators of gut microbiota composition and functions[41,42] and fail to be identified in healthy and elderly individuals[43]. In addition to diet, aging is also a considerable factor that changes the gut microbiota composition. Bacteria belonging to the *Bacteroidaceae*, *Lachnospiraceae* and *Ruminococcaceae* families are negatively correlated with aging independent of geographical region, lifestyle, and dietary habits[44-46]. Moreover, healthy aging showed increased microbial richness and higher numbers of *Bifidobacterium*, *Oscillospira*, *Akkermansia*, and *Christensenellaceae*[45]. Emerging metagenomic empirical evidence suggests that a healthier gut always has a more diverse microbiota population and that a healthy gut is essential to maintain human health[47,48].

GUT MICROBIAL COMMUNITY DYSBIOSIS

A change or alteration in gut microbial composition, which can be related to diseased conditions, is termed "dysbiosis"[49]. Gut microbiota composition varies from birth to death[50] and is influenced by various environmental factors[51-54]. Gut microbial dysbiosis also has a close connection with AFLD and NAFLD.

Gut microbiota alteration in AFLD

Persistent high intake of ethanol is the root cause of AFLD[55], as it disrupts the multilayered intestinal defense system involving physical, immunological, and humoral components[56]. Normally, the liver enzyme alcohol dehydrogenase and the ethanol-oxidizing system convert ethanol to acetaldehyde, which is toxic to hepatic cells. Acetaldehyde is immediately metabolized to acetate, released into the bloodstream, and used as a biological fuel by cells for energy production. In a persistently elevated ethanol consumption state, the accumulation of toxic acetaldehyde is increased in the liver, which leads to the production of highly reactive molecules that generate an oxidative stress milieu and contribute to liver injuries[16]. An increase in the flow of ethanol in the liver alters SIRT1 signaling and initiates fat accumulation in hepatocytes[57]. Ethanol reduces SIRT1 expression in the liver, which leads to the fat accumulation in liver cells by disrupting multiple SIRT1-dependent transcription factors and cofactors, such as peroxisome proliferator-activated receptor α, PPARγ coactivator-1α, AMP-activated kinase, lipin-1, β-catenin, forkhead transcription factor O1, sterol regulatory element-binding protein 1, nuclear factor activated T cells c4, and nuclear transcription factor-κB[57-59]. Ethanol facilitates the inhibition of SIRT1, which leads to various signaling network disruptions that increase the accumulation of fat in hepatocytes by decreasing β-oxidation and lipolysis, boosting lipogenesis and inflammation, and collectively leading to AFLD. Recently, human and animal models suggested that even a small intake of alcohol can harm intestinal barrier integrity and raise microbial byproduct levels in the circulation[60,61]. Moreover, there is adequate experimental evidence proving that the interrelationship between alterations in the intestinal microbiota and alcohol abuse and acute and

chronic alcohol exposure is primarily responsible for gut microbiota dysbiosis and can lead to AFLD through various pathways, as shown in Figure 1[62]. Animal model-based studies explain that alcohol-induced microbial dysbiosis in the intestine changes homeostasis in the gut-liver axis and that this altered intestinal microbiota plays a crucial role as a mediator in the production of the many negative effects of alcohol.

Animal studies have shown that 3 weeks of alcohol exposure causes a 'leaky gut', which increases the number of *Bacteroidetes* and *Verrucomicrobia* and decreases the growth of bacteria with anti-inflammatory activity, such as *Firmicutes* (genera such as *Lactobacillus, Lactococcus, Leuconostoc* and *Pediococcus*), in the cecum[63]. Another rodent alcohol-based model showed that changes in intestinal permeability associated with intestinal microbial alterations are related to the decreased expression of hypoxia-induced factor 1α. These studies showed a relative increase in *Actinobacteria* and *Proteobacteria* and a decline in the *Firmicutes* phylum. Moreover, these changes were restored by treatment with probiotic *Lactobacillus rhamnosus* (*L. rhamnosus*) GG therapy[64-67].

Interestingly, gnotobiotic animals have become an imperative alcoholic model to explore the relationship between the gut and the liver. A comparative study of gnotobiotic and wild-type rats showed less proinflammatory cytokine release and inflammation in gnotobiotic rats than in wild-type rats when treated with alcohol for one week. Moreover, fecal transplantation from alcohol-fed wild-type rats in gnotobiotic animals increased hepatic and intestinal inflammation, indicating the involvement of the intestinal microbiota in AFLD[68]. Chronic alcohol intake also changes the intestinal mucus composition, and mucin knockout animals have less bacterial overgrowth, minimal translocation of the bacteria and reduced intestinal inflammation when administered alcohol[69]. In other studies, the bacterial species *Akkermansia muciniphila* (*A. muciniphila*) from the *Verrucomicrobia* phylum showed potential anti-inflammatory properties in AFLD[70], and the depletion of *A. muciniphila* species was noticed in alcoholic animal models[71,72]. *A. muciniphila* improves intestinal markers such as gut barrier function and mucus thickness and diminishes the liver damage produced by alcohol[73]. Cumulatively, animal studies strongly indicate that alcohol intake considerably changes the intestinal microbial composition (as shown in Table 1), which can be responsible for producing early-onset AFLD by inducing proinflammatory changes, translocating the bacteria and bacterial material by reducing mucus thickness and increasing intestinal permeability.

Likewise, human studies also support the close association between alcohol intake and intestinal microbial dysbiosis in the onset of AFLD, similar to animal models. Prolonged alcohol intake markedly decreases the *Bacteroidetes* population and increases *Proteobacteria*, which leads to compromised intestinal permeability and an increase in the level of bacterial materials such lipopolysaccharides (LPS) and endotoxins in the hepatic circulation and ultimately causes liver injuries[74]. The families *Ruminococcaceae* and *Lachnospiraceae* and their ratio are considered to be protective, whereas *Enterobacteriaceae* to *Bacteroidaceae* and their ratio are believed to be potential pathobionts in the intestine, especially in those with a liver disease with an alcoholic etiology. Therefore, the overgrowth of potentially pathogenic species in the gut in chronic alcohol abuse conditions is related to the initiation of liver injuries[74-76]. The effect of these microbial alterations in the gut is not yet completely understood. However, the administration of *L. rhamnosus* GG improves the *Lachnospiraceae* population and limits the growth of *Enterobacteriaceae,* which leads to a decline in proinflammatory cytokines[77]. A reduction in *A. muciniphila* was observed in patients with alcoholic steatohepatitis compared with healthy controls, and this decline in *A. muciniphila* seems to be related to the severity of liver injuries[73]. Additionally, *A. muciniphila* was considered a health-boosting bacterial species along with *Bifidobacterium* spp., *Roseburia hominis*, and *Feacalibacterium prausnitzii*[78]. However, human and animal empirical data hinted that alcohol-induced gut microbial dysbiosis, especially ethanol consumption, but that some alcoholic beverages, such as red wine, could exert a positive impact on gut microbial ecology. A human crossover study demonstrated that red wine consumption increased the number of *Bacteroides, Enterococcus*, and *Bifidobacterium*[79].

Alterations in the gut microbiota due to persistent alcoholic intake are not restricted to bacterial species; the fungal composition also changes. Remarkably, alcoholic liver disease (ALD) patients have a high risk of bacterial infection, and patients with advanced cirrhosis are more prone to fungal infections. Moreover, fungal infections increased the mortality rate in cirrhosis and alcoholic hepatitis patients[80-82]. In an alcoholic murine model, increased fungal growth, particularly *Candida* spp., was observed and was related to an increase in liver damage[83]. The study results showed that liver inflammation was induced by β-glucan, which is a fungal cell wall component. β-Glucan binds with Kupffer cell C-type lectin-like receptor and

Table 1 Representative studies presenting gut microbial dysbiosis in alcoholic fatty liver disease

Ref.	Sequencing method	Overgrown microbes	Depleted microbes	Model
Yan *et al*[63] (2011)	Pyrosequencing	↑*Bacteroidales*	↓*Lactococcus*	Murine
		↑*Bacteroides*	↓*Pediococcus*	
		↑*Porphyromonadaceae*	↓*Lactobacillus*	
			↓*Leuconostoc*	
Otterson *et al*[64] (2013)	Pyrosequencing	↑*Corynebacterium*	↓*Bacteroides*	Murine
		↑*Alcaligenes*	↓*Tannerella*	
		↑*Listeria*	↓*unclassified Lachnospiraceae*	
		↑*Acetivibrio*	↓*undefined Ruminococcaceae*	
		↑*Allobaculum*		
Lowe *et al*[71] (2017)	16S rDNA	↑*Actinobacteria*	↓*Tenericutes*	Murine
		↑*Eubacteriaceae*	↓*Verrucomicrobia*	
			↓*Lachnospiraceae*	
			↓*Moraxellaceae*	
			↓*Akkermansia*	
Grander *et al*[73] (2018)	Illumina MiSeq	↑*Olsenella*	↓*Acinetobacter*	Murine
		↑*Eubacterium*	↓*Anaerotruncus*	
		↑*Acetivibrio*	↓*Akkermansia*	
			↓*Blautia*	
Kakiyama *et al*[74] (2013)	Pyrosequencing	↑*Enterobacteriaceae*	↓*Blautia*	Human
		↑*Veillonellaceae*	↓*Ruminococcaceae*	
			↓*Lachnospiraceae*	
			↓*Rikenellaceae*	
Bajaj *et al*[75] (2014)	Pyrosequencing	↑*Enterococcaeae*	↓*Clostridiales XIV*	Human
		↑*Staphylococcaceae*	↓*Ruminococcaceae*	
		↑*Enterobacteriaceae*	↓*Lachnospiraceae*	
Yang *et al*[83] (2017)	Illumina MiSeq	↑*Candida* spp.	↓*Epicoccum*	Human
		↑*Candida albicans*	↓*Unclassified fungi*	
		↑*Candida dubliniensis*	↓*Galactomyces*	
			↓*Debaryomyces*	
Ferrere *et al*[144] (2017)	Illumina MiSeq	↑*Actinobacteria*	↓*Bacteroidetes*	Murine
		↑*Firmicutes*	↓*Proteobacteria*	
		↑*Coriobacteriaceae*		
		↑*Odoribacteriacea*		
		↑*Clostridiaceae*		
		↑*Dorea*		
Wang *et al*[145] (2019)	Illumina MiSeq	↑*Verrucomicrobia*	↓*Bacteroidetes*	Monkey
		↑*Proteobacteria*	↓*Cytophagales*	
		↑*Optitutus*	↓*Flavobacteriales*	
		↑*Botrytis*	↓*Sphingobacteriales*	
		↑*Sporothrix*	↓*Lactobacillales*	
			↓*Nitrosomonadales*	

			↓*Opitutales*	
			↓*Helotiales*	
			↓*Ophiostomatales*	
Zhong *et al*[146] (2021)	Illumina MiSeq	↑*Proteobacteria*	↓*Ruminococcaceae*	Human
		↑*Fusobacteria*	↓*Faecalibacterium*	
		↑*Fusobacteriaceae*	↓*Lachnospira*	
		↑*Enterobacteriaceae*	↓*Agathobacter*	
		↑*Burkholderiaceae*	↓*Ruminococcus*	
		↑*Fusobacterium*		
		↑*Escherichia-Shigella*		

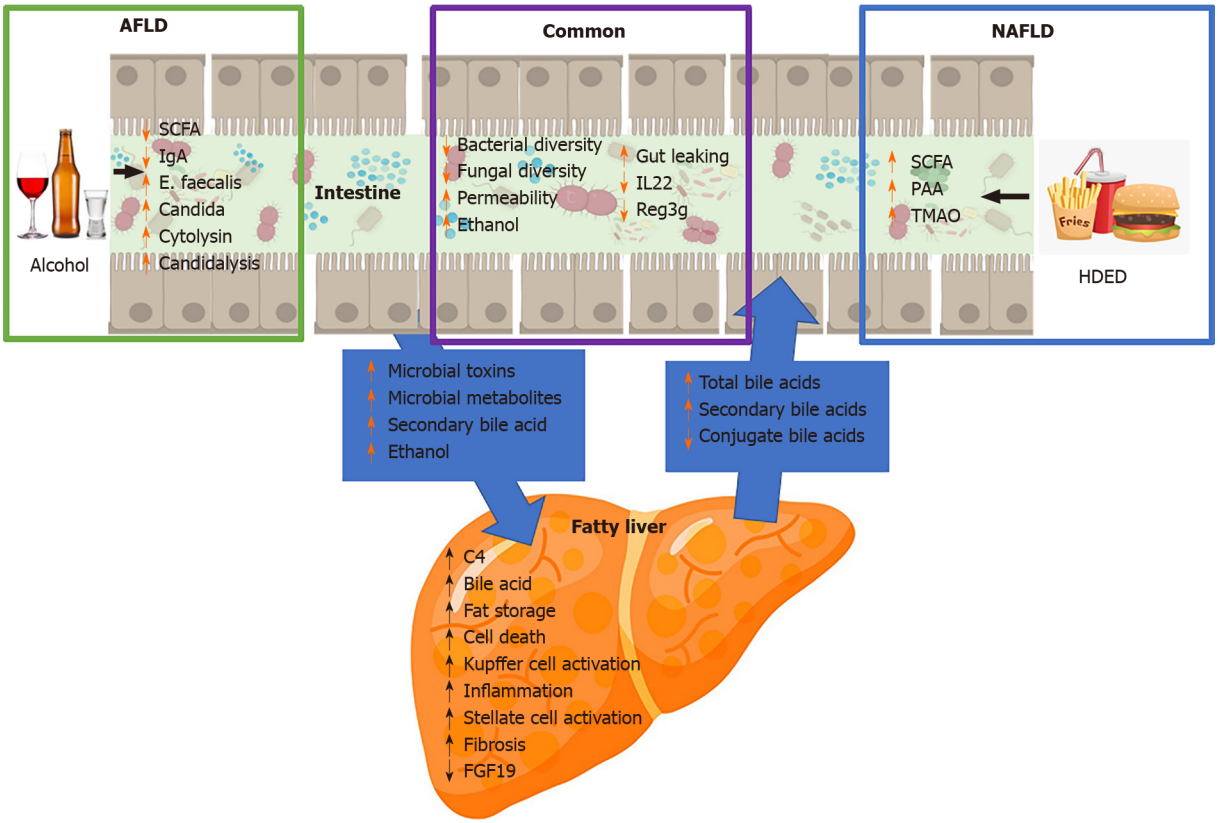

Figure 1 Gut microbiota role in alcoholic fatty liver disease and non-alcoholic fatty liver disease pathogenesis. Intestinal microbes have the potential relationship with fatty liver disease progression. Regular intake of alcohol and overnutrition altered the gut microbial composition which influence the various pathways and induce the liver injuries and produce the alcoholic fatty liver disease (AFLD) and non-alcoholic fatty liver disease (NAFLD). There are some common pathways found in both AFLD and NAFLD diseases (in the purple box) and others are specifically related to a particular disease. AFLD: Alcoholic fatty liver disease; NAFLD: Non-alcoholic fatty liver disease; HDED: High dense energy diet; SCFA: Short chain fatty acids; IgA: Immunoglobulin A; IL22: Interleukin 22; Reg3g: Regenerating islet-derived protein 3 gamma; C4: Precursor 7α-hydroxy-4-cholesten-3-one; FGF19: Fibroblast growth factor 19; PAA: Phenylacetic acid; TMAO: Trimethylamine N-oxide.

upregulates IL-1β. Similarly, ALD patients also showed an increased immune response to intestinal fungi compared to healthy controls. These findings suggest that the composition of nonbacterial gut microbes, such fungi, can also affect AFLD generation, progression, and final outcomes in patients with AFLD.

Gut microbiota alterations in NAFLD

The gut microbiota exacerbates and/or alleviates NAFLD conditions through several pathways (Figure 1). Animal and human studies have presented a causal involvement of the gut microbiota in NAFLD establishment[84-86] and its severity[87-89]; however, a robust correlation between the gut microbiota and NAFLD advancement has not yet been established. Differences in gut microbial composition at various hierarchical

levels have been recorded in NAFLD patients compared with healthy controls[90,91]. In NAFLD, alterations in the gut microbial community have been shown to start at the phylum level, where increased Proteobacteria have been reported in many studies[90,92,93]. Likewise, altered composition has also been observed at the family level, where an overgrowth of *Enterobacteriaceae*[84,92] and suppression of *Rikenellaceae*[84,94] and *Ruminococcaceae*[91,92,95] have been reported. Moreover, genera such as *Escherichia*[84,90], *Dorea*[94,95], and *Peptoniphilus*[84,94] were overpopulated, and *Anaerosporobacter* [91], *Coprococcus*[84,90,91], *Eubacterium*[84,90], *Faecalibacterium*[84] and *Prevotella*[90,96] were less populated.

In a comparative study, Wang *et al*[91] showed a higher proportion of gram-negative bacteria, including *Bacteroidetes*, and decreased *Firmicutes* in NAFLD patients when the gut microbiota was compared with lean healthy subjects. The decline in *Firmicutes* is associated with short-chain fatty acid (SCFA)-producing bacteria such as *Lactobacillaceae*, *Lachnospiraceae*, and *Ruminococcaceae*. An overgrowth of gram-negative bacterial species was seen in children with NAFLD, with an increased ratio of Gammaproteobacteria and Epsilonproteobacteria compared to their obese and lean counterparts[97]. In contrast, bacteria from the *Firmicutes* phylum (such as *Lactobacillus*, *Roseburia, Dorea*, and *Robinsoniella*) were found to be increased in the population of NAFLD patients in another study[95]. However, contradictory results were reported in other studies that showed increases in *Dorea* and *Ruminococcus* in NAFLD patients[94,98]. Variability in gut microbial composition was observed with different levels of NAFLD severity. Bacteria belonging to *Firmicutes* were more dominant in moderate NAFLD, while the prevalence of *Proteobacteria* was noted to be associated with the severity of disease, as in fibrosis[93]. The bacterial species that were dominant in mild NAFLD compared to severe NAFLD conditions were *Eubacterium rectale* and *Ruminococcus obeum*[93]. These human study results reflect the conflicting gut microbiota composition in NAFLD, which needs to be evaluated further by implementing a greater number of NAFLD patient-based gut microbial compositional studies.

The gut microbial composition was also assessed in severe NAFLD conditions such as fibrosis and/or in nonalcoholic steatohepatitis (NASH) to examine the functional role of gut microbial dysbiosis in fibrosis progression. The results of these comparative studies exhibited a decline in gram-negative bacterial abundance. Comparative analysis of the gut microbiota between individuals with severe NAFLD and healthy or less severe NAFLD conditions showed a decrease in the *Fusobacteria* phylum population and an increase in *Enterobacteriaceae* family bacteria such as the genera *Shigella*, *Ruminococcin* and *Bacteroides*[92,96]. Similarly, gram-positive bacteria from the *Firmicutes* phylum, the family *Prevotellaceae* and the genus *Prevotella* also showed increases in severe NAFLD conditions[93]. Recently, a study presented a significant alteration based on fibrosis severity in nonobese patients but not in obese patients, where *Ruminococcaceae* and *Veillonellaceae* were the leading microbes related to fibrosis severity in nonobese subjects[99]. Interestingly, oral microbes, including *Streptococcus* [76,100,101], *Veillonella*[91,100], and *Prevotella*[100,102], are discriminatory microbes for advanced NAFLD conditions (especially cirrhosis). Additionally, some microbe representations were constant in NAFLD patients compared with healthy individuals, but some showed conflicting tendencies[103]. Conclusively, microbial composition in NAFLD patients presented a drastic shift in taxonomic group composition by showing an increased ratio of pathogenic microbes and a decline in microbes that are considered metabolically beneficial microbes. Subsequently, this compositional shift in microbial composition might be responsible for NAFLD pathogenesis and exacerbate the severity of the disease from simple steatosis to NASH and from NASH to cirrhosis. Summarized information on gut microbial dysbiosis in NAFLD is listed in Table 2, which provides details regarding the increased and decreased populations of bacteria in NAFLD.

The gut microbiota composition in NAFLD presented a considerable contradiction, where some microbial taxa showed variability in their occurrence, as shown in Table 2. The underlying reasoning behind these contradictory compositional variabilities might be related to study design, clinical study end points, result interpretation, *etc*. Moreover, these underlying reasons could be fundamental restraints in the process of establishing a robust relationship between the gut and NAFLD. Thus, to determine the pathophysiological association between the gut and liver, these fundamental limitations should be resolved.

Table 2 Representative studies presenting gut microbial dysbiosis in non-alcoholic fatty liver disease

Ref.	Sequencing method	Overgrown microbes	Depleted microbes	Model
Michail *et al*[97] (2015)	Ion torrent	↑*Actinobacteria*	↓*Erysipelotrichia*	Human
		↑*Prevotella*	↓*Alphaproteobacteria*	
		↑*Clostridia*	↓*Verrucomicrobia*	
		↑*Fusobacteria*		
		↑*Epsilonproteobacteria*		
		↑*Gammaproteobacteria*		
Wang *et al*[91] (2016)	Pyrosequencing	↑*Bacteroidaceae*	↓*Lachnospiraceae*	Human
		↑*Prevotellaceae*	↓*Ruminococcaceae*	
			↓*Lactobacillaceae*	
Raman *et al*[95] (2013)	Pyrosequencing	↑*Alphaproteobacteria*	↓*Ruminococcaceae*	Human
		↑*Lactobacillaceae*	↓*Oscillibacter*	
		↑*Lachnospiraceae*	↓*Porphyromonadaceae*	
		↑*Veillonellaceae*		
Chierico *et al*[94] (2017)	Pyrosequencing	↑*Actinobacteria*	↓*Bacteroidetes*	Human
		↑*Bradyrhizobium*	↓*Oscillospira*	
		↑*Anaerococcus*	↓*Rikenellaceae*	
		↑*Peptoniphilus*		
		↑*Propionibacterium acnes*		
		↑*Dorea*		
		↑*Ruminococcus*		
Hoyles *et al*[90] (2018)	Shotgun	↑*Proteobacteria*	↓*Firmicutes*	Human
		↑*Actinobacteria*	↓*Euryarchaeota*	
		↑*Verrucomicrobia*		
Shen *et al*[92] (2017)	16S rDNA	↑*Proteobacteria*	↓*Bacteroidetes*	Human
		↑*Fusobacteria*	↓*Prevotellaceae*	
		↑*Lachnospiraceae*	↓*Ruminococcaceae*	
		↑*Enterobacteriaceae*	↓*Prevotella*	
		↑*Erysipelotrichaceae*		
		↑*Streptococcaceae*		
		↑*Escherichia Shigella*		
Zhu *et al*[84] (2013)	Pyrosequencing	↑*Bacteroidetes*	↓*Actinobacteria*	Human
		↑*Proteobacteria*	↓*Bifidobacteriaceae*	
		↑*Alcaligenaceae*	↓*Clostridiales family XI*	
		↑*Campylobacteraceae*	↓*Lachnospiraceae*	
		↑*Enterobacteriaceae*		
Chierico *et al*[94] (2017)	Pyrosequencing	↑*Coriobacteriaceae*	↓*Porphyromonadaceae*	Human
		↑*Bacteroidaceae*	↓*Rikenellaceae*	
Minicis *et al*[147] (2014)	Pyrosequencing	↑*Firmicutes*	↓*Bacteroidetes*	Murine
		↑*Actinobacteria*		
Lee *et al*[148] (2020)	16S rDNA	↑*Firmicutes*	↓*Bacteroidetes*	Murine
		↑*Deferribacters*	↓*Lactobacillus murinus*	

	↑*Helicobacter japonicus*
	↑*Mucispirillum schaedleri*
	↑*Flintibacter butyricus*

GUT ROLE IN FATTY LIVER

Trillions of microorganisms reside in the gut, including bacteria, archaea, fungi, and viruses, but liver-disease-related research primarily targets the bacterial community, which includes more than 10 bacterial phyla[104,105]. The gut microbiota includes more than 3 million genes collectively, in comparison to the 23000 genes of the human genome; however, human cells and gut bacterial cells are roughly equal in number [106]. This gut microbial genetic material certainly has a defining role in human pathophysiology through multiple mechanisms, especially in liver disease, due to its close relationship with the gut[6,107,108].

Gut microbial dysbiosis

Generally, the gut microbiota starts to be shaped at birth and becomes stable in early childhood. This balanced and stable gut microbiota acquires a unique quotient for each microbial species in a healthy state[38]. As discussed above, gut microbial dysbiosis, defined by diminished microbial diversity and distorted gut microbial composition, is observed in both AFLD and NAFLD patients compared to healthy controls[18,103]. Alcohol abuse and overnutrition deplete several bacterial species and shift the microbial composition toward gram-negative bacteria. Microbial species depleted in liver diseases are considered beneficial microbes, and overgrown microbial species are associated with liver pathophysiology and known as pathobionts[109]. Alcohol consumption is linked with diminished fungal diversity generated by an increased number of *Candida* species[83,110,111]. Moreover, gut viruses are the most abundant gut microbes; nonetheless, they have not yet been characterized in liver disease.

This gut compositional proclivity toward gram-negative bacterial species influences multiple pathways directly or indirectly that contribute to AFLD and NAFLD establishment[16,91,92]. The distorted gut microbiota alters various metabolic processes, such as bile acids, short-chain fatty acids, and energy harvesting, which leads to the initiation of fatty liver disease[112]. The distorted gut microbiota also damages gut barrier function, through which microbes and their metabolites can translocate and activate the inflammasome in the liver and cause fatty liver[112]. The detailed role of the gut microbiota in fatty liver pathogenesis is presented in Figure 1.

Leaky gut syndrome

In the intestine, there are multiple layers of barriers, including physical, biochemical, and immunological barriers, that restrict the translocation of microbes and their products. Chronic alcohol abuse causes gut barrier dysfunction by altering the gut microbial composition[113]. Thus, pathogen-associated molecular patterns, such as LPS, are able to translocate from the lumen of the intestine to the liver *via* the portal vein and are recognized by inflammasomes such as Toll-like receptors in the liver to stimulate hepatic inflammation, which leads to hepatocyte injuries and liver fibrosis [112]. Likewise, similar pathophysiological pathways are involved in NAFLD progression, but gut disruption and inflammation are stimulated by dietary factors other than alcohol[112]. Other types of inflammasomes, including NOD-like receptor protein 3 (NLRP3), also respond to LPS and cause liver inflammation. The activation of NLRP3 triggers the caspase 1 pathway and produces interleukin-1β and several other inflammatory cytokines, which cause apoptosis and fibrosis. Higher levels of inflammasomes such NLRP3 and others were found in severe fatty liver conditions induced by both alcohol and overnutrition[114,115]. The translocation of the microbial metabolites from the intestine to the liver because of the dysfunction of intestinal barriers and increased intestinal permeability could be the contributing factor for AFLD and NAFLD, but more studies are required to establish robust associations.

Bile acid dysregulation

Bile acid synthesis and secretion are essential functions performed by liver cells. Importantly, bile acids not only are crucial for dietary fat emulsification but also act as ligands for nuclear and G-protein coupled receptors and regulate various metabolic functions, including glucose and fat metabolism[116]. Therefore, smooth regulation of bile acids is important for maintaining a healthy metabolic profile, but gut microbial

dysbiosis is associated with bile acid dysregulation and is associated with fatty liver pathogenesis through metabolites[117].

Normally, conjugated bile acids are released from hepatocytes, carried by the biliary duct, and secreted into the intestine. After lipid emulsification, the remaining bile acids (primary, hydrophilic, and conjugated) are reabsorbed in the terminal ileum. Bile acid secretion from hepatocytes is primarily regulated by the farnesoid X receptor (FXR) negative feedback mechanism[118]. The release of primary bile acids in the intestine activates intestinal FXR, which precedes the transcription of fibroblast growth factor 19 (FGF19). The ileal hormone FGF19 is carried to the liver *via* the portal vein, where FGF19 suppresses CYP7A1 expression and controls bile acid secretion[119]. The disruption of bile acid homeostasis is the leading cause of fatty liver[119-121].

The change in the gut microbial composition induced by alcohol abuse and a high intake of energy-dense food cause the dysregulation of the bile acid system and instigate fatty liver diseases (AFLD and NAFLD)[122,123]. In AFLD and NAFLD, pathobionts increased in number and were responsible for the conversion of secondary bile acids from primary bile acids and reduced FXR signaling. This downregulation of FXR expression increased insulin resistance and altered glucose and lipid metabolism, which are the key regulatory pathways in AFLD and NAFLD generation. Moreover, AFLD and NAFLD patients showed higher levels of secondary bile acids than primary bile acids in feces and blood. Similarly, the dysregulation of FXR signaling and FGF19 is increased with the severity of the disease in both AFLD and NAFLD[124-127]. These outcomes suggest that FXR and bile acid compositional dysregulation are the metabolic features of AFLD and NAFLD and that the dysregulation of both metabolic factors (FXR and bile acids) increases with the severity of AFLD and NAFLD.

Although gut bacteria control bile acid metabolism, the involvement of intestinal bacteria or other gut microbes (including archaea, fungi, and viruses) in bile acid dysregulation in fatty liver patients is not completely understood, and more experimental evidence is required to fill the fundamental gaps.

Short-chain fatty acid dysregulation

Nondigestible carbohydrates in food are fermented by gut bacteria, and SCFAs are produced. Butyrate, acetate, and propionate are the most abundant SCFAs found in the intestine. SCFAs have many beneficial effects, including being used as an energy source by colonocytes and enterocytes, maintaining gut barrier function, suppressing hepatic cell proliferation, reducing inflammation, and lowering food intake by increasing satiety[128]. Considering the beneficial function of SCFAs in regulating metabolic pathways, their level in the body is crucial to maintain good health.

Chronic alcohol abuse is related to reduced SCFA levels in the stool[129]. The SCFA concentration and SCFA-producing bacterial concentration are decreased in the feces of alcoholic hepatitis patients[109]. The low circulatory butyrate level is also associated with serum endotoxin, inflammation, and more advanced liver diseases[130]. In contrast, a higher level of SCFAs and an increased number of SCFA-producing bacteria were found in NASH patients; however, the study population was small[131]. Additionally, increasing levels of SCFAs are related to immune regulation and NAFLD progression[131]. Higher fecal concentrations of propionate and butyrate were observed in mild to severe NAFLD patients, whereas higher fecal concentrations of acetate and formate were found in advanced fibrosis patients[132,133]. There is an insufficient amount of empirical proof to establish a concrete relationship between SCFAs and fatty liver diseases, and more studies are required to determine the association between SCFAs and fatty liver diseases.

Endogenous ethanol production

Microbial fermentation of dietary sugar increases the endogenous alcohol level in pediatric NASH patients[84]. Recently, a *Klebsiella pneumonia* strain was identified in a NASH patient fecal sample and was responsible for producing endogenous ethanol and increasing the blood ethanol level without alcohol consumption[134]. FMT from NASH patients to animals results in liver damage, and the elimination of alcohol-producing *Klebsiella pneumoniae* strains reduces liver damage. Additionally, NASH patient weight reduction was also related to a reduced ability to produce ethanol in the gut microbiome[134]. Another study focusing on comparing the gut microbial profile in pediatric NAFLD patients showed higher circulatory ethanol levels in diseased patients, which was related to the higher number of *Prevotella* and Gammaproteobacteria[97]. The results from another study showed that a higher circulatory level of ethanol in NAFLD patients could be the end result of ethanol dehydrogenase activity in insulin-dependent impairment conditions[135]. Thus, gut

microbial dysbiosis, which can lead to an increasing level of endogenous ethanol in the body, could be an underlying cause of NAFLD and could be responsible for producing the same histopathological characteristics as AFLD. However, some inconsistencies in the results were observed, and establishing an association between this endogenous ethanol phenomenon and NAFLD generation needs more experimental proof[16,136,137].

Gut microbial virulence factors

Virulence factors are microbial proteins and peptides that help pathobionts colonize and are associated with disease generation. A recent study recognized that cytolysin, a protein secreted (exotoxin) by *E. faecalis*, damages hepatocytes and is highly associated with increased mortality in alcoholic hepatitis patients[18,138]. Unfortunately, few studies have shown any further toxins or other proteins related to gut microbiota that can be associated with liver disease.

FUTURE PERSPECTIVES

Fatty liver disease (both AFLD and NAFLD) is intricately linked with the gut microbiota and its dysbiosis. Recent advancements in gut microbiome-based metagenomics studies related to liver disease have shown that an increase in and/or depletion of the specific microbial content could contribute greatly to liver injuries and is possibly the key regulator in fatty liver disease establishment and progression[112]. Growing evidence regarding the role of the gut microbiota in fatty liver disease generation and progression turns this noncommunicable disease into a communicable disease[139]. Therefore, targeting the gut microbiota through various techniques may be an approach for the management of liver disease in the future.

Prognostic and/or diagnostic biomarkers

As discussed above, the microbial composition in fatty liver diseases is different from that in healthy individuals. Gut microbes themselves or their microbial metabolites might be useful as prognostic and/or diagnostic tools for the early detection of fatty liver conditions. Generally, constant alcohol intake of more than 60 g per day leads to alcoholic hepatic steatosis, which also presents with higher levels of liver enzymes, such aspartate aminotransferase (AST) and alanine aminotransferase (ALT), and in NAFLD, daily alcohol intake is approximately 30 g per day. Typically, two to three times higher serum AST levels have been observed compared to serum ALT due to alcoholic liver injuries. Patients with AFLD also have higher serum gamma-glutamyl-transpeptidase levels[140]. Similarly, NAFLD also has noninvasive biomarker detection protocols, such as the NAFLD fibrosis score (including age, BMI, the AST-to-ALT ratio, impaired fasting glucose and diabetes, albumin and platelets), FIB-4 index (including age, ALT, AST, and platelets), and FibroTest (including total bilirubin, α2-macroglobulin, γ-glutamyl transferase, haptoglobin, and apolipoprotein A1 corrected for sex and age)[141]. These are the common diagnostic parameters used for AFLD and NAFLD diagnosis. However, there is a lack of conclusive biomarkers that can help in the early diagnosis of hepatic steatosis, and the repertoire of gut microbes and their metabolite profiles might help to fill this gap. Interestingly, a set of gut bacteria combined with age and BMI was used to identify liver disease, and a much more accurate diagnosis was able to be made with its use in patients with advanced fibrosis [93]. The gut microbes used as a marker in this study were first identified from NAFLD and advanced fibrosis patients *via* metagenomics analysis and then further used for diagnostic purposes[93]. In a separate study, the combination of metagenomic signature microbes with age and serum albumin levels precisely identified cirrhosis in patients with geographically different origins. Additionally, adding serum aspartate aminotransferase levels to these diagnostic tools increased diagnostic efficacy even in the early stage of fibrosis[142]. In other studies, gut-microbe-derived metabolites showed great potential as diagnostic markers for fatty liver diseases and other liver conditions[18,90,143].

Although gut microbes and their metabolites have the potential to be noninvasive prognostic and/or diagnostic tools for fatty liver and other liver diseases, larger population-based studies are still required to eliminate constraints related to geographical factors, ethnicity, and dietary factors. Further studies are also warranted to compare the diagnostic ability of gut microbes and their metabolites with contemporary in-use investigative practices such as biopsy and image-based approaches.

CONCLUSION

Gut microbiota is crucial in fatty liver diseases (in both AFLD and NAFLD), thus relevance of fatty liver disease specific gut microbial signatures should be further explored in longitudinal human studies. Where, a team of clinician and researchers can prospectively correlate the deterioration of liver with the alteration in the gut microbiota community. Merging fatty liver disease specific gut microbiota with microbial derived metabolites can be helpful in the future to diagnose and treat the AFLD and NAFLD patients.

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OPINION REVIEW

Surveillance for hepatocellular carcinoma at the community level: Easier said than done

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Abstract

Surveillance for hepatocellular carcinoma (HCC) in high-risk patients with semiannual ultrasound examinations is advocated by all international guidelines. However, as long as the identification of the population to be screened and the surveillance programs are not well implemented, the real-life impact of HCC surveillance in reducing mortality for HCC cannot be known. We propose a new approach that promotes the identification of cirrhotic patients by primary care physicians (PCPs) and referral of patients to the hepatologist for surveillance. Surveillance should be incorporated, when feasible, in a hub and spoke model of comprehensive hepatology care. Training PCPs to identify cirrhotic patients and performing surveillance in a subspecialist setting are equally important to improve the effectiveness of real-life surveillance and to decrease HCC mortality over time.

Key Words: Hepatocellular carcinoma; Cirrhosis; Ultrasound surveillance; Primary health care

Core Tip: Ultrasound surveillance for hepatocellular carcinoma is recommended by all official guidelines for high-risk patients, but its uptake at the community level is low. We discuss the obstacles hampering its implementation and propose a hub and spoke

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model network for the effective delivery of surveillance in the real world setting.

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INTRODUCTION

Primary liver cancer is the third leading cause of cancer related mortality worldwide [1]. It includes hepatocellular carcinoma (HCC), comprising of 75%-85% cases, intrahepatic cholangiocarcinoma (10%-15%), and other rare types[1]. The HCC death toll is the highest in East Asia and Africa, with an annual mortality rate greater than 20/100000 people[2]. Mortality is lower in Western Europe and in the United States (12-12.7/100000) but it has increased rapidly in the last decade[3]. On the other hand, Eastern Europe, Scandinavia, and India have the lowest mortality rate (less than 4/100.000). The great majority of HCC cases in the Western world are diagnosed in cirrhotic patients, while in Asia and Central Africa the most significant risk factor for HCC is chronic hepatitis B virus infection with or without cirrhosis. Therefore, patients with those risk factors (cirrhosis and/or chronic hepatitis B infection) should undergo surveillance, with the goal of identifying the tumor at an early stage when curative treatments are feasible. A randomized study of chronic hepatitis B patients in China [4], several longitudinal cohort studies in patients affected by cirrhosis of any etiology [5-7], and a meta-analysis[8] have demonstrated the benefit of ultrasound surveillance (US) in improving survival. In addition, several studies have shown that US is cost effective for the surveillance of patients affected by chronic hepatitis B and of cirrhotic patients. Indeed, HCC incidence in those populations is 0.2% and 1.5% per year respectively, which is considered acceptable to define cost effectiveness[9-11]. On the other hand, the effectiveness of US is still uncertain in young Non-Asian inactive carriers of hepatitis B surface antigen (HBsAg), in patients with chronic hepatitis C infection with advanced fibrosis, and by those with nonalcoholic fatty liver disease (NAFLD) without cirrhosis. For those reasons, the three most important international guidelines advocate periodic abdominal ultrasound scans with or without alpha-fetoprotein (AFP) in all cirrhotics and chronic hepatitis B patients, irrespective of antiviral treatment[2,12,13] as a cost effective and noninvasive surveillance modality. According to the guidelines, US should be performed on a semiannual basis, with the exclusion of decompensated cirrhotics not listed for liver transplantation, in which surveillance is considered futile.

Despite the recommendations, real-life implementation of surveillance programs is far from optimal. It has recently been shown that, in addition to an increase in the proportion of HCC diagnosed at an early stage, 64% of the cases detected in the United States between 2006 and 2012 were diagnosed at an intermediate or an advanced stage [14]. That is probably the result of the low implementation of surveillance programs, with less than 20% of cirrhotics undergoing regular surveillance[15]. Surveillance is hindered by several issues, insufficient identification of patients at risk, failure to order surveillance, failure to perform surveillance with effective tools, poor patient adherence to the surveillance program, failure to establish a care-unit network to provide effective treatment. This review aims to analyze these steps one-by-one in order to identify the obstacles hampering the implementation of surveillance in real life.

FAILURE TO IDENTIFY THE PATIENTS AT RISK

One of the most important factors responsible for the low uptake of surveillance is the failure of primary care physicians (PCPs) to identify patients at risk to be enrolled in surveillance programs. In two web-based surveys, PCPs reported lack of knowledge of current guidelines. They could not correctly identify the at-risk categories and ordered random surveillance of chronic liver disease patients[16,17]. It is noteworthy that cirrhosis is widely under diagnosed and that increased identification and surveillance

of cirrhotic patients would be very cost effective in detecting HCC at an early stage. As an example, assuming an ultrasound sensitivity of 65% for early-stage HCC[18], an increase in the detection rate of cirrhotic patients from 50% to 75% would result in a significant increase of early-stage HCC diagnosed by abdominal ultrasound. Thanks to the identification of high-risk patients, ultrasound screening would thus allow the identification of a number of early-stage HCC comparable to magnetic resonance imaging (MRI) use, which has a higher sensitivity (90%) but is more expensive and has a higher false-positive rate. Another negative consequence of the failure to identify cirrhotic patients is that the cause of liver damage is not treated, and consequently HCC would often be diagnosed in a critical patient not eligible for radical treatment anymore.

Fortunately, the performance of PCPs can be improved by training interventions[19] and by participation in specialist networks, as shown by the French experience in hepatitis C[20]. To address the problem, in 2006 we started an education program targeting 120 PCPs, aimed at the identification of cirrhotic patients and their surveillance for HCC[21]. The program was started in the southern part of the Province of Bergamo in two hospitals, Policlinico S. Marco, Zingonia, and Azienda Ospedaliera di Treviglio, that have hepatology clinics providing medium-complexity specialist care. More complex cases are referred to a tertiary care hospital in Bergamo, which has a liver transplant center. Two hepatologists had established a network of collaboration with PCPs for the management and referral of patients with liver disease, but also provided care for patients from the nearby provinces, where no specific network had been implemented. The training program consisted of a 1-year intensive course in 2006, followed by annual refreshers from 2007 to December 2013. PCPs were instructed to perform screening of all patients at risk of liver cirrhosis, *i.e.* hepatitis C virus (HCV) infection, HBsAg positivity, alcohol abuse (> 60 g/d in women and > 80 g/d in men), body mass index > 30 kg/m^2, and to refer them to the liver specialist for diagnostic confirmation and US planning[20]. Abdominal ultrasound was performed, and the data were analyzed, by hepatologists or by radiologists who worked at the same hospital and had strong expertise in liver cancer detection.

The results showed that 6 years after training PCPs, there was a significant increase in the number of HCC patients detected by US at an early stage and suitable for radical treatment (Figure 1). As a consequence, 3- and 5-year survival of HCC patients improved significantly from 35% to 48% and 20% to 40%, respectively ($P < 0.05$) after training. In contrast, both the proportion of HCCs detected by US, and patient survival did not change in areas where PCPs were not trained. The study has some limitations. Firstly, PCPs did not record the number of cirrhotic patients who were identified, but only the number of HCC patients who were diagnosed. Secondly, the cirrhosis criteria used by PCPs to refer the patient to the hepatologist were mainly ultrasound based using two of the following ultrasound criteria, liver surface nodularity, nonhomogeneous liver texture, left lobe or caudate lobe hypertrophy, distorted hepatic veins, spleen longitudinal diameter > 12 cm or one ultrasound criterion plus a platelet count < 130.000/mL. Currently, those criteria are obsolete, as the availability of new proprietary (*e.g.*, Fibrotest, Enhanced Liver Fibrosis, and Fibrometer) and nonproprietary biochemical scores (fibrosis-4, aspartate transaminase to platelet ratio index), and NAFLD fibrosis scores) have improved diagnostic accuracy. Nonetheless, the study showed that it is essential to establish a collaboration network among hepatologists and PCPs, and that strengthening PCP skills in identifying and referring suspected cirrhotic patients may result in an increased number of HCCs diagnosed at an early stage and improved patient survival. In the following years, the network of collaboration was extended to the whole province of Bergamo, and specific training aimed at the diagnosis of cirrhosis was incorporated into a comprehensive hepatology education program that involved 900 PCPs. The program consists of specific courses organized every 3 years by the local health authority and annual symposia organized by the tertiary care hospital of Bergamo. The percentage of HCCs diagnosed at an early stage in the following years was still high, indicating that the positive effect of training persisted in time. Indeed, 166 (77%) of 202 HCCs diagnosed in cirrhotic patients at Policlinico S. Marco Hospital from January 2014 through December 2020, were found at an early stage and could be effectively treated. It is worth noting that only 14 HCCs were diagnosed in noncirrhotic patients, seven with chronic hepatitis B and currently under surveillance, six were detected at an early stage, five had chronic hepatitis C infection, and two had noncirrhotic nonalcoholic steatohepatitis (NASH). Therefore, our real-life data confirm the adequacy of the official guidelines recommending US only in patients affected by cirrhosis or chronic hepatitis B.

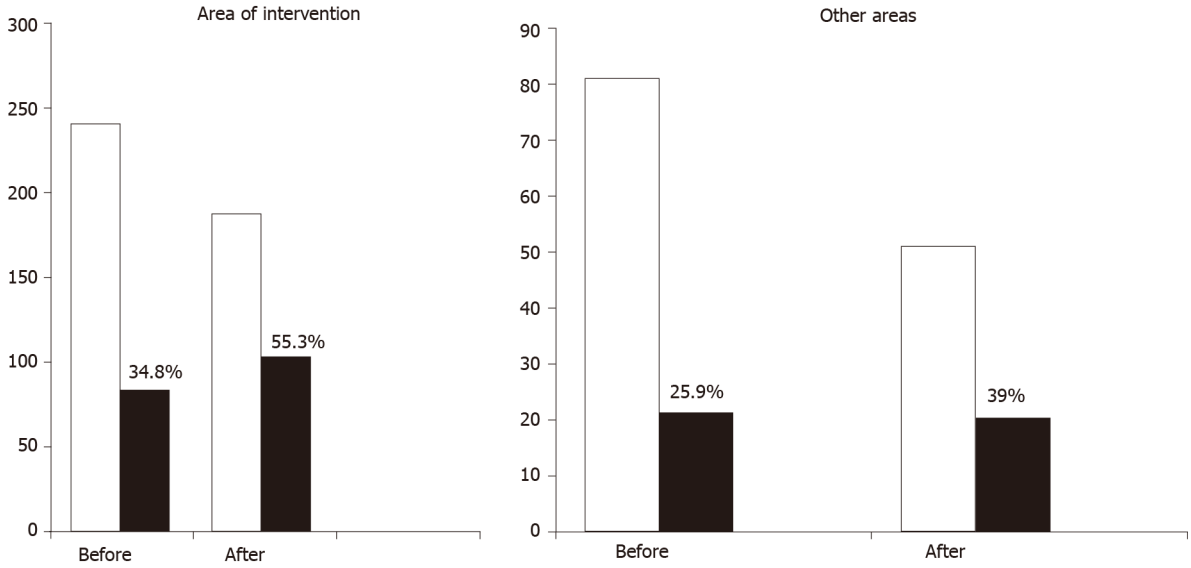

Figure 1 Hepatocellular carcinoma diagnosis before and after implementation of the training program in the area where primary care physicians were trained and in other areas where primary care physicians were not trained. White bars indicate the total number of hepatocellular carcinomas. Black bars indicate hepatocellular carcinomas diagnosed by surveillance. In the intervention area the diagnoses under surveillance increased from 85 of 244 (34.8%) to 105 of 190 (55%) after training (Δ = + 20.5%, $P < 0.001$). The diagnoses increased from 21 of 81 (25.9%) to 20 of 51 (39%) in other areas (Δ = + 13.1%, $P = 0.11$, not significant).

FAILURE TO ORDER SURVEILLANCE

The identification of cirrhotic patients for surveillance does not imply that surveillance will be effectively performed. In fact, surveillance is sometimes carried out by the PCP and ultrasound scans are performed by radiologists without specific expertise in liver disease. In that case, the patient is referred to the hepatologist or gastroenterologist only if a focal lesion is detected, a scenario called "population-based surveillance". In Italy and other countries, thanks to the widespread diffusion of ultrasound equipment and the motivation of an active medical sonography society, US is directly performed by hepatologists with sonographic skills and able to perform the examinations by themselves (*i.e.* specialist or subspecialist surveillance). Population-based surveillance is mainly implemented in the United States, while specialist surveillance is more widespread in Europe and Asia. A systematic review and meta-analysis comparing the two US modalities reported a much higher effectiveness of surveillance in the specialist setting (73.7%) rather than in population-based cohorts (8.8 %), with the lowest effectiveness in patients with alcoholic and NASH cirrhosis[22]. There are many reasons for the difference. PCPs and radiologists without specific expertise in liver disease may have limited knowledge of the international guidelines[17,23]. Concerns of other serious comorbidities, logistic problems such as poor accessibility of ultrasound facilities, and difficulties in the scheduling process. Consequently, the need to access low-cost, affordable ultrasound facilities may result in delays or absence of PCP surveillance[24]. Several interventions can be introduced to improve US programs, namely the use of automatic reminders for PCPs or the implementation of protocols that can be carried out by nurses or pharmacists[22]. Beste *et al*[25] reported that a primary care-oriented clinical reminder integrated into clinical records improved PCP surveillance from 18.2% to 27.6%[25], whereas no improvement was observed in the control sites (from 16.1% to 17.5%). Similarly, in a large study of 1800 cirrhotic patients, Singal *et al*[26] observed that mailed outreach invitations increased surveillance from 24% to a staggering 44.5% with no benefit seen by the addition of patient navigation [26]. Overall, it can be concluded that surveillance performed in subspecialist settings, if feasible, would be preferable to population surveillance. In the latter case, decreased use of US could be expected, and even after the interventions mentioned above (*i.e.* internal modalities such as medical reminders, nurse-based protocols, and patient navigation and outreach modalities such as mailed invitations), it was only slightly improved. Therefore, it is essential to ensure that PCPs are aware of the importance of appropriate specialist surveillance and to reinforce the need of specific education programs addressed to them.

FAILURE TO USE THE APPROPRIATE SURVEILLANCE TOOL

Liver ultrasound

Liver sonography is recommended by all existent guidelines as the standard HCC surveillance test to be used. A meta-analysis of 15 studies of cirrhotic patients showed that abdominal ultrasound had good sensitivity (84%) and excellent specificity (91%) for the detection of HCC at any stage[27]. However, the pooled sensitivity for the detection of early-stage HCC was as low as 47%, with only marginal improvement (53%) after exclusion of the studies performed before the introduction of high-density crystal probes and harmonic imaging. It is noteworthy that the sensitivity of ultrasound is characterized by a wide range of variation (from 21% to 81%), reflecting the operator dependency of US scans. In fact, in a single study performed at a university-based tertiary care center, the sensitivity of abdominal ultrasound was 82% [28]. Similarly, in a large Italian cohort study, where examinations were performed in both outside facilities and hospital wards, the sensitivity rose to 66%[18]. It is important to consider that ultrasound examinations in the United States were performed by technicians who recorded the images, which were subsequently reviewed and interpreted by radiologists. That can affect the accuracy of the examinations reported in those studies, as real-time interpretation of ultrasound findings is of paramount importance in ensuring the adequacy of the examination, especially in patients who are difficult to examine. In conclusion, it seems that pooled study results underestimate the sensitivity of ultrasound for the detection of early-stage HCC. It is more likely that the sensitivity of ultrasound carried out with high quality scanners and by well-trained doctors in a real-time manner, would be as high as 70%. Admittedly, even in optimal conditions, US fails to detect early-stage HCC in approximately one-third of the patients[18]. Two studies have addressed the causes of surveillance failure. Overall, both studies found that male sex, obesity, and advanced liver disease were associated with failure to detect HCC at an early stage[18,29]. Fatty liver, often associated with obesity and more frequently observed in males, may hinder good visualization of the liver, while advanced liver disease may produce a coarse echo pattern that masks early-stage tumors. It is important to distinguish poor visibility of the tumor by abdominal ultrasound, which represents a true limitation of this technology, from biologically aggressive tumors, which may show an infiltrative or rapidly growing pattern that may be difficult to detect regardless of the diagnostic tool.

Computed tomography and magnetic resonance imaging

The use of CT or MRI can obviate the above-mentioned technical limitations of ultrasound, but they rarely can identify an aggressive tumor at an early stage. In one study, 20% of the ultrasound examinations were considered technically inadequate for excluding HCC[29]. In another study, 32% of HCCs were detected beyond the early stage by semiannual US, 12% of them showing biologically aggressive features, *i.e.*, AFP > 1000 ng/mL, vascular thrombosis, distant metastasis, or infiltrative pattern[18]. Therefore, technical limitations of US to detect early-stage HCC can be deemed to be around 20%, hence it is important that ultrasound reports include a statement about the quality of the examination and the potential recommendation to perform second-level imaging (CT or MRI). MRI has a sensitivity of 83.7% for the detection of early-stage HCC[30] and should be preferred to CT, which has lower sensitivity (62.5%), similar to that of ultrasound[31]. However, it is noticeable that biologically aggressive tumors are poorly detected by MRI (16% failure to detect HCC) as well as by US (12% failure). Those tumors thus represent a hardcore of HCCs eluding both kinds of imaging surveillance. The good news is that only a small percentage of HCCs have those characteristics. However, it should be recognized that the pattern of HCC growth is heterogeneous[32] and many indolent tumors can develop an aggressive pattern with time, as shown by an increase of the percentage of biologically aggressive tumors detected by annual compared with semiannual surveillance, (*i.e.* 28% *vs* 12%) [18]. The possibility that indolent HCCs might transform into more aggressive tumors with time has raised some concerns of the common real-life practice of performing annual instead of semiannual MRI to reduce surveillance cost

Contrast-enhanced ultrasound

Contrast-enhanced US with intravascular agents (*e.g.*, Levovist, Sonovue) has a limited role in surveillance as it is impossible to scan the whole liver in the arterial phase when small tumors are detected[33]. On the other hand, perfluorocarbon (Sonazoid) seems to a promising agent[34], as it is taken up and retained by Kupffer cells for 60 min after

bolus injection, allowing a scan of the entire liver in the late phase when HCC appears as a black hole. Sonazoid-enhanced US might therefore be useful to detect HCC developing in livers with a coarse echotexture, but it cannot be of any advantage in obese patients or severe fatty livers, where CT or MRI remain the only option to accurately exclude HCC.

AFP

The sensitivity of AFP to detect early-stage HCC is too low to be used alone as a surveillance tool[35]. Only the Asian-Pacific Guidelines recommend the use of AFP in surveillance programs, with a cutoff of 200 ng/mL[2]. This cutoff is highly specific but reduces AFP sensitivity to a level as low as 22%[12]. However, new and effective pharmacological treatment of hepatitis B and C may remove false-positive AFP results caused by necro-inflammatory activity and enable the use of lower cutoff levels, providing better AFP sensitivity without compromising its specificity. Another method to improve AFP diagnostic accuracy is to monitor its changes over time[36]. An algorithm including AFP variations has been created and was tested in the Hepatitis C Antiviral Long-Term Treatment Against Cirrhosis Trial cohort, with good results in identifying patients at increased risk of HCC[37]. A recent meta-analysis showed that the addition of AFP levels to abdominal ultrasound improved the overall sensitivity for the detection of early-stage HCC from 45% to 63%[27], but as pointed out before, the low sensitivity of US in the meta-analysis raises doubts on the quality of the ultrasound examinaions. It therefore remains an open question whether AFP can improve the performance of properly executed sonography. It is important to stress that the addition of AFP screening to abdominal sonography could adversely affect the already low level of surveillance application by PCPs because of the logistic and financial burden introduced by duplicating the surveillance tool. In our real-life practice we assess AFP levels alongside abdominal US only in patients with cured hepatitis C or well-controlled chronic hepatitis B and optimal adherence to follow-up visits. A change in AFP values over time prompts performing second-level imaging, particularly in cases with a coarse echo pattern on sonography. On the other hand, stable AFP levels permit advising the continuation of standard US, avoiding additional cost.

New biochemical markers

In recent years, new markers have been proposed for the diagnosis of early HCC. Each of them by itself does not seem to confer any benefit in diagnostic accuracy compared with US with or without AFP[35]. However, a combination of AFP-L3, AFP, and des-gamma-carboxy prothrombin was tested in a large study and proved to have a sensitivity greater than 60% for early HCC, which is similar to the sensitivity of currently approved surveillance tools[38]. The algorithm is interesting, and if confirmed could be used as an alternative to ultrasound in the setting of population surveillance, especially where ultrasound scanners are not available or not accessible.

THE PROBLEM OF ADHERENCE

Patient adherence is key to the effectiveness of the surveillance program. Racial and ethnic minorities have poor awareness of surveillance programs, probably because of their low socioeconomic status. Patients also report difficulties scheduling surveillance ultrasound, concerns of the cost, need of off-work days, and difficulties reaching the facilities where the exam is performed[39]. The difficulties have a greater impact on subspecialty-based than on population-based surveillance, and should be addressed by appropriate interventions. In our hospital, the great majority of examinations are performed by hepatologists (PD and SL) and each appointment is directly booked by the specialist, thus making the scheduling process much easier. Visits and US examinations are reimbursed by the National Health Care System, with no additional cost for the patient. They are both performed at the same time to minimize off-work time, and if the patient is under antiviral treatment, the drug can be collected from the hospital pharmacy on the same day. Table 1 shows adherence to surveillance in 362 cirrhotic patients followed from January 2013 through December 2020 in our outpatient clinic. Adherence was defined as the performance of regular annual or semiannual ultrasound examinations in the last 24 months. Overall adherence to surveillance was rather good and only slightly lower than that observed in studies from other subspecialty clinics, (*i.e.* 65% *vs* 73%)[22]. It should be highlighted that in 2020 Northern Italy was heavily hit by the coronavirus disease 2019 pandemic and that the liver clinic was

Table 1 Adherence to surveillance by 362 cirrhotic patients followed from January 2013 through December 2020 at the hepatology unit of Policlinico S. Marco

Etiology of cirrhosis	Adherent	Semiannual	Annual	Nonadherent
Alcoholic (103)	55 (52%)	31 (56%)	24 (44%)	48 (46%)
HBV (53)	39 (73%)	35 (90%)	4 (10%)	14 (27%)
HCV (164)	119 (72%)	101 (85%)	18 (15%)	45 (28%)
NAFLD (42)	24 (57%)	17 (71%)	7 (29%)	18 (43%)
Total: 362	237 (65%)	184 (77%)	53 (23%)	125 (35%)

Adherence was defined as the performance of regular annual or semiannual ultrasound examinations in the previous 24 mo. HBV: Hepatitis B virus; HCV: Hepatitis C virus; NAFLD: Nonalcoholic fatty liver disease.

closed down for 2 months until April 2020. The restrictions and the fear of being infected and contracting the disease might have induced patients to avoid medical appointments, as observed for other screening programs during the pandemic. Despite that, nearly two-thirds of the patients continued surveillance, which in 77% of the cases was semiannual and in line with the official recommendations. It should be emphasized that the definition of adherence is not unequivocal, as some investigators used restrictive definitions while others, as we did, use operational definitions. Patients who underwent at least 1 or 2 surveillance exams in the last 12 months were considered adherent to the surveillance program. Moreover, as adherence decreases over time, studies with longer follow-up show lower adherence rates and cannot be compared with studies that have a shorter follow-up. To solve this conundrum it has been proposed to define adherence to the surveillance program as the proportion of time up-to-date with screening[22], although in retrospective studies complete patient data are often lacking, and this parameter may be difficult to achieve. In accord with other studies, we found a lower adherence to surveillance programs in alcoholic and metabolic cirrhotic patients (NAFLD). In those patients, the time interval between screening exams was more frequently annual than semiannual. On the other hand, patients affected by cirrhosis secondary to hepatitis B and C, had a higher adherence to the surveillance programs (72% and 73% respectively) and exams were almost exclusively performed with semiannual periodicity. The reasons for the low adherence in the former categories are multifactorial. Lack of family support and low socio-economic status are important and can hinder the access to the health care system as well as social stigma associated with alcohol abuse and obesity. It is well known that marginalized and stigmatized patients are more dependent on a good relationship with the healthcare provider than on getting the right information from scientific sources or mail reminders. They may also overestimate the prescription of drugs compared with regular screening tests, and perceive the periodicity of ultrasound examinations as a mere waste of time. It is therefore important to establish an empathic nonjudgmental relationship with those patients. The ultrasound examination should be integrated with the hepatologist consultation, a longer time for the visit should be scheduled, medical issues should be discussed, and drugs prescribed if necessary. It is important to make the patient understand that surveillance is essential to take care of their liver disease. For that purpose, it would be helpful to establish a close collaboration with Alcoholics Anonymous or other self-help groups, that could help the patients to be actively involved and aware of the importance of surveillance.

CONCLUSION

In conclusion, despite generalized consensus of all scientific guidelines on the utility of surveillance for HCC in high-risk patients, the implementation of surveillance at the community level is far from ideal. Low access to surveillance programs is associated with a lower number of HCC diagnosed at an early stage and decreased survival. Therefore, it is important to study the obstacles hampering the adherence to surveillance of the high-risk population in order to implement appropriate interventions.

Adherence to surveillance programs is higher in hospital/subspecialty-based surveillance compared with population-based surveillance, in which tests are ordered by PCPs and performed by general radiologists. Subspecialty-based surveillance should therefore be the first choice when feasible. However, the geographic location of the facilities and the distance from the hospital may result in population surve-illance as the only available choice. In both cases, lack of identification of the at-risk categories by PCPs, (*i.e.* cirrhotic patients and chronic hepatitis B patients), is the most important issue hindering implementation of adequate surveillance. Education programs targeted to PCPs to improve their skills in identifying the patients at risk can facilitate prompt referral to the hepatologist/gastroenterologist for US. PCPs should be encouraged to use biochemical scores to raise the suspicion of cirrhosis, and if feasible, portable elastography scanners. Once the diagnosis of cirrhosis has been established by transient elastography, acoustic radiation force impulse elastography, or other modalities, the patient should be followed up in a specialty setting. Regarding the surveillance tools, real-life experience confirms that semiannual ultrasound examination with the addition of sequential AFP monitoring in selected cases is the most appropriate method, with a sensitivity of about 70%. Ultrasound examinations should be of high quality and hence preferably performed by radiologists with good expertise in liver imaging, or when feasible, by the hepatologist himself. When ultrasound examinations are unsatisfactory (about 20% of cases), it should be explicitly noted in the sonographic report and the patient preferably surveilled with an annual MRI, which brings the total detection rate of early-stage HCC up to 85%. Fifteen percent of HCCs are aggressive tumors that cannot be diagnosed at an early stage with the currently available tools. Adherence to scheduled examinations is still a problem, particularly in some categories of patients, *i.e.* alcoholic and NAFLD cirrhotic patients. In order to improve patient adherence, a less complex approach that reducing the number of visits to the hospital and reducing bureaucratic procedures should be used. Once a diagnosis of HCC is made. It should be promptly treated in the appropriate setting, with evaluation of the necessity to refer the patient to a tertiary care hospital for more advanced treatments or to be evaluated for liver transplantation. Figure 2 shows the hub and spoke model of care of cirrhotic patients implemented in our province. According to the model, cirrhotic patients are identified by PCPs using appropriate screening tests. The patients are then referred to the liver clinics of general hospitals (Spokes) for diagnosis confirmation and implementation of surveillance programs. Therapeutic interventions are performed locally when feasible or the patient is referred to the tertiary referral hospital (Hub) for further treatment.

KEY TAKE AWAY MESSAGES

Surveillance for HCC in cirrhotic patients and chronic hepatitis B patients allows detection of the tumor at an early stage and improves survival.

International guidelines recommend semiannual ultrasound with or without AFP monitoring in these patients, but surveillance implementation at the community level is low.

Education programs targeting PCP and aiming at improving the identification and referral of patients at risk of HCC should be implemented.

Subspecialty-based surveillance performed by the hepatologist or gastroenterologist in the setting of the liver clinic of a general hospital is the preferred model, when feasible.

Ultrasound and MRI should be used sequentially in case of inadequate sonography.

Surveillance should be incorporated into the specific care and follow-up provided by the liver clinic to enhance adherence.

The liver clinic of the general hospital should be integrated into a hub and spoke model of care alongside PCPs and tertiary referral hospitals to ensure proper access to care.

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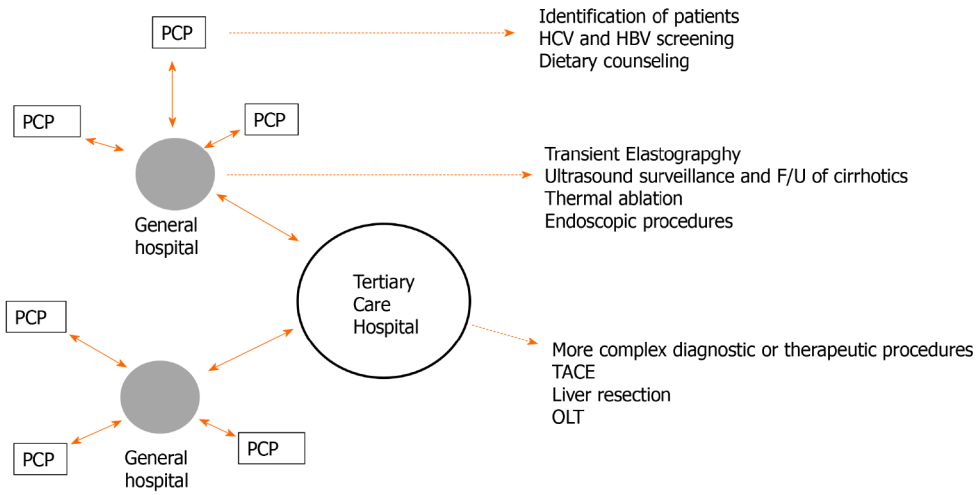

Figure 2 Hub and spoke model for the diagnosis and follow-up of cirrhotic patients by primary care physicians in the province of Bergamo. In the model, ultrasound surveillance and follow-up of cirrhotics are performed by hepatologists in general hospitals. HBV: Hepatitis B virus; HCV: Hepatitis C virus; OLT: Orthotopic liver transplantation; PCP: Primary care physicians.

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REVIEW

Challenges and opportunities in the application of artificial intelligence in gastroenterology and hepatology

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Abstract

Artificial intelligence (AI) is an umbrella term used to describe a cluster of interrelated fields. Machine learning (ML) refers to a model that learns from past data to predict future data. Medicine and particularly gastroenterology and hepatology, are data-rich fields with extensive data repositories, and therefore fruitful ground for AI/ML-based software applications. In this study, we comprehensively review the current applications of AI/ML-based models in these fields and the opportunities that arise from their application. Specifically, we refer to the applications of AI/ML-based models in prevention, diagnosis, management, and prognosis of gastrointestinal bleeding, inflammatory bowel diseases, gastrointestinal premalignant and malignant lesions, other nonmalignant gastrointestinal lesions and diseases, hepatitis B and C infection, chronic liver diseases, hepatocellular carcinoma, cholangiocarcinoma, and primary sclerosing cholangitis. At the same time, we identify the major challenges that restrain the widespread use of these models in healthcare in an effort to explore ways to overcome them. Notably, we elaborate on the concerns regarding intrinsic biases, data protection, cybersecurity, intellectual property, liability, ethical challenges, and transparency. Even at a slower pace than anticipated, AI is infiltrating the healthcare industry. AI in healthcare will become a reality, and every physician will have to engage with it by necessity.

Key Words: Artificial intelligence; Machine learning; Gastroenterology; Hepatology; Artificial neural networks; Support vector machine

Core Tip: The opportunities that arise from the application of artificial intelligence/machine learning-based models in gastroenterology and hepatology include the

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establishment of targeted screening programs through the identification of patients prone to develop cancer, the development of non-invasive diagnostic tools, the improvement of the diagnostic accuracy, the development of treatment allocation frameworks based on predictions of outcomes for different treatment modalities, the development of models to ensure cost-effective use of resources, the development of triage tools for higher levels of care and decision-making tools for further treatment, based on individualized patient outcome predictions, and finally the development of predictive models of prognosis for patient and family counseling.

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INTRODUCTION

Artificial intelligence (AI) is an umbrella term to describe any application where computer systems are used to perform tasks normally associated with human intelligence[1,2]. AI is a cluster of interrelated fields, including machine learning (ML) and probabilistic reasoning, planning and decision making, fuzzy systems, computer vision, natural language processing, knowledge representation, and neural networks (NN)[1]. Despite their differences, these fields are all driven by advancements in computing power and Big Data.

ML could be described as a model that learns from past data in order to predict future data[3]. The application of ML in healthcare was catalyzed by several healthcare trends, such as electronic healthcare records and patient summaries, genomic analyses and biomedical research, routine imaging, and telemedicine, that have transformed the healthcare industry into a data-rich science with data as an omnipresent concept[4-6]. Specifically, while in 2013, the healthcare industry produced 153 exabytes (10^{18} gigabytes) of data, it has been projected to reach 2314 exabytes in 2020[7]. Therefore, the healthcare industry generates an enormous amount of data that conform with the features that define Big Data: Volume, high-velocity, high-variety, and veracity, which cannot be analyzed or managed by traditional software[5,8,9]. AI promises to process and analyze these extensive repositories of data and turn them into meaningful insights.

Several studies have described AI as the potential solution to long-standing healthcare challenges such as increasing diagnostic accuracy, enhancing telemedicine, providing substantial cost reduction, promoting evidence-based medicine, facilitating targeted literature search, and delivering individualized care[10-14]. More importantly, AI could substantially alleviate the burden of diseases by reducing the associated mortality and morbidity through optimizing patient outcomes[15,16].

In gastroenterology and hepatology, physicians handle large amounts of clinical data and an extensive repository of imaging data generated from endoscopy, ultrasound, and computed tomography (CT). Therefore gastroenterology and hepatology are data-rich fields, and thus fruitful ground for AI applications with extensive research conducted in these fields, particularly regarding the prevention, diagnosis, management, and prognosis of diseases[17,18]. We aim to comprehensively review the applications of AI in gastroenterology and hepatology and identify the current challenges of utilizing AI in healthcare in an effort to explore ways to overcome them.

SEARCH STRATEGY

We conducted a comprehensive literature review of the Medline, Cochrane, and Scopus databases using the following algorithm: [(artificial intelligence OR machine learning OR deep learning OR neural networks OR support vector machine OR computer-assisted OR computer-aided) AND (gastroenterology OR hepatology OR

esophageal OR small bowel OR large bowel OR gastric cancer OR capsule endoscopy OR polyps OR colonoscopy OR colorectal cancer OR gastrointestinal bleeding OR inflammatory bowel disease OR Crohn's disease OR celiac disease OR ulcers OR hepatocellular carcinoma OR cholangiocarcinoma OR liver fibrosis OR fatty liver OR chronic liver disease OR cirrhosis)]. Articles were reviewed for eligibility by the two authors independently (CC, GT), and disagreements were solved by a discussion between the two authors. Finally, the reference lists of eligible articles were reviewed to identify further related literature, including articles, books, and other forms of publication. We excluded studies written in a language other than English and publications of abstracts. The review of the literature was completed on January 27, 2021.

AI CLASSIFIERS

Before discussing AI's current applications and challenges in gastroenterology and hepatology, we briefly describe the AI classifiers in ML models that we identified as the most commonly used among the eligible articles. Specifically, we describe the basic features of the support vector machine (SVM), the artificial NN (ANN), and the convolutional NN (CNN). ML is divided into supervised and unsupervised based on whether the training data is labeled or not[19]. In other words, through supervised ML, a new data set is classified for an outcome based on previous data sets that trained the ML model, while in unsupervised ML, there is no outcome but rather an attempt to detect unknown patterns and correlations within the data[20].

SVMs are supervised learning models with associated learning algorithms trained to assign classes to new cases. SVMs require a training set of data where each case is pre-labeled regarding the outcome classification. The data points of the features of each case are treated as points in a high-dimensional space[21]. Based on these mapped points, separating hyperplanes are drawn, aiming to distinguish these points upon their labeled class[22]. The hyperplane with the maximum distance from the mapped data points, known as the maximum functional margin, is selected since it provides the SMV's full potential to classify new examples correctly[21,22]. SVMs typically perform linear classification analysis. For non-linear analysis, kernel function could introduce additional dimensions to the raw data; and thus turning a non-linear problem into a linear problem in a higher-dimensional space[23].

An ANN is an ML model inspired by the human brain's neuronal connections that consist of an input layer, an output layer, and a hidden layer between them[18]. ANNs are applied both in supervised and unsupervised ML[24]. When multiple hidden layers are inserted between the input and output layers, and the network's architecture becomes more complex with multiple interconnections, the concept of deep NN (DNN) emerges[24,25]. During DNN training, the model adjusts the weighted correlations between the nodes of the input layer and the nodes of the multiple hidden layers[18,20]. Within DNN, CNNs are biologically variants inspired by multi-layer perceptrons[26]. CNNs have been extensively applied for medical image analysis[17,18]. During the CNN model development, the images are preprocessed using multiple filters, and multiple feature maps are created in a process called convolution[26].

APPLICATIONS OF AI IN GASTROENTEROLOGY

AI/ML-based models have been extensively applied in the prevention, diagnostics, management, and prognosis of gastrointestinal (GI) diseases, including GI bleeding (GIB), inflammatory bowel diseases (IBDs), malignant and premalignant lesions, and other nonmalignant lesions/diseases such as gastroesophageal reflux, *Helicobacter pylori* (*H. pylori*) infection, ulcers, celiac disease, and intestinal hookworms.

Prevention

Table 1 summarizes the findings of the identified studies applying AI/ML models in the prevention of gastroenterological diseases. AI/ML-based software could be used in screening programs to identify high-risk patients not currently identified by standard screening guidelines. In a recent study, researchers developed seven different AI/ML models to identify patients at high risk of developing gastric cancer following the eradication of *H. pylori* infection[27]. The extreme gradient boosting (GB) model demonstrated the highest performance[27]. A CNN was developed in a different study to classify patients at low, moderate, and high risk of developing gastric cancer using

Table 1 Artificial intelligence applications in gastroenterology: Prevention

Ref.	Parameters employed	AI classifier	Sizes of the training/validation sets	Outcomes	Performance
Leung *et al* [27]	Laboratory results, clinicopathological parameters	Several	64238/25330 patients	Risk of gastric cancer development following *H.pylori* eradication	0.53-0.97[2,6], 59.3-98.1[3,6], 51.5-93.6[4,6]
Nakahira *et al*[28]	Laboratory results, clinicopathological parameters, endoscopic images	CNN	7826/454 patients	Stratify risk of gastric cancer development	---
Taninaga *et al*[29]	Laboratory results, clinicopathological parameters, endoscopic images	CART	1144/287	Prediction of future gastric cancer	63.4-94.8[1,6], 0.736-0.874[2,6]
Goshen *et al*[31]	Laboratory results, clinicopathological parameters	DT, RF, GB	688 flagged patients	High risk of CRC development	----

[1]Accuracy (%).
[2]Area under the receiver operating curve or c-index.
[3]Sensitivity (%).
[4]Specificity (%).
[5]Training.
[6]Internal validation.
[7]External validation/testing. CART: Classification and regression tree; CNN: Convolutional neural network; CRC: Colorectal cancer; DT: Decision tree; GB: Gradient boosting; RF: Random forest.

endoscopic images[28]. A similar study used multiple classification and regression trees (CART) to develop a model that predicts future gastric cancer development[29]. Another notable example is the ColonFlag test, an ML algorithm that uses age, sex, and complete blood count data to identify patients at high risk of developing colorectal cancer[30,31]. In a recent study, among 254 individuals who underwent colonoscopy, 19 cancers (7.5%) and 22 (8.7%) advanced adenomas were identified in a population of patients who would otherwise have avoided screening[31]. These studies demonstrate how AI/ML-based models could be used in a real clinical setting to establish targeted screening programs.

Diagnostics

Table 2 summarizes the findings of the identified studies applying AI/ML models to diagnose gastroenterological diseases. AI/ML-based software is applied in Computed Aided Detection or Diagnosis (CAD) systems used in radiology to augment medical images' interpretation accuracy. A CAD typically includes the stages of preprocessing, extraction of features, and feature selection and classification[32]. CAD systems could also aid endoscopists in navigating through the different anatomical locations of the GI tract. Specifically, a model based on a CNN employed esophagogastroduodenoscopy images to recognize the anatomical locations with outstanding performance[33].

Regarding the diagnosis of gastroesophageal reflux, an ANN model was developed as a non-invasive diagnostic tool by employing only clinical data[34]. For patients with Barrett's esophagus, a deep learning-based (DL) CAD system was developed to differentiate patients with malignant from patients with nondysplastic Barret's esophagus [35]. The CAD system also identified the optimal site to perform a biopsy with an accuracy between 92% and 97%[35]. An SVM model was developed in another study employing white-light endoscopic imaging to identify early neoplastic lesions[36]. In a recent study, images from volumetric laser endomicroscopy were used to develop a CAD system to detect neoplastic lesions for patients with Barret's esophagus[37]. A similar study using volumetric laser endomicroscopy developed an ML model for detecting neoplastic lesions[38]. Notably, the model outperformed the experts in volumetric laser endomicroscopy[38]. Finally, to diagnose esophageal squamous cell carcinoma, a CNN model was developed by employing endocytoscopic images as an alternative to biopsy[39].

For diagnosing patients with *H. pylori* infection, a CNN was developed in a study employing gastroscopic images[40]. A different CNN for *H. pylori* diagnosis was developed in a prospective pilot study analyzing images taken from the stomach's lesser curvature using either white light imaging, blue light imaging, or linked color imaging[41]. Notably, employing blue light and linked color imaging yielded significantly higher performance than white light imaging[41]. In a similar study, a

Table 2 Artificial intelligence applications in gastroenterology: Diagnosis

Ref.	Diagnostic Modality	AI classifier	Sizes of the training/validation sets	Outcomes	Performance
Takiyama *et al* [33]	Esophago-gastro-duodenoscopy imaging	CNN	1750/435[7]	Anatomical classification among larynx, esophagus, stomach, and duodenum	0.99-1.00[2,7]
Pace *et al*[34]	Laboratory results, clinicopathological parameters	ANN	159 patients	Diagnosis of gastroesophageal reflux disease	67.86-100[1,6]
de Groof *et al* [35]	Esophageal endoscopic images	DNN	1247/297[6]/80[7]/80[7] patients	Classification of malignant from nondysplastic Barret's esophagus	88.2[1,6], 87.5-88.8[1,7], 87.6[3,6], 90.0-92.5[3,7], 88.6[4,6], 82.5-87.5[4,7]
van der Sommen *et al*[36]	White-light endoscopic imaging	SVM	44 patients with Barret's esophagus	Diagnosis of early neoplastic lesions	Per image: 62-90[3,6], 65-90[4,6], Per patient: 52-100[3,6], 74-96[4,6]
Struyvenberg *et al*[37]	Volumetric laser endomicroscopy imaging	Several	29 patients with Barret's esophagus	Diagnosis of neoplastic lesions	0.83-0.94[2,6]
Swager *et al*[38]	Volumetric laser endomicroscopy imaging	Several	60 images	Diagnosis of neoplastic lesions	0.89-0.95[2,6]
Kumagai *et al* [39]	Endocytoscopic imaging	CNN	4715/1520[7]	Diagnosis of esophageal squamous cell carcinoma	90.9[1,7], 0.72-0.90[2,7], 39.4-46.4[3,7], 98.2-98.4[4,7]
Zheng *et al*[40]	Endoscopic images	CNN	1507/452 patients	Diagnosis of *H.pylori* infection	84.5-93.8[1,6], 0.93-0.97[2,6], 81.4-91.6[3,6], 90.1-98.6[4,6]
Nakashima *et al* [41]	Endoscopic images	CNN	162/60 patients	Diagnosis of *H.pylori* infection	0.66-0.96[2,6]
Itoh *et al*[42]	Endoscopic images	CNN	149/30 images	Diagnosis of *H.pylori* infection	0.956[2,6], 86.7[3,6], 86.7[4,6]
Shichijo *et al*[43]	Endoscopic images	CNN	32308/11481[7]	Diagnosis of *H.pylori* infection	83.1-87.7[1,7], 81.9-88.9[3,7], 83.4-87.4[4,7]
Kanesaka *et al* [45]	NBI	SVM	126/81 NBI images	Diagnosis of gastric cancer	96.3[1,6], 96.7[3,6], 95.0[4,6]
Hirasawa *et al* [46]	Endoscopic images	CNN	13584/2296[7]	Diagnosis of gastric cancer	92.2[3,7]
Zhu *et al*[47]	Laboratory results, clinicopathological parameters, cancer biomarkers	GB/DT	496/213 patients	Diagnosis of gastric cancer	85.9[1,5], 831[,6], 0.91[2,6], 88[3,5], 87[3,6], 83.4[4,5], 84.1[4,6]
Tenório *et al*[48]	Laboratory results, clinicopathological parameters	Several	178/38	Diagnosis of celiac disease	71.5-80[1,6], 0.71-0.84[2,6], 69-82[3,6], 67-80[4,6]
Caetano Dos Santos *et al*[49]	Endomysial autoantibody test for IgA-class antibodies images	SVM	2597 images (training:validation = 7:3)	Diagnosis of celiac disease	96.8-98.85[1,6], 82.84-98.91[3,6], 98.81-99.40[4,6]
Hujoel *et al*[50]	Laboratory results, clinicopathological parameters	Several	408 undiagnosed patients	Diagnosis of celiac disease	0.49-0.53[2,6]
Manandhar *et al* [51]	Gut microbiome data	RF	1429 fecal 16S metagenomic data subjects	Diagnosis of IBD	0.80-0.82[2,6]
Wei *et al*[52]	Single nucleotide polymorphisms data	Several	60828 samples	Classification of CD and UC	0.782-0.866[2,6]
Mossotto *et al* [53]	Capsule endoscopy, histologic imaging	SVM	239/48[7] pediatric patients	Classification of CD, UC, and unclassified IBD	71-82.7[1,5], 0.78-0.87[2,5], 83.3[1,7], 83-85[3,7]
Xia *et al*[58]	Capsule endoscopy imaging	CNN	697/100[7] patients, 822590/201365[7], images	Classification among different types of gastric lesions	77.1-86[1,7], 0.80-0.90[2,7], 96.2-100[3,7], 56.5-76.2[4,7]
Seguí *et al*[59]	Capsule endoscopy imaging	CNN	50 videos	Classification of small bowel mobility events	96[1,6]
Park *et al*[60]	Capsule endoscopy imaging	CNN	139 videos, 200000 images (training:validation:test = 6:2:2)	Small bowel lesion identification	80.29-98.34[1,6], 0.999[2,5], 0.998[2,6,7]

Hwang *et al*[61]	Capsule endoscopy imaging	CNN	7556/5760[7] images	Classification of hemorrhagic and ulcerative lesions	96.62-96.83[1,7], 95.07-97.61[3,7], 96.04-98.18[4,7]
Otani *et al*[62]	Capsule endoscopy imaging	DNN	167/40[7] patients	Classification among different types of small bowel lesions	0.950-0.996[2,6], 0.884-0.928[2,7]
Yuan *et al*[63]	Capsule endoscopy imaging	SVM	20 patients, 340 images (training:validation = 8:2)	Diagnosis of peptic ulcers	92.65[1,6], 94.12[3,6], 91.18[4,6]
Karargyris *et al*[64]	Capsule endoscopy imaging	SVM	80 frames	Diagnosis of peptic ulcers	75[3,6], 73.3[4,6]
He *et al*[65]	Capsule endoscopy imaging	CNN	11 patients, 440000 images	Diagnosis of intestinal hookworms	88.5[1,6], 0.895[2,6], 84.6[3,6], 88.6[4,6]
Leenhardt *et al*[66]	Capsule endoscopy imaging	CNN	600/600 images	Diagnosis of gastrointestinal angiectasia	100[3,6], 96[4,6]
Zhou *et al*[67]	Capsule endoscopy imaging	CNN	21 videos	Diagnosis of celiac disease	100[3,6], 100[4,6]
Yamada *et al*[68]	Colon capsule endoscopy imaging	CNN	15933/4784[7]	Diagnosis of colorectal neoplasias	83.9[7], 0.902[2,7], 79[3,7], 87[4,7]
Wang *et al*[69]	Colonoscopy imaging	CNN	5545 images/27113[7] images/612[7] images/138[7] videos/54[7] videos	Identification of colorectal polyps	0.984[2,7], 88.24-100[3,7], 95.40-95.92[2,7]
Misawa *et al*[70]	Colonoscopy imaging	CNN	411/35 short videos	Identification of colorectal polyps	76.5[1,6], 0.87[2,6], 90[3,6], 63.3[4,6]
Urban G *et al*[71]	Colonoscopy imaging	CNN	8641 images/20[7] videos	Identification of colorectal polyps	96.4[1,7], 0.991[2,7]
Ozawa *et al*[72]	Colonoscopy imaging	CNN	20431/7077[7] images	Identification of colorectal polyps, Classification of colorectal polyps	90-97[3,7], 47-98[1,7]
Mori *et al*[73]	NBI and methylene blue staining images	SVM	466 diminutive polyps	Classification of diminutive rectosigmoid adenomas	NPV(%): 93.7-96.5
Tischendorf *et al*[74]	NBI	SVM	209 colorectal polyps	Classification of colorectal polyps	90[3,6], 70.2[4,6]
Gross *et al*[75]	NBI	SVM	434 colorectal polyps	Classification of small colorectal polyps	93.1[1,6], 95.0[3,6], 90.3[4,6]
Kominami *et al*[76]	NBI	SVM	118 colorectal polyps	Classification of colorectal polyps	93.2[1,6], 93.0[3,6], 93.3[4,6]
Misawa *et al*[77]	NBI endocytoscopy	SVM	979/100 endocytoscopy, images	Classification of colorectal polyps	90[1,6], 84.5[3,6], 97.6[4,6]
Takeda *et al*[78]	NBI endocytoscopy	SVM	5543/200 endocytoscopy, images	Diagnosis of invasive CRC	94.1[1,6], 89.4[3,6], 98.9[4,6]
Chen *et al*[79]	NBI	CNN	2157/284[7]	Classification neoplastic from hyperplastic polyps	96.3[3,7], 78.1[4,7], NPV(%): 91.5[7]
Komeda *et al*[80]	NBI	CNN	1200/600 images	Classification of adenomatous from non-adenomatous polyps	75.1[1,6]
Byrne *et al*[81]	NBI	CNN	223/40[7] videos	Classification of adenomas from hyperplastic polyps	94[1,7], 0.95[2,7], 98[3,7], 83[4,7], NPV(%): 97[7]

[1]Accuracy (%).
[2]Area under the receiver operating curve or c-index.
[3]Sensitivity (%).
[4]Specificity (%).
[5]Training.
[6]Internal validation.
[7]External validation/testing. ANN: Artificial neural network; CD: Chron's disease; CNN: Convolutional neural network; CRC: Colorectal cancer; DNN: Deep neural network; DT: Decision tree; GB: Gradient boosting; IBD: Inflammatory bowel disease; NBI: Narrow-band imaging; NPV: Negative predictive value; RF: Random forest; SVM: Support vector machine; UC: Ulcerative colitis.

CNN model employed images from upper GI endoscopy to detect *H. pylori* infection

[42]. Finally, Shichijo *et al*[43] developed two different CNN models to diagnose *H. pylori* infection. The first, a 22-layered deep CNN, performed comparably with endoscopists, while the second, which classified images based on their location in the stomach, performed comparably with endoscopists in terms of sensitivity and specificity but demonstrated a significantly higher accuracy[43]. The time needed to analyze all the images was 3 min and 18 s and 3 min and 14 s for the two CNNs, while for endoscopists, the average time needed was 230.1 min[43]. A recent meta-analysis evaluating the performance of CNNs to diagnose infection with *H. pylori* concluded that it is currently equivalent to physicians[44].

Regarding the diagnosis of gastric cancer, an SVM-based CAD model was developed to identify early gastric cancer features in narrow-band imaging (NBI) gastroscopy[45]. In a different retrospective study, a CNN was developed to detect gastric cancer from endoscopic images. Even though the model managed to correctly classify 71 of the 77 gastric cancer lesions (92.2% sensitivity), it also falsely identified 161 non-cancerous lesions as gastric cancer, yielding a low positive predictive value of 30.6%[46]. Approximately half of these lesions were gastritis with an irregular mucosal surface or changes in color tone[46]. Finally, in a recent study, a non-invasive GB/decision tree (DT) model employing only nonendoscopic parameters was developed to diagnose gastric cancer[47].

Regarding the diagnosis of celiac disease, five different AI/ML-based models were developed in a study using clinical data as a base for non-invasively diagnosing celiac disease[48]. Interestingly, the models were tested using 13 different algorithms and different input variables variations, resulting in 270 different tested models[48]. Among them, a model based on the Bayesian classifier demonstrated the highest performance[48]. A different study developed an SVM that employed images from the endomysial autoantibody test for IgA-class antibodies to classify patients with celiac disease[49]. Finally, in a different study, the authors aimed to develop several AI/ML-based models, which employ clinical data, to predict celiac disease in a group of patients who remained undiagnosed[50]. The models were unsuccessful, with only two models slightly outperforming random chance in predicting celiac disease[50].

In the diagnosis of IBDs, a recent study developed five different AI/ML-based models using data from the gut microbiome to classify patients with IBD[51]. The Random Forest (RF) model demonstrated the highest performance with AUROCs of 0.80 when bacterial taxa were used and 0.82 when operational taxonomic features were used[51]. Finally, the models were tested in distinguishing between Chron's disease (CD) and ulcerative colitis (UC), with the RF model demonstrating an AUROC > 0.9[51]. A multicentered, genome-wide association study used data from single-nucleotide polymorphisms to develop several AI/ML-based models to classify patients with CD and UC[52]. For pediatric IBDs, an SVM-based model employed data from histologic and endoscopic images to classify pediatric patients with CD, UC, or unclassified IBD[53]. Other studies have employed images from wireless capsule endoscopy to build SVM models that identify patients with CD with reported accuracies between 80.2% and 100%[54-57].

Focusing on capsule endoscopy, in a recent study, magnetically controlled capsule endoscopy imaging was employed to develop a CNN that provides an automatic detection and classification system for gastric lesions[58]. These lesions included erosions, polyps, ulcers, submucosal tumors, normal mucosa, and xanthomas[58]. A different study used images generated by a wireless capsule to develop a CNN able to classify the small bowel motility among six distinct intestinal motility events[59]. In a recent study, an AI-assisted capsule endoscopy reading model was developed to assist lesion identification[60]. Notably, the model significantly shortened the reading time of images by trainees[60].

To distinguish between hemorrhagic and ulcerative lesions, a CNN was developed using images from small bowel capsule endoscopy[61]. In a similar, recent study, a DNN model used capsule endoscopy images to classify different small bowel lesions (erosions, ulcers, tumors, and vascular lesions)[62]. SVM-based models were developed in two other studies employing images from capsule endoscopy to identify peptic ulcers[63,64]. In three different studies, CNNs have been developed, employing images from capsule endoscopy to diagnose intestinal hookworms, GI angiectasia, and celiac disease[65-67]. Finally, a CNN was developed in a recent study to detect colorectal neoplasias in images from colon capsule endoscopy[68].

Regarding the identification of colorectal polyps in coloscopy, a CNN model was designed to analyze colonoscopy images and videos and identify colorectal polyps [69]. Interestingly, the model's sensitivity to identify flat isochromatic, less than 0.5 cm polyps, which are typically associated with a higher missing rate, was estimated at 91.65% per image[69]. A different study, employing short videos from colonoscopies,

developed a CNN model that identifies colorectal polyps[70]. In another study, a CNN was developed employing videos and images from coloscopy to detect colorectal polyps in real-time[71]. Interestingly, when experts reviewed the colonoscopy videos, they managed to identify additional non-removed polyps with the assistance of the CNN model[71]. In a recent study, an automated polyp detection model based on CNN was developed to identify and then classify colorectal polyps into adenomas, hyperplastic, sessile serrated adenomas, and cancer[72]. The model managed to process the images at a speed of 20 m per frame[72].

Specifically for colorectal polyps classification, in a prospective study, an SVM-based model was developed using NBI and methylene blue staining images to classify diminutive rectosigmoid adenomas in real-time[73]. A prospective pilot study developed an SVM model that employed NBI images to detect and classify colorectal polyps based on vascularization features as neoplastic and non-neoplastic[74]. In a comparative study, an SVM model demonstrated comparable performance at classifying the neoplastic nature of diminutive polyps (< 10 mm) to that of experts in NBI colonoscopy but surpassed the performance of non-experts[75]. Two other studies developed SVM models that employed NBI images to classify colorectal lesions (neoplastic or non-neoplastic)[76,77]. Finally, an SVM model was developed in a retrospective study to diagnose invasive colorectal cancer based on NBI endocytoscopy images[78].

Except for SVMs, several studies employed CNNs for colorectal polyps classification. One comparative study developed a CNN employing NBI colonoscopy images to classify neoplastic polyps[79]. Notably, the model's performance was found comparable to that of experts but superior to the performance of non-experts[79]. In another study focusing on NBI colonoscopy, a CNN model was developed to classify adenomatous from non-adenomatous polyps[80]. A different study developed a CNN model to classify adenomas from hyperplastic polyps during NBI colonoscopy[81].

The performances reported by the majority of the AI/ML-based models surpass both the NPV threshold recommended by the American Society of Gastrointestinal Endoscopy (90%) for adenoma detection and the estimated pooled NPV reported in a meta-analysis conducted by the society (91%)[82,83]. Finally, we should mention that currently, the majority of the CAD systems that we reported have the shortcoming of manual segmentations of lesions. The endoscopists should identify the areas of interest before the model could analyze and attempt to classify. This weakness has been acknowledged by the European Society of Gastrointestinal Endoscopy[84]. Other obstacles to developing CAD systems constitute the lack of large datasets and the lack of variability in images. A recent study aimed to resolve this by developing a CNN that "adds" polyps to the images to increase the repository of images for training and advance the development of automated polyp detection models[85].

Management

Table 3 summarizes the findings of the identified studies applying AI/ML models for the management of gastroenterological diseases. In a recent study, data from baseline impedance, nocturnal baseline impedance, and acid exposure time were used as a base for a DT model to predict the treatment response with proton pump inhibitors for patients with gastroesophageal reflux disease[86]. The aim was to establish a decision-making framework for treatment allocation[86].

AI/ML-based models have been used in the management of malignant GI lesions. Regarding gastric cancer, a CNN has been developed to predict whether the early gastric cancer has invaded the mucosa and submucosa layers of the stomach and act as a decision tool for endoscopic resection[87]. Interestingly, the CNN model outperformed endoscopists[87]. In the same concept, a DNN was developed to classify gastric cancer based on invasion depth as a basis for treatment allocation[88]. Specifically, the model demonstrated accuracy for predicting T1, T1a, T1b T2, T3, T4, and an overall accuracy of 77.2%, 68.9%, 63.6%, 49.1%, 51.0%, 55.3%, and 64.7%, respectively[88]. In colorectal cancer, the identification of microsatellite instability significantly impacts the treatment allocation process. A DL model was developed to identify microsatellite instability directly from hematoxylin and eosin-stained whole slide image[89]. Notably, the model outperformed a group of pathologists[89]. Finally, an SVM model that predicted the lymph node metastasis status of patients with colorectal cancer was designed as a tool to identify patients who would benefit from additional treatment following the endoscopic resection of T1 tumors[90]. Notably, the model outperformed the staging systems endorsed by current guidelines[90].

Regarding the management of GIB, in a recent study, an ML model was developed using data from patients admitted to the intensive care unit (ICU) following a GIB to predict the need for transfusion[91]. The authors suggested that it could potentially be

Table 3 Artificial intelligence applications in gastroenterology: Treatment

Ref.	Parameters employed	AI classifier	Sizes of the training/validation sets	Outcomes	Performance
Rogers *et al* [86]	Data from baseline impedance, nocturnal baseline impedance, and acid exposure time	DT	335 patients	Prediction of treatment response with proton pump inhibitors for patients with gastroesophageal reflux disease	0.31-0.938[2,6]
Zhu *et al* [87]	Endoscopic images	CNN	790/203[7] images	Invasion of gastric cancer at the mucosa and submucosa layers of the stomach	89.16[1,7], 0.94[2,7], 76.47[3,7], 95.56[4,7]
Kubota *et al* [88]	Endoscopic images	DNN	800/90 images	Invasion depth of gastric cancer	64.7[1,6]
Yamashita *et al*[89]	Hematoxylin and eosin-stained WSI	DNN	100/15[6]/484[7]	Identificication of CRC microsatellite instability	0.931[2,6], 0.779[2,7], 76[3,7], 66.6[4,7]
Ichimasa *et al*[90]	Laboratory results, clinicopathological parameters	SVM	590/100[7]	Prediction of lymph node metastasis status	69[1,7], 0.821[2,7], 100[3,7], 66[4,7]
Levi *et al* [91]	Laboratory results, clinicopathological parameters	RFE	14620 patients	Prediction of the need for transfusion following GIB	50.21-74.88[1,6], 0.7858-0.8141[2,6], 69.17-92.77[3,6], 35.02-79.82[4,6]
Chu *et al* [92]	Laboratory results, clinicopathological parameters	Several	122/67 patients	Prediction of the source of GIB	69.7-94.3[1,6], 0.658-0.999[2,6], 90.1-98.0[3,6], 89-100[4,6]
				Prediction of the need for blood resuscitatio	64.7-94.1[1,6], 0.381-0.993[2,6], 90.3-93.9[3,6], 18.4-95.5[4,6]
				Prediction of the need for emergent endoscopy	62.7-83.3[1,6], 0.404-0.913[2,6], 80.1-89.1[3,6], 13.8-85.7[4,6]
				Prediction of disposition	58.4-89.7[1,6], 0.324-0.972[2,6], 81.9-92.9[3,6], 18.4-90.9[4,6]
Das *et al*[93]	Laboratory results, clinicopathological parameters	ANN	194/193[6]/200[7] patients	Prediction of major stigmata of recent hemorrhage	89[1,3,4,6], 77[1,7], 96[3,7], 63[4,7]
				Prediction of the need for emergent endoscopy	81[1,3,6], 61[1,7], 94[3,6], 82[4,6], 48[4,7]
Augustin *et al*[94]	Laboratory results, clinicopathological parameters	CART	164/103[7] patients	Stratification of risk of rebleeding and mortality following acute variceal hemorrhage	0.81-0.83[2,7]
Loftus *et al* [95]	Laboratory results, clinicopathological parameters	ANN	103/44 patients	Prediction of severe lower GIB	0.979[2]
				Prediction of the need for surgical intervention	0.954[2,6]
Ayaru *et al* [96]	Laboratory results, clinicopathological parameters	GB	170/130[7]	Prediction of severe lower GIB	78[1,6], 83[1,7]
				Prediction of recurrent bleeding	88[1,6], 88[1,7]
				Prediction of the need for intervention	88[1,6], 91[1,7]

[1]Accuracy (%).
[2]Area under the receiver operating curve or c-index.
[3]Sensitivity (%).
[4]Specificity (%).
[5]Training.
[6]Internal validation.
[7]External validation/testing. ANN: Artificial neural network; CART: Classification and regression tree; CNN: Convolutional neural network; CRC: Colorectal cancer; DT: Decision tree; DNN: Deep neural network; GB: Gradient boosting; GIB: Gastrointestinal bleeding; RFE: Recursive feature elimination; WSI: Whole-slide image.

used as a decision-making tool to triage patients to the ICU or the ward[91]. A different study developed several AI/ML models for patients with acute GIB to predict four different outputs: The source of bleeding, the need for urgent blood transfusion, the need for urgent endoscopy, and the disposition[92]. The study aimed to establish a decision-making framework for the efficient management of GIB. The RF model outperformed all seven other models for all four outputs[92].

Focusing on the upper GIB, an ANN model was developed as a non-invasive triage tool for patients with upper GIB[93]. In the external cohort, the ANN performed similarly to the complete Rockall Score (includes endoscopic variables) in predicting stigmata of recent hemorrhage[93]. A different study developed a CART model regarding acute variceal hemorrhage to predict rebleeding and mortality and achieved the discrimination of three distinct prognostic groups of low, intermediate, and high risk[94]. Its performance was significantly superior to Child-Pugh and Model for End-Stage Disease (MELD) but comparable to a conventional logistic regression (LR) model [94].

Focusing on the lower GIB, two different ANN models were developed in a study aiming to predict severe acute lower GIB and the need for surgical intervention[95]. The first ANN significantly outperformed the Strate prediction rule for predicting severe bleeding (AUROCs: 0.98 *vs* 0.66)[95]. A different study employed nonendoscopic variables to develop a model based on a GB classifier to predict severe lower GIB, recurrent bleeding, and the need for clinical intervention[96]. On external validation, the model was found equally accurate to a conventional LR model for recurrent bleeding and the need for clinical intervention but superior in predicting severe lower GIB[96].

Prognosis

Table 4 summarizes the findings of the identified studies applying AI/ML models regarding the prognosis of gastroenterological diseases. Several SVM-based nomograms were developed in a study to predict distant metastasis for operated patients with oesophageal squamous cell carcinoma[97]. A different study employed clinicopathological data to develop an ANN model to predict the survival of patients with esophageal cancer operated with curative intends[98]. The model surpassed the Tumor–Node–Metastasis (TNM) model[98]. Regarding gastric cancer, a recent study developed five different AI/ML-based models to predict recurrence in operated patients[99]. Among the models, the RF demonstrated the best performance[99].

An ANN model was developed for patients with IBD, which used meteorological data to predict seasonal variations of onset and relapse in patients with CD and UC [100]. In the validation cohort, the model predicted the onset frequency and the frequency of relapse of the IBD with a mean absolute percentage error of 37.58% and 17.1%, respectively[100]. A study focusing on CD developed an ANN model to predict mucosal remission for patients treated with azathioprine 16 wk following treatment [101]. In a study focusing on UC, an ANN was developed employing clinical data to predict the patients with UC treated with cytoapheresis, who will eventually require operation[102].

Regarding the prognosis of GIB, a CART model was developed to predict in-hospital mortality of cirrhotic patients presenting with upper GIB[103]. In a multicentered study, an ANN model was developed employing pre-endoscopic variables to predict 30-d mortality in patients with non-variceal upper GIB[104]. Similarly, a prospective, multicentered study employed pre-endoscopic variables to develop an ANN model that predicts 30-d mortality in patients with non-variceal upper GIB[105]. The ANN significantly outperformed the Rockall scoring system (AUROCs: 0.95 *vs* 0.67)[105].

Regarding colorectal cancer, a recent study developed several ML models to predict the RAS and BRAF mutation status for patients with advanced colorectal cancer[106]. Notably, the ANN demonstrated the best performance[106]. In a different study employing clinicopathologic variables and data generated from immunochemistry, a least absolute shrinkage and selection operator regression model was developed to predict the lymph node metastasis status in a cohort of operated patients for T1 colorectal cancer[107].

Opportunities of AI application in gastroenterology

Therefore, the opportunities that arise from applying AI/ML-based software in gastroenterology include:

AI/ML models could be developed and integrated into the clinical setting to employ routinely collected data directly from the patient's electronic health records and flag patients at high risk of developing certain GI diseases in real-time. Current efforts include the prevention of gastric[27-29] and colorectal cancer[30,31]. Such ML models could become the basis for tailoring targeted screening programs.

Endoscopy is the gold standard for the diagnosis of a plethora of GI diseases. AI/ML models employing non-invasive parameters that provide reliably accurate diagnosis could substitute endoscopy or significantly minimize its use, thus ameliorating the impact of endoscopy-related complications, significantly decreasing

Table 4 Artificial intelligence applications in gastroenterology: Prognosis

Ref.	Parameters employed	AI classifier	Sizes of the training/validation sets	Outcomes	Performance
Yang *et al*[97]	Laboratory results, immunomarkers, clinicopathological parameters	SVM	319/164 patients	Distant metastasis of oesophageal squamous cell carcinoma following surgery	69.5-80.1[1,6], 44.7-67.2[3,6], 81.6-97.7[4,6]
Sato *et al*[98]	Laboratory results, clinicopathological parameters, tumor characteristics	ANN	395 patients (training:validation:test = 53:27:20)	1-year and 5-year survival of patients with esophageal cancer following surgery	0.883-0.884[2,7], 78.1-80.7[3,7], 84.7-86.5[4,7]
Zhou *et al*[99]	Laboratory results, clinicopathological parameters, tumor characteristics	Several	2012 patients (training:validation = 8:2)	Recurrence of gastric cancer following surgery	0.790-0.962[2,5], 0.771-0.795[2,6]
Peng *et al*[100]	Meteorological data	ANN	901 patients	Variations of onset and relapse of IBDs	----
Hardalaç *et al* [101]	Clinicopathological parameters, treatment data	ANN	129 patients (training:validation:test = 80:10:10)	Prediction of mucosal remission for CD patients treated with azathioprine	58.1-79.1[1,6], 0.527-0.883[2,6]
Takayama *et al*[102]	Clinicopathological parameters, treatment data	ANN	54/36 patients	Prediction of the need for operation for UC patients treated with cytoapheresis	96[3,6], 97[4,6]
Lyles *et al* [103]	Laboratory results, clinicopathological parameters	CART	884 patients	Prediction of in-hospital mortality of upper GIB in cirrhotic patients	----
Grossi *et al* [104]	Laboratory results, clinicopathological parameters	ANN	807 patients	30-d mortality of patients with non-variceal upper GIB	81.2-89.0[1,6], 0.87[2,6], 81.5-93.3[3,6], 80.9-84.7[4,6]
Rotondano *et al*[105]	Laboratory results, clinicopathological parameters	ANN	2380 patients	30-d mortality of patients with non-variceal upper GIB	96.8[1,6], 0.95[2,6], 83.8[3,6], 97.5[4,6]
Shi *et al*[106]	CT radiomics	Several	124/35 patients	Prediction of the presence of RAS and BRAF mutations in CRC	ANN: 87[1,5], 71[1,6], 0.90-0.95[2,5], 0.79[2,6]
Kang *et al* [107]	Laboratory results, immunomarkers, clinicopathological parameters, tumor characteristics	LASSO	221/95 patients	Prediction of lymph node metastasis status in operated patients for T1 CRC	0.795[2,5], 0.765[2,6]

[1]Accuracy (%).
[2]Area under the receiver operating curve or c-index.
[3]Sensitivity (%).
[4]Specificity (%).
[5]Training.
[6]Internal validation.
[7]External validation/testing. ANN: Artificial neural network; CART: Classification and regression tree; CD: Chron's disease; CRC: Colorectal cancer; CT: Computed tomography; GIB: Gastrointestinal bleeding; IBD: Inflammatory bowel disease; LASSO: Least absolute shrinkage and selection operator; SVM: Support vector machine; UC: Ulcerative colitis.

the cost for diagnosis, and providing an alternative to an unpleasant intervention for the patient. Current efforts include the diagnosis of gastroesophageal reflux disease [34], gastric cancer[47], celiac disease[48,50], and IBDs[51].

Expect from replacing endoscopy; AI/ML models could also improve its efficacy. CAD systems could facilitate navigating the GI tract and serve as the second "observer" for the endoscopist, an "observer" non-susceptible to distraction, which identifies lesions missed by the endoscopist. Particularly for capsule endoscopy, a CAD system that automatically detects and classifies lesions could significantly decrease the time required to evaluate the images by endoscopists while increasing diagnostic accuracy. Current efforts include the diagnosis of IBDs[53-57], gastric lesions[58], small bowel mobility disorders[59], small bowel lesions such as hemorrhagic and ulcerative lesions[60-64], intestinal hookworms[65], GI angiectasia[66], celiac disease[67], and colorectal cancer[68].

Physicians could use CADs to augment the accuracy of classifying polyps based on their neoplastic nature. As a result, the morbidity and mortality associated with failing to remove a neoplastic polyp could be lessened. At the same time, the complications related to removing a non-neoplastic polyp could be avoided. Therefore, CADs could significantly increase the cost-effectiveness of polyp management. Current efforts include the development of SVMs[73-76] and CNNs[79-81] for the classification of the neoplastic nature of colorectal polyps during NBI colonoscopy.

AI/ML models that accurately predict the response to different treatments could be used as a basis for individualized treatment allocation. Current efforts include the response of treatment with proton pump inhibitors for patients with gastroesophageal reflux disease[86], the mucosal remission for CD patients treated with azathioprine [101], and the need for operation for UC patients treated with cytoapheresis[102].

Particularly for GIB management, AI/ML models could be used to identify the source of bleeding[92] and as frameworks for decision-making, including the need to transfuse patients[92], perform emergent endoscopy[92,93] or emergent surgery[95, 96], and finally, triage patients with severe GIB to the ICU[95,96].

Finally, AI/ML models could be used as predictive tools that stratify the risk of complications and predict overall survival and recurrence following treatment. Such models could be used to tailor individualized follow-up schedules and for patient and family counseling. Current efforts include the prediction of overall survival for patients with esophageal cancer[98], the risk of recurrence of operated patients with gastric cancer[99], and 30-d mortality of patients with non-variceal GIB[104,105].

APPLICATIONS OF AI IN HEPATOLOGY

Prevention

Table 5 summarizes the findings of the identified studies applying AI/ML models for the prevention of disease in the field of hepatology. In a recent study, a prospective cohort of apparently healthy volunteers was enrolled in a study, which developed a DT-based model to classify patients based on their risk of developing non-alcoholic fatty liver disease (NAFLD) and liver fibrosis[108]. A similar study used routinely collected laboratory and clinical parameters to develop several AI/ML-based models to identify patients with NAFLD in the general population[109]. In a different study, several AI/ML models were developed as potential tools for targeted screening for NAFLD[110]. The Bayesian network model demonstrated the highest accuracy, followed by an SVM model[110]. Notably, an LR model outperformed all the models. These studies are examples of how AI/ML-based models could be used in primary care as tools for detecting chronic liver diseases. In a different study, multiple AI/ML models were developed as a basis for a non-invasive tool for assessing the level of fibrosis progression in NAFLD patients[111]. Notably, the RF model demonstrated the highest performance[111]. A different study managed to develop an ML model for patients at high risk of developing NAFLD to classify patients with non-alcoholic fatty liver (NAFL) and non-alcoholic steatohepatitis (NASH)[112]. Several AI/ML models were developed in a different study to classify healthy individuals from patients with NAFL and NASH[113]. Among them, a GB tree model demonstrated the highest performance, followed by an RF model[113]. In another study, four different AI/ML-based models were developed utilizing data from biochemical and enzyme-linked immunosorbent assays to classify patients with NAFLD and alcoholic liver disease with or without cirrhosis[114].

AI/ML models that utilize data directly from the electronic health records from patients infected with hepatitis B (HBV) and hepatitis C (HCV) could be used as the basis for targeted screening tools for liver carcinomas. A GB model was developed in a study using only serum markers aiming to predict fibrosis and classify the stage of fibrosis in two cohorts of patients with HBV and HCV[115]. An ANN model was developed in another study that employed only routine clinical data to predict significant fibrosis for patients with HBV[116]. In a similar study, an ANN model was developed by employing only routine laboratory data from HBV patients to identify patients with cirrhotic liver[117]. A different study developed an ANN model employing only non-invasive data to predict advanced liver fibrosis in HCV patients [118]. A similar study developed several AI/ML-based models to develop a non-invasive tool for identifying HCV patients with advanced fibrosis[119]. Among the developed models, a model based on alternating DTs demonstrated the highest performance[119]. These studies demonstrate how AI/ML models using routine clinical data could be used to identify patients who will benefit from a sustained

Table 5 Artificial intelligence applications in hepatology: Prevention

Ref.	Parameters employed	AI classifier	Sizes of the training/validation sets	Outcomes	Performance
Goldman *et al*[108]	National database of routine annual health check-ups	DT-based	12019 patients	Risk of NAFLD and cirrhosis	84.50-85.73[1,6], 0.7740-0.8486[2,6]
Yip *et al*[109]	Laboratory results, clinicopathological parameters	Several	500/422	Identify patients with NAFLD	0.87-0.90[2,5], 0.78-0.88[2,6], 55.48-94.52[3,5], 51.69-92.37[3,6], 58.47-91.53[4,5], 50.99-90.46[4,6]
Ma *et al*[110]	Laboratory results, clinicopathological parameters	Several	10508 patients (training:validation = 9:1)	Identify patients with NAFLD	49.47-82.92[1,6], 20.2-68.0[3,6], 54.4-94.6[4,6]
Sowa *et al* [111]	Laboratory results, clinicopathological parameters	Several	126 morbidly obese patients (training:validation = 9:1)	Fibrosis in NAFLD patients	79[1,6], 30.8-60.0[3,6], 77.0-92.2[4,6]
Canbay *et al* [112]	Laboratory results, clinicopathological parameters	EFS	164/122 obese patients	Classification of NAFLD and NASH	0.7339[2,5], 0.7028[2,6]
Fialoke *et al* [113]	National database of routine annual health check-ups	Several	108139 patients (training:validation = 4:1)	Classification among healthy, NAFLD, and NASH	77.2-79.7[1,6], 0.842-0.876[2,6], 74.5-77.4[3,6]
Sowa *et al* [114]	Data from biochemical and enzyme-linked immunosorbent assays	Several	133 patients (training:validation = 9:1)	Classification of NAFLD and ALD	DT: 89.02-95.1[1,6], 74.19-94.12[3,6], 96.08-98.04[4,6], RF: 0.8932-0.9846[2,6], SVM: 0.9058-0.9118[2,6]
Wei *et al* [115]	Laboratory results, clinicopathological parameters	GB	576 HBV patients, (training:validation = 7:3), 368[7] HCV patients	Classification of fibrosis/cirrhosis in HBV patients	0.904-0.974[2,5], 0.871-0.918[2,6], 79-88[3,5], 78-84[3,6], 86-92[4,5], 85[4,6]
				Classification of fibrosis/cirrhosis in HCV patients	0.797-0.849[2,7]
Wang *et al* [116]	Laboratory results, clinicopathological parameters	ANN	226/113[6]/116[7] HBV patients	Classification of significant fibrosis	0.883[2,5], 0.884[2,6], 0.920[2,7]
Raoufy *et al* [117]	Laboratory results, clinicopathological parameters	ANN	86/58 HBV patients	Classification of liver cirrhosis	91.38[1,6], 0.898[2,6], 87.5[3,6], 92[4,6]
Piscaglia *et al*[118]	Laboratory results, clinicopathological parameters	ANN	414/96 HCV patients	Classification of significant fibrosis	45.8-86.5[1,6], 0.87[2,5], 0.93[2,6], 30.4-100[3,6], 30.1-98.6[4,6]
Hashem *et al* [119]	Laboratory results, clinicopathological parameters	Several	22690/16877 HCV patients	Classification of significant fibrosis	66.3-84.4[1,6], 0.73-0.76[2,6]
Ioannou *et al* [120]	Clinical/laboratory data extracted directly from electronic health records	DNN	48151 patients with HCV-related cirrhosis (training:validation = 9:1)	HCC development in HCV cirrhosis	0.759-0.806[2,6]
Emu *et al* [121]	Laboratory results, clinicopathological parameters	Several	1385 patients HCV (training:validation = 4:1)	Stage of liver cirrhosis	97.228-97.831[1,6]

[1]Accuracy (%).
[2]Area under the receiver operating curve or c-index.
[3]Sensitivity (%).
[4]Specificity (%).
[5]Training.
[6]Internal validation.
[7]External validation/testing. ALD: Alcoholic liver disease; ANN: Artificial neural network; DNN: Deep neural network; DT: Decision tree; EFS: Ensemble feature selection; GB: Gradient boosting; HBV: Hepatitis B; HCC: Hepatocellular carcinoma; HCV: Hepatitis C; NAFLD: Non-alcoholic fatty liver disease; NASH: Non-alcoholic steatohepatitis RF: Random forest; SVM: Support vector machine.

virological response (SVR) to delay chronic liver disease progression.

Regarding the development of HCC, a recent study investigated if a DNN could outperform conventional LR models in predicting HCC development in patients with HCV[120]. A different study using variables including demographic, laboratory results, and clinical findings, developed RF, DNN, and LR models to reliably predict

the stage of liver cirrhosis in patients infected with HCV[121]. Accurately predicting patients with HCV prone to develop HCC could help identify patients who would be benefited from a targeted screening and are in a greater need of antiviral treatment to achieve an SVR.

Diagnostics

Table 6 summarizes the findings of the identified studies applying AI/ML models for diagnosis in the field of hepatology. Regarding chronic liver disease, a study used CT imaging data of patients with a confirmed liver fibrosis diagnosis to develop a CNN model for the staging of liver fibrosis[122]. Notably, the model outperformed the radiologists' interpretation[122]. A different study employed ultrasound imaging of patients with fatty liver disease to develop an SVM model and an extreme learning machine model (a type of ANN) for diagnosis and risk stratification[123]. A different study that employed ultrasound shear wave elastography features developed an SVM model to identify patients with chronic liver disease[124]. Another study used images from real-time tissue elastography to develop four different AI/ML-based models to classify liver fibrosis[125]. Notably, the RF model outperformed the rest, followed by the KNN and the SVM models[125].

In a study, CT imaging was employed to develop an ANN model that differentiates between HCC, intrahepatic peripheral cholangiocarcinoma (CCA), hemangioma, and metastasis[126]. Interestingly, when radiologists evaluated the images, their performance significantly increased when they considered the ANN's output, from an AUROC of 0.888 to one of 0.934[126]. Focusing on HCC diagnosis, a recent retrospective study developed a CNN that employed MRI images of patients with HCC. The model was trained with a combination of images that met the Liver Imaging Reporting and Data System (typical) and with images that did not (atypical)[127]. In a multicenter, retrospective study, a CNN was developed that employed MRI scans to identify HCC lesions[128]. Notably, the model surpassed the performance of less experienced radiologists in the diagnosis of small HCC lesions[128]. The model was able to analyze 100 images in just 3.4 s[128]. Finally, a different study aimed to develop a non-invasive ANN model that predicts the presence of microscopic vascular invasion and the tumor grade of HCC[129].

Regarding diagnosis of CCA and pancreatic adenocarcinoma, a study developed an ANN model using data generated by metabolomic and proteomic analyses of bile from patients undergoing endoscopic retrograde cholangiopancreatography aiming to classify patients with and without cancer[130]. A different study used data from the plasma levels of bile acids to develop six different AI/ML-based models to classify patients as having CCA or a benign biliary disease[131]. Among the six developed models, a model based on the Naive Bayes classifier demonstrated the highest performance[131]. Finally, in another study, an ANN model was designed to analyze images from magnetic resonance cholangiopancreatography to diagnose CCA with a reported accuracy of 92.8%[132].

Management

Table 7 summarizes the findings of the identified studies applying AI/ML models to manage hepatic diseases. With the guidance of AI/ML-based software trained by data generated from gene mutation biomarkers, serum markers, imaging, and the clinical setting, high-quality, evidence-based, and individualized treatments could be employed regarding chemotherapy, radiotherapy, and immunotherapy. In HBV management, a recent study developed several AI/ML-based models that use soluble immune markers to predict early virological relapse after discontinuation of nucleoside analogs treatment[133]. The model could be used to exclude patients at high risk of virological relapse from treatment cessation. In HCV management, a study utilized data from full-length HCV genome sequencing of variants of HCV to develop multiple AI/ML-based models that classify HCV anti-viral resistance variants[134]. Notably, the SVM model demonstrated the highest performance[134].

In HCC management, a recent study used data from DNA methylation profiling to develop an RF-based model that predicts 6-mo progression-free survival. Such models could be used to personalize patient surveillance[135]. In an international, multi-institutional study, a CART model was developed that aimed to create a framework beyond the Barcelona-Clinic-Liver-Cancer (BCLC) staging system, which is currently endorsed by guidelines for treatment allocation[136,137]. The model defined six distinct prognostic groups of patients based on predictive factors of overall survival that could be used as a framework for treatment allocation[137]. Notably, the radiologic tumor burden score, which is not integrated into the BCLC staging system, was found as the best predictor of long-term outcome for BCLC stage B patients[137].

Table 6 Artificial intelligence applications in hepatology: Diagnosis

Ref.	Diagnostic Modality	AI classifier	Sizes of the training/validation sets	Outcomes	Performance
Choi *et al*[122]	CT imaging	CNN	7461/421[6]/298[7]/172[7] patients	Liver fibrosis staging (F0-F4)	83.1[1,5], 80.8[1,6], 74.4-80.2[1,7]
				Classification among significant fibrosis, advanced fibrosis, and cirrhosis	92.1-95.0[1,6,7], 0.95-0.97[2,6,7], 84.6-95.5[3,6,7], 89.9-96.6[4,6,7]
Kuppili *et al*[123]	US imaging	ELM, SVM	63 patients	Diagnosis of FLD	ELM: 81.7-92.4[1,6], 0.81-0.92[2,6], 85.10-91.30[3,6], 78.52-92.10[4,6], SVM: 76.14-86.42[1,6], 0.74-0.86[2,6], 76.80-88.20[3,6], 74.52-86.30[4,6]
Gatos *et al*[124]	US shear wave elastography imaging	SVM	126 patients	Classification of chronic liver disease from healthy patients	87.3[1,6], 0.87[2,6], 93.5[3,6], 81.2[4,6]
Chen *et al*[125]	Real-time tissue elastography imaging, age, sex	Several	513 patient (training:validation = 3:1)	Classification of liver fibrosis	80.44-82.87[1,6], 79.67-92.97[3,6], 46.25-82.50[4,6]
Matake *et al*[126]	Clinicopathological parameters, CT imaging	ANN	120 patients	Classification among four types of focal liver lesions	0.961[2,6]
Oestmann *et al*[127]	Multiphasic MRI scans	CNN	150/10 patients	Classification of HCC and non-HCC lesions	94.1[1,5], 87.3[1,6], 0.912[2,6]
					For HCC: 92.7[3,6], 82.0[4,6]
					For non-HCC: 82.0[3,6], 92.7[4,6]
Kim *et al*[128]	MRI scans	CNN	455[5,6]/54[7] patients	HCC detection	0.97[2,6], 94[3,6], 99[4,6], 0.90[2,7], 87[3,7], 93[4,7]
Cucchetti *et al*[129]	Laboratory results, clinicopathological parameters, radiological data, histological data	ANN	175/75 patients	MVI	0.92[2,5], 91.0[1,6]
				Histopathological Grade	0.94[2,5], 93.3[1,6]
Urman *et al*[130]	Metabolomic and proteomic analyses of bile	Several	139 patients	Classification of CCA and pancreatic adenocarcinoma	0.98-1.00[2,6], 88-94.1[3,6], 92.3-100[4,6]
Negrini *et al*[131]	Plasma bile acids profiles	Several	112 patients (training:validation = 4:1)	Classification of CCA and benign biliary disease	68.2-86.4[1,6], 0.77-0.95[2,6], 64-79[3,6], 63-100[4,6]
Logeswaran[132]	MRCP	MLP	55/593[7] images	CCA diagnosis	92.8-96.3[1,6], 83.64-90.14[1,7]

[1]Accuracy (%).
[2]Area under the receiver operating curve or c-index.
[3]Sensitivity (%).
[4]Specificity (%).
[5]Training.
[6]Internal validation.
[7]External validation/testing. ANN: Artificial neural network; CCA: Cholangiocarcinoma; CNN: Convolutional neural network; CT: Computed tomography; ELM: Extreme learning machine; FLD: Fatty liver disease; HCC: Hepatocellular carcinoma; MLP: Multi-layer perceptron; MRCP: Magnetic resonance cholangiopancreatography MRI: Magnetic resonance imaging; MVI: Microvascular invasion; SVM: Support vector machine; US: Ultrasound.

These findings could help us reevaluate HCC management into a multidisciplinary, individualized approach that goes beyond the BCLC criteria[138].

In the management of patients with CCA, in an international study, a CART model was developed with solely preoperative variables to identify patients who would be more likely to benefit from surgery[139]. The model managed to isolate four distinct prognostic groups of patients with similar patient outcomes[139]. The authors concluded that this model could be used to inform presurgical decisions-for example, the use of neoadjuvant therapy for patients with poor prognoses[139]. In a different study, the researchers developed a DNN model to establish an AI framework through which specific prognostic groups could be used to identify which patients were more likely to benefit from different treatment modalities such as neoadjuvant chemotherapy or transarterial chemoembolization[140]. The framework was found to be

Table 7 Artificial intelligence applications in hepatology: Treatment

Ref.	Parameters employed	AI classifier	Sizes of the training/validation sets	Outcomes	Performance
Wübbolding *et al*[133]	Analyze soluble immune markers	Several	28/49[7] HBV patients	Prediction of early virological relapse	0.73-0.89[2,6], 0.59-0.67[2,7]
Haga *et al*[134]	WGS of HCV	Several	86/87 HCV patients	Classification of HCV variants resistant to antiviral drugs	0.5-0.937[2,5], 0.597-0.954[2,6]
Bedon *et al*[135]	DNA methylation profiling	RF-based	300/74 HCC specimens	6-mo progression-free survival	67.1-80.6[1,5], 64.8-80.2[1,7]
Tsilimigras *et al* [137]	Laboratory results, clinicopathological parameters, tumor characteristics	CART	976 HCC patients	Determining factors of prognostic weigh preoperatively within the BCLC staging system	---
Tsilimigras *et al* [139]	Laboratory results, clinicopathological parameters, tumor characteristics	CART	1146 CCA patients	Determining factors of prognostic weigh preoperatively	---
Jeong *et al*[140]	Laboratory results, clinicopathological parameters	DNN	1421/234[7]	Intrahepatic CCA susceptible to adjuvant therapy following resection	0.84[2,5], 0.78[2,7]
Shao *et al*[141]	Clinicopathological parameters	ANN	288 CCA patients (training:validation = 8:2)	Predict early occlusion following bilateral plastic stent placement	0.9648[2,5], 0.9544[2,6]

[1]Accuracy (%).
[2]Area under the receiver operating curve or c-index.
[3]Sensitivity (%).
[4]Specificity (%).
[5]Training.
[6]Internal validation.
[7]External validation/testing. ANN: Artificial neural network; BCLC: Barcelona clinic liver cancer; CART: Classification and regression tree; CCA: Cholangiocarcinoma; DNN: Deep neural network; HCC: Hepatocellular carcinoma; HCV: Hepatitis C; RF: Random forest; WGS: Whole-genome sequencing.

significantly more accurate than the current guidelines of the American Joint Committee of Cancer[140]. Finally, a study developed an ANN to predict which patients with inoperable hilar CCA will develop early occlusion following a bilateral plastic stent placement[141].

Prognosis

Table 8 summarizes the findings of the identified studies applying AI/ML models regarding prognosis in the field of hepatology. An ANN model was developed in a study to identify patients with HBV cirrhosis at a high risk of developing esophageal varices[142]. In a different study, two different RF models were created using clinical data, the first to identify esophageal varices and the second to classify patients with esophageal varices that require treatment[143].

Regarding HCC, several studies have focused on developing AI/ML-based models that would reliably predict patient outcomes (survival and recurrence). A retrospective study compared the performance among an ANN, an LR model, and a DT to predict the 1-, 3-, and 5-year disease-free survival in patients with HCC following resection [144]. A similar study used a nationwide database to compare an ANN and an LR model in predicting the 5-year survival of patients with HCC following hepatic resection and concluded that the ANN model surpassed the performance of the LR model[145]. In a different study by the same department, an ANN model and an LR model were compared, but instead, the outcome was in-hospital mortality, and the ANN was found superior to the LR model[146]. Interestingly, the study reported that the surgeon volume was the best single predictor of in-hospital mortality[146]. Regarding predictors, a different study comparing ANN and LR models reported that the ANN model identified a greater number of significant predictors than the LR model, except for outperforming it in survival predictions[147]. In a prospective study that aimed to compare an ANN model's performance to the performance of traditional staging systems in predicting survival for patients with early HCC, the ANN model outperformed the Hepato-Pancreato-Biliary Association's, the TNM 6th, and the BCLC staging systems with higher reported AUROCs in all training, internal validation, and external validation cohorts[148]. In a recent study, a GB survival classifier-based

Table 8 Artificial intelligence applications in hepatology: Prognosis

Ref.	Parameters employed	AI classifier	Sizes of the training/validation sets	Outcomes	Performance
Hong *et al* [142]	Laboratory results, clinicopathological parameters	ANN	197 HBV patients (training:validation = 4:1)	Development of esophageal varices in HBV cirrhosis	87.82[1,6], 93.75[3,6], 71.70[4,6]
Dong *et al* [143]	Laboratory results, clinicopathological parameters	RF	238/109[7]	Identification of esophageal varices	0.84[2,5], 0.82[2,7]
				Classification of esophageal varices requiring treatment	0.74[2,5], 0.75[2,7]
Ho *et al*[144]	Laboratory results, clinicopathological parameters, surgery parameters	ANN, DT	427, 354, and 297 patients for 1-, 3-, and 5-year survival (training:validation = 8:2)	1-, 3-, and 5-year disease-free survival Following surgical resection	ANN: 0.963-0.989[2,5], 93.5-96.3[3,5], 91.6-97.9[4,5], 0.774-0.864[2,6], 70.0-78.7[3,6], 54.2-92.7[4,6]
					DT: 0.675-0.825[2,5], 19.6-94.8[3,5], 45.8-97.9[4,5], 0.561-0.718[2,6], 0-88.5[3,6], 37.5-96.4[4,6]
Shi *et al*[145]	Laboratory results, clinicopathological parameters, tumor characteristics	ANN	22926 patients	5-year survival following surgical resection	96.57[1,6], 0.885[2,6], 97.43[1,7], 0.871[2,7], 74.23[3,7],
Shi *et al*[146]	Laboratory results, clinicopathological parameters, surgery parameters	ANN	22926 hepatectomies	In-hospital mortality following surgical resection	97.28[1,6], 0.84[2,6], 95.93[1,7], 0.82[2,7], 78.40[3,7], 94.57[4,7]
Chiu *et al* [147]	Laboratory results, clinicopathological parameters, tumor characteristics	ANN	434, 341, and 264 patients for 1-, 3-, and 5-year survival, (training:validation = 8:2)	1-, 3-, and 5-year overall survival, following surgical resection	98.5-99.5[1,5], 0.980-0.993[2,5], 99.7-100[3,5], 96.2-99.2[4,5], 72.1-85.1[1,6], 0.798-0.875[2,6], 71.4-88.6[3,6], 50.0-82.1[4,6]
Qiao *et al* [148]	Laboratory results, clinicopathological parameters, tumor characteristics	ANN	362/181[6]/104[7] patients	Survival following surgical resection	0.855[2,5], 80.00[3,5], 73.40[4,5], 0.832[2,6], 78.67[3,6], 75.70[4,6], 0.829[2,7], 77.42[3,7], 78.08[4,7]
Liu *et al*[149]	Laboratory results, data from immunochemistry of peripheral blood mononuclear cells, tumor characteristics	GB survival classifier	136/56[6]/105[7]	Risk of HCC-related death	0.844[2,5], 0.827[2,6], 0.806[2,7]
Zhong *et al* [150]	ALBI/CTP stage	ANN	319 / 61[7] / 124[7]	Survival of patients treated with chemoembolization and sorafenib	ALBI-based: 0.716[2,7], 0.823[2,7]
					CTP-based: 0.779[2,7], 0.693[2,7]
Divya and Radha[152]	Laboratory results, clinicopathological parameters, tumor characteristics	APO, SVM, RF	152 patients	Recurrence following RFA	95.5[1,6], 95.1[3,6], 95.8[4,6]
Yamashita *et al*[153]	Hematoxylin and eosin-stained WSI	CNN	299 / 53[6]/198[7] WSIs	Recurrence following Surgical Resection	0.724[2,6], 0.683[2,7]
Liang *et al* [154]	Laboratory results, clinicopathological parameters	SVM	83 patients	Recurrence following RFA	73-82[1,6], 0.60-0.69[2,6], 77-86[3,6], 73-82[4,6]
Eaton *et al* [155]	Laboratory results, clinicopathological parameters	GB-based	509/278 patients with primary sclerosing cholangitis	Classify risk of primary sclerosing cholangitis-related complications	0.96[2,6], 0.90[2,7]
Andres *et al* [156]	Laboratory results, clinicopathological parameters, donor characteristics	PSSP system	2769 patients	Survival following transplantation for primary sclerosing cholangitis	----
Rodriguez-Luna *et al* [157]	Genotyping data from microsatellite mutations/deletions	ANN	19 transplated patients	Post-transplant HCC recurrence	89.5[1,6]
Lau *et al*[158]	Laboratory results, clinicopathological parameters, donor characteristics	ANN, RF	90/90 transplants	Graft failure/primary nonfunction	ANN: 0.734-0.835[2,6]
					RF: 0.787-0.818[2,6]
				3-mo graft failure	ANN: 0.559[2,6], R6: 0.715[2,6]
Briceño *et al* [160]	Laboratory results, clinicopathological parameters,	ANN	1003 liver transplants	3-mo graft failure	0.806-0.821[2,6]

	surgical parameters, donor characteristics

[1]Accuracy (%).
[2]Area under the receiver operating curve or c-index.
[3]Sensitivity (%).
[4]Specificity (%).
[5]Training.
[6]Internal validation.
[7]External validation/testing. ALBI: Albumin-bilirubin; ANN: Artificial neural network; APO: Artificial plant optimization; CNN: Convolutional neural network; CTP: Child–Turcotte–Pugh; DT: Decision tree; GB: Gradient boosting; HBV: Hepatitis B; HCC: Hepatocellular carcinoma; PSSP: Patient-specific survival prediction; RF: Random forest; RFA: Radiofrequency ablation; SVM: Support vector machine; WSI: Whole-slide image.

model was developed to stratify the risk of an HCC-related death into three distinct categories[149]. Finally, in another recent study, an ANN identified albumin-bilirubin grade as the most important prognostic factor for the survival of patients with HCC treated with the combination of transarterial chemoembolization and sorafenib as initial treatment[150]. A systematic review aimed to compare the performance of AI/ML-based software and that of traditional linear prediction models in predicting survival for patients with HCC concluded that AI/ML models provided enhanced accuracy[151].

Except for survival, other studies have developed AI/ML models to predict the recurrence of HCC following therapeutical treatment. An interesting study employed several AI/ML methods, including Artificial Plant Optimization, SVM, and RF to predict HCC recurrence following radiofrequency ablation[152]. A recent study developed a CNN employing histopathologic images to predict recurrence in operated HCC patients[153]. A different study developed an SVM model to predict the recurrence in a group of patients who underwent radiofrequency ablation[154].

Regarding primary sclerosing cholangitis, a team of researchers derived and validated a risk estimate tool based on GB algorithms to predict the outcomes of the disease[155]. In a different study, an ML model was developed to predict survival curves for patients with primary sclerosing cholangitis following liver transplantation [156]. The P value of the χ^2 test of the distributional calibration was 1, indicating excellent calibration of the model[156].

Focusing on liver transplantation, a team developed an ANN model combined with genotyping for microsatellite mutations/deletion to predict HCC recurrence in a cohort of patients receiving a liver transplant[157]. Several other studies have focused on the survival of individual liver grafts following transplantation[158-160]. In a multi-centered study, the authors developed an ANN model to predict the 3-mo graft survival and loss[160]. Interestingly, their model outperformed all extensively validated scores, including the MELD, the donor risk index (DRI), the survival outcome following liver transplantation, and the balance of risk, with their performance significantly lower (AUROCs range: 0.42-0.67)[160]. In a different study, an RF model was developed to predict the 30-d failure graft. Notably, this model too outperformed MELD and DRI[158]. These findings could help reevaluate our thinking regarding the current models of recipient-donor matching.

Opportunities of AI application in hepatology

Therefore, the opportunities that arise from applying AI/ML-based software in hepatology include:

Regarding primary care, ML models could be integrated into the clinical setting and flag individuals in the general population at high risk of developing chronic liver disease in real-time by employing routinely collected data from the electronic health records. Current efforts include models that identify patients at high risk to develop NAFLD, NASH, fibrosis, and cirrhosis[108-114]. These models could be used to design targeted screening programs.

Particularly for patients with chronic HBV and HCV infection, AI/ML models could be used to stratify each individual's risk to progress through the several stages of cirrhosis and develop HCC. Multiple studies have been conducted in this regard for both HBV-related[115-117] and HCV-related[115,118-121] cirrhosis. Such models could be used to identify patients in greater need of SVR and tailor individualized follow-up schedules.

In diagnosis, AI/ML models provide the opportunity for increased diagnostic accuracy of various hepatic diseases and in multiple diagnostic modalities (such as

US/CT/MRI imaging and histologic images). Current efforts include CAD models for the diagnosis of chronic liver disease, HCC[127-129], and CCA[130-132].

AI/ML models that accurately and reliably predict the response to specific treatments could be used to tailor evidence-based frameworks for individualized treatment allocation. Current efforts include the development of frameworks for the management of HCC[136,137] and CCA[139,140].

Regarding prognosis, AI/ML models could be used to reliably predict complications, in-hospital mortality, overall survival, and recurrence following treatment. Current efforts include the prediction of in-hospital and HCC-related death[146,149], of overall survival of HCC patients following surgery[144,145,147,148], and HCC recurrence following surgery or RFA[152-154]. Such models could be used to tailor individualized follow-up schedules and for patient and family counseling.

Particularly for liver transplantation, AI/ML models could be used to predict graft failure or cancer recurrence for patients transplanted for cancer. Such models could be used to reevaluate and optimize our current practices regarding recipient-donor matching for graft allocation[157-160].

CURRENT CHALLENGES OF AI APPLICATIONS

Intrinsic bias and accuracy

The level of accuracy of AI systems is mostly dependent on the quality of the training dataset. Existing biases and prejudices in the training data set will inadvertently be built into the algorithms limiting the AI/ML-based software's accuracy[161]. Discrepancies in the data collection process, imperfections of standardization, and incorrectly labeled cases become part of the algorithms' training and are thus integrated into the end product. Two of the most common biases found in these models are: (1) Spectrum bias; and (2) Overfitting. Spectrum bias occurs when the patients (whose generated data were used during the training and internal validation of these models) do not constitute a representative sample of the target population[162]. On the other hand, overfitting refers to the tendency of models to be customized for the training data [162]. The performance of the model is thus exaggerated for the training dataset but is significantly inferior in new datasets[162]. CNNs, which are extensively used in gastroenterology and hepatology, are particularly vulnerable to overfitting[26]. Another substantial bias source is that physicians often misrecord data in the electronic health records, sometimes even the chief complaint[163]. These biases significantly impact the performance of AI/ML-based models and undermine their applications. A measure to alleviate the impact of biases is the standardization of data collection methods while establishing evaluation systems that scrutinize underlying biases and check data accuracy[15,164]. Another crucial step to tackle these biases is the external validation of models, in a clinical setting, from data generated from patients prospectively enrolled in the study[165]. Initial implementation of AI/ML-based models in the clinical setting should occur on a small scale, similar to phase I and phase II of the clinical trials[166]. Existing tools, such as the PROBAST tool, could be used during the development of AI/ML-based models to comprehensively assess the risk of bias[164,167]. The American Medical Association has recently acknowledged the need to identify and address bias in data when testing or deploying AI/ML-based software to avoid introducing or exacerbating health care disparities [168]. Therefore, the real challenge is to develop an AI/ML-based software that taps into the true potential hidden by the data without picking up the biases.

Several other limitations affect the performance of these models. An example is the limited resolution of capsule endoscopy images compared to other types of endoscopy [18]. A different limitation is the in silico nature of most of the currently developed models, which significantly impacts the expectations of similar performances in a real clinical setting. Another limitation of these algorithms is that they utilize a series of variables that in the real clinical setting are derived in a series of careful decisions made by physicians and are not readily available as a complete set of data. Therefore, these variables' presence or absence could become a significant shortcoming of these models in a real clinical setting, making them impractical to use. Finally, there is a lack of consistency in the metrics used to assess performance (sensitivity, specificity, AUROC, accuracy, *etc.*), limiting the ability to draw meaningful comparisons between the models[17].

Data protection and cybersecurity

Similarly, with all patient data, those utilized in AI/ML-based software should

conform with the seven principles described in Article 5 of the General Data Protection Regulation. Specifically, the personal data are required to: (1) Be processed fairly, lawfully, and transparently; (2) Be relevant, adequate, and limited to the intended purpose; (3) Be collected for explicit, specific, and lawful purposes; (4) Be accurate and up to date; (5) Permit identification for only as long as necessary; (6) Ensure appropriate security; and (7) Demonstrate compliance and accountability[169]. A particular challenge for AI is the resistance to the concept of utterly electronic tracking of healthcare records due to the belief that it exposes the vast amount of stored sensitive health record data to massive disclosures[170]. These concerns are not entirely unjustifiable if one considers examples such as the transfer of data from 1.6 million patient records from the Royal Free Nathional Health Service Foundation Trust to Google DeepMind, which was later ruled illegal[171]. Nevertheless, any effort or policy towards paper-based data due to data protection concerns could substantially undermine the application of AI modes in healthcare.

The healthcare industry is a particularly attractive target for cyberattacks as it contains sensitive personal data and financial information. Several physical and technical safeguards have been implemented under the Health Insurance Portability and Accountability Act to protect against the breach of sensitive patient data[172]. However, from the application of AI technology in the healthcare industry, new vulnerabilities and dangers emerge except traditional cybersecurity concerns. If ML models can learn from data, they could also be fooled by data. Data could be introduced malevolently in the algorithms to manipulate the developed AI/ML models into making wrong decisions with currently unknown ramifications to patient outcomes[173]. In a recent study, the authors demonstrated how attackers could use DL to add or remove lung cancer tumors in CT scans[174]. This study demonstrated how both a group of radiologists and a state-of-the-art deep AI model were particularly susceptible to the attack[174]. How could we, therefore, be confident that the AI/ML model has not been compromised? Evidently, healthcare facilities will have to develop additional information technology infrastructure to shield the healthcare system from these new threats.

Intellectual property

The Food and Drug Administration (FDA) recently acknowledged that it receives a high volume of submissions regarding AI/ML-based software marketing, with the list of already approved algorithms increasing rapidly[175,176]. A challenging point will be determining the law framework and regulatory standards that AI/ML-based software should follow. The key is classifying AI/ML software as "medical device", "service", or as "product", and achieving this requires a careful evaluation of the intentive use of AI/ML-based software. AI/ML-based software that aims to assist physicians in diagnosing, interpreting, and treatment decisions could be classified as medical devices and fall under the respective regulations[12]. In 2019, the FDA announced its aim to review AI/ML-based software regulation and has recently published the AI/ML– Based Software as a Medical Device Action Plan[175,177]. The action plan published by the FDA focuses on five pillars aimed to facilitate innovation and advance AI/ML-based software that are classified as medical devices. These include: (1) A tailored regulatory framework; (2) Good ML practice that could be achieved by consensus standards efforts; (3) A patient-centered approach incorporating transparency to users that takes into account usability, trust, equity, and accountability; (4) Regulatory science methods related to algorithm bias and robustness, and finally; and (5) Real-world performance[175]. When it comes to law and regulation, the real challenge is finding the golden snitch between too much regulation that strangles innovation and creation and too little regulation, which could have unexpected and devastating consequences for healthcare, and by extension, the well-being of patients.

Another interesting point regarding intellectual property and safety is the substantial divergence of the AI/ML-based software from the original product years after its approval and distribution[178]. What are the rights of developers on the product following its purchase? Since the original product constantly changes by learning from the clinical setting's data, the deviated model years after the purchase and the original software could be seen as two entirely different products. The first is protected under copyright law. However, the latter has now produced intellectual property on its own since it encompasses data generated by the healthcare facility [173]. Who could therefore claim legal rights over the final product? Also, who ensures the credibility and safety of the model as it deviates from the original product? Clearly, there is a need for lifecycle regulation of AI/ML-based software that ensures postapproval guardrails such as built-in audits[165]. Alternatively, time-limited authoriz-

ations could be employed to allow the FDA to perform periodic audits to review the accumulated modifications to the initial product[165].

Liability

Except for intellectual property, another legal challenge that arises from applying AI/ML-based software in the clinical setting is liability. A quite intriguing concept is that AI's findings and decisions could become legally binding in the future. As AI/ML-based models evolve and become more sophisticated, it is fair to assume that they will eventually surpass physicians, at least in specific tasks. How could then, the physicians justify ignoring the decision presented by AI? Especially when their decisions are made solemnly based on data and lack any sense of subjectivity. And who is liable when the followed decision made by the model causes injury? Currently, there is no legal precedent that assigns liability in a case where the injury was inflicted on a patient due to an erroneous output generated by AI/ML-based software[179].

To avoid malpractice liability, a physician is required to provide medical care at the same level as a competent physician of the same specialty while considering the available resources[180]. However, when an AI/ML-based software recommendation is involved, the concept of liability becomes more complicated. In an insightful recent legal analysis, the authors provide eight scenarios based on the combinations of whether the AI recommendation follows standard care and/or is accurate, the physician follows or rejects that recommendation, and whether a medical injury occurs [179]. The authors conclude that since current law shields physicians from liability when the standard care is followed, it also incentivizes physicians to minimize AI's actual usefulness, transforming AI into a confirmatory tool rather than a tool to augment the level of care[179]. Until a comprehensive legal framework regarding liability is developed, healthcare facilities would justifiably hesitate to adopt AI technologies due to the fear and unawareness of how they will expose the facility and its staff to liability[181].

Ethical challenges and transparency

AI could become the third participant in the physician-patient relationship and potentially undermine the trust between them. First, the idea that data are shared with third parties for AI model development could lead patients to withhold information from physicians and become less transparent[182]. Second, AI/ML-based models cannot act like compassionate human beings, which is an integral part of a physician's clinical life. Therefore, AI-driven decision-making neither encompasses an understanding of the patient's needs nor respects the patient's wishes nor demonstrates empathy, nor realizes when a patient feels discomfort or requires some rest or a hand to hold on to. Therefore, retaining the trust in the physician-patient relationship could be proven challenging in the AI era.

Another challenge is patient consent in AI applications. Clearly, it is practically impossible to acquire informed consent from each and every patient whose data are used during the development and validation of AI/ML-based software. In any case, we cannot predict how the algorithms would use specific data points during the training of the model and whether data of a specific patient have any significant impact on the model as a whole[183]. However, when AI/ML-based models are used in the clinical setting, especially for patient recording (in computer vision), patients should be adequately informed, and explicit consent should be acquired[184].

Achieving equilibrium between ensuring a high level of care and avoiding privacy encroachment could be challenging. For example, with computer vision advancement, monitoring could be used to detect any deviations from the optimal bedside practices such as patient mobilization and hand hygiene practices, which leaves patients vulnerable to identification[185,186]. Practices such as data minimization (collecting the least data needed) could address these challenges[185].

Changing the physicians' and patients' stance towards AI technologies could be proven a herculean task. Currently, around 2/3 of the population feels uncomfortable using their data to improve care quality and are unfavorable to AI/ML-based software performing tasks typically performed by physicians[183]. There are justifiable concerns that certain biases included in the training data due to racism, sexism, and socioeconomic inequality would be integrated into the AI/ML-based software. A notorious example is the COMPAS algorithm, which was found to falsely flag black people as likely to re-offend[187]. This was aggravated when developers made the argument that the algorithm was protected under intellectual property law and thus is not open to scrutiny[187]. These examples could erroneously undermine the trust towards AI altogether. A trustworthy AI/ML-based software should be built around the principles of transparency, credibility, auditability, reliability, and recoverability

[188].

The last but not least challenge in applying AI/ML-based software in healthcare is the lack of transparency, which is currently a major concern. The lack of transparency is demonstrated by the non-interpretability associated with these algorithms, described as a "black-box", where the inner logic is hidden[18]. This violates a fundamental tenet of medical ethics that physicians should comprehend at least the basic features of the devices they use and could undermine the trustworthiness of these technologies[189]. Thus, those who develop AI/ML-based models and physicians should collaborate to reach at least a degree of explainability on how AI/ML-based algorithms reach decisions. Attention maps and saliency region are examples of methods that could lessen the lack of interpretability of these models[190]. A slightly different and intriguing point of view is that withholding the widespread application of AI/ML-based software, due to their opacity, when their application could significantly benefit patients is, in fact, unethical[183]. With most of the population being prejudiced against AI, developing trust for these technologies would require several steps, including addressing bias, increasing transparency, communicating with the patients their role in provided care, protecting data privacy, and developing a robust regulatory framework.

CONCLUSION

Even though multiple efforts for AI's integration into healthcare have been made, they all originate from high-income countries[191]. Understandably, since all AI/ML-based software is data-driven, the opportunities to participate in the AI ecosystem for low- and middle-income countries, where the healthcare system does not generate extensive electronic, standardized data, are significantly limited. Not being included in the development of these models would introduce a significant spectrum bias, which could undermine these models' application in low- and middle-income countries. Thus, despite being described as the key to healthcare equity, AI could become another brick in the wall of inequality. A challenge for the future would be to include low- and middle-income countries in AI model development initiatives.

Even though these AI/ML-based software's central idea is that they could surpass physicians in performing certain tasks, the existing literature comparing AI/ML-based models and physicians' performance is limited[192]. A recent meta-analysis, which included multidisciplinary comparative studies, identified only 25 studies meeting their inclusion criteria from all medical specialties[193]. The authors concluded that currently, DL models' diagnostic performance in detecting diseases from medical imaging is equivalent to that of healthcare professionals[193]. Clearly, there is a contrast between the abundance of studies developing and validating ML algorithms and the lack of studies comparing these models' performance to physicians' performance at the investigated task. Thus, there is a crystalline need for further comparative studies in the clinical setting.

Another challenge for the future would be to combine AI with other emerging fields, such as 3D printing and bioprinting, augmented reality, novel biomarkers such as microRNAs, and robotics[194-198]. Envision a world where all these technologies and their applications are unified and integrated into everyday clinical practice. AI could be the means through which this new sophisticated, complex clinical setting is handled. The future AI clinician rejects oversimplifying an inherently complex field but instead embraces the complexity.

AI can outperform physicians in the ability to precisely quantify correlations even in domains where physicians possess in-depth knowledge[173]. However, AI models are simply tools like any other and should be treated as such. Tools with their accuracy, sensitivity, and specificity in performing certain tasks, tools that carry biases, and whose findings should be evaluated in conjunction with other clinical and paraclinical findings. Reliance on AI should not exclude non-quantifiable information from decision-making[183]. Clinical reasoning and critical thinking should not be subsumed by AI at the altar of technological advancement. AI's integration into healthcare should not replace the physicians' intelligence but rather augment it. Thus, we should aim for AI-assisted and not AI-driven clinical practice[14]. Finally, AI systems should be applied in healthcare along with equally advanced evaluation systems, which properly assess their ramifications within the clinical practice environment, investigate their unintended consequences, and, most notably, evaluate their impact on patient outcomes[163]. An example of an evaluative initiative is the Digital Health Innovation Action Plan launched by the FDA that aims to assess medical software under

development based on five excellence criteria: product quality, patient safety, clinical responsibility, cybersecurity responsibility, and proactive culture[199].

Despite being on the front line for several decades, AI still comes short of delivering the presumable solutions to long-standing healthcare problems. AI winters, where AI funding is significantly reduced, demonstrate the frustration that AI is not evolving at a pace investors would feel comfortable sustaining the funding[1]. AI may seem like a field that constantly overpromises but usually underdelivers, given its current impact on healthcare. In this review, we have highlighted the current challenges that we believe restrain the extensive application of AI in healthcare in an attempt to explore ways to overcome them.

Nevertheless, even at a slower pace, AI will eventually "infiltrate" not only the hospital setting but rather the healthcare industry as a whole, from central healthcare facilities to private practice and telemedicine, from medical schools and teaching hospitals to pharmaceutical companies, and even healthcare policy-makers in government. AI will become a reality; everyone will have to conform with, to avoid becoming obsolete. Therefore, sooner or later, physicians will have to engage with the field of AI by necessity. The role of physicians in this upcoming "revolution", however, remains to be seen. Will we be shaping this "revolution", or will we be mere observers?

Limitations

Finally, our review has few limitations. First, our study is a narrative review, and despite our best effort to follow a carefully designed search strategy to provide a comprehensive review of the current literature, our study is prone to selection bias due to its nature. Second, each study described in our study carries its own risk of bias and faces several limitations described in each study. We did not use any bias risk assessment tool to systematically evaluate if a study was worth inclusion rather than the followed search strategy and our judgment. Again this makes our review prone to selection bias. In addition, the majority of our included studies were performed in silico, and the performances reported could deviate substantially when the models are applied in a real clinical setting. Finally, with few exceptions, the majority of results report a superior or at least equivalent performance of AI/ML-based algorithms compared to the performance of conventional statistic models, widely used staging systems, extensively investigated and validated scores, and physician knowledge and reasoning, which have dominated decision-making in healthcare for decades. Thus, at least in the concept of publication bias, our findings should be interpreted with caution.

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MINIREVIEWS

Impact of *Helicobacter pylori* infection on gut microbiota

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Abstract

A number of studies have revealed the association between *Helicobacter pylori* (*H. pylori*) infection and the gut microbiota. More than half of the investigations on the impact of *H. pylori* on the gut microbiota have been the sub-analyses of the influence of eradication therapy. It was observed that *H. pylori* eradication altered gut microbiota within a short period after eradication, and majority of the alterations took a long period of time to reverse back to the original. Changes in the gut microbiota within a short period after eradication may be attributed to antibiotics and proton pump inhibitors. Modification of gastric acidity in the stomach caused by a long-term *H. pylori* infection alters the gut microbiota. Analysis of the gut microbiota should be conducted in a large population, adjusting for considerable biases associated with the composition of the gut microbiota, such as age, sex, body mass index, diet and the virulence of *H. pylori*.

Key Words: *Helicobacter pylori*; Gut microbiota; Atrophic gastritis; Eradication; Proton pump inhibitor

Core Tip: *Helicobacter pylori* (*H. pylori*) eradication alters gut microbiota within a short period after eradication; this is attributed to antibiotics and proton pump inhibitors. However, most of these alterations reverse back to baseline levels over a long period of time. Modification of acidity in the stomach with mucosal atrophy caused by *H. pylori* infection alters the gut microbiota. As the human gut microbiome is diverse among individuals, a large population size is needed to study. Adjustment of biases associated with the composition of the gut microbiota is also crucial for accurate evaluation of the association between *H. pylori* infection and the gut microbiota.

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INTRODUCTION

In recent years, a number of studies related to gut microbiota have been published, shedding light on the association between gut microbiota and human health. The human microbiota consists of as many as 10-100 trillion symbiotic microbial cells harbored in the intestinal tract of every person[1]. The gut microbiota plays a pivotal role of in the metabolic, physiological, and immunological systems of the human body [2], and its structure is closely associated with an individual's health and past illnesses [3].

Accordingly, research on the association between *Helicobacter pylori* (*H. pylori*) infection and the microbiota has also increased[4]. Most of the studies, including our previous studies that revealed the influence of *H. pylori* infection on the gut microbiota, have focused on the gastric microbiota, while only a few studies have investigated the gut microbiota harbored in the intestinal tract of patients with *H. pylori* infection[5,6]. Subsequently, some published studies have revealed new findings and have improved our understanding of this phenomenon. Therefore, the current review aims to summarize the recent evidence on the influence of *H. pylori* infection on the gut microbiota, while focusing on the gut microbiota in the intestinal tract, and to discuss the mechanisms underlying the *H. pylori* mediated alterations in the gut microbiota.

H. PYLORI AND GUT MICROBIOTA

More than half of the investigations on the impact of *H. pylori* on the gut microbiota have been the sub-analyses of the influence of eradication therapy on the gut microbiota[7-11] (Table 1). Two earlier studies were based on in situ hybridization and bacterial culturing using fecal samples. A study showed that the gut microbiota of *H. pylori*-positive patients was characterized by an increase in the growth of acid-tolerant *Lactobacillus acidophilus*[7]. Another study found that the total amount of *Anaerobes* and *Clostridia* present in *H. pylori*-positive patients was significantly lower as compared to that of *H. pylori*-negative subjects[8]. Subsequent studies were based on the analysis of the fecal 16S rRNA. The analysis of the fecal 16S rRNA from 70 *H. pylori*-positive subjects and 35 *H. pylori*-negative subjects showed a decrease in the abundance of *Clostridia* as well as total anaerobes in the fecal samples of *H. pylori*-positive individuals[9]. In a study on young adults, the microbial diversity of the gut microbiota was higher in patients infected with *H. pylori* than in healthy controls. Moreover, at the phylum level, the relative abundance of *Proteobacteria* significantly increased in patients infected with *H. pylori*[10]. In contrast, only the study by Martín-Núñez *et al*[11] revealed that in comparison with uninfected individuals, the alpha diversity of gut microbiota was significantly lower in patients infected with *H. pylori*. In these studies, the composition of the gut microbiota between subjects infected and uninfected was not the primary endpoint. Moreover, the number of subjects taken into consideration was relatively small. As the diversity of the human gut microbiome varies among individuals, a large population size is needed.

A few studies have been conducted to investigate the influence of *H. pylori* infection on the gut microbiota[5,6,12,13]. Our large population study performed using 16S rRNA amplification from fecal samples revealed that *Lactobacillus* in the human gut microbiota may be influenced by *H. pylori* infection[5]. In a small-sample study, Dash *et al*[12] showed that the gut microbiota of *H. pylori*-infected individuals were enriched with members of *Succinivibrio*, *Coriobacteriaceae*, *Enterococcaceae*, and *Rikenellaceae* families. Furthermore, several studies have suggested that the composition of the human gut microbiota changes with age, body mass index (BMI), and sex[14-16]. Therefore, we excluded the influence of these factors using the propensity score matching, which has not been considered in previous studies. We compared 214 *H. pylori*-positive subjects and 214 matched *H. pylori*-negative subjects from a large population study and found a higher gut microbial diversity and a different gut microbiota composition in subjects with *H. pylori*[6]. Furthermore, at the genus level,

Table 1 Studies for the influence of *Helicobacter pylori* infection on gut microbiota

Ref.	Study groups *H. pylori* (+) *vs* (-)	Aim	Main findings for *H. pylori* positive subject
Bühling *et al*[7], 2001	51 *vs* 27	Sub analysis for eradication study	*L. acidophilus* ↑
Myllyluoma *et al* [8], 2007	39 *vs* 19	Sub analysis for eradication study	*Clostridia* ↓, *Anaerobes* ↓
Chen *et al*[9], 2018	70 *vs* 35	Sub analysis for eradication study	Diversity ↑, *Nitrospirae* ↓, the relative abundance of 19 pathways were significantly different between *H. pylori*-negative and *H. pylori*-positive patients
Iino *et al*[5], 2018	226 *vs* 524	Analysis of microbiota without eradication	*Lactobacillus* ↑
He *et al*[10], 2019	10 *vs* 7	Sub analysis for eradication study	Diversity ↑, *Proteobacteria* ↑
Iino *et al*[6], 2020	214 *vs* 214	Analysis of microbiota without eradication	Diversity ↑, *Haemophilusu* ↑, *Streptococcus* ↑, *Gemella* ↑, *Actinomyces* ↑
Martín-Núñez *et al* [11], 2019	40 *vs* 20	Sub analysis for eradication study	Diversity ↓, *Oscillospira* ↓
Dash *et al*[12], 2019	12 *vs* 48	Analysis of microbiota without eradication	Diversity ↑, *Succinivibrio* ↑, *Coriobacteriaceae* ↑, *Enterococcaceae* ↑, *Rikenellaceae* ↑, *Candida glabrata* ↑
Frost *et al*[13], 2019	212 *vs* 212	Analysis of microbiota without eradication	Diversity ↑, *Prevotella* ↑, *Bacteroidetes* ↓, *Parasutterella* ↑, *Holdemanella* ↑, *Betaproteobacteria* ↑, *Pseudoflavonifractor* ↓, *Alisonella* ↑, *Howardella* ↑

Helicobacter pylori: *H. pylori*.

the abundance of *Actinomyces*, *Gemella*, *Streptococcus*, and *Haemophilus* was significantly higher in the gut microbiota of *H. pylori*-infected subjects. Another recent study conducted by Frost *et al*[13] assessed the microbiota composition of 212 *H. pylori*-positive subjects and 212 matched negative controls. Similar to our study, all control samples were matched with respect to age, sex, BMI, alcohol consumption, smoking, proton pump inhibitor (PPI) usage, history of peptic ulcer disease, and dietary habits. This study demonstrated that *H. pylori* infection was associated with alterations in fecal microbiota and an overall increase in fecal microbial diversity. A later study on the long-term effects of *H. pylori* eradication demonstrated that the structure of the gut microbiota is more closely associated with subject-specific parameters, such as age or BMI, than with the eradication therapy itself[17]. Therefore, adjusting for biases associated with the composition of the gut microbiota is crucial for accurate evaluation of its composition. Diet is a key modifiable factor affecting the composition of the gut microbiota[18]. However, only one study has addressed this parameter[13].

THE INFLUENCE OF *H. PYLORI* ERADICATION ON GUT MICROBIOTA

A number of published studies have investigated the changes in the gut microbiota after *H. pylori* eradication. A recent systematic review of 24 articles examining the effect of *H. pylori* eradication on the gut microbiota revealed that most studies identified a significant decrease in the alpha diversity of the gut microbiota within a short period after eradication but no further alterations were observed for over 6 mo after *H. pylori* eradication[19]. Additionally, the abundance of *Proteobacteria* increased during a short-term follow-up whereas that of *Lactobacillus* decreased; *Enterobacteriaceae* and *Enterococcus* increased during the short-term and interim follow-up. Moreover, a more recent study evaluating the long-term effects of *H. pylori* eradication found out that the composition of the gut microbiota was restored to baseline status over the 2 years after eradication, and the relative abundances of the microbial species at the genus level before and after eradication did not differ significantly[17]. However, modest differences in the taxonomic composition were observed before and after eradication. The findings of this study where diversity of the microbiota tends to decrease in the short period after eradication and returns to baseline thereafter, it was consistent with the findings of most studies[9,10,20-26]. However, the taxonomic composition before and after eradication varied among the studies[21,22]. Some studies demonstrated that the relative abundance of all genera was restored to baseline

levels. Other studies revealed notable changes at the genus level[10,24-26]. Thus, it may be assumed that after the microbial diversity returns to baseline, the levels of each strain might demonstrate minor variations following the eradication of *H. pylori*.

THE MECHANISMS UNDERLYING *H. PYLORI* INFECTION INDUCED GUT MICROBIOTA

Although the mechanisms underlying *H. pylori* infection associated alterations in gut microbiota are still unknown, some studies have suggested possible contributing factors; these included host immune responses, virulence factors, physical contact and modification of gastric acidity[4,27]. A previous study performed using a transgenic *Drosophila* model revealed that the virulence factor, cytotoxin-associated gene A (CagA), of *H. pylori* may contribute to gut microbiota dysbiosis[28]. CagA, which is translocated into host epithelial cells after bacterial attachment, impairs cell polarity and affects host signaling pathways, thereby promoting inflammation[29]. Vacuolating cytotoxin A (VacA) is also an important virulence factor of *H. pylori*. VacA is a secreted toxin that lead to damages of gastric epithelial cells, and promotes cell death[30]. CagA and VacA counter-regulate each other to manipulate host cell responses[31]. CagA and VacA can alter the gastric microbiota and immune phenotypes previously attributed to *H. pylori* infection in the stomach[28]. Therefore, CagA and VacA have been associated with important requirements for long-term sequelae in humans. As such, ongoing crosstalk between *H. pylori* and gastric commensal microbiota may affect the host immune response. The altered host immune response may also modulate the gut microbiota[9,32]. A previous review suggested the possibility of a direct interaction of *H. pylori,* which migrates from the stomach towards the intestinal tract, with the local gut microbiota[4]. However, this hypothesis is yet to be proven. In fact, in our previous study, the presence of *H. pylori* in the intestinal tract was found to be rare even in subjects with *H. pylori* infection[6]. Therefore, the influence of *H. pylori* in the intestine on gut microbiota seems to be limited.

Modification of gastric acidity as a result of *H. pylori* infection is one of the variable effects on altered gut microbiota. PPIs, which decrease gastric acidity, affect the gut microbiota[33,34]. Reduced gastric acid promotes the passage of acid-sensitive bacteria and changes the intestinal environment[35]. Similar to the interference with the action of PPIs, *H. pylori* can regulate gastric luminal acidity. *H. pylori* infection is generally acquired during childhood and persists for life unless eradicated by treatment. In the initial stages of *H. pylori* infection, acute gastritis temporarily leads to impaired gastric acid secretion[36]. In later stages, a significant decrease in gastric acid secretion is observed in individuals who develop severe atrophic gastritis[36,37]. Previous studies investigating the gut microbiota following PPI administration detected an increase in the *Lactobacillus* population in the gut microbiota[33,34]. We evaluated *Lactobacillus* according to the degree of gastric atrophy in subjects with and without *H. pylori* infection[5]. The relative abundance of *Lactobacillus* in the human gut microbiota significantly increased after the development of severe atrophic gastritis. In another study, after adjusting for biases, we demonstrated that among *H. pylori*-infected subjects, a significant increase in the abundance of the genus *Streptococcus* was observed in subjects with severe atrophic gastritis[6]. These results support the hypothesis that severe atrophic gastritis reduces gastric acid secretion and affects the composition of the gut microbiota, similar to the results of PPI administration. Most previous studies examining the association between *H. pylori* infection and gut microbiota have not considered the influence of gastric mucosal atrophy. However, atrophic gastritis may be an important mechanism associated with the changes in the gut microbiota induced by *H. pylori* infection.

In previous studies, although *H. pylori* eradication was observed to alter gut microbiota within a short period, most of the changes induced tended to return to baseline levels over a long periods after eradication therapy[9,10,20-26]. The changes in the gut microbiota within a short period after eradication may be attributed to antibiotics and PPIs that were administered for *H. pylori* eradication. This finding was represented by a study that demonstrated a decrease in gut microbial diversity within a short period after eradication therapy in both patients with and without successful eradication[38]. The changes in the diversity within a short period after eradication may be attributed to the eradication therapy itself. Hence, the influence of eradication therapy on the gut microbiota would diminish over a long period of time. After eradication, the influence of host immune responses towards *H. pylori,* virulence factors, and physical contact with *H. pylori* could decrease or disappear. In contrast, the

modification of gastric acidity depends on the degree of mucosal atrophy. Hence, after *H. pylori* eradication, gastric acid secretion gradually improves in patients without gastric mucosal atrophy[39]. However, this improvement is not observed in patients with severe atrophic gastritis[40]. Further studies demonstrating minor changes in the gut microbiota over a long period need to be conducted using a large number of subjects with severe atrophic gastritis. Especially after *H. pylori* eradication, mechanisms other than gastric acid modification would not have a significant impact on the gut microbiota.

CONCLUSION

Although all the studies demonstrated a compositional change in the gut microbiota of *H. pylori*-infected patients, the results of these studies were not consistent with each other. This incompatibility may be attributed to several factors. Although remarkable variations are observed in the gut microbiota among individuals, the sample size considered in these studies was relatively small, and subjects were included regardless of biases associated with the composition of the gut microbiota, such as, age, gender, BMI and diet. Therefore, an analysis of gut microbiota in a large population should be conducted while adjusting to considerable biases. Particularly, it is necessary to evaluate of the degree of atrophic gastritis, which is associated with gastric acid production, when investigating the influence of *H. pylori* infection on the gut microbiota. Additionally, the virulence of *H. pylori* differs depending on the status of CagA. The prevalence of CagA-positive *H. pylori* infection varies, and the prevalence of the most virulent strain, eliciting the East Asian-type CagA phenotype, is dependent on geographical area[41]. Therefore, the investigation of the influence of *H. pylori* infection on the gut microbiota may yield different results depending on the area in which the study is conducted. Future studies should consider these points and predict the mechanisms underlying *H. pylori* infection induced changes in the gut microbiota.

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MINIREVIEWS

Therapeutic drug monitoring in inflammatory bowel disease: The dawn of reactive monitoring

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Abstract

Inflammatory bowel disease (IBD) is a chronic condition that significantly affects the quality of life of its patients. Biologic drugs have been the mainstay treatment in the management of IBD patients but despite their significant contribution, there remains a proportion of patients that do not respond or lose response to treatment. Therapeutic drug monitoring (TDM) involves measuring levels of serum drug concentrations and anti-drug antibodies. TDM of biologic drugs initially emerged to understand treatment failure in other immune mediated inflammatory diseases. This was then introduced in IBD to rationalize primary non-response or secondary loss of response, given that low serum drug concentrations or the formation of anti-drug antibodies are variably associated with treatment failure. The aim of this narrative review is to provide an overview regarding the current use of TDM in clinical practice and to present the evidence available regarding its use in both proactive and reactive clinical settings in preventing and managing treatment failure. This review also presents the existing evidence regarding the association of various clinical outcomes with specific thresholds of drug concentrations, in everyday practice. A narrative review of published articles and conference abstracts regarding the use of TDM in IBD management, through an electronic search using PubMed and ScienceDirect. TDM has proven to be superior and more cost effective in guiding management of

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patients with treatment failure compared to empiric dose escalation or change in treatment. Despite a trend towards an association between clinical outcomes and drug concentrations, proactive TDM based strategies have not been shown to achieve clear benefit in long-term outcomes. In the clinical setting, TDM has proven to be useful in managing IBD patients, and its use in the reactive setting, as an additional tool to help manage patients with treatment failure, is being promoted as newer guidelines and consensus groups implement TDM as part of the management plan.

Key Words: Therapeutic drug monitoring; Inflammatory bowel disease; Biologic therapies; Loss of response; Reactive; Proactive

Core Tip: In this review, we discuss the existing studies that looked at both proactive and reactive therapeutic drug monitoring (TDM) and concluded that in current practice, reactive TDM has been shown to be useful. When used as an adjunct to clinical assessment and biomarkers in patients with treatment failure, TDM has proven to be a valuable tool for subsequent management.

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INTRODUCTION

The management of patients with inflammatory bowel disease (IBD) has changed dramatically over the past decade, more so since the use of biologic agents[1]. Biologics such as anti-tumor necrosis factor (anti-TNF) agents have revolutionized the treatment of patients with IBD during this time in addition to newer classes of biologic agents, such as selective adhesion molecule and interleukin 12 and 23 inhibitors that are also being used. Furthermore, with advances in the treatment of IBD, goals of therapy have also changed as specialists worldwide have adopted the 'treat to target' approach in the management of IBD[2]. Despite these advances, treatment failure still occurs in a significant proportion of IBD patients[3], prompting a need in dose intensification or discontinuation of therapy with a change to another class of drug[4,5]. In the past, treatment failure in IBD, defined as either a primary non-response (PNR) or secondary loss of response (sLOR) to a drug, has been difficult to explain and predict. But as a result of a better understanding of the pharmacodynamics (PD) and pharmacokinetics (PK) of biologic drugs, the idea of therapeutic drug monitoring (TDM) emerged as an important tool. It plays a role in not only identifying the mechanism of loss of response but also in guiding clinicians in their treatment approach[6,7].

TDM is defined as the measurement of serum drug and/or anti-drug antibody (ADA) concentrations. ADAs refer to antibodies that are formed in response to the immune system's recognition of biologic drugs. In several studies, ADAs have been found to be associated with treatment failure because of an up-regulated clearance of the drug[8,9]. Another mechanism of treatment failure relates to a non-immune mediated clearance, resulting in sub-therapeutic levels of the drug.

Over the years, TDM was initially used in the reactive setting for patients, when there was a suspected loss of response to a biologic drug, mainly the anti-TNF agents. Based on the serum drug levels and/or presence of ADAs, the management was changed with the aim of optimizing their current treatment, thus avoiding unnecessary dose intensification or targeted switching between anti-TNF agents or out of class. However, preliminary results from recently published data have shown that TDM used in the proactive setting, by preemptively targeting specific thresholds of serum drug levels, may in fact result in more favorable clinical outcomes[10-17]. Despite growing evidence supporting the use TDM in IBD, there still exist limitations

of its use, such as when to use it and how best to apply it. This is evident in the fact that routine use of TDM in the management of IBD patients is not recommended in guidelines, but rather made as suggestions by organizations when faced with a patient with suspected treatment failure[18-22].

This review article summarizes the latest evidence with regards to the use of TDM in both the reactive and proactive settings in addition to its limitations and how it is currently being used in clinical practice.

BACKGROUND OF TDM

The idea of TDM involves measuring the serum concentration of a drug, to maintain an adequate dose that would ensure drug efficacy and to avoid drug toxicity[23]. Use of TDM in clinical practice has existed for many years, even before the development of biologics. In the past, TDM was used for a variety of medications, such as antibiotics and immunosuppressants. More recently, it has been applied to biologics mainly to monitor drug efficacy and to guide management in suspected treatment failure for IBD patients on biologics. In IBD, TDM involves the measurement serum drug levels, in addition to the measurement of ADAs, both of which are related to drug efficacy[8,9]. Although many studies have proved its utility, many issues exist such as timing of TDM, identification of target thresholds for serum drug levels and ADAs, and the practical application of the results. Data looking into this shows that there is considerable variability in target thresholds because of multiple factors that come to play, such as the different methods and assays used in TDM measurements, or the desired clinical outcome.

Lastly, given that the first class of biologics were anti-TNF agents, the use of TDM was first applied for patients who were treated with either infliximab or adalimumab, thus most studies looking into TDM revolved around anti-TNFs. With time, as newer classes of biologics emerged for their use in IBD, more studies were done regarding the application of TDM with vedolizumab or ustekinumab.

OVERVIEW OF TECHNICAL ASPECTS OF TDM

In TDM, the measurement of serum drug levels involves measuring the trough level (TL), meaning the lowest concentration of the drug just before the next dose. With the TL, the goal is to maintain a level sufficient enough for the drug to reach its maximal efficacy[24], and this is where the issue of target thresholds comes to play[25]. Before discussing the clinical relevance of these targets, it is important to know the technical aspects of TL and ADA measurements.

Assays used for TDM

Current tests for TL and ADA concentrations include various commercially available assays such as enzyme-linked immunoassay (ELISA), radioimmunoassay, high pressure liquid chromatography based homogeneous mobility assay, with ELISA being the most commonly used[26,27]. Drug assays use the drug as a calibrator while ADA assays use a monoclonal ADA as a calibrator, with titers often expressed as milligrams *per* liter. Several studies comparing various drug assays showed good correlation, however since assay methodologies and sensitivities differ, it is best to use the same assay when applying TDM[28]. Additionally, it is important to note that in the case of ELISA assays, the presence of the drug in the serum interferes with the detection of ADAs, thus not adequately quantifying ADA concentrations[29,30]. Various modified ELISA methods have been constructed to circumvent this problem, *e.g.*, by improved puffer technologies, labeled 'drug resistant' assays for ADA detection.

Additionally, other factors that may influence results include human factors such as appropriate collection, handling, and storage. Newer studies looking into improving TDM have suggested the idea of point of care (POC) testing in the near future, where TDM measurements are made available on spot, without the need for a laboratory-based testing method[30].

MECHANISMS OF TREATMENT FAILURE IN IBD

To better comprehend the application of TDM in IBD, it is essential to understand the idea of drug PD and PK, as they are significant in understanding the mechanism of treatment failure[24]. Various factors affect a patient's response to treatment, including low or sub-therapeutic drug levels related to increased clearance, whether it is immune or non-immune mediated in addition to the underlying pathway targeted by the drug.

PNR vs sLOR

Biologic agents, namely anti-TNF drugs, have significantly advanced the management of IBD patients. However, despite this, up to 30% of IBD patients fail to show an initial response after the induction period, also known as PNR, and up to 50% showing sLOR during the maintenance phase, especially during the first year 3-5]. Those with sLOR are patients who had initially responded after the induction phase, but then started to develop symptoms of disease activity, suggestive of treatment failure. Pharmacokinetic mechanisms underlining PNR and sLOR are thought to be due to inadequate serum drug concentrations as evidence has shown that those with low serum drug concentrations during induction or maintenance are less likely to achieve clinical response[31,32].

One study that signified this is the personalized anti-TNF therapy in Crohn's disease (CD) study (PANTS), which was a prospective observational study carried out in the United Kingdom that included 955 patients with active luminal CD[33]. Results of the study revealed that anti-TNF failure is highly dependent on low drug concentration and that this was associated with immunogenicity and the development of ADAs, especially during induction[33]. This finding mimics other studies that also revealed that higher drug concentrations early on is associated with a reduced risk of PNR and in preventing sLOR[34,35].

Clinical relevance of ADAs

One important aspect of treatment failure in IBD revolves around the idea of immunogenicity, which is an important downside of biologics. Immunogenicity deals with the fact that the immune system recognizes protein aspects of the drug as foreign and forms antibodies in response. These antibodies then form complexes with the drug, resulting in increased drug clearance and lower concentrations of the drug, rendering it ineffective[30]. However, it is important to note that not all antibodies work in the same way. In fact, two types of antibodies exist, neutralizing (NAb) *vs* non-neutralizing antibodies (non-NAb). Although they both bind to the drug, each render it ineffective in a different way[36]. NAb inhibits the pharmacological function of the drug, thus preventing target binding, whereas non-NAb promotes increased clearance of the drug[37]. This is relevant to the understanding of TDM since there is evidence pointing to a correlation between ADA formation and low serum drug levels[38-40].

One study that evaluated the clinical significance of ADAs was by Baert *et al*[32] that looked at a cohort of 125 CD patients receiving episodic infusions of infliximab. Results of this study revealed an association between the development of antibodies against infliximab response to treatment, as low trough infliximab level and high ADA level was found in 83% (10/12) with complete loss of response to infliximab. This finding reflects results of other studies that have also shown that ADA association was independently associated with LOR. There was also a large proportion of patients (61%) that had detectable ADAs and that there was an inverse relationship between ADA titers and duration of response ($P < 0.0001$)[32]. Moreover, they also noted that the median duration of clinical response was longer in patients with low ADA concentrations compared to those with higher ADA concentrations (71 d *vs* 35 d; $P < 0.001$). Similar conclusions were drawn in a study by Reinhold *et al*[41], where 16/104 (14%) patients had positive ADA titers and 15 of them had sub-therapeutic TLs, reflecting the increased drug clearance effect of ADAs[41].

As explained, ADAs play a significant role in treatment failure of IBD patients on biologics as antibodies neutralize the effect of the biologic agent, but the evidence linking ADA to LOR is not straightforward. This was demonstrated in a prospective study by Gonczi *et al*[42] that followed 112 IBD patients who were on therapy with adalimumab. Results of this study revealed that ADA positivity was significantly associated with LOR ($P = 0.007$), but the association between low TL and ADA positivity was not statistically significant ($P = 0.054$). They concluded that despite the development of ADA, low TL was not associated with LOR and that mainly ADA development should be considered a predictor for treatment failure[42]. This reiterates

the significance of NAb in that they inhibit the pharmacological function of the drug resulting in clinical non-response. As opposed to non-NAb, which affect drug efficacy by increased drug clearance thus resulting in low drug levels. For this reason, interpretation of ADA and TL when dealing with LOR should be made with caution.

Another clinically relevant aspect involves patients who develop infusion reactions. These are often found in those with persistently low drug concentrations and a significant concentration of ADAs[32,43], and this was demonstrated in the same study by Reinhold *et al*[41]. In this retrospective study that followed 104 IBD patients treated with infliximab or adalimumab, the authors delineated a positive correlation between the presence of ADAs and the development of infusions reactions as 44% (7/13) of patients with infusion reactions had positive ADA titers[41].

In conclusion, it has become clear that different ADAs exist and that not all types of antibodies affect drug clearance. Available data suggests that the presence of ADAs has a potential negative impact on clinical outcomes. Additionally, the relationship between ADA and treatment failure is not clear cut and that the impact of ADAs, may be more relevant to certain biologics than others[44-46].

ASSOCIATION OF SERUM DRUG CONCENTRATIONS WITH CLINICAL OUTCOMES

Numerous studies have demonstrated an association between drug levels and favorable outcomes[33,47-50]. Additionally, recent data has shown that higher serum drug concentrations during induction especially, is strongly associated with positive outcomes[34]. However, it is important to note that serum drug concentrations of different biologics vary as a result of many factors, such as patient demographics (age and body size), degree of underlying inflammation, and severity of disease[51]. As a result, there exists a large variation in target thresholds of TLs for each biologic drug. Current thresholds for serum drug concentrations are determined from observational studies and post HOC analyses, by looking at a variety of drug concentrations that have been found to be associated with specific outcomes; these can be seen in Table 1 [48,52-56].

In a retrospective multi-center study carried out by Juncadella *et al*[47], 98 IBD patients on therapy with adalimumab, underwent TDM and were followed to determine factors associated with outcomes such as biochemical, endoscopic, and histological remission. Results revealed that higher drug concentration during maintenance associated with good clinical outcomes and drug concentration > 12 μg/mL were associated with endoscopic (P = 0.003) and histological remission (P = 0.012) in CD[47]. Similar results were found in another retrospective study by Ungar *et al*[57], where serum drug levels of both infliximab and adalimumab were significantly higher in patients with mucosal healing compared to those with active disease on endoscopy (P = 0.002 for infliximab and P = 0.01 for adalimumab)[57].

Moreover, results of the PANTS study, showed that the only factor independently associated with PNR was a low infliximab (IFX) concentration at week 14 and that drug concentrations values associated with remission at weeks 14 and 54 were > 7 μg/mL for infliximab and > 12 μg/mL for adalimumab[33].

In terms of disease severity, for CD patients with peri-anal fistula, current evidence supports the idea of targeting higher serum drug concentrations in those patients compared to those without fistulizing disease[56,58,59]. A study by Davidov *et al*[56], showed a positive association between infliximab TLs during induction and closure of perianal fistula in CD patients, further suggesting that those patients may benefit from a drug level guided treatment.

Interestingly, despite evidence that shows a positive association of higher serum drug levels with clinical outcomes, preliminary results from the SERENE CD and SERENE ulcerative colitis (UC) trials did not show an added benefit. In both trials, patients with CD and UC, were randomized to either induction by standard dosing (SIR) of adalimumab or by an intensified dosing regimen (HIR). Results of both trials revealed that there was no added benefit from those who underwent an intensified regimen even though that subset of patients had higher serum drug concentrations[60,61]. Additionally, in terms of primary endpoints, the rates of clinical and endoscopic remission were similar among both groups.

Table 1 Association between various thresholds for serum drug concentrations and clinical outcomes

Drug	Serum drug level	Clinical outcome
Infliximab (IFX)		
IFX	< 2 μg/mL for CD/UC at week 14	Increased incidence of IFX antibodies[52]
IFX	> 2.1 μg/mL at week 14 in UC	Associated with mucosal healing[53]
IFX	≥ 3 μg/mL during maintenance	Clinical remission[48]
IFX	> 3 μg/mL at week 14 or 22 in CD	Sustained response[54]
IFX	3-7 μg/mL during maintenance	Remission[16]
IFX	≥ 7 μg/mL during maintenance	Mucosal healing[48]
IFX	< 7 μg/mL at week 14 in luminal CD	Absence of primary response[53]
IFX	> 9.2 μg/mL at induction week 2 in CD	Fistula response at weeks 14 and 30[56]
IFX	≥ 10 μg/mL at induction week 6	Clinical response[48]
IFX	> 15 μg/mL at week 6 in UC	Associated with mucosal healing[53]
IFX	≥ 20 μg/mL at induction week 2	Clinical response[48]
IFX	≥ 22 μg/mL at week 6 in UC	Clinical response at week 8[55]
IFX	≥ 25 μg/mL at induction week 2	Mucosal healing[48]
Adalimumab (Ada.)		
Ada.	≥ 3 μg/mL during maintenance	Clinical response[48]
Ada.	≥ 5 μg/mL post induction (week 14)	Clinical response[48]
Ada.	≥ 7 μg/mL post induction (week 14)	Mucosal healing[48]
Ada.	≥ 8 μg/mL during maintenance	Mucosal healing[48]
Ada.	< 12 μg/mL at week 14 in luminal CD	Absence of primary response[53]
Ustekinumab (UST)		
UST	≥ 1 μg/mL during maintenance	Clinical response[48]
UST	≥ 3.5 μg/mL post induction (week 8)	Clinical response[48]
UST	≥ 4.5 μg/mL during maintenance	Mucosal healing[48]
Vedolizumab (VDZ)		
VDZ	≥ 12 μg/mL during maintenance	Clinical response[48]
VDZ	≥ 14 μg/mL during maintenance	Mucosal healing[48]
VDZ	≥ 15 μg/mL post induction (week 14)	Clinical response[48]
VDZ	≥ 17 μg/mL post induction (week 14)	Mucosal healing[48]
VDZ	≥ 24 μg/mL at induction (week 6)	Clinical response[48]
VDZ	≥ 28 μg/mL at induction (week 2)	Clinical response[48]

Many studies that looked at exposure response relationship studies of anti-tumor necrosis factor drugs have demonstrated that specific drug concentration thresholds are associated with certain clinical outcomes, such as clinical response, improvement in biochemical markers, endoscopic remission, and mucosal healing.

MANAGEMENT OF TREATMENT FAILURE IN IBD USING A TDM BASED STRATEGY-REACTIVE TDM

Prior to TDM, the standard of care for suspected treatment failure, after exclusion of secondary causes such as infections and non-compliance, was either by empiric dose escalation, change to an alternative anti-TNF, or change to a different class of biologic.

The empiric approach of managing loss of response is considered frequently suboptimal and may lead to additional costs. The TDM-based strategy, by applying measurements of anti-TNF drug levels and anti-drug antibodies at the time of treatment failure offers an alternative. With time, more studies looking into benefits of

TDM emerged. These studies highlighted that TDM may be a useful adjunct to optimize the treatment of IBD patients, more cost effective than the standard of practice of empiric changes in management and can be useful in identifying patients who may be supra-therapeutic and instead, may benefit from dose reduction[6,62-64].

A multi-center study carried out by Guidi *et al*[65], was one of the first prospective studies carried out in a clinical practice setting that compared the management of sLOR by a TDM based algorithm to empiric dose escalations for IBD patients on infliximab. Results of their study demonstrated that the group of patients who underwent TDM based management of LOR resulted in lower rates of dose escalations with similar rates of clinical response, in addition to the fact that the TDM approach was more cost-effective[65]. Another study that reflected similar results was a randomized controlled study (RCT) carried out by Steenholdt *et al*[66], which recruited 69 Danish patients with CD on treatment with infliximab, who had developed sLOR. Patients were randomized to either routine infliximab dose or TDM based interventions. Rates of clinical response, measured by CD activity index were the same in both groups, 53% *vs* 58% ($P = 0.81$) respectively, however the costs *per* patients was 34% lower in TDM based group ($P < 0.001$)[66].

An important study that supported the use of reactive monitoring was by Afif *et al*[67], which looked at measurements of serum drug and ADA levels of 155 IBD patients who were on treatment with infliximab. Results of this study highlighted the importance of TDM in the management of partial or loss of response, as it provided insight into who might benefit from a change in drug or dose escalation. In this study, it can be seen that in ADA positive patients, the decision to change to another anti-TNF resulted in a complete or partial response in 92% of patients compared to 17% for those who underwent dose escalation. Additionally, with regards to drug levels, those who underwent dose escalation because of sub-therapeutic drug levels had a higher rate of complete or partial response compared to those who changed to an alternate drug[67]. Similarly, Yanai *et al*[6] demonstrated the utility of TDM in managing loss of response, as results from their study showed that patients with high ADA levels had longer duration to response when there was a drug change compared to dose escalation ($P = 0.03$) and that dose escalation was found to be more effective for those with low or undetectable ADA levels[6].

Additionally, in a study carried out by Kelly *et al*[63], which followed primary responders on infliximab who initially presented with clinical disease activity, authors revealed that those who underwent TDM based escalation had significantly higher clinical response ($P < 0.01$) and lower rates of hospitalization (22% TDM *vs* 35% non-TDM, $P = 0.025$) compared to the non-TDM based group[63].

A study by Ungar and colleagues that looked at infliximab levels during induction, brought to light an important finding that may help better manage patients with acute severe UC. Results of their study indicated that at day 14, those with acute severe UC had lower serum drug levels compared to patients with moderately severe UC. This is significant as it suggests that patients with acute severe UC may benefit from an intensified regimen as drug efficacy may have been affected by the high degree of inflammation[68].

In terms of cost-effectiveness, Steenholdt *et al*[66] was able to demonstrate that an individualized approach using reactive TDM was in fact more effective[66]. Results of this multi-center RCT showed that the costs for the intention to treat patients were substantially lower in the TDM based group compared to various infliximab dosing regimens (€ 6038 *vs* € 9178, $P < 0.001$). Similar results were also replicated in a study by Velayos *et al*[62], again demonstrating that dose adjustments made based on a TDM based algorithmic approach is more cost effective than empiric dose escalation[62].

The key recommendations at sLOR regarding the clinical scenarios of different combinations of TL and ADA level results at TDM are consistent based on the above studies. The suggested therapeutic algorithm for the optimal management of anti-TNF treatment failure based on TDM results is summarized in Figure 1[69].

As mentioned earlier, TDM can be approached in one of two ways, either in the reactive or proactive setting. Although much debate exists around the many issues surrounding the use of TDM, in current practice, it is mainly used in the reactive setting. Prior to TDM, standard of practice for the management of treatment failure in IBD is through empiric dose escalation. But with growing evidence highlighting the utility of reactive TDM in guiding subsequent management and that it may in fact be more cost effective, more specialists are using it in their practice[62,66].

As a result of numerous exposure-response relationships linking target TLs to therapeutic outcomes, the idea of proactive approach emerged in the hopes of targeting a specific threshold to avoid PNR or sLOR.

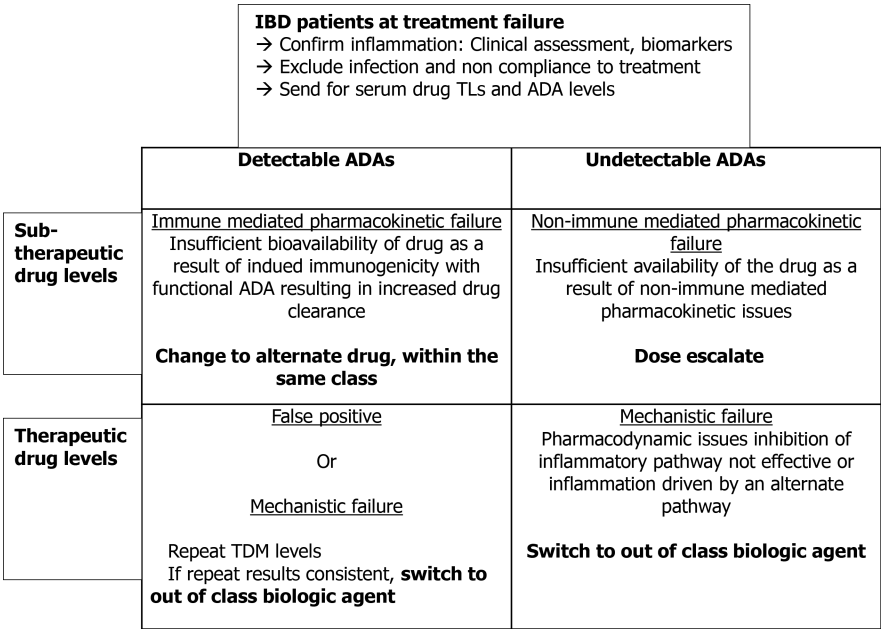

Figure 1 Therapeutic drug monitoring based approach to treatment failure. IBD: Inflammatory bowel disease; ADA: Anti-drug antibody.

PROACTIVE TDM

The idea of using a proactive approach stemmed from preliminary data revealing that targeting a specific drug concentration at various intervals is associated with better clinical outcomes, compared to empiric dose escalation and TDM done in the reactive setting[13,16,47-50]. A pivotal trial that demonstrated the potential benefits of proactive TDM is the landmark TAXIT trial (Trough Level Adapted Infliximab Treatment). This was a single center RCT of 263 IBD patients on infliximab, whose progress was followed to compare clinical benefit and cost effectiveness of concentration *vs* clinical based approach. As a result of the design study, the primary end point, of clinical and biochemical remission after 1 year, was not met. However, important findings included lower rates of undetectable drug levels and disease relapse in the proactive group[16].

More recently, Fernandes *et al*[15] carried out a prospective study of 205 IBD patients with results strongly supporting the proactive approach. Patients were randomized to two groups, those who were treated with IFX without TDM (data collected retrospectively) and those who underwent proactive TDM. In terms of outcomes, they looked at mucosal healing, need for hospitalization, and surgery. Results demonstrated that there were higher rates of treatment escalation in the TDM group ($P < 0.001$) with less need for surgery in addition to higher rates of mucosal healing ($P < 0.0001$), concluding that the proactive approach significantly decreased odds of reaching any unfavorable outcomes[15].

In a multi-center, retrospective cohort study of 102 IBD patients on infliximab, Papamichael and colleagues compared outcomes associated between those who underwent reactive *vs* proactive drug monitoring. The study demonstrated that those who underwent proactive testing after initial reactive testing was associated with greater drug persistence in addition to fewer IBD related hospitalizations (HR 0.18, CI 0.05-0.99; $P = 0.007$)[10].

Another multi-center study carried out by Papamichael *et al*[11], involving IBD patients on adalimumab also showed promising results with regards to the proactive approach as results also demonstrated that those who underwent proactive TDM had a reduced risk of treatment failure compared to the standard of care[11].

A recent study that strongly supports the proactive approach is the PAILOT study (Pediatric CD Adalimumab-Level based Optimization Treatment) by Assa *et al*[14]. This was a RCT of 78 children with CD who were on treatment with adalimumab and their primary end point was sustained steroid free remission. Authors of the study reached their primary endpoint with 82% in the proactive *vs* 48% in the reactive ($P = 0.002$) in addition to lower biochemical markers in the proactive group ($P = 0.003$)[14]. This study is considered a step forward supporting proactive TDM as this was the first proactive RCT whose primary endpoint was reached.

The TAILORIX trial (Tailored Treatment with Infliximab for Active CD) by D'Haens *et al*[12], was another important proactive trial carried out with the aim of promoting the treat to trough approach. This trial followed 122 biologic naïve patients with active CD who received induction with infliximab and were randomized to three different groups of maintenance infliximab. The three various groups comprised of various dose intensifications based on clinical assessment, biomarkers and/or serum infliximab levels with the primary outcome being sustained corticosteroid free clinical remission at one year. In the end, this trial failed to show the benefit of the proactive approach as results demonstrated that increasing infliximab dose based on combination of symptoms, biomarkers, and drug concentrations does not lead to increased rates of steroid free remission (P = 0.50) compared to dose escalation based on symptoms alone[12]. Interestingly, it is worth noting that less than 50% of patients in each group failed to reach the primary endpoint of corticosteroid free remission in addition to the fact that dose optimization was only carried out at week 14.

Interestingly, new evidence looking at proactive TDM was revealed through several studies including both the SERENE-CD and SERENE-UC trials. In the post induction phase of both trials, authors followed patients into the maintenance phase following induction with high dose *vs* standard dose adalimumab in the treatment of patients with moderate to severe CD and UC[60,70]. In the maintenance phase of SERENE-CD study, clinical responders from the induction phase were recruited and the authors explored outcomes after 44 wk on adalimumab using a clinical based assessment with the use of biomarkers or through proactive TDM with specified target thresholds. Preliminary results presented at United European Gastroenterology conference (UEG) showed that in terms of efficacy endpoints, the rates were the same in both arms and that the addition of TDM as a criteria showed no added benefit as the rates of clinical response, steroid free clinical remission, and endoscopic remission were similar[70]. A study by Bossuyt *et al*[71], presented at UEG 2020, showed similar results in terms of clinical endpoints, by comparing outcomes of two groups, ultra-proactive TDM *vs* reactive TDM. In this study, at the end of one year, there was no difference noted between both groups in terms of composite endpoints of: IBD related hospitalizations, IBD related surgeries, and change of treatment[71].

On the other hand, the SERENE-UC trial looked at outcomes of patients who were randomized to either a high dose or standard dose adalimumab maintenance regimen. Colombel *et al*[60], concluded that there was a higher number of patients who achieved clinical remission at week 52 in the high dose group, compared to the standard dose group. However, despite the clear numerical difference, it was not statistically significant. Here, the hypothesis was that with an intensified regimen, there would be higher drug bioavailability, which could result in positive outcomes. This theory was based on a previous trial that an intensive regimen in patients with severe UC resulted in lower rates of patients requiring colectomy[72]. Although drug levels were higher in the intensified regimen in both trials, the rates of clinical endpoints were the same.

Although more studies are demonstrating results in favor of proactive TDM, the range of studies remain limited in that the majority are retrospective in nature. Additionally, even with the current use of TDM, used by IBD specialists in the reactive setting, multiple factors are considered in the subsequent management of treatment failure, and not simply by a treat to trough concentration approach.

TDM IN CLINICAL PRACTICE

Despite the issues surrounding the application of TDM involving how and when it should be used, it has proven to be a valuable tool. The current use of TDM as one of many tools of clinical assessment has helped in advancing the care of these patients. As such, the use of TDM has been recommended in certain guidelines and by consensus groups as an adjunct to manage patients with suspected treatment failure. Furthermore, because of the lack of evidence from proactive trials to support the idea of improved clinical outcomes associated with this approach, many support the use of TDM in the reactive setting. For further information, refer to Table 2 for a list of the main TDM based trials including both reactive and proactive[6,10-12,14-16,66,67,70,71,73-77].

As evident, the debate over when to apply TDM is still ongoing because of limited prospective large trial studies. For this reason, TDM has not been fully incorporated into guidelines but rather hinted at as suggestions to help guide treatment in those with suspected treatment failure. One example is the guideline by the American

Table 2 A list of the main trials looking at both reactive and proactive therapeutic drug monitoring

Ref.	Study design; *n*	Population studied	Type of intervention	Primary outcome	Results
Vande Casteele *et al*[16], 2015 (Proactive)	Prospective single center study RCT, *n* = 263	Adults with mod to severe UC responders to infliximab (IFX)	IFX. Target 3-7 μg/mL during maintenance phase. Clinical *vs* concentration-based dose escalation	Clinical and biochemical remission	Fewer flares in concentration-based group. No difference in remission rates at 1 yr
Papamichael *et al*[73], 2017 (Proactive)	Retrospective multi-center RCT, *n* = 264	Adults with CD + UC	IFX 5-10 μg/mL	Treatment failureNeed for IBD related hospitalization or surgery. Adverse events	Proactive was associated with better clinical outcomes, including greater drug durability, less need for IBD-related surgery or hospitalization
Perinbasekar *et al*[74], 2017 (Proactive)	Retrospective single center study, *n* = 127	Adult IBD patients initiating treatment with either IFX or adalimumab (Ada)	IFX target ≥ 3 μg/mL; Ada target ≥ 5 μg/mL	Clinical response at 1 yr. Endoscopic response. Persistence with anti-TNF at 1 yr	Persistence with therapy and clinical and endoscopic response were superior for proactive compared to control patients treated with infliximab
Bernardo *et al* [75], 2017 (Proactive)	Retrospective single center study, *n* = 117	Adult IBD patients on treatment with infliximab	Clinical based *vs* proactive TDM. (1) Target IFX CD 3-7 μg/mL; (2) Target IFX UC 5-10 μg/mL; (3) Target Ada CD 5-7 μg/mL; and (4) Target Ada UC 7-9 μg/mL	At 48 wk (1) Clinical remission; (2) Rates of hospitalizations; (3) Rates of surgery; and (4) Therapeutic failure	No difference noted in relation to outcomes. Higher rates of drug escalation in proactive group. Longer period of remission in proactive group
D'Haens *et al* [12], 2018 (Proactive)	Prospective multi-center RCT, *n* = 122	Adults with mod to severe luminal CD biologic naïve on infliximab maintenance	Dose escalation using combined approach of clinical + TDM *vs* symptom-based approach. IFX target > 3 μg/mL during maintenance phase	Sustained steroid-free clinical remission at weeks 22-54 and mucosal healing at week 54	No difference in terms of rates of steroid-free remission
Papamichael *et al*[10], 2018 (Proactive *vs* reactive)	Retrospective multicenter study, *n* = 102	Adult IBD patients on infliximab	Reactive TDM followed by subsequent proactive TDM *vs* reactive testing IFX target 5-10 μg/mL	Treatment failure. IBD related hospitalization and surgery	Proactive monitoring after reactive testing associated with greater drug persistence and fever IBD related hospitalizations
Papamichael *et al*[11], 2019 (Proactive)	Retrospective multicenter study, *n* = 382	IBD patients on maintenance therapy with adalimumab	Proactive *vs* reactive TDM. Ada > 10 μg/mL	Treatment failure	Proactive associated with lower risk of treatment failure
Assa *et al*[14], 2019 (Proactive)	Prospective multi-center RCT, *n* = 78	Ages 6-17 yr with CD with response to adalimumab	Ada target trough levels ≥ 5 μg/mL	Sustained steroid-free clinical remission (weeks 8-72)	Higher rates of steroid free clinical remission in proactive group
Strik *et al*[76], 2019 (Proactive)	Retrospective multi-center RCT, *n* = 80	UC + CD in clinical remission on infliximab maintenance therapy	Dashboard driven dose escalation with TDM *vs* non TDM. IFX level > 3 μg/mL	Clinical remission	Dashboard-guided dosing resulted in a significant higher proportion of patients who maintained clinical remission during 1 yr of treatment
Danese *et al* [70], 2020 (Proactive)	Prospective multi-center RCT, *n* = 184	Clinical responders from induction phase of SERENE-CD	Clinical based group *vs* proactive TDM (TL 5-10 μg/mL) adalimumab every week or every other week	Clinical remission and endoscopic response and remission at 1 yr	No difference in terms of clinical end points
Fernandes *et al* [15], 2020 (Proactive)	Prospective study, *n* = 205	IBD patients completing infliximab induction therapy	Prospective arm (TDM-based dose escalation) *vs* retrospective arm (non-TDM). IFX levels 3-7 μg/mL CD; IFX levels 5-10 μg/mL UC	Need for surgery, hospital admission, treatment endrates of mucosal healing at 2 yr of treatment	Proactive TDM associated with fewer surgeries and higher rates of mucosal healing
Bossuyt *et al* [71], 2020 (Proactive *vs* reactive)	Prospective multi-center RCT	All IBD patients on infliximab therapy > week 14	Using point of care testing at the time of infusion > proactive *vs* reactive TDM	Clinical remissionDiscontinuation of infliximab. Composite end points of IBD related hospitalizations and surgeries, change of treatment	No difference in terms of rate of clinical remission or treatment discontinuationUltra-proactive not superior to reactive
Afif *et al*[67], 2010 (Reactive)	Retrospective study, *n* =155	IBD patients who had infliximab	Measurements of human anti-chimeric antibodies	Loss of response. Change in treatment	Measurement of both antibody and drug levels lead to

			(HACAs) and infliximab concentrations		improved response
Steenholdt *et al* [66], 2014 (Reactive)	Prospective RCT, *n* = 69	CD patients failing on infliximab therapy	Infliximab intensification *vs* algorithm defined using TDM	Clinical and economic outcomes at week 20	Lower healthcare costs in algorithm-based group. Similar rates of clinical response and remission
Kelly *et al*[63], 2017 (Reactive)	Retrospective study, *n* = 312	Primary responders on infliximab who underwent dose escalation	TDM *vs* clinical based dose escalation of infliximab	Endoscopic remissionClinical response	Higher rates of endoscopic remission with TDM
Pouillon *et al* [77], 2018 (Reactive)	Retrospective single center study, *n* = 226	IBD patients who completed maintenance phase of TAXIT	Clinical based *vs* trough concentration-based dosing of infliximab, infliximab level 3-7 μg/mL	IBD related hospitalization and surgery. Steroid use. Mucosal healing	Similar rates of mucosal healing in both groups. Higher rates of treatment discontinuation in clinic-based group

TDM: Therapeutic drug monitoring; TNF: Tumor necrosis factor; IBD: Inflammatory bowel disease; RCT: Randomized controlled study; IFX: Infliximab; Ada: Adalimumab.

Gastroenterological Association, in which the use of TDM is suggested for patients with active IBD on anti-TNFs but not recommended for those with quiescent IBD[18]. Table 3 lists the various recommendations and statements made by various gastroenterology guidelines and consensus groups[18-22].

PRACTICAL ASPECTS-HOW BEST TO APPLY TDM

Although much evidence exists supporting the use of TDM in the proactive setting, it has become clear that current evidence has not supported its use as routine practice in the management of IBD patients. Instead of dividing TDM into two separate entities, TDM should be considered as a useful tool in the management of IBD patients. As clinicians, we treat patients as a whole, in the sense that we treat their conditions by looking at various aspects including their clinical assessment in addition to biomarkers. Therefore, TDM should be considered as another adjunct that will help guide management as opposed to looking at it as the sole means of ensuring clinical response and remission.

Given the fact that various thresholds for serum drug levels and ADAs exist, it can be difficult to choose which one to target, especially since another variable that complicates this is the type of assay used for these measurements. However, despite the wide range, in current practice, certain thresholds have been followed to ensure clinical response in IBD patients who are on biologic agents. An example of these thresholds to target during maintenance therapy can be seen clearly in Table 4[16,41,78,79] The values listed in the table have been determined by a panel of IBD specialists that have carefully looked at existing literature relating to TDM[79]. Interestingly, the cut-off values listed by this group differs from the suggestions put forth in the AGA 2017 guideline[18]. It is important to note that these cut-off values are not absolute and should always be made in context with the clinical picture and that higher values might be needed for more rigorous clinical outcomes[48]. In the end, optimal TLs are difficult to define because of the multiple factors discussed above and because of the limited data from observational studies and post HOC analyses, thus it is important to be aware that the idea of 'one size' fits all may not be appropriate.

LIMITATIONS OF TDM

In research, RCTs are considered the gold standard in providing evidence for causal relationships and to support changes to clinical practice, and this seems to be lacking with regards to TDM. Thus, the majority of data available regarding TDM has been based mainly on prospective and retrospective observational studies or post HOC analyses. In addition to the timing of TDM and how often it should be carried out, other issues around TDM have to do with how to best interpret the results given the difficulty in obtaining results in a timely manner and being able to implement changes immediately[79]. Also, the bulk of evidence supporting TDM revolves mainly around

Table 3 Recommendations and statements made by various gastroenterology guidelines and consensus groups

Guideline/Consensus group	Recommendation
AGA[18,19]	Active IBD with anti-TNF → suggest use of reactive TDM
	Quiescent IBD with anti-TNF → not recommended
Inflammatory bowel disease Sydney/Australian Inflammatory bowel disease consensus working group (2017)[20]	Use of TDM preferred in (1) Upon suspected treatment failure; (2) Following successful induction; and (3) When completed drug holiday
	For those in clinical remission, consider TDM periodically only if it will change management
British guidelines (2019)[21]	Good practice recommendation → ALL IBD patients should be reviewed 2-4 wk post loading dose to assess response and check drug levels and anti-drug antibodies
	Use of serum drug trough & anti-drug antibody concentrations to be incorporated when deciding in change of therapy (dose escalation *vs* switch to other anti-TNF drug or out of class change)
ECCO (2020)[22]	CD in remission on anti-TNF → insufficient evidence to recommend FOR or AGAINST TDM
	CD patients who have lost response → insufficient evidence

AGA: American Gastroenterology Association; ECCO: European Colitis & Crohn's Organization; TDM: Therapeutic drug monitoring; TNF: Tumor necrosis factor; IBD: Inflammatory bowel disease.

Table 4 Therapeutic drug monitoring thresholds used in current practice (Using enzyme-linked immunoassay)

Drug	Cut-off for serum drug concentration	Cut-off for detectable ADA
Infliximab	> 3 μg/mL	Present if > 10 μg/mL
Adalimumab	> 5 μg/mL	Present if > 10 μg/mL
Certolizumab	> 15 μg/mL	-
Ustekinumab	Insufficient evidence to make a suggestion	-
Vedolizumab	Insufficient evidence to make a suggestion	-

ADA: Anti-drug antibody.

anti-TNF and less so with the other biologics such as vedolizumab and ustekinumab [80-82]. These newer biologics are now being used more in IBD thus it is imperative that well designed studies are done looking into outcomes associated with TDM based approach using these drugs.

Nonetheless, results from recent trials are promising and provide hope that more guidance may come soon. One example of such trials, is the PRECISION trial by Strik *et al*[76], which was the first prospective trial that demonstrated clinical benefit of a more personalized approach involving a dashboard system that incorporates patient features and TDM[76]. The ultra-proactive trial put forth by Bossuyt *et al*[71] is another example, making it one of the first studies to involve the use of POC testing, suggesting that this technology may be feasible to facilitate future research and the application of TDM. Lastly, the NOR-DRUM study also brings hope as this will be the first large sized RCT looking at the safety and effectiveness of TDM in patients receiving anti-TNF for a range of immune-mediated diseases, including IBD[83].

CONCLUSION

In the end, despite its issues, TDM has evolved the management of IBD patients and is being used more in clinical practice in the hopes of preventing loss of response and to ensure maximal use of biologic drugs. Even though more studies are showing results that support proactive TDM usage, many of them have methodological issues, making their data less reliable to be able to implement changes in clinical practice. Hence making it difficult to prove that proactive TDM is associated with better therapeutic outcomes. At present, TDM has been shown to be a useful tool in managing patients

suspected of loss of response and although it has not been fully implemented in guidelines, its usefulness in current practice brings hope that this might soon change.

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Basic Study

Increased systemic RNA oxidative damage and diagnostic value of RNA oxidative metabolites during *Shigella flexneri*-induced intestinal infection

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Abstract

BACKGROUND

Shigella flexneri (*S. flexneri*) is a major pathogen causing acute intestinal infection, but the systematic oxidative damage incurred during the course of infection has not been investigated.

AIM

To investigate the incurred systemic RNA oxidative damage and the diagnostic value of RNA oxidative metabolites during *S. flexneri*-induced intestinal infection.

METHODS

In this study, a Sprague-Dawley rat model of acute intestinal infection was established by oral gavage with *S. flexneri* strains. The changes in white blood cells (WBCs) and cytokine levels in blood and the inflammatory response in the colon were investigated. We also detected the RNA and DNA oxidation in urine and tissues.

RESULTS

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S. flexneri infection induced an increase in WBCs, C-reactive protein, interleukin (IL)-6, IL-10, IL-1β, IL-4, IL-17a, IL-10, and tumor necrosis factor α (TNF-α) in blood. Of note, a significant increase in urinary 8-oxo-7,8-dihydroguanosine (8-oxo-Gsn), an important marker of total RNA oxidation, was detected after intestinal infection (P = 0.03). The urinary 8-oxo-Gsn level returned to the baseline level after recovery from infection. In addition, the results of a correlation analysis showed that urinary 8-oxo-Gsn was positively correlated with the WBC count and the cytokines IL-6, TNF-α, IL-10, IL-1β, and IL-17α. Further detection of the oxidation in different tissues showed that *S. flexneri* infection induced RNA oxidative damage in the colon, ileum, liver, spleen, and brain.

CONCLUSION

Acute infection induced by *S. flexneri* causes increased RNA oxidative damage in various tissues (liver, spleen, and brain) and an increase of 8-oxo-Gsn, a urinary metabolite. Urinary 8-oxo-Gsn may be useful as a biomarker for evaluating the severity and prognosis of infection.

Key Words: 8-oxo-7,8-dihydroguanosine; *Shigella flexneri*; Infection; Oxidative damage

Core Tip: *Shigella flexneri* (*S. flexneri*) is a major pathogen causing acute intestinal infection, but the systematic oxidative damage incurred during the course of infection has not been investigated. By establishing a Sprague-Dawley rat model with *S. flexneri* infection in the intestine, we found that 8-oxo-7,8-dihydroguanosine (8-oxo-Gsn) levels were markedly increased in the colon, ileum, liver, spleen, and brain after infection, showing that *S. flexneri* can exacerbate systemic RNA oxidation. The changing trend in levels of urinary 8-oxo-Gsn (as the RNA oxidative metabolites) was consistent with the changes in the white blood cell count, C-reactive protein level, and cytokine levels during infection. Urinary 8-oxo-Gsn may be useful as a biomarker for evaluating the severity and prognosis of infection.

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INTRODUCTION

Shigella infection is a major public health concern around the world, causing acute diarrhea, known as shigellosis or bacillary dysentery. There are almost 165 million cases of diarrhea caused by *Shigella* each year, of which 163 million occur in developing countries[1]. People of all ages can be infected by *Shigella*, but children under five years old are most susceptible[2].

Shigella flexneri (*S. flexneri*) is the most frequently isolated *Shigella* species worldwide [2]. The pathogenesis of *S. flexneri* involves invasion of the mucosa by bacteria, which replicate within and spread between the mucosal epithelial cells, resulting in a subsequent severe inflammatory response and epithelial destruction in the colon tissue. However, the oxidative damage in the colon during *S. flexneri*-induced infection has not been investigated. In the present study, the oxidative stress changes in the intestine during *S. flexneri*-induced acute intestinal infection were investigated.

Shigella infection can cause inflammatory responses in the intestine, which is also a potential source of inflammation-related cytokines[3]. Researchers have established cell models/animal models and observed the infected patients to investigate the inflammation-related cytokine production. In cell models, *S. flexneri* was able to cause an increase in interleukin (IL)-1, IL-10, and tumor necrosis factor α (TNF-α) in macrophage-like cells[4]. Outer membrane protein A of *S. flexneri* can trigger B cells to

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secret the cytokines IL-6 and IL-10[5]. The production of the cytokines IL-4, IL-6, and IL-8 is increased in HeLa cells with *S. flexneri* infection[6]. Researchers also found that the TNFα and IL-1β production is reduced in mouse macrophages with an *S. flexneri msbB* gene mutation[7]. In mouse models, *S. flexneri* infection can cause an increase in the IL-1β production[8]. In *S. flexneri*-infected patients, the TNF-α, IL-1, IL-4, IL-6, and interferon-γ levels increased significantly after infection[3].

In the present study, we established an *S. flexneri*-infected rat model to explore the changes in the levels of the above-mentioned cytokines (IL-1, IL-6, IL-10, and TNF-α) as well as IL-17α, which plays important roles in host immunity against intracellular pathogens.

Urinary 8-oxo-7,8-dihydroguanosine (8-oxo-Gsn) and 8-oxo-7,8-dihydro-2-deoxyguanosine (8-oxo-dGsn) are oxidative stress by-products of RNA and DNA, respectively, which are widely used as markers of oxidative stress[9,10]. Previous studies have reported their utility as biomarkers for predicting aging-related diseases[11] and neurodegenerative diseases[12]. Our lab found that 8-oxo-Gsn may have potential utility as a marker for evaluating the severity of *Salmonella*-related infection[13]. The present study investigated the diagnostic value of 8-oxo-Gsn and 8-oxo-dGsn in intestinal infection induced by *S. flexneri*. In addition, we investigated the correlation between cytokine levels and oxidative production of 8-oxo-Gsn.

Accumulating evidence suggests that intestinal infection caused by Gram-negative bacteria may also influence other organs. For example, the Gram-negative bacterium *Escherichia coli* can form extracellular amyloid, which may play a role in causing sporadic Alzheimer's disease[14]. *S. dysenteriae*, a causative agent of bloody diarrhea, may cause diarrhea-associated hemolytic uremic syndrome and neurological disorders [15]. Acute intestinal infection induced by *Salmonella* can cause oxidative damage to the intestine, liver, and spleen[13]. However, the effects of *Shigella*-induced intestinal infection on other tissues have not been reported.

We hope that the rat infection model established in the present study will help improve our understanding of how vertebrates regulate their response to *S. flexneri* infection. We further intended to explore whether or not *S. flexneri* infection in the intestine can cause oxidative damage to other organs, such as the liver, spleen, and brain.

MATERIALS AND METHODS

Bacterial strains

The *S. flexneri* strain used in this study (CICC 21534) was purchased from the China Center of Industrial Culture Collection (CICC; Beijing, China). The source of the CICC 21534 strain was Guizhou Health and Epidemic Prevention Station, China. The serotype of CICC 21534 was F1a.

S. flexneri strain CICC 21534 was stored at -80 °C. The isolate was subcultured on blood agar plates twice and then suspended in Trypticase Soy Broth (bioMérieux SA, 69280 Marcy I'Etoile, France). The bacterial concentration was measured spectrophotometrically and confirmed by serial dilution on blood agar.

Animal experimental design

Twenty-four 4-wk-old healthy male Sprague-Dawley (SD) rats were purchased from Vital River (Beijing, China). All appropriate measures were taken to minimize the pain or discomfort of the rats. The rats were housed in a temperature-controlled room (24 ± 2 °C). Drinking water and food were provided *ad libitum*. There were three rats in each cage. The rats were not pretreated with any antibiotics and were all healthy before treatment, with normal white blood cell (WBC) counts and a normal flora in the feces.

The rats were divided randomly into either an infection group or a control group. In the infection group (n = 12), rats were infected by gavage with 1 dose of 1×10^9 colony-forming units (CFU) of the *S. flexneri* strain. In the control group (n = 12), rat gavage was performed with phosphate-buffered saline (PBS). On days 1, 5, 9, and 18, three rats per group were euthanized by intraperitoneal injection of pentobarbital sodium (200 mg/kg), and tissues were obtained.

For the establishment of the infection model, before infection, the rats were starved overnight. To neutralize the gastric acid, 400 μL of 3% $NaHCO_3$ was administered orally 15 min before infection. The rats in the infection group were then orally administered with 1×10^9 CFU of *S. flexneri* in a volume of 1 mL. The establishment of infection was verified by the continuous detection of *S. flexneri* in feces, an increased WBC count, and obvious lymphocyte infiltration in the colon. In the control group,

rats were also starved overnight and administered with 3% $NaHCO_3$ orally 15 min before PBS administration.

We took care of and sacrificed the animals strictly according to animal welfare laws and regulations. This study was approved by the Ethical Committee of the Institute of Medical Biotechnology, Chinese Academy of Medical Sciences (approval number: IMB-201803140206).

Body temperature and body weight of rats

The body temperature (rectal temperature) and body weight of the rats were measured every morning.

Microbiological detection

For the qualitative detection, conventional bacterial culture methods were used to detect *S. flexneri* in feces. Samples were collected and inoculated onto blood agar, China blue agar plates (Jinzhang Science and Technology Development Co. Ltd, Tianjin 300190, China), and Salmonella-Shigella (bioMérieux SA, 69280 Marcy I'Etoile, France) agar plates within 2 h of collection. *S. flexneri* was identified using morphologic observation on the selective medium, combined with triple-iron slants and Vitek 2 (bioMérieux, Inc., NC, United States) detection.

For the quantitative detection of *S. flexneri*, the feces and tissues were weighed after collection and then diluted in 1 mL of PBS and homogenized with a tissue homogenizer (PRO Scientific Inc., CT, United States). After centrifugation, the supernatant was diluted and inoculated onto Salmonella-Shigella (bioMérieux SA, Marcy I'Etoile, France) agar plates. The plates were incubated at 37 °C overnight. The number of *Shigella* colonies was counted on each plate. *Shigella* was quantitatively detected in the infection and control groups at fixed time points on days 1, 3, 5, 7, 9, 13, 15, and 18.

White blood cell count

WBCs in blood samples were counted manually. A 20-μL blood sample was added to 380 μL of 2% ethanoic acid solution. Then, WBCs were counted in a hemacytometer using a conventional light microscope.

C-reactive protein and cytokine detection

Blood samples were collected by the tail vein sampling method. Serum C-reactive protein (CRP) levels were detected with a Rat CRP ELISA kit (Immunology Consultants Laboratory, Inc., OR, United States). The CRP test kit is a highly sensitive two-site ELISA for measuring CRP in rat biological samples. The assay for quantification of CRP in samples requires that each test sample be diluted before use. For the serum samples of the rats in our study, 1/12000 dilution was appropriate for detection. The dilution ratio was determined based on the data provided by the kit and the data of the preliminary experiment.

The cytokines TNF-α, IL-6, IL-10, IL-1β, IL-4, and IL-17α were measured using a commercially available MILLIPLEX MAP Kit (Rat Cytokine/Chemokine Magnetic Bead Panel, 96-Well Plate Assay, Cat. #RECYTMAG-65K, RECYMAG65K27PMX, RECYMAG-65PMX27BK; Millipore Corporation, MA, United States) according to the manufacturer's protocol. In this study, 1/2 dilution of serum was appropriate for detection. A Luminex 200 System (Luminex Corporation, TX, United States) was used for quantification.

Detection of urinary 8-oxo-dGsn and 8-oxo-Gsn levels

The manual bladder expression method was used to collect the urine samples of rats. Urine samples were stored at -80 °C. Urinary 8-oxo-dGsn and 8-oxo-Gsn were detected by the liquid chromatography with tandem mass spectrometry method as reported [13]. In brief, all urine samples were analyzed using an Agilent 1290 Infinity UHPLC instrument equipped with an Agilent 6490 triple-quadrupole mass spectrometer with a Jet Stream ESI source and iFunnel (Agilent, United States; parameters are shown in Supplementary Tables 1-3). The concentrations of 8-oxo-dGsn and 8-oxo-Gsn were then normalized by urinary creatinine to correct for the impact of water intake/excretion on urine. The results are presented as the ratio of the concentrations of nucleosides (8-oxo-dGsn and 8-oxo-Gsn) and creatinine.

Hematoxylin and eosin staining of tissues

Histopathological tests were performed using standard laboratory procedures. Tissue samples were fixed in 10% neutral-buffered formalin and embedded in paraffin. The paraffin-embedded tissue was then sliced into 4-μm-thick sections with a microtome

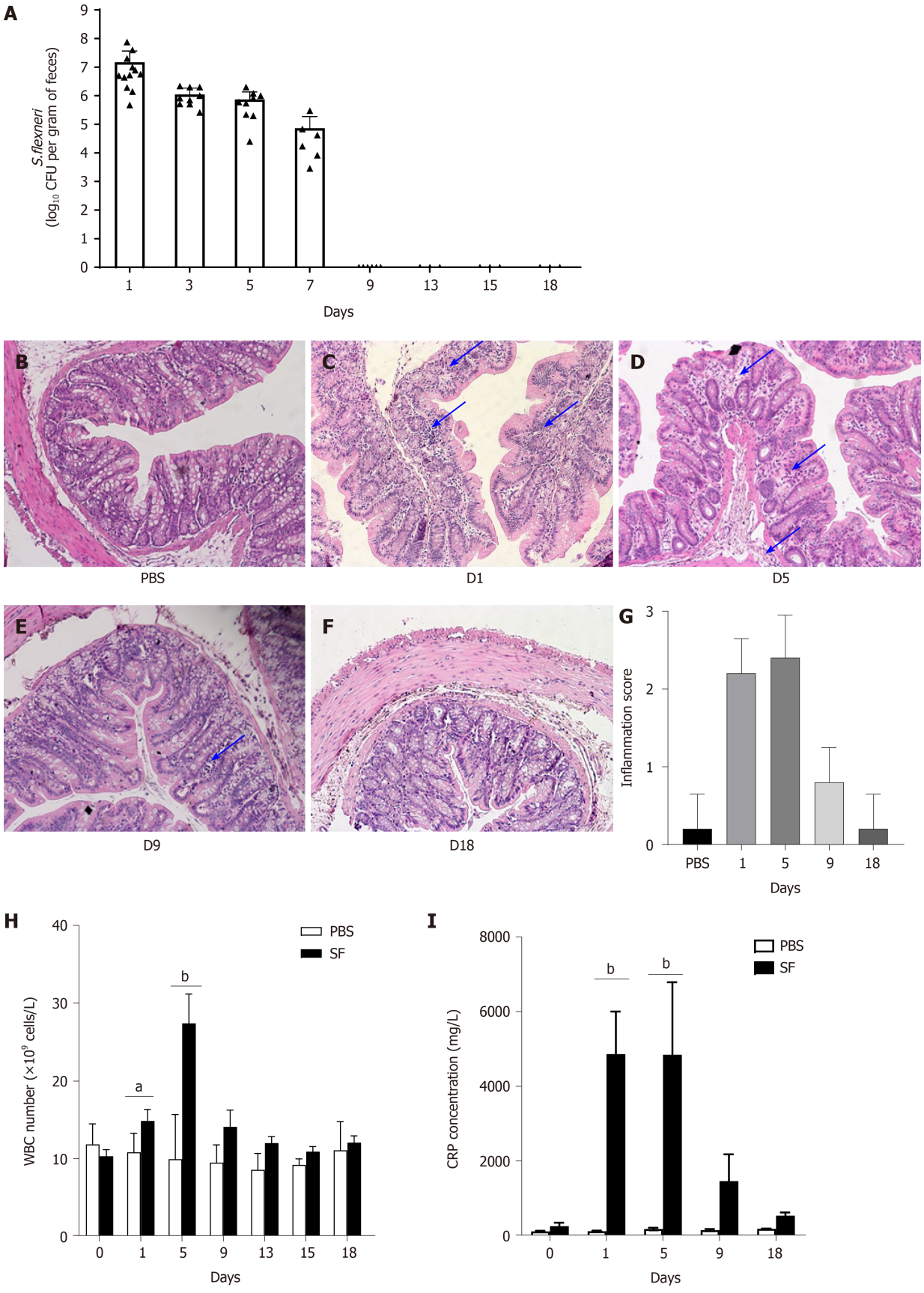

Figure 1 Pathogen colonization, intestinal inflammation, and changes in the white blood cell count and C-reactive protein level after *Shigella flexneri* infection in rats. A: Quantitative detection of *Shigella flexneri* (*S. flexneri*) in feces of Sprague-Dawley rats after infection; B-G: Representative hematoxylin & eosin (H&E) staining (100 × magnification) of colon tissue from control and *S. flexneri*-infected groups; B: H&E staining of normal colon tissue in the control group; C: H&E staining of colon tissue after *S. flexneri* infection at day 1; D: H&E staining of colon tissue on day 5; E: H&E staining of colon tissue on day 9; F: H&E staining of colon tissue on day 18; G: Inflammation scores of the control and infection groups at different time points; H: White blood cells counts in the *S.*

flexneri infection and control groups; I: C-reactive protein in the *S. flexneri* infection and control groups. [a]*P* < 0.05; [b]*P* < 0.01; and [c]*P* < 0.001. Scale bar, 100 μm. Black arrows indicate inflammatory cell infiltration. PBS: Phosphate-buffered saline; WBC: White blood cell; CRP: C-reactive protein; CFU: Colony-forming units; *S. flexneri*: *Shigella flexneri*.

and placed onto glass slides for further experiments. The tissue sections were deparaffinized with xylene, hydrated through an alcohol series, and then stained with hematoxylin and eosin (H&E). After dehydration and clearing, the mounting tissue sections were observed under a light microscope. H&E Stain kit was purchased from ZSGB-BIO (Beijing, China).

Immunohistochemical detection of 8-oxo-Gsn and 8-oxo-dGsn

Immunohistochemical staining was performed to detect the expression of 8-oxo-Gsn in the ileum, colon, liver, spleen, and brain. In brief, paraffin-embedded tissue sections were deparaffinized in xylene and rehydrated through a series of graded alcohols. Antigen retrieval was performed in citric acid buffer at 95 °C for 40 min. DNase I (TaKaRa Biotechnology Co., Ltd., Dalian, China) was added to slides to remove genomic DNA from samples. The slides were washed with 1 × PBS, and 3% hydrogen peroxide was added to eliminate endogenous peroxidases. Slides were then blocked with goat serum working solution. Primary monoclonal 15A3 antibody (ab62623; Abcam, Cambridge, United Kingdom) was added, and slides were incubated at 37 °C for 2 h (antibody dilutions: Colon, 1:700; ileum, 1:2000; liver, 1:700; spleen, 1:700; brain, 1:2000). The sections were incubated with HRP-conjugated secondary antibody (PV-6002; ZSGB-BIO) for 20 min. Coloration was performed with DAB (ZSGB-BIO), and the sections were observed under a microscope[16].

To detect the expression of 8-oxo-dGsn in tissue, tissue sections were treated with RNase A (TaKaRa Biotechnology Co., Ltd.) to remove RNA from samples. The primary monoclonal antibody was N45.1 (ab48508; Abcam). The dilutions of primary antibody were 1:2000 for the colon, 1:3000 for the ileum, 1:3000 for the liver, 1:500 for the spleen, and 1:2000 for the brain.

Statistical analysis

The SPSS 16.0 software program was used for the statistical analyses. Quantitative data are expressed as the mean ± SD and were analyzed by a *t*-test or one-way analysis of variance (ANOVA) after the normality test. Pearson's correlation coefficient (*r*) was used when comparing the linear relationship between the variables with a normal distribution. An area under the receiver operating characteristic (ROC) curve was created to assess the predictive value of urinary 8-oxo-Gsn and cytokines for the presence of infection. The results were considered significant only when $P < 0.05$.

RESULTS

Orally administered S. flexneri can provoke pathogen colonization and intestinal inflammation in rats

The body temperature showed no significant changes during the infection progression. Furthermore, the body weight increase of the rats was not significantly different between the infection and control groups.

After the intragastric administration of *S. flexneri*, serial fecal cultures were performed at fixed time points (days 1, 3, 5, 7, 9, 13, 15, and 18) in the two groups. In the control group, *S. flexneri* was undetectable during the experiment. In the infection group, *S. flexneri* was detectable in the feces of all infected rats after infection (day 1) and remained until day 7, whereas it was undetectable on day 9 (Figure 1A). In the intestine, liver, and spleen, *S. flexneri* was detectable on days 1 and/or 5. *S. flexneri* was undetectable in the brain at all time points.

Shigella can invade the colonic epithelial cells and provoke an intense inflammatory response. Compared with the control group (Figure 1B), obvious leukocyte infiltration was observed in the colonic mucosa on day 1 ($P < 0.001$ *vs* control; Figure 1C and G) and day 5 ($P < 0.001$ *vs* control; Figure 1D and G) after infection with *S. flexneri*, as detected by H&E staining. Intestinal inflammation was decreased on day 9 (Figure 1E), which was accompanied by pathogen elimination. By day 18, the colonic mucosa had returned to normal (Figure 1F).

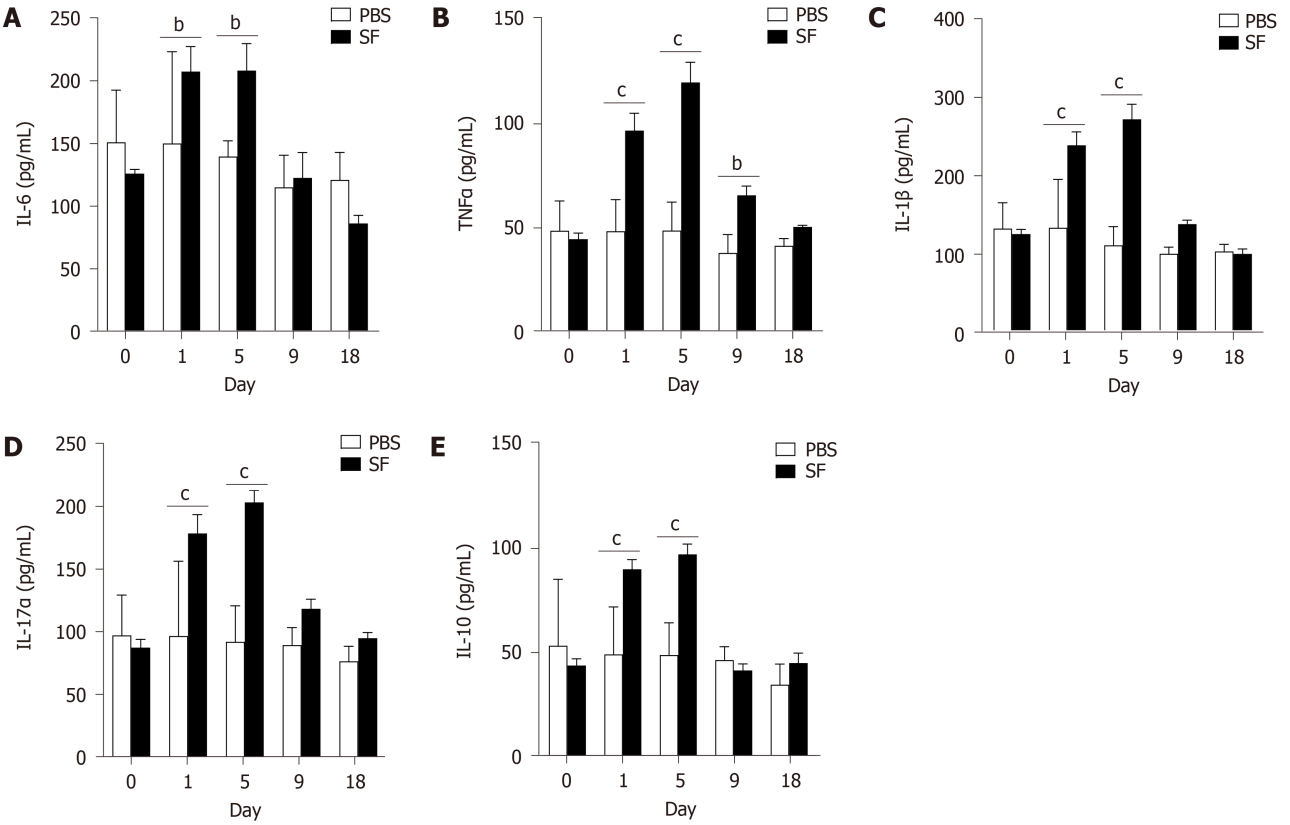

Figure 2 Inflammatory cytokines interleukin-6, tumor necrosis factor-α, interleukin-1β, interleukin-17α, and interleukin-10 detected in serum samples of *Shigella flexneri* infected rats. A: Interleukin (IL)-6; B: Tumor necrosis factor-α; C: IL-1β; D: IL-17α; E: IL-10. 0: Before infection. 1-18: The number of days after the establishment of the *Shigella flexneri* infection model. n = 12 per group on day 0 and day 1; n = 9 per group on day 5; n = 6 per group on day 9; n = 3 per group on day 18. Data are presented as the mean ± SEM. $^{a}P < 0.05$; $^{b}P < 0.01$; and $^{c}P < 0.001$ (two-tailed unpaired Student's t-test). PBS: Phosphate-buffered saline; IL: Interleukin; TNF: Tumor necrosis factor.

Changes in WBC count and CRP levels during the course of infection

The WBC count is often used to assess bacterial infections. The changes in the WBC count after the establishment of intestinal infection are shown in Figure 1H. The WBC count increased after oral exposure to *S. flexneri* and peaked on day 5 ($P < 0.0001$, $WBC_{day5} = 27.42 \pm 3.77 \times 10^9$ cells/L). The WBC count then decreased and returned to baseline after day 13. In the control group, there were no significant changes in the WBC count during the experiment.

Furthermore, it was shown that the level of CRP, an acute-phase response protein commonly used as a marker of inflammation, was significantly increased after exposure to *S. flexneri* on day 1 (CRP = $4.87 \pm 1.59 \times 10^3$ mg/L, $P = 0.005$), and the level remained high when it was detected on day 5 (CRP = $4.85 \pm 1.41 \times 10^3$ mg/L, $P = 0.002$). The CRP level then decreased and returned to baseline on day 18 (Figure 1I).

Induction of inflammatory cytokine production

The expression of IL-6, an important mediator of the acute-phase response, was significantly increased in the infection group on day 1 (207.26 ± 19.93 pg/mL, $P = 0.02$) and remained high when detected on day 5 (207.99 ± 21.49 pg/mL, $P = 0.01$; Figure 2A). After pathogen clearance on day 9, the levels of IL-6 decreased and returned to baseline.

The expression of TNF-α, a cell-signaling protein involved in systemic inflammation and one of the cytokines that make up the acute phase reaction, was also significantly increased after infection (day 1: 96.07 ± 8.29 pg/mL, $P < 0.001$) and peaked on day 5 (119.11 ± 9.69 pg/mL, $P < 0.001$). The TNF-α level then decreased and returned to the baseline at the end of the experiment (Figure 2B).

Consistent with this trend, increased levels of proinflammatory IL-1β (Figure 2C) and IL-17α (Figure 2D) were observed after infection and peaked on day 5. IL-10, a cytokine with multiple, pleiotropic effects in immunoregulation and inflammation, was also significantly produced after bacterial infection (Figure 2E).

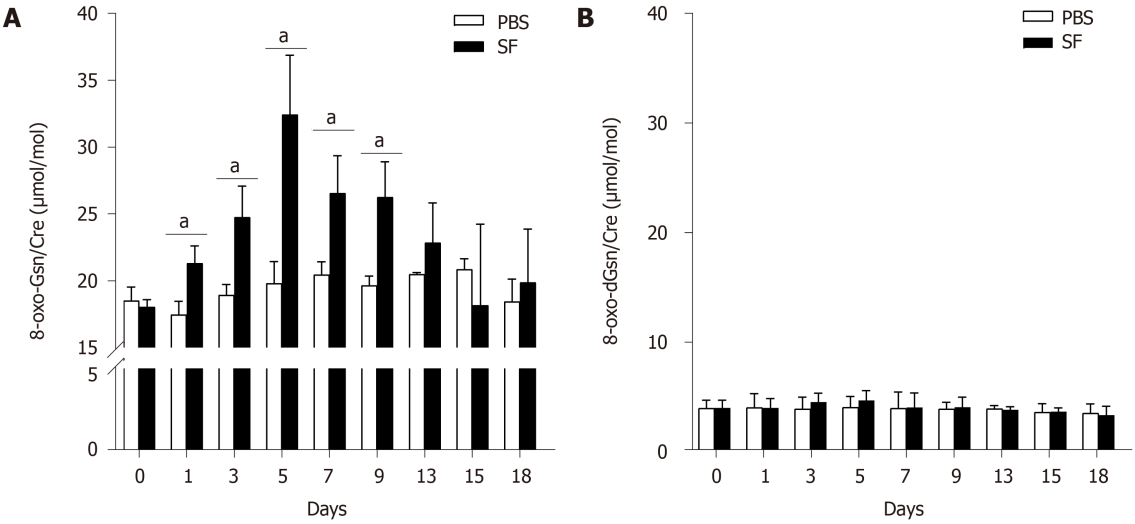

Figure 3 Liquid chromatography-tandem mass spectrometry measurement of 8-oxo-7,8-dihydroguanosine and 8-oxo-7,8-dihydro-2-deoxyguanosine in the urine of Sprague-Dawley rats. A: Urinary 8-oxo-7,8-dihydroguanosine; B: Urinary 8-oxo-7,8-dihydro-2-deoxyguanosine. *n* = 12 per group on day 0 and day 1; *n* = 9 per group on day 3 and day 5; *n* = 6 per group on day 9; *n* = 3 per group on day 13, day 15, and day 18. 0: Before infection. 1-18: The number of days after the establishment of the *Shigella flexneri* infection model. [a]*P* < 0.05. 8-oxo-Gsn: 8-oxo-7,8-dihydroguanosine; 8-oxo-dGsn: 8-oxo-7,8-dihydro-2-deoxyguanosine.

High levels of oxidized guanosine in urine after Shigella infection

8-oxo-Gsn levels were increased after oral exposure to *S. flexneri*. Compared with the control group, significantly increased levels of 8-oxo-Gsn were observed from day 1 to day 9 ($P < 0.05$), with the level peaking on day 5 ($P = 0.017$). After day 5, the level of 8-oxo-Gsn began to decrease and returned to baseline on day 15 (Figure 3A). The trends in the changes in the urinary 8-oxo-Gsn level were consistent with the changes in colonic tissue inflammation, the WBC count, and the CRP level, suggesting that urinary 8-oxo-Gsn may serve as a biomarker for assessing the severity of the infection.

In the control group, no significant changes in the 8-oxo-Gsn level were observed at any time point. For 8-oxo-dGsn, no significant changes were observed at any time point (Figure 3B).

Shigella infection leads to RNA oxidative damage in tissues

Immunohistochemical staining showed that the 8-oxo-Gsn staining intensity in the infected colon tissue was significantly altered compared with normal colon tissue ($P < 0.05$; Figure 4A). In infected colon tissues, antibodies directed against the RNA damage marker 8-oxo-Gsn produced more intense staining than uninfected colon tissues, thereby indicating a general increase in oxidative stress and RNA damage. Although 8-oxo-Gsn was more highly expressed on day 1 than on day 5, the difference between the two time points was not statistically significant. In the ileum (Figure 4B), liver (Figure 4C), spleen (Figure 4D), and brain tissues (Figure 4E), the expression of 8-oxo-Gsn was significantly higher on days 1 and day 5 than that in uninfected tissues.

The levels of the DNA damage marker 8-oxo-dGsn were significantly increased in the colon and brain tissues after infection (Figure 5A and E). In the infection group, there was no significant change in the 8-oxo-dGsn expression in the ileum, liver, or spleen (Figure 5B-D).

Correlation of 8-oxo-Gsn with WBC, CRP, and inflammatory cytokines

The 8-oxo-Gsn concentration in the urine of the infection group was positively correlated with the WBC count ($r = 0.652$, $P < 0.001$) and levels of IL-6 ($r = 0.461$, $P = 0.005$), TNF-α ($r = 0.541$, $P = 0.001$), IL-10 ($r = 0.359$, $P = 0.032$), IL-1β ($r = 0.402$, $P = 0.017$), and IL-17α ($r = 0.431$, $P = 0.009$). No significant correlation was found between 8-oxo-Gsn and CRP ($r = 0.289$, $P = 0.203$).

HE staining and fecal discharge are the gold standards for diagnosing the presence of infection. The predictive value of 8-oxo-Gsn in identifying the presence of intestinal infection was assessed using a ROC curve. The area under the ROC curve of urinary 8-oxo-Gsn was 0.750 (95%CI, 0.615–0.885, $P = 0.001$) (Figure 6A).

The predictive value of the WBC count and CRP for identifying intestinal infection was also assessed using a ROC curve. The area under the ROC of the WBC count was

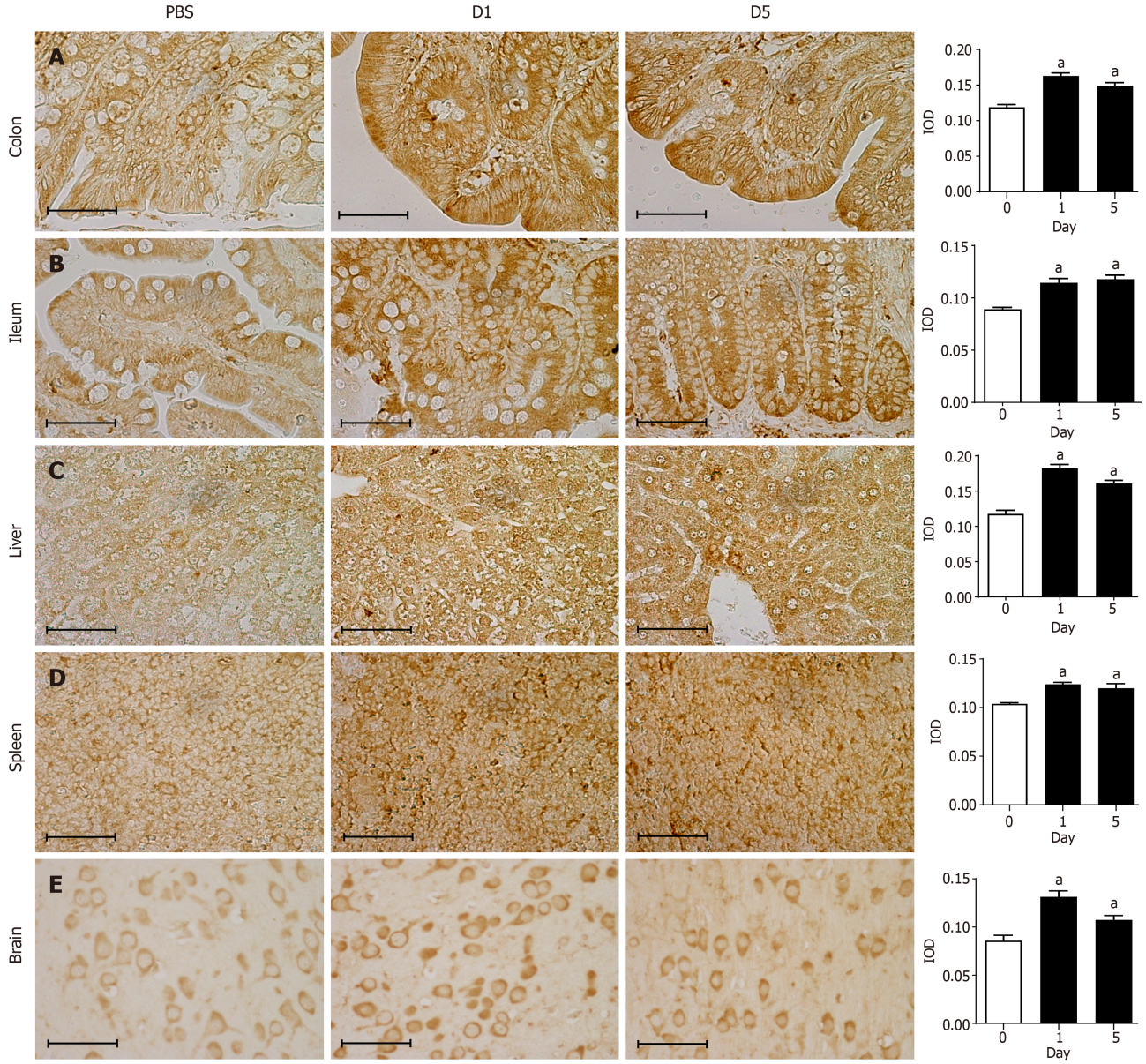

Figure 4 Immunohistochemical staining for 8-oxo-7,8-dihydroguanosine in tissues. A: Representative images showing immunohistochemical staining (400 × magnification) of 8-oxo-7,8-dihydroguanosine (8-oxo-Gsn) in paraffin-embedded colon sections from rats. Left panel images (PBS) indicate the results of the control group. Middle panel images (D1) indicate the results one day after *Shigella flexneri* (*S. flexneri*) infection. Right panel images (D5) indicate the results five days after *S. flexneri* infection; B: Immunohistochemical staining for 8-oxo-Gsn in ileum sections; C: Immunohistochemical staining for 8-oxo-Gsn in liver sections; D: Immunohistochemical staining for 8-oxo-Gsn in spleen sections; E: Immunohistochemical staining for 8-oxo-Gsn in brain sections. Histogram: The quantitative analysis of immunohistochemical staining of 8-oxo-Gsn in tissues of the infection and control groups. Scale bar, 50 μm. $^{a}P < 0.05$. The immunohistochemical images were analyzed using the Image Pro-Plus (IPP) software. The integrated optical density (IOD) is a representative parameter for assessing the immunostaining quantification in the IPP analyses, and it indicates the total amount of staining material in that area. IOD: Integrated optical density.

0.792 (95%CI, 0.674–0.911, $P < 0.001$) (Figure 6B), and that for CRP was 0.733 (95%CI, 0.544–0.923, $P = 0.014$) (Figure 6C).

For inflammatory cytokines, the area under the ROC of IL-6 (Figure 6D), TNF-α (Figure 6E), IL-1β (Figure 6F), IL-17α (Figure 6G), and IL-10 (Figure 6H) was 0.838 (95%CI, 0.698–0.978, $P = 0.003$), 0.980 (95%CI, 0.948–1.012, $P < 0.001$), 0.948 (95%CI, 0.882–1.014, $P < 0.001$), 0.964 (95%CI, 0.917–1.012, $P < 0.001$), and 0.864 (95%CI, 0.752–0.977, $P < 0.001$), respectively.

DISCUSSION

The present findings suggested that urinary 8-oxo-Gsn can be used to identify the presence of infection and to assess the severity of infection. In this study, *Shigella* infection in the gastrointestinal tract induced significant increases in the 8-oxo-Gsn

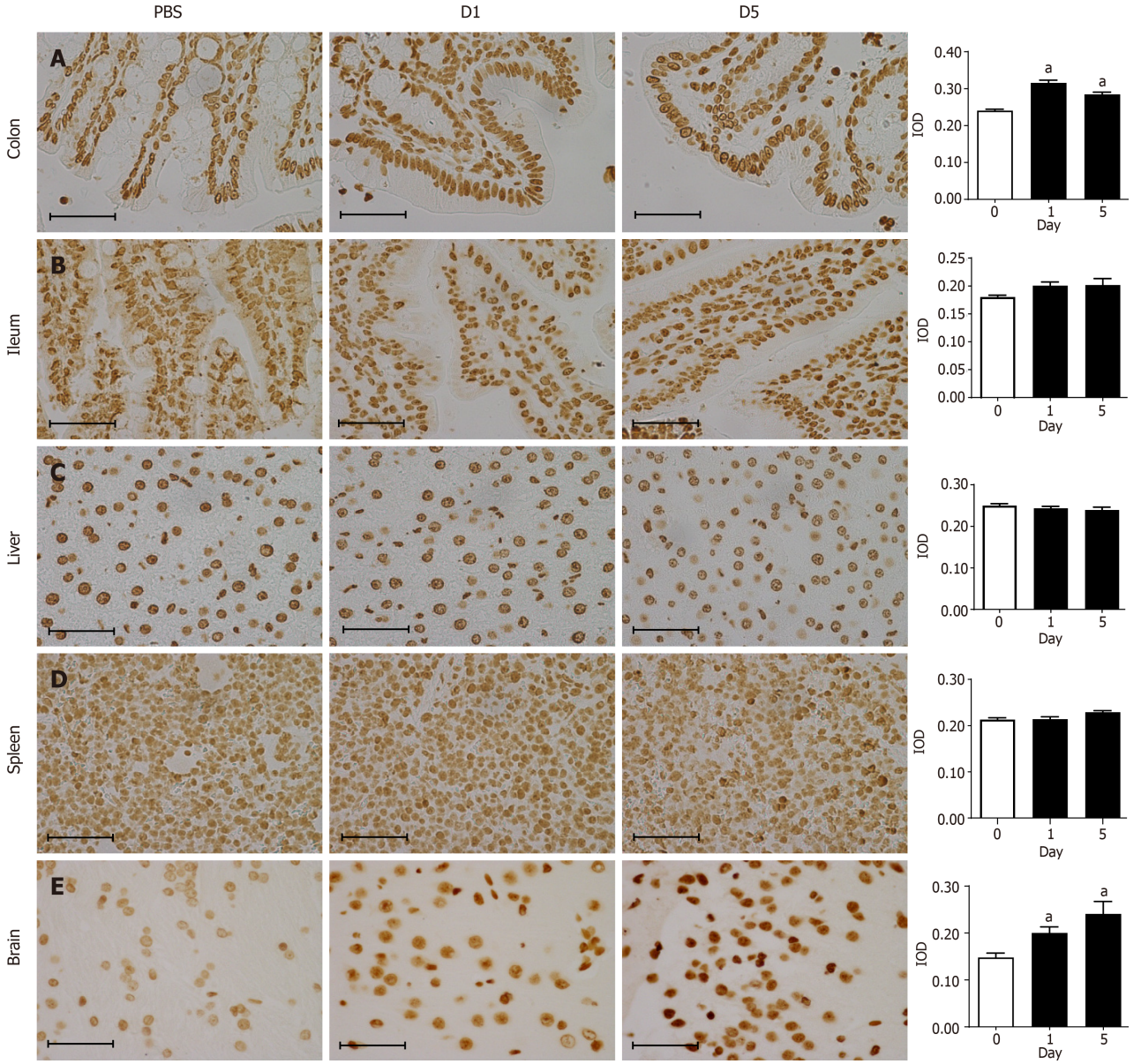

Figure 5 Immunohistochemical staining for 8-oxo-7,8-dihydro-2-deoxyguanosine in tissues. A: Representative images showing immunohistochemical staining (400 × magnification) of 8-oxo-7,8-dihydro-2-deoxyguanosine (8-oxo-dGsn; a DNA oxidative damage marker) in colon sections of rats. Left panel images (PBS) indicate the results of the control group. Middle panel images (D1) indicate the results one day after *Shigella flexneri* (*S. flexneri*) infection. Right panel images (D5) indicate the results five days after *S. flexneri* infection; B: Immunohistochemical staining for 8-oxo-dGsn in ileum sections; C: Immunohistochemical staining for 8-oxo-dGsn in liver sections; D: Immunohistochemical staining for 8-oxo-dGsn in spleen sections; E: Immunohistochemical staining for 8-oxo-dGsn in brain sections. Histogram: The quantitative analysis of immunohistochemical staining of 8-oxo-dGsn in tissues from the infection and control groups. Scale bar, 50 μm. $^{a}P < 0.05$. IOD: Integrated optical density.

concentration, the WBC count, and the CRP level. The WBC count is widely used to evaluate the severity of bacterial infections. CRP, an acute-phase protein of hepatic origin, is a non-specific marker of inflammation and one of the most sensitive acute-phase reactants.

The levels of 8-oxo-Gsn, a marker of oxidative stress, as well as the WBC count and the CRP level, which are markers of inflammation, were all increased significantly on day 1 and peaked on day 5. Following the clearance of the pathogen, the values for these biomarkers returned to the baseline level.

The correlation analysis showed that 8-oxo-Gsn was positively correlated with the WBC count. Although no significant correlation was found between 8-oxo-Gsn and CRP, the trend of the urinary 8-oxo-Gsn concentration was consistent with the changes of the WBC count and CRP level. The sharp increase in the CRP level on day 1 (20 times higher than that on day 0) may be the reason for the absence of a significant correlation. The predictive value of the 8-oxo-Gsn concentration, WBC count, and CRP level for differentiating between infection and non-infection was assessed using the

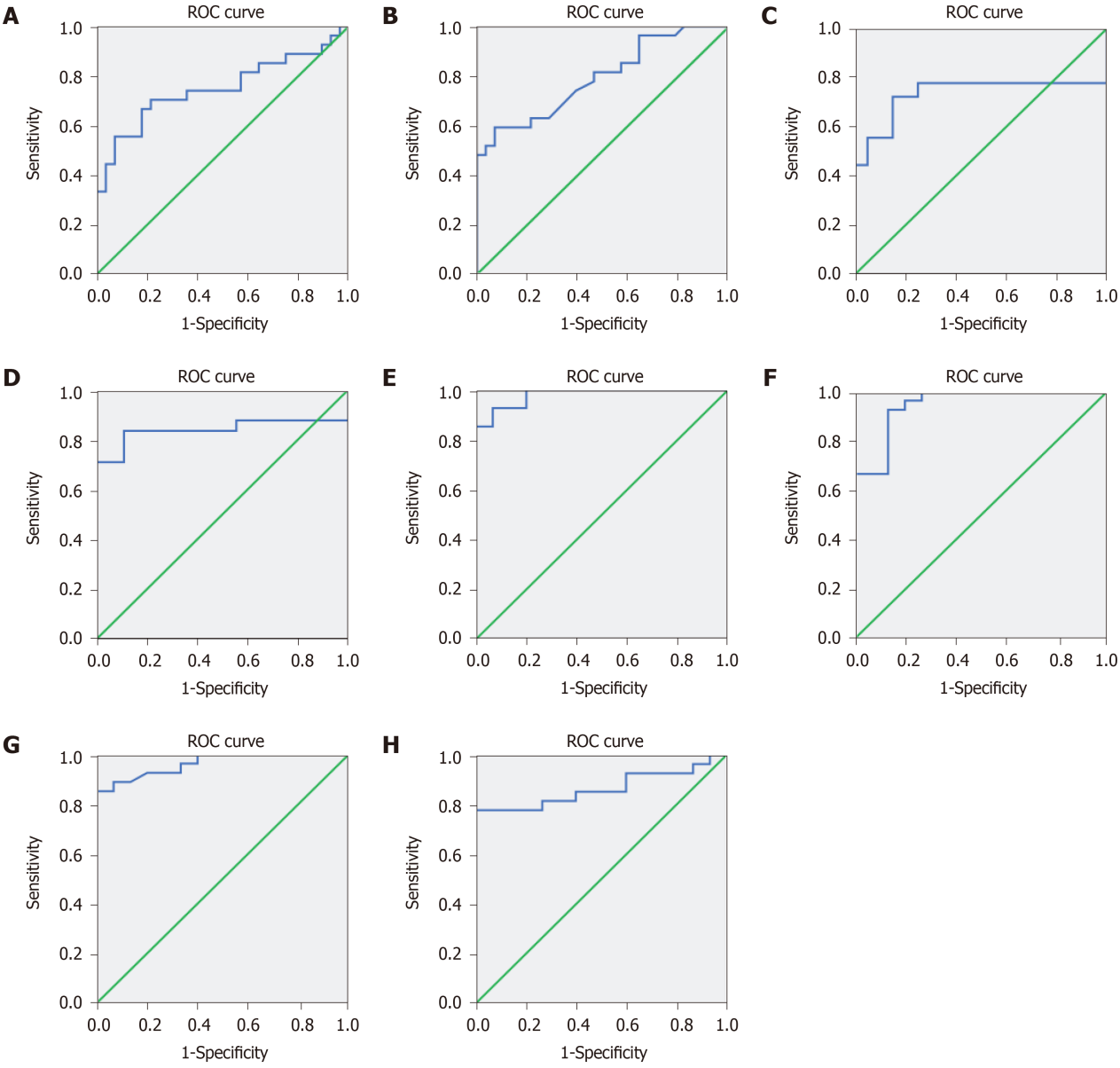

Figure 6 Receiver operator characteristic curves of 8-oxo-7,8-dihydroguanosine, white blood cell count, C-reactive protein, interleukin-6, tumor necrosis factor α, interleukin-1β, interleukin-17α, and interleukin-10 for assessing the infection status. A: Receiver operator characteristic (ROC) curve of urinary 8-oxo-7,8-dihydroguanosine; B: ROC curve of white blood cell count; C: ROC curve of C-reactive protein; D: ROC curve of interleukin (IL)-6; E: ROC curve of tumor necrosis factor α; F: ROC curve of IL-1β; G: ROC curve of IL-17α; H: ROC curve of IL-10. ROC: Receiver operator characteristic.

ROC curve. The results showed that 8-oxo-Gsn achieved a fair accuracy in classifying subjects as infected or uninfected. When the area under the ROC curve for the 8-oxo-Gsn concentration was compared to those of the WBC count and CRP level, all values ranged from 0.70 to 0.80, indicating that these diagnostic markers have a similar discrimination ability for the infection status[17]. Furthermore, urinary 8-oxo-Gsn is a noninvasive biomarker and can be measured more frequently than blood biomarkers, such as the WBC and CRP.

The immunohistochemistry results showed that in addition to intestinal tract tissue, 8-oxo-Gsn (RNA oxidative product) levels were increased in the liver, spleen, and brain tissues. Previous studies have shown that *Shigella* has a predilection to infect and colonize the large bowel[2,18]. The increased oxidative stress in other tissues might be induced by endo- or exotoxin produced by *Shigella* strains, according to previous studies[14,15,19]. Our study suggested that diarrhea caused by *Shigella* bacteria could induce a transient elevation in systemic oxidative stress.

The levels of the DNA oxidative product 8-oxo-dGsn showed no significant changes in urine, ileum, liver, or spleen tissues. The increase was only detected in the colon and brain. The results suggested that DNA oxidative damage occurred less frequently than

RNA oxidative damage in the course of acute infection. This might be because DNA molecules have a double-stranded structure, which is protected by histones and the cell nucleus[11]. All of these protections reduce the DNA oxidative damage during acute infection. Previous studies have shown that, in the course of chronic infection, DNA oxidative damage occurred more frequently, which helps promote inflammation-mediated carcinogenesis[20,21].

The present study showed that the levels of the cytokines IL-6, TNF-α, IL-1β, IL-17α and IL-10 were significantly increased after infection. Previous studies also showed that *S. flexneri* induced the release of both inflammatory (IL6, IL-8, and IL1β) and anti-inflammatory cytokines (IL-10)[22-24]. However, changes in the levels of IL-17α in *S. flexneri*-related infection have not been investigated. IL-17 has emerged as a central player in the mammalian immune system[25]. To promote inflammation, IL-17 acts in concert with IL-1β and TNF-α[26], with IL-1β being required to mediate inflammation in *S. flexneri* infection[8]. The present results suggested that *S. flexneri* infection activates inflammatory cytokines. Our findings also showed that the cytokine levels were positively correlated with the concentration of 8-oxo-Gsn, a marker of RNA oxidation, which proved again that 8-oxo-Gsn was useful as a diagnostic biomarker in infection.

CONCLUSION

In summary, during the early stage of *Shigella* infection, tissue inflammation occurs at the site of pathogen colonization, accompanied by an increase in reactive oxygen species (ROS) and the release of inflammatory cytokines. With the clearance of *Shigella*, the levels of oxidative stress markers and inflammatory cytokines decline, and the tissue inflammation recovers. The results suggested that although an increase in ROS can induce RNA oxidative damage in the colon and various tissues, this is temporary and differs from DNA oxidative damage, which occurs more frequently under conditions of chronic infection[20]. Thus, the combination of oxidative stress and inflammatory cytokines promotes infection recovery. A previous study showed that the RNA oxidative nucleic acid 8-oxoGTP increased protein variation[27]. The influence of various mutated proteins in infection, especially in the course of chronic infection, should be investigated in future studies.

ARTICLE HIGHLIGHTS

Research background

Shigella infection is a major public health concern around the world. *Shigella flexneri* (*S. flexneri*) is a major pathogen causing acute intestinal infection and is more frequently fatal than other *Shigella* species. The white blood cell (WBC) count and levels of C-reactive protein (CRP) and cytokines have always been used to evaluate the severity of *Shigella*-induced infection. Because RNA oxidation plays a substantial role in the progression of multiple diseases, the RNA oxidative product 8-oxo-7,8-dihydroguanosine (8-oxo-Gsn) may be useful as a biomarker for predicting infectious disease progression and severity.

Research motivation

There is no information regarding the diagnostic value of urinary 8-oxo-Gsn and 8-oxo-7,8-dihydro-2-deoxyguanosine (8-oxo-dGsn) in intestinal infection induced by *S. flexneri*. In addition, the correlation of blood inflammatory-related cytokines with RNA oxidative metabolites is unclear. Furthermore, the systematic tissue oxidative damage during *Shigella*-induced intestinal infection has not been investigated.

Research objectives

To determine the systemic RNA oxidative damage incurred in *S. flexneri*-induced intestinal infection and to evaluate the diagnostic value of the RNA oxidative metabolites 8-oxo-Gsn in urine.

Research methods

Sprague-Dawley rats were used to establish an acute intestinal infection model by oral gavage of *S. flexneri* strains. The CRP level was measured by ELISA, and cytokines

were detected using the MILLIPLEX MAP Kit according to the manufacturer's protocol. Hematoxylin and eosin staining was performed to detect tissue inflammation. Liquid chromatography with tandem mass spectrometry was used to determine the 8-oxo-dGsn and 8-oxo-Gsn levels in urine. Immunohistochemical staining was performed to detect the expression of 8-oxo-Gsn and 8-oxo-dGsn in the ileum, colon, liver, spleen, and brain.

Research results

Intestinal infection induced by *S. flexneri* caused an increase in inflammation-related markers in the blood [WBCs, CRP, tumor necrosis factor α (TNF-α), interleukin (IL)-6, IL-10, IL-1β, IL-4, and IL-17a] and RNA oxidative damage-related markers in the urine (8-oxo-Gsn). The urinary 8-oxo-Gsn level increased after infection and returned to the baseline level after recovery from infection. A correlation analysis showed that urinary 8-oxo-Gsn was positively correlated with the WBC count and the cytokines IL-6, TNF-α, IL-10, IL-1β, and IL-17α. The evaluation of oxidation in different tissues showed that intestinal infection caused by *S. flexneri* induced an increase in RNA oxidative damage in various tissues, including the intestine, liver, spleen, and brain.

Research conclusions

This study demonstrated that acute intestinal infection induced by *S. flexneri* not only causes RNA oxidative damage in the intestine but also other tissues as well (liver, spleen, and brain). Furthermore, the urinary metabolite of RNA oxidation 8-oxo-Gsn was shown to be useful as a diagnostic index to evaluate the infection status.

Research perspectives

Urinary 8-oxo-Gsn can be used as a noninvasive diagnostic biomarker to evaluate the severity and prognosis of infection in clinical practice.

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ORIGINAL ARTICLE

Retrospective Cohort Study

Hepatitis B virus persistent infection-related single nucleotide polymorphisms in HLA regions are associated with viral load in hepatoma families

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Author contributions: Tai DI is the guarantor and designed the study; Hsieh AR and Fann CSJ participated in the statistical analysis and data interpretation; Lin HC, Tai J, Hsieh SY and Tai DI participated in the data acquisition; Fann CSJ revised the manuscript critically for important intellectual content.

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Institutional review board statement: The study was approved by the institutional review board of Chang Gung Memorial Hospital, Taiwan (IRB 104-2596).

Informed consent statement: Written informed consent was obtained from all participants before the study. All experiments

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Abstract

BACKGROUND

Genome-wide association studies from Asia indicate that HLA-DP and HLA-DQ loci are important in persistent hepatitis B virus (HBV) infections. One of the key elements for HBV-related carcinogenesis is persistent viral replication and inflammation.

AIM

To examine genetic and nongenetic factors with persistent HBV infection and viral load in families with hepatocellular carcinoma (HCC).

METHODS

The HCC families included 301 hepatitis B surface antigen (HBsAg) carriers and 424 noncarriers born before the nationwide vaccination program was initiated in 1984. Five HBV-related single nucleotide polymorphisms (SNPs) — rs477515, rs9272105, rs9276370, rs7756516, and rs9277535 — were genotyped. Factors associated with persistent HBV infection and viral load were analyzed by a generalized estimating equation.

RESULTS

In the first-stage persistent HBV study, all SNPs except rs9272105 were associated with persistent infection. A significantly higher area under the reciprocal operating characteristic curve for nongenetic factors *vs* genetic factors ($P < 0.001$)

suggests that the former play a major role in persistent HBV infection. In the second-stage viral load study, we added 8 HBsAg carriers born after 1984. The 309 HBsAg carriers were divided into low (n = 162) and high viral load (n = 147) groups with an HBV DNA cutoff of 10^5 cps/mL. Sex, relationship to the index case, rs477515, rs9272105, and rs7756516 were associated with viral load. Based on the receiver operating characteristic curve analysis, genetic and nongenetic factors affected viral load equally in the HCC family cohort (P = 0.3117).

CONCLUSION

In these east Asian adults, the mechanism of persistent HBV infection-related SNPs was a prolonged viral replication phase.

Key Words: Generalized estimating equation; Genetic polymorphism; Genome-wide association study; Hepatitis B surface antigen; Hepatitis B virus; Replication

Core Tip: Hepatitis B virus (HBV)-related single nucleotide polymorphisms (SNPs) have been identified in East Asians. We evaluated five SNPs and nongenetic factors associated with HBV infection in a hepatocellular carcinoma family cohort. The factors were correlated with hepatitis B surface antigen (HBsAg) in the first-stage and with HBV viral load in the second-stage. The SNPs, sex, generation, and index case HBsAg contributed to persistent HBV infection. Neonatal tolerance and SNPs in the HLA loci were both independently associated with persistent HBV infection. A prolonged HBV replication phase in parents could be the main mechanism of persistent HBV infection in children in East Asia.

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INTRODUCTION

Chronic hepatitis B is a global disease, with the highest prevalence in Africa and Asia [1,2]. Hepatitis B virus (HBV) is highly infectious[3,4], and those who are infected early in life are likely to develop a persistent infection[5-7]. Intra-familial spread of infection is common, resulting in the clustering of chronic hepatitis B surface antigen (HBsAg) carriers and hepatocellular carcinoma (HCC) in families[8-10]. Recent genome-wide association studies (GWASs) in Japan, Korea, Saudi Arabia, China, and Taiwan have consistently shown that single nucleotide polymorphisms (SNPs) at the HLA-DP and HLA-DQ loci play important roles in persistent HBV infection[11-19]. However, risk alleles of HBV-related SNPs are not present in the majority of Africans[20,21], so the high prevalence of HBsAg carriers in Africa cannot be completely explained by the SNPs.

It is well known that clearance of the hepatitis B e antigen (HBeAg) occurs earlier in African than in Asian HBsAg carriers[22-25]. In east Asia, the annual HBeAg seroconversion rate is < 2% in children younger than 3 years of age and around 5% in children older than 3 years of age[22,23]. On the contrary, an HBeAg annual clearance rate of 14%-16% has been found in Euro-Mediterranean and African children[24,25]. HBeAg clearance is associated with a decreased viral load and results in a decrease of perinatal infections and the development of chronic persistent HBV infection[7,23]. We propose that persistent HBV infection-related SNPs may be one of the reasons for the prolonged HBV replication phase in east Asians. To evaluate this hypothesis, we analyzed the HBV-related SNP and demographic data obtained from HCC families. HCC families are known to have higher perinatal transmission and a longer HBV replication phase than the general population[9,10]. We expect that the genetic and nongenetic factors characteristic of HCC families may help us to understand the

and data comparisons were carried out in compliance with relevant laws and guidelines, and in accord with the ethical standards of the Declaration of Helsinki.

Conflict-of-interest statement: The authors declare that they have no conflicting interests.

Data sharing statement: No additional data are available.

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nature of persistent HBV infection.

MATERIALS AND METHODS

Ethics statement

Our study was approved by the institutional review board of Chang Gung Memorial Hospital, Taiwan (IRB 104-2596). Written informed consent was obtained from all participants. All experiments and data comparisons were carried out in compliance with relevant laws and guidelines, and complied with the ethical standards of the Declaration of Helsinki.

Study participants

Patients with HCC who were diagnosed at Chang Gung Memorial Hospital, Lin-Kou Medical Center were included as index cases. From 2003 to 2007, relatives of the patients were prospectively invited to complete a liver disease survey. The details of the survey can be seen in our previous report[10]. Briefly, after confirmation of their relation to the index HCC patient, the relatives received a structured questionnaire and underwent assessments of their liver biochemistry, alpha-fetoprotein, viral markers, and HBV genotyping. Peripheral blood samples were collected for host genome analysis.

Study size

We calculated sample sizes and statistical power to detect genetic effects in the study. The calculation considered the impact the minor allele frequency (MAF, from 0.1 to 0.4), odds ratio (OR, from 1.05 to 3), statistical power (from 0.5 to 0.9) and measurement error (type I error = 0.05) have on sample size. Power calculations were performed with QUANTO power calculator, version 1.2.4 (https://preventivemedicine.usc.edu/download-quanto/).

SNP selection and genotyping

Four genetic variants (rs477515, rs9276370, rs7756516, rs9277535) associated with persistent HBV infection that were previously identified[17] were included in the analysis. One additional HCC-related SNP (rs9272105) previously identified in China was also included[26]. Genomic DNA was extracted from peripheral blood cells using MagNA Pure LC DNA isolation kits with automated DNA isolation instruments (MagNA Pure LC II; Roche Diagnostics, Mannheim, Germany). Triple-SNP (rs477515, rs9272105, rs9277535) genotyping was performed with *Taq*Man Genotyping assays (Applied Biosystems, Foster City, CA, United States). Two SNPs (rs7756516, rs9276370) were genotyped with a Sequenom MassARRAY System (Sequenom, San Diego, CA, United States). The *Taq*Man assays were carried out by Vita Genomics (New Taipei City, Taiwan), and the Sequenom MassARRAY assays were performed by the Academia Sinica National Genotyping Center (Taipei, Taiwan). The overall genotype call rate was > 95%.

Statistical analysis

The statistical analyses were performed with SAS version 8.2 for UNIX (SAS Institute, Cary, NC, United States), PLINK (http://zzz.bwh.harvard.edu/plink/) (http://zzz.bwh.harvard.edu/plink/summary.shtml), R 2.15.1 (http://www.r-project.org/), and the Family-Based Association Test software (http://www.biostat.harvard.edu/~fbat/fbat.htm)[27]. A two-tailed P value < 0.05 was considered statistically significant. All associations were controlled for confounding factors. SNP data was quality controlled using the following criteria: (1) Call rate > 0.95; (2) MAF > 0.01; and (3) Deviation from Hardy-Weinberg equilibrium P > 0.001.

Individual locus analysis: We assessed the association of SNPs with persistent HBV infection or viral load in an additive genetic model using univariate and multivariate logistic regression of the data from unrelated male participants. In the family analysis, relatives included individuals living in the same household. First- and second-stage analyses were conducted with a generalized estimating equation (GEE) that included data correlated with a binary response (*e.g.*, to HBsAg status and HBV DNA level) using an exchangeable working correlation structure[28,29]. Univariate and multivariate analysis of the first- and second-stage results were assessed using the GEE method combined with the PROC GENMOD procedure in SAS 9.3 (SAS Institute). ORs were reported with 95% confidence intervals (CIs).

Weighted genetic risk score calculation: The weighted genetic risk score (WGRS) was calculated for the SNPs that were significantly associated with persistent infection or viral load. We assumed that each SNP was independently associated with risk according to an additive genetic model. The WGRS was calculated by multiplying the number of risk alleles at each polymorphic locus (0, 1, or 2) by each person for the corresponding relative logarithm of the OR (w_i) from the multivariate individual locus analysis and rescaling it with the factor $m/\sum_i w_i$, as follows: $WGRS = (m/\sum_i w_i) \cdot \sum w_i n_i$, where m is the number of statistically significant SNPs and n_i is the number of risk alleles for SNPi[30]. We divided the continuous WGRS into quartiles (Q1-4) and compared the risks among them.

Evaluation of genetic and nongenetic factors: We analyzed factors associated with persistent HBV infection or viral load using the logistic regression model unrelated participants and the GEE method for family data. Three prediction models were used: (1) The genetic model included only SNPs and WGRS; (2) The nongenetic model included only demographic data; and (3) The mixed model included both genetic and nongenetic variables. The contribution of the WGRS was evaluated using the area under the receiver operating characteristic curve (AUC), net reclassification improvement (NRI) method[31], and integrated discrimination improvement (IDI)[32] with the prediction model with and without the WGRS. To assess the demographic impact of including the WGRS in the model, an AUC of 0.5 indicated no discrimination and an AUC of 1 indicated perfect discrimination. The NRI indicated the proportion of subjects reclassified correctly (NRI > 0) or incorrectly (NRI < 0) into the various risk categories. An IDI > 0 indicated a statistically significant prediction of improvement as a result of adding variables to the model.

RESULTS

The HCC family cohort included 835 participants (Figure 1), of whom 301 HBsAg-positive and 424 of HBsAg-negative family members were selected for the first-stage HBV infection-persistence analysis. We excluded those born after the nationwide vaccination program was initiated in 1984. In the second-stage viral load study, we added 8 HBsAg carriers born after 1984 (Figure 1). A cohort of 309 HBsAg carriers was divided into high (n = 147) and low (n = 162) viral load groups using an HBV DNA cutoff of 10^5 cps/mL.

First stage: Factors associated with persistent HBV infection

Risk factors associated with being an HBsAg carrier were identified in the first-stage analysis. Demographic factors, which included age, sex, index case sex, relation to the index case, index HBsAg, and maternal HBsAg, are shown in Table 1. Age (OR = 1.018, P = 0.0013), sex (OR = 1.641, P = 0.0001), relation to the index case (OR = 3.203, P < 0.0001; index generation compared with children and grandchildren), index HBsAg (OR = 4.913, P < 0.0001), maternal HBsAg (OR = 3.31, P < 0.0001), and serum glutamic pyruvic transaminase (SGPT) (OR = 1.017, P < 0.0001) were significantly associated with persistent HBV infection. The associations remained significant after controlling for sex and age.

The SNPs rs477515 (OR = 1.377, P = 0.0274), rs9276370 (OR = 1.790, P = 0.0012), rs7756516 (OR = 1.654, P = 0.0048), and rs9277535 (OR = 1.519, P = 0.0004) were significantly associated with chronic HBV infection (Table 1). The ORs remained statistically significant after controlling for sex and age. HCC families carrying more risk alleles had an increased OR (Table 2, upper panel). Compared with participants with a WGRS in Q1, those with scores in Q2 and Q3–4 had higher risks of HBsAg positivity (Q2 OR = 1.878, P = 0.0014; Q3-4 OR = 2.538, P < 0.0001).

Results of the multivariate GEE analysis of the risk factors associated with persistent HBV infection are shown in Table 3. In the nongenetic model, sex, index generation, and index and maternal index HBsAg were associated with persistent HBV infection. In the genetic model, rs9277535 and WGRS were associated with persistent HBV infection. In the mixed model, all the risk factors were significant (male sex P = 0.0205; index generation P = 0.0001; index HBsAg P < 0.0001; maternal HBsAg P = 0.0072; rs9277535 P = 0.0029; WGRS P = 0.0012; Table 3).

The AUC for persistent HBV infection (Table 3) was 0.786 (P < 0.0001) in the nongenetic model and 0.620 (P < 0.0001) in the genetic model. Although the SNPs were identified by GWAS in unrelated subjects, the AUC data suggest that nongenetic factors were more important than genetic factors for the development of persistent

Table 1 Factors associated with persistent hepatitis B virus infection in the hepatocellular carcinoma family cohort

	HBsAg					
Category	**Positive**	**Negative**	**OR (95%CI)**	**Adjusted OR (95%CI)[1]**	***P* value**	**Adjusted *P* value[2]**
Total family members, *n*	301	424				
Age in yr, mean ± SD	44.23 ± 13.84	41.25 ± 14.97	1.018 (1.007-1.03)	1.017 (1.006-1.028)	0.0013	0.0030
Sex, *n* (%)						
Male	182 (60.47)	203 (47.88)	1.641 (1.279-2.107)	1.57 (1.225-2.011)	0.0001	0.0004
Female	119 (39.53)	221 (52.12)	1	1		
Index sex, *n* (%)						
Male	226 (75.08)	309 (72.88)	1.207 (0.726-2.006)	1.147 (0.681-1.93)	0.4685	0.6061
Female	75 (24.92)	115 (27.12)	1	1		
Relation to index, *n* (%)						
Children and grandchildren	146 (48.50)	319 (75.24)	1	1		
Parent generation	7 (2.33)	15 (3.54)	0.7 (0.302-1.622)	1.472 (0.533-4.065)	0.4059	0.4559
Index generation	148 (49.17)	90 (21.23)	3.203 (2.282-4.498)	4.861 (2.923-8.083)	< 0.0001	< 0.0001
Index status, *n* (%)						
HBsAg-	70 (23.26)	257 (60.61)	1	1		
HBsAg+	231 (76.74)	167 (39.39)	4.913 (3.209-7.522)	5.928 (3.747-9.377)	< 0.0001	< 0.0001
Mother's status, *n* (%)						
HBsAg-	85 (28.24)	239 (56.37)	1	1		
HBsAg+	91 (30.23)	45 (10.61)	3.31 (1.894-5.783)	3.296 (1.891-5.746)	< 0.0001	< 0.0001
Unknown	125 (41.53)	140 (33.02)	2.305 (1.568-3.39)	1.87 (1.202-2.91)	<.0001	0.0055
SGPT, mean ± SD	49.98 ± 65.39	25.83 ± 24.92	1.017 (1.011-1.022)	1.015 (1.009-1.020)	< 0.0001	< 0.0001
rs477515 (MAF = 0.1552) Chr6: 32601914[1]						
TT (reference)	5 (1.66)	20 (4.72)				
TC	59 (19.60)	116 (27.36)				
CC	237 (78.74)	288 (67.92)	1.377 (1.036-1.831)	1.38 (1.034-1.842)	0.0274	0.0285
rs9272105 (MAF = 0.4282) Chr6: 32632222[1]						
GG (reference)	54 (17.94)	84 (19.86)				
GA	137 (45.51)	207 (48.94)				
AA	110 (36.54)	132 (31.21)	1.054 (0.859-1.293)	1.031 (0.844-1.261)	0.6126	0.7639
rs9276370 (MAF = 0.1159) Chr6: 32739518[1]						
GG (reference)	3 (1.00)	13 (3.07)				
GT	39 (12.96)	97 (22.88)				
TT	259 (86.05)	314 (74.06)	1.790 (1.258-2.547)	1.759 (1.228-2.519)	0.0012	0.0021
rs7756516 (MAF = 0.1166) Chr6: 32756140[1]						
CC (reference)	3 (1.00)	13 (3.07)				
CT	42 (13.95)	95 (22.41)				
TT	256 (85.05)	316 (74.53)	1.654 (1.166-2.346)	1.612 (1.123-2.313)	0.0048	0.0096
rs9277535 (MAF = 0.3234) Chr6: 33087084[1]						
AA (reference)	21 (6.98)	61 (14.39)				
AG	114 (37.87)	191 (45.05)				
GG	166 (55.15)	172 (40.57)	1.519 (1.204-1.916)	1.493 (1.182-1.886)	0.0004	0.0008

[1]Genome build GRCH38.
[2]Adjusted for sex and age. HBsAg: Hepatitis B surface antigen; MAF: Minor allele frequency; SGPT: Serum glutamic pyruvic transaminase; CI: Confidence interval; OR: Odds ratio.

Table 2 Cumulative effect of the genetic-risk alleles associated with hepatitis B viral load or persistent hepatitis B virus infection

Study	WGRS quartile	OR (95%CI)	*P* value
Family first stage: Persistent HBV infection	Q1 (WGRS ≤ 6.166)	1	
	Q2 (WGRS = 6.166-7.083)	1.878 (1.277-2.762)	0.0014
	Q3,4 (WGRS > 7.083)[1]	2.538 (1.742-3.698)	< 0.0001
	Cochran-Armitage trend test		< 0.0001
Family second stage: Viral load	Q1 (WGRS ≤ 4.583)	1	
	Q2 (WGRS = 4.583-5.291)	2.204 (1.253-3.878)	0.0061
	Q3,4 (WGRS > 5.291)[2]	3.156 (1.780-5.595)	< 0.0001
	Cochran-Armitage trend test		< 0.0001

[1]The number of hepatitis B surface antigen negative individuals in Q4 was < 5, so Q3 and Q4 were combined.
[2]The number of individuals with hepatitis B virus DNA < 10^5 cps/mL in Q4 was < 1, so Q3 and Q4 were combined. The cumulative effect was calculated from: Four single nucleotide polymorphisms (SNPs) (rs9272105, rs9276370, rs7756516, and s9277535) in unrelated male hepatitis B surface antigen (HBsAg) carriers; four SNPs (rs477515, rs9276370, rs7756516, and rs9277535) in the first-stage hepatocellular carcinoma (HCC) family cohort analysis; and three SNPs (rs477515, rs9272105, and rs7756516) in HBsAg-positive carriers in the second-stage HCC family cohort analysis. CI: Confidence interval; OR: Odds ratio; Q: Quartile; WGRS: Weighted genetic risk score; HBV: Hepatitis B virus.

HBV infection (P < 0.0001; Figure 2). The combination of genetic and nongenetic factors resulted in an AUC of 0.795 (P < 0.0001; Figure 2 and Table 3). The IDI was 0.017 (95%CI: 0.009-0.026, P < 0.0001) and the NRI was 0.330 (95%CI: 0.192-0.467, P < 0.0001). The IDI and NRI values indicated statistically significant predicted improvement in the mixed, relative to the nongenetic model (Table 3).

Second stage: Factors associated with HBV viral load in HBsAg-positive HCC families

Factors associated with the HBV viral load were evaluated in HBsAg-positive families (Table 4). In that group, male sex (OR = 1.922, P = 0.0078), relation to the index case (OR = 2.033, P = 0.0029), index HBsAg (OR = 2.508, P = 0.0036), and SGPT (OR = 1.010, P = 0.0105) were significantly associated with the HBV viral load. The associations remained statistically significant after controlling for sex. HBV genotypes were also evaluated in HCC families, and of the participants with known HBV genotypes, the prevalence of genotype C was higher in those with high viral loads (41/143, 28.7%) than in those with low viral loads (15/90, 16.7%, P = 0.0431). The difference was marginally significant in multivariate analysis (P = 0.0515; Table 4).

Of the five SNPs included in the analysis, rs477515 (OR = 3.107, P = 0.0002), rs9272105 (OR = 1.747, P = 0.0009), and rs7756516 (OR = 1.951, P = 0.0272) were significantly associated with HBV viral load. The associations remained significant after controlling for sex (Table 4). Participants carrying more risk alleles had higher ORs for HBV viral load (Table 2, lower panel) and compared with patients having a WGRS in Q1, those in Q2 (OR = 2.204, P = 0.0061) and Q3-4 (OR = 3.156, P < 0.0001) had higher odds of having an HBV viral load.

The results of multivariate GEE analysis of factors associated with the HBV viral load in the genetic, nongenetic, and mixed models are shown in Table 5. In the nongenetic model, the risk of HBV viral load was higher in males (OR = 1.955, P = 0.0162) and in those with index HBsAg positivity (OR = 2.219, P = 0.0187). In the genetic model, the risk allele rs477515 (OR = 2.246, P = 0.0159) and the WGRS (OR = 1.644, P < 0.0001) were significantly different between the groups with high and low viral loads. In the mixed model, sex, rs477515, and WGRS were significantly different in the groups with high and low viral loads (Table 5).

The AUC of the HBV viral load was 0.674 (P < 0.0001) for the nongenetic model, 0.632 (P < 0.0001) for the genetic model, and 0.704 (P < 0.0001) for the mixed model (Figure 3 and Table 5). The results suggest that both genetic and nongenetic factors

Table 3 Multivariate generalized estimating equation and area under the curve for hepatitis B surface antigen status in the hepatocellular carcinoma family cohort

	Nongenetic model		Mixed model	
Variable	**OR (95%CI)**	***P* value**	**OR (95%CI)**	***P* value**
Sex, male	1.458 (1.048-2.027)	0.0250	1.514 (1.075-2.133)[1]/1.487 (1.063-2.081)[2]	0.0177[1]/0.0205[2]
Index sex, male	0.915 (0.532-1.573)	0.7475	0.853 (0.496-1.466)[1]/0.838 (0.488-1.442)[2]	0.5648[1]/0.5238[2]
Age in yr	1.001 (0.984-1.018)	0.9392	1.000 (0.982-1.017)[1]/0.998 (0.981-1.016)[2]	0.9608[1]/0.8582[2]
Relation to index				
Parent generation	0.523 (0.176-1.555)	0.2434	0.560 (0.182-1.719)[1]/0.603 (0.198-1.833)[2]	0.3108[1]/0.3726[2]
Index generation	3.385 (1.836-6.239)	< 0.0001	3.344 (1.766-6.331)[1]/3.493 (1.860-6.559)[2]	0.0002[1]/0.0001[2]
Index's HBsAg+	5.077 (3.103-8.308)	< 0.0001	4.919 (2.980-8.119)[1]/4.756 (2.912-7.766)[2]	< 0.0001[1]/< 0.0001[2]
Mother's status				
HBsAg+	2.597 (1.332-5.064)	0.0051	2.459 (1.270-4.760)[1]/2.517 (1.284-4.933)[2]	0.0076[1]/0.0072[2]
Unknown	1.395 (0.788-2.469)	0.2538	1.412 (0.790-2.522)[1]/1.413 (0.795-2.511)[2]	0.2444[1]/0.2387[2]
AUC (95%CI)	0.786 (0.752-0.820)	< 0.0001		
	Genetic model			
rs477515	1.303 (0.969-1.753)	0.0802	1.121 (0.770-1.631)	0.5507
rs9276370	2.741 (0.766-9.812)	0.1211	3.040 (0.623-14.839)	0.1693
rs7756516	0.592 (0.171-2.042)	0.4064	0.516 (0.104-2.554)	0.4177
rs9277535	1.575 (1.244-1.995)	0.0002	1.535 (1.157-2.035)	0.0029
AUC (95%CI)	0.632 (0.593-0.671)	< 0.0001	0.798 (0.765-0.831)	< 0.0001
WGRS	1.322 (1.162-1.505)	< 0.0001	1.269 (1.099-1.465)	0.0012
AUC (95%CI)	0.620 (0.580-0.660)	< 0.0001	0.795 (0.762-0.829)	< 0.0001
IDI (95%CI)			0.017 (0.009-0.026)	< 0.0001
NRI (95%CI)			0.330 (0.192-0.467)	< 0.0001

[1]Each single nucleotide polymorphism was included in the mixed model.
[2]The weighted genetic risk score was added in the mixed model. AUC: Area under the receiver operating characteristic curve; CI: Confidence interval; OR: Odds ratio; GEE: Generalized estimating equation; IDI: Integrated discrimination improvement; NRI: Net reclassification improvement; WGRS: Weighted genetic risk score.

had an effect on HBV viral load. Both the IDI (0.042, 95%CI: 0.019-0.065, $P = 0.0003$) and the NRI (0.440, 95%CI: 0.236–0.644, $P < 0.0001$) indicated that the mixed model represented a significant improvement (Table 5).

DISCUSSION

In this HCC family cohort, we found that both genetic and nongenetic factors were significantly associated with persistent HBV infection. In addition, HBV-related SNPs in the HLA-DP and HLA-DQ regions were associated with HBV viral load. GWASs conducted in diverse Asian populations have revealed that the HLA-DP and -DP loci play roles in persistent HBV infection[10-19]. We evaluated persistent HBV infection in the first-stage HCC family study. Expression of four of the five HBV-related SNPs differed significantly between the HBsAg carriers and the noncarriers. When only the risk alleles of the four SNPs were included in the univariate analysis, the OR for

Table 4 Factors associated with hepatitis B viral load in a hepatitis B surface antigen-positive hepatocellular carcinoma family cohort

	HBV DNA					
Category	**≥ 10^5 cps/mL**	**< 10^5 cps/mL**	**OR (95%CI)**	**Adjusted OR (95% CI)[2]**	***P* value**	**Adjusted *P* value[2]**
Total members	147	162				
Age in yr, mean ± SD	45.03 ± 14.18	41.82 ± 14.21	1.017 (1-1.035)	1.017 (0.999-1.035)	0.0538	0.0668
Sex, *n* (%)						
Male	100 (68.03)	88 (54.32)	1.922 (1.188-3.111)	1.914 (1.187-3.087)	0.0078	0.0078
Female	47 (31.97)	74 (45.68)	1	1		
Relation to index, *n* (%)						
Children and grandchildren generation	58 (39.46)	95 (58.64)	1	1		
Parent generation	5 (3.4)	2 (1.23)	3.683 (0.866-15.656)	5.056 (1.259-20.3)	0.0775	0.0223
Index generation	84 (57.14)	65 (40.12)	2.033 (1.274-3.246)	1.845 (1.144-2.977)	0.0029	0.0121
Index's status, *n* (%)						
HBsAg-	21 (14.29)	49 (30.25)	1	1		
HBsAg+	126 (85.71)	113 (69.75)	2.508 (1.351-4.657)	2.492 (1.324-4.692)	0.0036	0.0047
Mother's status, *n* (%)						
HBsAg-	43 (29.25)	43 (26.54)	1	1		
HBsAg+	46 (31.29)	51 (31.48)	0.874 (0.467-1.634)	0.91 (0.485-1.707)	0.6724	0.7693
Unknown	58 (39.46)	68 (41.98)	0.855 (0.491-1.49)	0.857 (0.488-1.503)	0.5804	0.5898
HBV genotype (BGT230), *n* (%)						
Unknown[3]	3 (2.05)	72 (44.44)	0.03 (0.009-0.104)	0.029 (0.008-0.1)	< 0.0001	< 0.0001
B	102 (69.86)	75 (46.3)	1	1		
C	41 (28.08)	15 (9.26)	2.042 (1.023-4.079)	2.066 (0.995-4.288)	0.0431	0.0515
SGPT, mean ± SD	63.92 ± 79.63	37.02 ± 45.22	1.010 (1.002-1.018)	1.009 (1.001-1.017)	0.0105	0.0260
rs477515 (MAF = 0.1149) Chr6: 32601914[1]						
TT (reference)	1 (0.68)	4 (2.47)				
TC	15 (10.2)	46 (28.4)				
CC	131 (89.12)	112 (69.14)	3.107 (1.708-5.653)	3.195 (1.746-5.847)	0.0002	0.0002
rs9272105 (MAF = 0.4078) Chr6: 32632222[1]						
GG (reference)	20 (13.61)	36 (22.22)				
GA	59 (40.14)	81 (50)				
AA	68 (46.26)	45 (27.78)	1.747 (1.256-2.428)	1.75 (1.247-2.456)	0.0009	0.0012
rs9276370 (MAF = 0.07605) Chr6: 32739518[1]						
GG (reference)	1 (0.68)	3 (1.85)				
GT	14 (9.52)	25 (15.43)				
TT	132 (89.8)	134 (82.72)	1.747 (0.933-3.272)	1.679 (0.901-3.131)	0.0811	0.1029
rs7756516 (MAF = 0.08091) Chr6: 32756140[1]						
CC (reference)	0 (0)	4 (2.47)				
CT	16 (10.88)	26 (16.05)				
TT	131 (89.12)	132 (81.48)	1.951 (1.078-3.53)	1.875 (1.029-3.417)	0.0272	0.0400
rs9277535 (MAF = 0.2589) Chr6: 33087084[1]						
AA (reference)	13 (8.84)	8 (4.94)				
AG	45 (30.61)	73 (45.06)				

GG	89 (60.54)	81 (50)	1.235 (0.849-1.797)	1.303 (0.888-1.911)	0.2703	0.1767

[1]Genome Build ARCH38.
[2]Adjusted by sex.
[3]Eight cases not tested. CI: Confidence interval; HBsAg: Hepatitis B surface antigen; OR: Odds ratio; HBV: Hepatitis B virus.

Table 5 Multivariate generalized estimating equation and area under the curve hepatitis B viral loads in a hepatocellular family cohort

	Nongenetic model		Mixed model	
Variable	**OR (95%CI)**	***P* value**	**OR (95%CI)**	***P* value**
SNP				
Sex, male	1.955 (1.132-3.376)	0.0162	1.918 (1.101-3.341)[1]/1.911 (1.097-3.328)[2]	0.0214[1]/0.0223[2]
Index sex, male	0.903 (0.481-1.699)	0.7527	0.914 (0.488-1.713)[1]/0.912 (0.482-1.725)[2]	0.7786[1]/0.7771[2]
Age in yr	1.012 (0.987-1.037)	0.3363	1.007 (0.983-1.032)[1]/1.008 (0.983-1.033)[2]	0.5658[1]/0.5426[2]
Relation to index				
Parent generation	4.182 (0.68-25.731)	0.1228	4.343 (0.81-23.285)[1]/4.091 (0.75-22.316)[2]	0.0866[1]/0.1036[2]
Index generation	1.7 (0.844-3.423)	0.1372	1.851 (0.912-3.756)[1]/1.797 (0.894-3.611)[2]	0.0881[1]/0.0999[2]
Index HBsAg+	2.219 (1.142-4.31)	0.0187	1.734 (0.853-3.526)[1]/1.816 (0.907-3.636)[2]	0.1283[1]/0.0918[2]
Mother's status				
HBsAg+	0.828 (0.407-1.684)	0.6021	0.766 (0.361-1.623)[1]/0.763 (0.360-1.619)[2]	0.4862[1]/0.4814[2]
Unknown	0.537 (0.275-1.045)	0.0673	0.549 (0.272-1.107)[1]/0.559 (0.278-1.125)[2]	0.094[1]/0.1030[2]
AUC (95%CI)	0.674 (0.614-0.734)	< 0.0001		
	Genetic model			
rs477515	2.246 (1.164-4.333)	0.0159	2.242 (1.113-4.515)	0.0238
rs9272105	1.386 (0.965-1.991)	0.0775	1.266 (0.866-1.849)	0.2232
rs7756516	1.385 (0.765-2.509)	0.2826	1.379 (0.753-2.524)	0.2977
AUC (95%CI)	0.638 (0.579-0.698)	< 0.0001	0.705 (0.648-0.763)	< 0.0001
WGRS	1.644 (1.317-2.052)	< 0.0001	1.567 (1.250-1.965)	< 0.0001
AUC (95%CI)	0.632 (0.573-0.692)	< 0.0001	0.704 (0.646-0.761)	< 0.0001
IDI (95%CI)			0.042 (0.019-0.065)	0.0003
NRI (95%CI)			0.440 (0.236-0.644)	< 0.0001

[1]Each single nucleotide polymorphism was added in the mixed model.
[2]The weighted genetic risk score was added in the mixed model. AUC: Area under the receiver operating characteristic curve; GEE: Generalized estimating equation; IDI: Integrated discrimination improvement; NRI: Net reclassification improvement; CI: Confidence interval; HBsAg: Hepatitis B surface antigen; OR: Odds ratio.

persistence was significant if the WGRS was > 7 (Table 2, upper panel). In the genetic model, multivariate GEE analysis found that expression of one SNP (rs9277535) and the WGRS were significantly different between HBsAg carriers and noncarriers, and the differences remained significant in the presence of nongenetic factors (Table 3). Regression analysis showed that HBV-related SNPs were associated with persistent HBV infection in these HCC families. This is the first study to confirm that SNPs identified by GWAS were associated with persistent HBV infection in a family cohort.

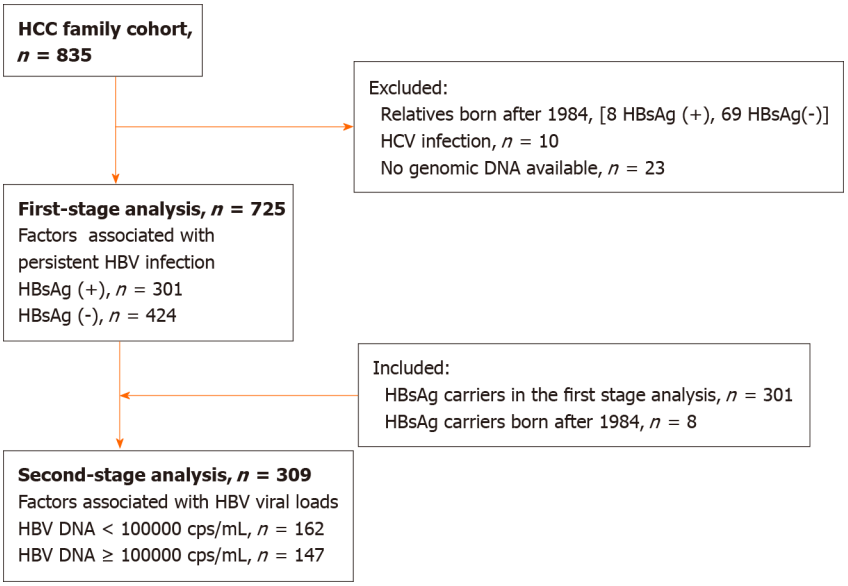

Figure 1 Study flow chart. Hepatitis B virus persistent infection and viral load were analyzed in a hepatocellular carcinoma family cohort. HCC: Hepatocellular carcinoma; HBV: Hepatitis B virus; HCV: Hepatitis C virus; HBsAg: Hepatitis B surface antigen.

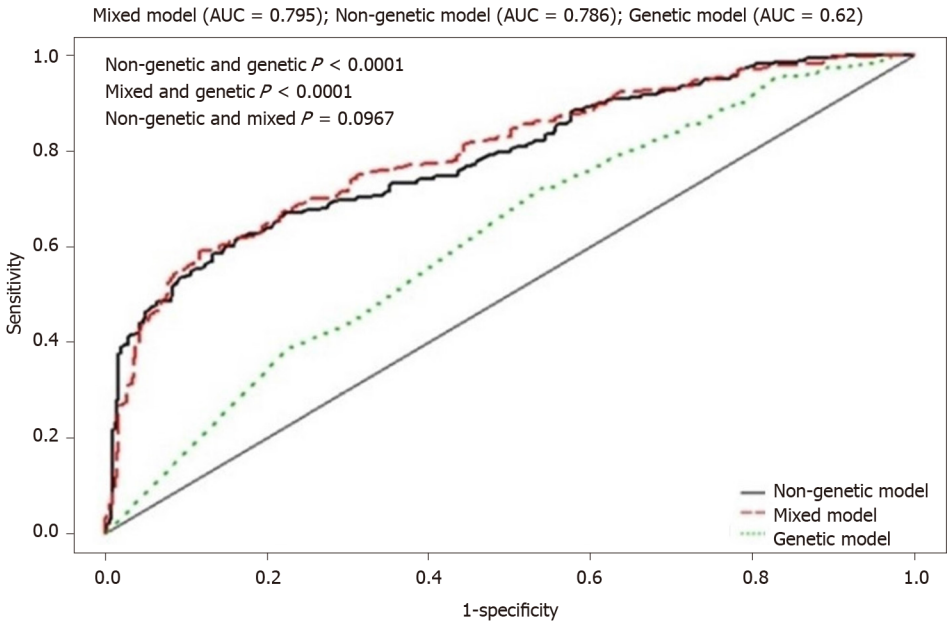

Figure 2 First-stage persistent hepatitis B virus infection. Genetic, nongenetic, and combined risk factors for persistent hepatitis B virus (HBV) infection were evaluated by area under the receiver operating characteristic curves derived from generalized estimating equation regression models. Significantly higher areas under the curve for nongenetic compared with genetic factors ($P < 0.001$) suggest that nongenetic factors played a major role in persistent HBV infection. AUC: Area under the receiver operating characteristic curve.

Nongenetic factors also affected persistent HBV infection. Age, sex, generation, index HBsAg status, and maternal HBsAg status all differed significantly between HBsAg carriers and noncarriers (Table 1). The AUC was 0.786 in the nongenetic model, 0.620 in the genetic model, and 0.795 in the mixed model (Table 3). The ROC analysis thus implied that nongenetic factors contributed more to persistent HBV infection than genetic factors did (genetic *vs* nongenetic factors $P < 0.0001$ and mixed *vs* nongenetic factors $P < 0.0001$; Figure 2). The results are consistent with exposure to HBV early in life and an important influence on the persistence of HBV infection[5-7]. Our overall findings indicate that the HCC family members may have been exposed to HBV early in life because of the high HBsAg prevalence in the index cases and/or their mothers. Accounting for both genetic and nongenetic cofactors, the prevalence of HBsAg was 41.5% (301/725) in this HCC family cohort.

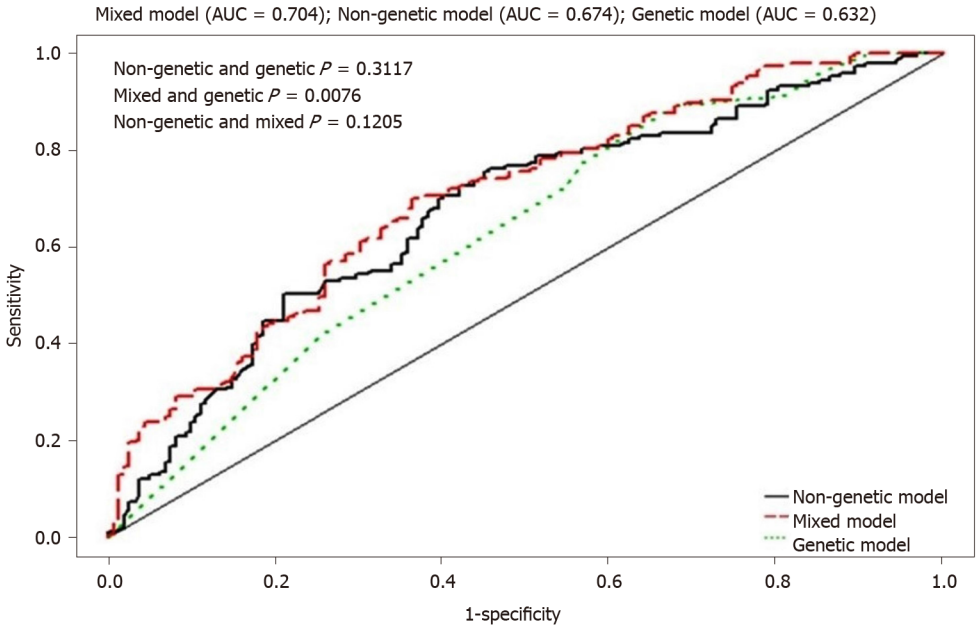

Figure 3 Second-stage hepatitis B virus viral load. The genetic, nongenetic, and combined risk factors for hepatitis B virus (HBV) viral load were evaluated by area under the receiver operating characteristic curves derived from generalized estimating equation regression models. The difference between the receiver operating characteristic curves of genetic and nongenetic factors was not significant (P = 0.3117). The finding suggests that both factors contributed to the HBV viral load. AUC: Area under the receiver operating characteristic curve.

In the presence of SNPs identified in a GWAS, nongenetic factors remain important in persistent HBV infection. The persistence of infection induced by the SNPs might depend on a delay in clearance of the HBeAg. It is known that in HBsAg carriers, HBeAg clearance occurs earlier in African than in Asian populations[22-25]. That means East Asians of reproductive age are likely to have higher HBV viral loads and a higher rate of perinatal HBV infection of their babies[7,10,22,23]. Perinatal infection usually persists as a chronic infection[7,23]. As African women usually clear HBeAg before reproductive age[24,25], the viral load during pregnancy is likely to be lower than that in East Asians, which would decrease the chance of perinatal HBV infection [24]. We suspect that a prolonged HBV replication phase in parents could be the mechanism of persistent HBV infection associated with SNPs.

Univariate analysis of the factors associated with HBV viral load in the HCC family cohort revealed that three of the five SNPs (rs477515, rs9272105, rs7756516) differed significantly between the high and low viral load groups (Table 4). The cumulative effect of the WGRS was also greater in the high viral load group (Table 3, lower panel). Multivariate GEE analysis found that the rs477515 SNP (OR = 2.242, P = 0.0238) and WGRS (OR = 1.567, P < 0.0001) were independently associated with a high viral load in the mixed model (Table 5). Our data thus support the prevailing view that the SNPs associated with persistent HBV infection promote persistent HBV replication. The mean ages of our study groups ranged from 41.25-45.03 years (Tables 1 and 4). Persistent high viral loads in these age groups were likely to have resulted in perinatal transmission of chronic HBV infection during the reproductive age.

Our previous study demonstrated that nongenetic factors influenced the HBV viral load in HCC families[10]. In this study, we observed that sex, generation, and index HBsAg cases were associated with a high viral load in the nongenetic model (Table 4). We also compared the relative contributions of genetic and nongenetic factors associated with viral load in the HCC family cohort. The AUCs of the viral load were 0.674 in the nongenetic model and 0.632 in the genetic model. The AUC in the mixed model was up to 0.704 (Table 5). Therefore, both genetic and nongenetic factors were associated with HBV viral load in the HCC family cohort. It should be noted that we included only SNPs in the HLA region. The association of other loci, such as polymorphisms of interferon gamma, complement factor B, CD40, and INST10, which have also been reported to be associated with HBV viral load, was not investigated[33-35].

One of the five SNPs we evaluated, rs9277535, was reported by Tao *et al*[36] to be associated with more aggressive liver disease, but it was reported by Li *et al*[37] not to be associated with disease progression. Our previous GWAS revealed that rs9276370 was associated with HBV therapeutic response[17]. Univariate analysis found that the

two SNPs were not significantly associated with viral load in this HCC family cohort. Two previous studies found that rs477515 was associated with HBV vaccine response [38,39], and that SNP was found to be associated with viral load in this cohort. Li *et al* [26] reported that rs9272105 was associated with HCC in a GWAS, and univariate analysis found that it was associated with viral load in this HCC family cohort. All these previous reports suggest that a single SNP provides a small contribution to HBV viral loads. Persistent HBV replication seems to be determined by multiple genetic and nongenetic risk factors.

This study provides information that may help to establish more accurate models of disease through the incorporation of genetic and nongenetic factors, but it was limited by the relatively small number of HCC families. Another limitation was that HBV genotype studies were not available in patients with low viral loads. HBV genotype C has been associated with a lower HBeAg clearance rate than genotype B[40]. We found a high adjusted OR (2.066, P = 0.0515) for the association of genotype C with a high viral load relative to a low viral load in this HCC family cohort (Table 4).

CONCLUSION

We conclude that SNPs associated with persistent HBV infection prolong the replication phase in the parent generation and increase the burden of persistent infection in the offspring generation.

ARTICLE HIGHLIGHTS

Research background

Genome-wide association studies (GWASs) in Asian populations indicate that the HLA-DP and HLA-DQ loci are involved in the persistence of hepatitis B virus (HBV) infections. Persistent viral replication and inflammation are key influencers in HBV-related carcinogenesis.

Research motivation

HBV-related single nucleotide polymorphisms (SNPs) have been identified in east Asian populations but are uncommon in African populations. Different mechanisms may drive persistent infection in those regions.

Research objectives

We examined genetic and nongenetic factors associated with persistent HBV infection and viral load in families with hepatocellular carcinoma (HCC).

Research methods

HCC families were enrolled. Five HBV-related SNPs (rs477515, rs9272105, rs9276370, rs7756516, and rs9277535) were genotyped. Factors associated with persistent HBV infection and viral load were identified with the use of generalized estimating equations.

Research results

In the first-stage persistent HBV study, all SNPs except rs9272105 were associated with persistent infection. A significantly higher contribution of nongenetic than genetic factors (P < 0.001) to persistent HBV infection was found. In the second-stage viral load study, sex, relationship with index case, rs477515, rs9272105, and rs7756516 were associated with viral load. Receiver operating characteristic curve, and genetic and nongenetic factors had equal effects on viral load in the HCC family cohort (P = 0.3117).

Research conclusions

GWAS identified SNPs that have roles in persistent HBV infection and HBV viral loads in an HCC family cohort. Nongenetic factors were more important than genetic factors in persistent HBV infection but had equal contributions to HBV viral load. HBV-related SNPs resulting in high viral loads in parents may drive persistent infection in East Asian populations. The mechanism of persistent HBV infection-related SNPs involves a prolonged viral replication phase in East Asian adults.

Research perspectives

Termination of the HBV replication phase before pregnancy will be a therapeutic goal in East Asian countries.

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ORIGINAL ARTICLE

Retrospective Study

Recently acquired hepatitis C virus infection among people living with human immunodeficiency virus at a university hospital in Taiwan

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Abstract

BACKGROUND

Little is known about the engagement in hepatitis C virus (HCV) care and completion of HCV treatment in people living with human immunodeficiency virus (HIV) (PLWH) who have HCV coinfection in the Asia-Pacific region. Examining the HCV care cascade can identify barriers to the completion of HCV treatment and facilitate achievement of HCV micro-elimination in PLWH.

AIM

To investigate the care cascade of incident HCV infections among PLWH in

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METHODS

PLWH with incident HCV infections, defined as HCV seroconversion, were retrospectively identified by sequential anti-HCV testing of all archived blood samples at National Taiwan University Hospital between 2011 and 2018. All PLWH with incident HCV infections were followed until December 31, 2019. The care cascade of HCV examined included all incident HCV-infected patients, the percentages of anti-HCV antibodies detected by HIV-treating physicians in clinical care, plasma HCV RNA load tested, HCV RNA positivity diagnosed, referral to treatment assessment made, anti-HCV treatment initiated, and sustained virologic response achieved. Those who had HCV seroconversion during the interferon (IFN) era (2011–2016) and the direct-acting antiviral (DAA) era (2017–2018) were analyzed separately. The duration of HCV viremia—from the date of seroconversion to viral clearance by treatments or until the end of observation—and the incidence of sexually transmitted infections (STIs) during the HCV viremic period were estimated.

RESULTS

During the study period, 287 of 3495 (8.2%) PLWH (92.3% being men who have sex with men) who were HCV-seronegative at baseline developed HCV seroconversion by retrospective testing of all archived blood samples. Of the 287 incident HCV infections, 277 (96.5%) had anti-HCV antibodies detected by HIV-treating physicians, 270 (94.1%) had plasma HCV RNA determined and 251 (87.5%) tested positive for HCV RNA. Of those with HCV viremia, 226 (78.7%) were referred to treatment assessment, 215 (74.9%) initiated anti-HCV treatment, and 202 (70.4%) achieved viral clearance. Compared with that in the IFN era, the median interval from HCV seroconversion by retrospective testing to detection of HCV seropositivity by HIV-treating physicians was significantly shorter in the DAA era {179 d [interquartile range (IQR) 87-434] *vs* 92 d (IQR 57-173); $P < 0.001$}. The incidence rate of STIs in the DAA *vs* the IFN era was 50.5 per 100 person-years of follow-up (PYFU) and 38.5 per 100 PYFU, respectively, with an incidence rate ratio of 1.31 (95% confidence interval 0.96-1.77), while the duration of HCV viremia was 380 d (IQR 274-554) and 735 d (IQR 391-1447) ($P < 0.001$), respectively.

CONCLUSION

While anti-HCV therapies are effective in achieving viral clearance, our study suggests more efforts are needed to expedite the linkage of PLWH diagnosed with incident HCV infections to HCV treatment.

Key Words: Recent hepatitis C virus infection; Cascade of care; Direct-acting antivirals; People living with human immunodeficiency virus; Sustained virologic response; Sexually transmitted infections

Core Tip: We examined the hepatitis C virus (HCV) care cascade among people living with human immunodeficiency virus who acquired incident HCV infections at a university hospital in Taiwan between 2011 and 2018. We observed high rates of linkage to HCV care and retention in care in both interferon (IFN, 2011 to 2016) and direct-acting antiviral (DAA, 2017 to 2018) eras. The rate of referral to treatment assessment had increased from the IFN era to the DAA era. Moreover, the duration of HCV viremia was markedly shortened because of early diagnosis and linkage to effective treatment in the DAA era compared to that in the IFN era.

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INTRODUCTION

Viral hepatitis is a major public health threat and approximately 2.3 million people living with human immunodeficiency virus (HIV) (PLWH) are coinfected with hepatitis C virus (HCV) globally[1-3]. Injection drug users (IDUs) have been the major contributors to HCV transmission[4]. In Asia-Pacific region, the prevalence of HCV coinfection among PLWH ranges from 3.8% to 42.6%, and may be as high as 80.8% to 98.5% among IDUs[5,6]. Since 2000, incident HCV infections among PLWH who are MSM have continued to increase in the United States, European countries, and Australia[7-9]. Similar increasing trends were observed among PLWH who are MSM in Asia-Pacific region[10,11]. The causes of the increases of HCV transmission among MSM are multifactorial and are related to concurrent sexually transmitted infections (STIs), unprotected anal intercourse, traumatic sexual contacts, and use of recreational drugs by inhalation or injection[12].

The evolution of HCV treatment from interferon (IFN)-based therapy to IFN-free direct-acting antivirals (DAAs) has significantly improved tolerability and effectiveness in achieving viral clearance[13]. The rates of viral clearance with DAAs are similarly high for HCV-monoinfected patients and HCV/HIV-coinfected patients in clinical trials and real-world experience[14-18]. With the introduction of effective DAAs, ending the HCV epidemic is considered an achievable goal by scaling up diagnosis and treatment of HCV infection[1]. Among the interventional strategies proposed to achieve hepatitis elimination, the continuum assessed by the "cascade of care" on the population level is suggested to evaluate the status of hepatitis treatment services and the level of engagement in care[19,20]. The proportion of patients in each step of the cascade—infected, tested, status confirmed, engagement in care, treatment initiated, cured, and followed up for chronic care—reflects the coverage of diagnosis, access to treatment, and quality of care.

In Taiwan, pegylated IFN plus ribavirin (PEG-IFN/RBV) has become the standard anti-HCV treatment regimen since 2004 and has been fully reimbursed by the National Health Insurance (NHI) since 2009[21,22]. DAAs were not available until 2017, when the regimens were restricted to individuals with chronic HCV infection with evidence of significant hepatic fibrosis. Generic versions of patented DAAs could be purchased for individual use from Bangladesh[23]. Before 2019, referral to a hepatologist for hepatitis C evaluation and treatment was required according to NHI regulations. The reimbursement restrictions on the use of DAAs were lifted in January of 2019 by allowing HCV viremic patients to initiate DAAs regardless of liver fibrosis status. Subsequently, PLWH with HCV viremia could be evaluated and DAAs initiated by HIV-treating physicians at infectious disease clinics.

In this study, we aimed to examine the HCV care cascade among PLWH who received a diagnosis of incident HCV infection at a university hospital in Taiwan.

MATERIALS AND METHODS

Study design and data collection

We retrospectively reviewed the medical records of all PLWH aged 18 years or older seeking medical care for HIV at the National Taiwan University Hospital (NTUH), the largest designated hospital for HIV inpatient and outpatient care in Taiwan, from January 2011 to December 2018; and those included were followed until death, loss to follow-up, transfer of care, or the end of the study on December 31, 2019, whichever occurred first. The study period (2011-2018) spanned two different eras of anti-HCV treatment, from the IFN/RBV era (2011-2016) to the DAA era (2017-2018) before restricted access to HCV treatments were completely lifted in early 2019. In this study, the IFN-based regimen was PEG-IFN plus weight-based RBV with response-guided treatment duration. From 2016 to 2018, generic versions of patent IFN-free DAAs (sofosbuvir/velpatasvir) were used for treatment of recently acquired HCV infection.

Anti-HCV antibodies were detected using a fourth-generation enzyme immunoassay (Dia.Pro Diagnostic Bioprobes Srl. Italy)[11] and the HCV serostatus of the first available blood samples during the study period were regarded as the baseline serostatus. For those with a negative anti-HCV status at baseline, anti-HCV testing of

all their archived blood samples were performed retrospectively on an annual basis to detect HCV seroconversion. Patients who developed HCV seroconversion within the past 12 mo were identified as having recently acquired HCV infection and were included in this study.

The date of HCV seroconversion was arbitrarily assigned as the mid-point between the dates of the first positive and the last negative anti-HCV test. Using a standardized case record form, we collected data on patient demographics, HIV transmission risk groups, and laboratory test results, including CD4 lymphocyte count and plasma HIV RNA load (PVL), hepatitis B surface antigen (HBsAg), rapid plasma reagin (RPR) titer, plasma HCV RNA load and genotype when HCV viremia was detected.

The study was approved by the Research Ethics Committee of the hospital (registration number: 201605103RINC and 201605128RINC) and informed consent was obtained from all the participants.

Definitions

The HCV care cascade comprised the following steps of care: (1) "HCV infected" included all seroconverters in the past 12 mo identified by testing of archived blood samples; (2) "antibody detected" defined as a confirmed anti-HCV-positive test identified by HIV-treating physicians and documented in the medical records; (3) "HCV RNA tested" as the performance of a plasma HCV RNA test either after a positive anti-HCV test or when HCV viremia is detected during an acute infection with a negative anti-HCV test; (4) "HCV RNA positivity" as detectable plasma HCV RNA at the time of testing; (5) "Referred to treatment assessment" as successful referral of HCV-infected individuals to a hepatology clinic for evaluation of liver fibrosis or HCV treatment; or completion of pretreatment blood testing in infectious disease clinics as required by NHI regulation in 2019, including evaluation of liver fibrosis, renal function, HCV genotype, and plasma HCV RNA load[16]; (6) "Treatment initiated" as patients who had been prescribed HCV treatment medications; and (7) "Sustained virologic response (SVR) achieved" as the state of persistently undetectable HCV RNA until 24 wk after completion of IFN-based regimens and 12 wk after completion of DAA treatments.

The durations between two consecutive steps of the care cascade were recorded. Those who had HCV seroconversion during the IFN era (2011–2016) and the DAA era (2017–2018) were analyzed separately for comparison of the proportion of patients attaining each step and the interval between two consecutive steps of the cascade. The total duration of HCV viremia was defined as the interval between the estimated date of HCV seroconversion to the end of HCV treatment in those with undetectable HCV RNA; those who did not achieve SVR were followed until the completion of re-treatment with undetectable HCV RNA, the end of observation, or loss to follow-up, whichever occurred first.

The number of STIs, including syphilis (defined as a four-fold increase of RPR titers, consistent clinical symptoms, or administration of syphilis treatment), and gonorrhea (defined as culture-confirmed *Neisseria gonorrhoeae* infection or consistent clinical symptoms and administration of gonorrhea treatment), during the defined intervals of HCV viremia was calculated. We used occurrences of STIs as surrogate markers for unprotected sex contacts of the included PLWH during the HCV viremic period without effective treatment to indicate the risk for onward transmission of HCV.

Statistical analysis

The cascade of care after diagnosis of recently acquired HCV infection was examined by each step using descriptive analysis. Comparisons of categorical variables were performed by chi-square analysis and those of continuous variables by a Mann-Whitney *U* Test. The incidence rate of STIs during HCV viremia was calculated as the number of STIs per 100 PYFU. All analyses were two-tailed, and a $P < 0.05$ was considered statistically significant. All statistical analyses were performed using Stata/SE, version 14.0. The statistical methods of this study were reviewed by the staff of National Taiwan University Hospital–Statistical Consulting Unit (NTUH-SCU).

RESULTS

Between January 2011 and December 2018, 3,495 PLWH had a negative anti-HCV antibody test at baseline (Figure 1) and HCV seroconversion was detected in 294 (8.4%) PLWH. After excluding 7 PLWH who were lost to follow-up when seroconversion occurred, a total of 287 PLWH were included in the analysis of the HCV care

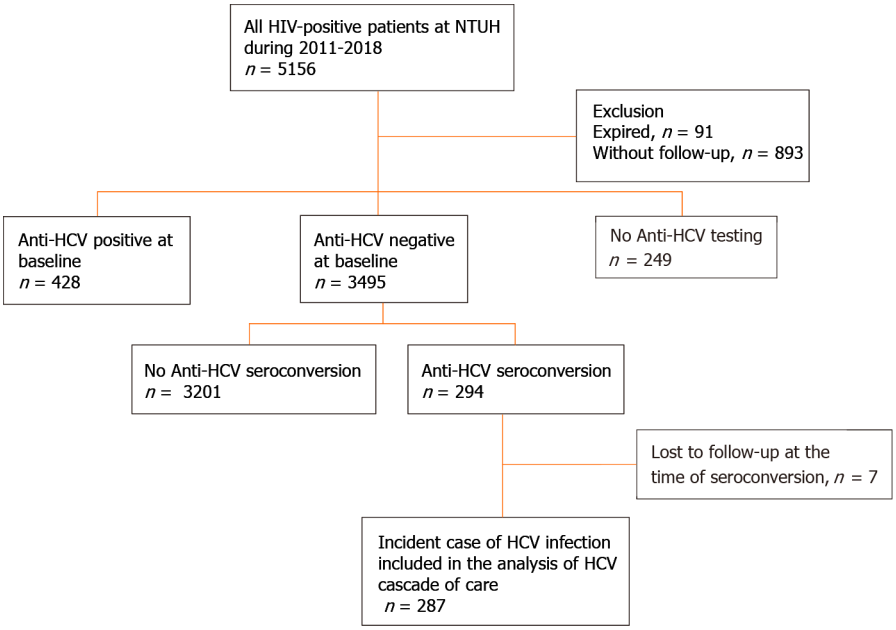

Figure 1 Patient flow. Between 2011 and 2018, 3495 people living with human immunodeficiency virus (HIV) (PLWH) had negative anti-hepatitis C virus (HCV) antibody tests at baseline and HCV seroconversion was detected in 294 (8.4%) PLWH. After excluding 7 PLWH who were lost to follow-up when HCV seroconversion occurred, a total of 287 PLWH were included in the analysis of the care cascade of incident HCV infections. HIV: Human immunodeficiency virus; NTUH: National Taiwan University Hospital; HCV: Hepatitis C virus.

cascade.

The clinical characteristics of the included 287 PLWH with recently acquired HCV infection are shown in Table 1. All of them were male, with a mean age of 34.5 years [standard deviation (SD) 7.6]. The mean PVL was 1.72 $\log_{10}$ copies/mL (SD 1.09) and mean CD4 count was 597.7 cells/mm^3 (SD 251.1) at the time of HCV seroconversion. The HBsAg seropositivity rate was 14.9% and active or previous syphilis was present in 89.2%. The risk groups of HIV acquisition included 265 (92.3%) MSM, 5 (1.8%) heterosexuals, 4 (1.4%) IDUs, and 13 (4.5%) unknown. The mean plasma HCV RNA load was 5.72 $\log_{10}$ copies/mL (SD 1.50). Twenty-two (7.7%), 84 (29.3%), 119 (41.5%), 6 (2.1%), and 34 (11.8%) PLWH had infection with HCV genotypes 1a, 1b, 2a, 3, and 6, respectively, and 3 (1%) had mixed infections of genotypes 1b and 2.

Of the 287 PLWH, 277 (96.5%) were found to have HCV seroconversions by HIV-treating physicians, including 8 diagnosed at the stage of acute infection with positive HCV RNA and negative anti-HCV antibody. Plasma HCV RNA load was determined in 270 (94.1%) PLWH after seroconversion, with 19 (12.5%) clearing viremia spontaneously and 251 (87.5%) remaining viremic (Table 2). Overall, 226 (78.7%) PLWH were referred to hepatology clinics for treatment assessment, and anti-HCV treatments were initiated in 215 (74.9%) PLWH, of whom 202 (70.4%) achieved SVR (Table 2).

Compared with PLWH who had HCV seroconversion in the IFN era (2011–2016), those who seroconverted in the DAA era (2017–2018) had higher achievement rates of all sequential steps of the care cascade (Figure 2), though not reaching statistical significance. The rate of antibody diagnosis within one year of HCV seroconversion was significantly higher in the DAA era than that in the IFN era (88.2% *vs* 69.5%; $P < 0.001$) (Figure 2). In the IFN era, the major gap of engagement in care was from HCV RNA positivity to referral for assessment and treatment, which resulted in a loss of 11.8% of those found to be HCV viremic.

The median interval from seroconversion to detection of HCV seropositivity by HIV-treating physicians was 130 d [interquartile range (IQR), 80-295], which was significantly shorter in the DAA era than in the IFN era [92 d (IQR, 57-173) *vs* 179 d (IQR 87-434); $P < 0.001$] (Table 2, Figure 3). From detection of HCV seropositivity to HCV RNA testing, the interval was 21 d (IQR 6-39) and 12 d (IQR 6-68) in the IFN and DAA eras, respectively ($P = 0.19$). The intervals from HCV RNA testing to treatment assessment and from assessment to treatment initiation were longer in the DAA era compared with those in the IFN era, 81 d (IQR 14-169) *vs* 26 d (IQR 7-208), and 42 d (IQR 18-84) *vs* 35 d (IQR 27-90), which were not statistically significantly different ($P = 0.25$ and $P = 0.55$, respectively). The duration of viremia was 735 d (IQR 391-1447) in the IFN era, which was significantly longer than that in the DAA era (380 d; IQR 274-

Table 1 Clinical characteristics of people living with human immunodeficiency virus who had incident hepatitis C virus infection from 2011 to 2018

Clinical characteristics	*n* = 287
Age, mean ± SD, yr	34.5 ± 7.6
Male sex, *n* (%)	287 (100)
Risk group, *n* (%)	
Men who have sex with men	265 (92.3)
Heterosexuals	5 (1.8)
Injecting drug users	4 (1.4)
Unknown	13 (4.5)
HIV PVL, mean ± SD, log_{10} copies/mL	1.72 ± 1.09
CD4 count, mean ± SD, cells/mm^3	597.7 ± 251.1
HBsAg-positive, *n* (%)	42 (14.9)
Syphilis, *n* (%)	256 (89.2)
Plasma HCV RNA, mean ± SD, log_{10} copies/mL	5.72 ± 1.50)
HCV genotype, *n* (%)	
1a	22 (7.7)
1b	84 (29.3)
2a	119 (41.5)
3	6 (2.1)
6	34 (11.8)
Mixed genotype 1b+2	3 (1.0)
No data	19 (6.6)
Year of seroconversion, *n* (%)	
2011	23 (8.0)
2012	28 (9.8)
2013	27 (9.4)
2014	24 (8.4)
2015	31 (10.8)
2016	43 (14.9)
2017	54 (18.8)
2018	57 (19.9)

HBsAg: Hepatitis B surface antigen; HCV: Hepatitis C virus; HIV: Human immunodeficiency virus; PVL: Plasma viral load; RNA: Ribonucleic acid.

554; $P < 0.001$).

Among the HCV seroconverters in the IFN era, a total of 165 episodes of STIs were observed in the entire HCV viremic duration of 428.2 PYFU, resulting in an incidence rate of 38.5 per 100 PYFU. In the DAA era, the HCV seroconverters acquired a total of 55 episodes of STIs during 108.8 PYFU of viremia, leading to an incidence rate of 50.5 per 100 PYFU. The incidence rate ratio (IRR) of STIs in the DAA era *vs* the IFN era was 1.31 (95%CI 0.96-1.77).

Figure 4 demonstrates the breakdown of HCV treatment uptake by year. The annual number of PLWH with HCV viremia who received IFN-based therapy was unchanged from 2012 to 2016. With the introduction of DAAs in 2016, the number of PLWH receiving DAA treatment increased year by year, especially in 2019, when restrictions on DAA reimbursement were lifted and all HCV viremic patients could be treated with DAAs.

Table 2 Care cascade of incident hepatitis C infections among people living with human immunodeficiency virus in the interferon and direct-acting antiviral eras

	Total 2011-2018, *n* = 287	Seroconversion in		*P* value
		2011-2016 IFN era, *n* = 176	2017-2018DAA era, *n* = 111	
Antibody detected, *n* (%)[1]	277 (96.5)	167 (94.9)	110 (99.1)	0.09
HCV RNA tested, *n* (%)[1]	270 (97.5)	162 (97.0)	108 (98.2)	0.08
HCV RNA positivity, *n* (%)[1]	251 (93.0)	152 (93.8)	99 (91.7)	0.48
Referred to treatment assessment, *n* (%)[1]	226 (90.0)	134 (88.2)	92 (92.9)	0.17
Treatment initiated, *n* (%)[1]	215 (95.1)	131 (97.8)	84 (91.3)	0.81
SVR achieved, *n* (%)[1]	202 (94)	120 (91.6)	82 (97.6)	0.19
Interval between each step, median days (IQR)				
Seroconversion to antibody detected	130 (80-295)	179 (87-434)	92 (57-173)	< 0.001
Antibody detected to HCV RNA tested	19 (6-81)	21 (6-93)	12 (6-68)	0.19
HCV RNA tested to treatment assessment	43 (11-181)	26 (7-208)	81 (14-169)	0.25
Treatment assessment to treatment initiation	36 (21-90)	35 (27-90)	42 (18-84)	0.55
Duration of viremia[2]	502 (325-945)	735 (391-1447)	380 (274-554)	< 0.001
Events of STIs during HCV viremia	220	165	55	< 0.001
Incidence rate of STIs during HCV viremia, (per 100-PYFU)	41.0	38.5	50.5	0.09

[1]Percentage that moved from the previous step to current step of care cascade.

[2]From the time of seroconversion to end of treatment. DAA: Direct-acting antiviral; IFN: Interferon; IQR; Interquartile range; PYFU: Person-years of follow-up; STIs: Sexually transmitted infections; SVR: Sustained virologic response.

DISCUSSION

In this study, we observed high rates of linkage to HCV care and retention in the care system among PLWH at our institution, in both the IFN and DAA eras. The retention rates between each step of the care cascade after detection of incident HCV infection primarily exceeded 90% (Figure 2). A major barrier noted in the IFN era was referral to treatment assessment (88.2%) after detection of HCV viremia, while this gap seemed diminished in the DAA era. In addition, the duration of HCV viremia, from seroconversion to completion of HCV treatment, was markedly shortened in the DAA era compared to that in the IFN era (380 d *vs* 735 d; $P < 0.001$). The improvement was mainly due to early diagnosis of recently acquired HCV infection by HIV-treating physicians because the diagnostic rate within one year after seroconversion increased from 69.5% (116/167) to 88.2% (97/110), and the median time from seroconversion to antibody diagnosis was shortened from 179 d to 92 d ($P < 0.001$).

The occurrence of STIs during HCV viremia represented risky sexual behavior, potentially resulting in onward transmission of HCV to their contacts. The incidence rate of STIs observed in the DAA era was higher than that in the IFN era, though not reaching statistical significance (50.5 *vs* 38.5 per 100 PYFU; IRR 1.31, 95%CI 0.96-1.77). In contrast, the total number of STI episodes was far fewer in the DAA era *vs* the IFN era (55 episodes among 111 patients *vs* 165 episodes among 176 patients, respectively), likely due to a shorter duration of HCV viremia. The curtailing of HCV viremia with the initiation of effective DAAs may indicate reduced opportunities to transmit HCV during the viremic period.

There are limited data to describe the care cascade after incident HCV infections were diagnosed both in the general population and in HCV-coinfected PLWH in the Asia-Pacific region. In our study, we tested archived blood samples to include all patients who had recent HCV infections in the previous year and estimated the time of seroconversion. In doing so, we could assess the rate of antibody diagnosis of HCV infection by treating physicians, the interval of delay in clinical diagnosis, and the duration of HCV viremia before effective anti-HCV treatments were initiated. Prior population-level studies of the care cascade either estimated the seroprevalence of

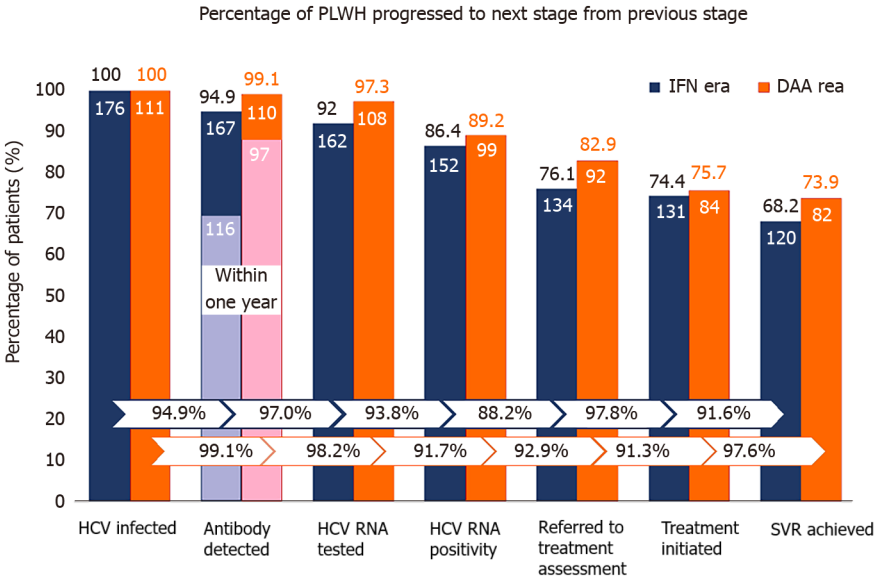

Figure 2 Care cascade of incident hepatitis C virus infections in the interferon and direct-acting antiviral eras. In the interferon era (blue columns), 176 people living with human immunodeficiency virus (HIV) (PLWH) had incident hepatitis C virus (HCV) infections by retrospective testing of all archive blood samples; 167 (94.9%) were found to have HCV seroconversion by HIV-treating physicians; and the diagnostic rate within one year after seroconversion was 69.4% (116 out of 167). Plasma HCV RNA was tested in 162 (97.0%) PLWH after seroconversion, of which 152 (93.8%) were viremic. A total of 134 (88.2%) viremic PLWH were referred to hepatology clinics; anti-HCV treatments were initiated in 131 (97.8%), and 120 (91.6% of all treated PLWH) achieved sustained virologic response (SVR). In the direct-acting antiviral era (orange columns), 111 PLWH had incident HCV infections; 110 (99.1%) were found to have HCV seroconversion by HIV-treating physicians; and the diagnostic rate within one year after seroconversion was 88.2% (97 out of 110). Plasma HCV RNA was detected in 108 (98.2%) PLWH after seroconversion and 99 (91.7%) were viremic. A total of 92 (92.9%) viremic PLWH received treatment assessment, anti-HCV treatments were initiated in 84 (91.3%), and 82 (97.6% of all treated patients) achieved SVR. IFN: Interferon; DAA: Direct-acting antiviral; HCV: Hepatitis C virus; PLWH: People living with human immunodeficiency virus.

HCV[24,25] or described the cascade since the diagnosis of anti-HCV positivity[26]. In single-center studies focusing on HCV-coinfected PLWH, the actual numbers of HCV infections were not shown in the care cascade[27,28].

Previous population-based care cascade assessments for HCV infection in the IFN era revealed low rates of retention in care and treatment initiation, probably related to concerns about inconvenience, lower effectiveness, intolerability, and higher rates of adverse effects with IFN/RBV treatment. The antibody diagnostic rate was 50% to 75%; HCV RNA testing was performed in 30 to 50% of patients; less than 20% of patients received HCV treatment; and the final SVR rate was lower than 10%[24,25]. At our institution during the IFN era, the antibody diagnosis, HCV RNA testing, treatment initiation, and SVR rates were 94.9%, 92.0%, 74.4%, and 68.2%, respectively, much higher than those of previous population-level studies. We believe that regularly scheduled follow-up visits for fully-reimbursed HIV antiretroviral treatments in the majority of PLWH might have provided an opportunity to facilitate HCV testing and increase engagement of HCV care[29,30]. However, mandatory referrals to a hepatologist for HCV pretreatment assessment and management may have raised the barrier to anti-HCV treatment initiation and achievement of viral clearance, as such a requirement negatively impacts convenience and costs, thereby potentially increasing the risk of dropout from HCV care. This barrier has diminished since 2019 when NHI approved HIV-treating physicians to participate in HCV assessment and treatment for PLWH at infectious disease clinics. The rate of treatment assessment after HCV RNA positivity increased from 88.2% (134/152) in the IFN era to 92.9% (92/99) in the DAA era.

The introduction of DAAs has been shown to improve engagement of HCV care and treatment uptake. The British Columbia Hepatitis Tester Cohort demonstrated improvement of the care cascade in the DAA era. Of the patients who tested anti-HCV-positive, 83% had HCV RNA testing done; and, of those who were HCV viremic and were genotyped, 61% received DAA therapy, with 90% achieving SVR[31]. In a report from seven HCV elimination studies among PLWH in the early DAA era, the average treatment uptake increased to 48% and treatment completion rates reached 96%[32]. Our current study showed a significantly higher rate of linkage to care, with > 90% of PLWH consistently advancing from each cascade step to the next in the DAA era, which resulted in a total of 84.8% (84/99) viremic patients initiating anti-HCV

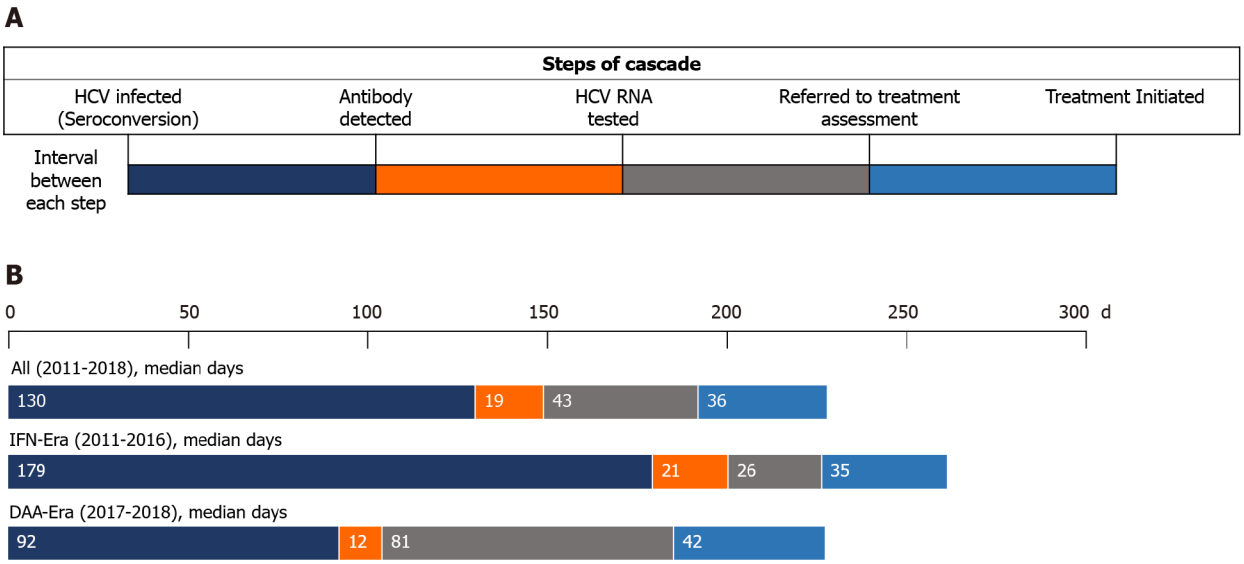

Figure 3 Intervals between steps of the care cascade of incident hepatitis C virus infections. A: Demonstration of the intervals between each step of the care cascade; B: In all included people living with human immunodeficiency virus (HIV) (PLWH), the median interval from seroconversion to detection of hepatitis C virus (HCV) seropositivity by HIV-treating physicians was 130 days, which was significantly shorter in the direct-acting antiviral (DAA) era (median 92 d) than that in the interferon (IFN) era (median 179 d) ($P < 0.001$). In the IFN era, the median interval from detection of HCV seropositivity to HCV RNA testing was 21 d, that from RNA testing to referral to treatment assessment was 26 d, and that from assessment to treatment initiation was 35 d. In the DAA era, the median interval from detection of HCV seropositivity to HCV RNA testing was 12 d, that from RNA testing to referral to treatment assessment was 81 d, and that from assessment to treatment initiation was 42 d. The differences in the intervals after antibody diagnosis were not statistically significant between PLWH included in the IFN era and those in the DAA era. HCV: Hepatitis C virus; IFN: Interferon; DAA: Direct-acting antiviral.

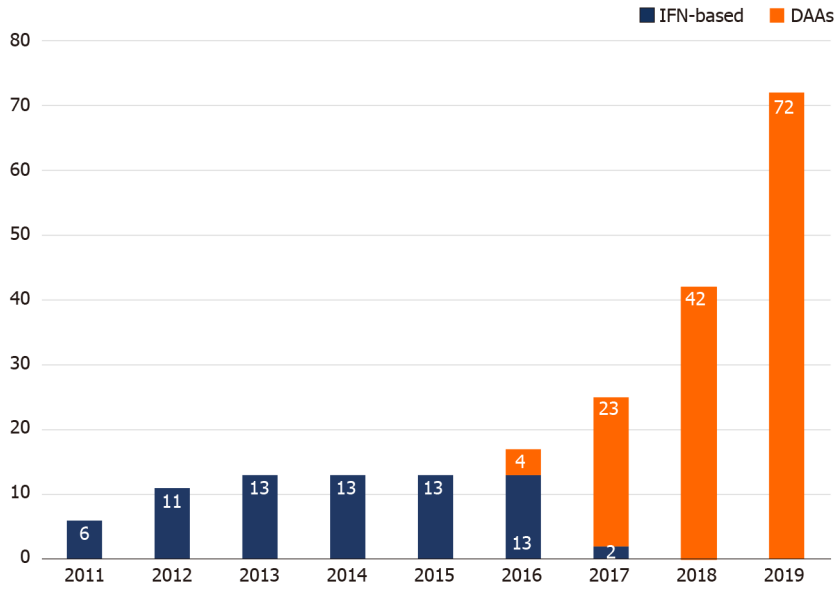

Figure 4 Hepatitis C virus treatment uptake by year from 2011 to 2019. The annual numbers of people living with human immunodeficiency virus (PLWH) who received interferon-based therapy were unchanged from 2012 to 2016. With the introduction of direct-acting antivirals (DAAs) since 2016, the number of PLWH receiving DAAs increased annually, especially in 2019, when the restrictions on the DAA reimbursement were lifted and all hepatitis C virus viremic patients could be treated with DAAs. DAA: Direct-acting antiviral; IFN: Interferon.

treatments, and 97.6% (82/84) achieving SVR. In the Swiss HIV Cohort Study, the treatment incidence increased from 4.5 per 100 PYFU before the availability of DAAs, to 22.4 per 100 PYFU after the introduction of second-generation DAAs[33]. These findings were in line with observations from our study. While the annual number of IFN-based therapy remained unchanged from 2012 to 2016, the number of DAA treatment initiated progressively increased from 2016 to 2018, which further rocketed in 2019, when the restriction on DAA reimbursement was lifted (Figure 4). During the period between 2016 and 2018, when access to DAA through NHI reimbursement

remained limited, generic DAAs could be purchased overseas by PLWH themselves; however, accessibility was limited by the cost incurred. During this period, we found that the median intervals from HCV RNA testing to referral to treatment assessment, and those from referral to treatment initiation were longer than those in the IFN era, though not reaching statistical significance (Table 2 and Figure 3).

There are several limitations in this study. First, the cascade of HCV care from this single-center study might not be generalizable to other institutions in Taiwan, given that discrepancies in the patients' demographic and clinical characteristics and socio-economic status may exist. At our institution, PLWH with recent HCV infection were mainly MSM, accounting for more than 90% of the included patients, while the percentages of heterosexual and IDUs were both lower than 2%. Prior studies suggested from an assessment of the HCV care cascade in IDUs that active drug users had poorer treatment adherence, resulting in lower rates of viral clearance[34-36]. Second, our study did not investigate factors that may be associated with failure of engagement in the HCV care cascade. Third, HCV reinfection, which can occur in this high-risk group[37-39], were not included in the analyses of the HCV care cascade.

CONCLUSION

In conclusion, we describe the HCV care cascade at a referral hospital in Taiwan from the IFN era to the DAA era. The rates of engagement in each step of the HCV care cascade were high in both eras and the barriers to referral and reimbursement diminished over time. However, given both the longer intervals of HCV viremia observed in the DAA era and a higher incidence of STIs potentially contributing to onward transmission of HCV, more efforts are needed to expedite the linkage of PLWH diagnosed with incident HCV infections to HCV treatment.

ARTICLE HIGHLIGHTS

Research background

Recently acquired hepatitis C virus (HCV) infections are increasingly reported in people living with human immunodeficiency virus (HIV) (PLWH) who are men who have sex with men. With availability of highly effective direct-acting antivirals (DAAs) for the treatment of HCV, microelimination HCV is considered an achievable goal in this at-risk population.

Research motivation

To achieve microelimination of HCV in PLWH, each step of the continuum of HCV care, from diagnosis, linkage to and engagement in care, initiation of anti-HCV treatment, treatment completion, to prevention against reinfection, is crucial. Examining the HCV care cascade can identify barriers to the completion of HCV treatment and facilitate achievement of HCV micro-elimination in PLWH.

Research objectives

The study aimed to evaluate the care cascade of PLWH with recently acquired HCV infections at a university hospital designated for HIV care in Taiwan.

Research methods

The authors retrospectively reviewed the medical records of all PLWH testing negative for anti-HCV at baseline who developed anti-HCV seroconversion between 2011 to 2018 and were observed till the end of 2019. The number of people in each step of HCV care cascade was assessed and the duration between two sequential steps was estimated.

Research results

A total of 287 PLWH recently acquired HCV infections during the study period. High rates of linkage to HCV care and retention in the care were observed in our cohort. Compared with the interferon (IFN, 2011-2016) era, the barrier of referral to anti-HCV treatment assessment was diminished and the total duration of HCV viremia marked decreased in the direct-acting antiviral (DAA, 2017-2018) era.

Research conclusions

The achievement rates of engagement in each step of the HCV care cascade were high in both IFN and DAA eras and the barriers to referral and treatment initiation diminished over time.

Research perspectives

The impact of scale-up of HCV testing and DAA treatment after lifting the restriction on DAA reimbursement on the trends of incident HCV infections in PLWH warrants more long-term observation.

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ORIGINAL ARTICLE

Observational Study

Helicobacter pylori in gastric cancer: Features of infection and their correlations with long-term results of treatment

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Abstract

BACKGROUND

Helicobacter pylori (*H. pylori*) is a spiral-shaped bacterium responsible for the development of chronic gastritis, gastric ulcer, gastric cancer (GC), and MALT-lymphoma of the stomach. *H. pylori* can be present in the gastric mucosa (GM) in both spiral and coccoid forms. However, it is not known whether the severity of GM contamination by various vegetative forms of *H. pylori* is associated with clinical and morphological characteristics and long-term results of GC treatment.

AIM

To establish the features of *H. pylori* infection in patients with GC and their correlations with clinical and morphological characteristics of diseases and long-term results of treatment.

METHODS

Of 109 patients with GC were included in a prospective cohort study. *H. pylori* in the GM and tumor was determined by rapid urease test and by immunohistochemically using the antibody to *H. pylori*. The results obtained were compared with the clinical and morphological characteristics and prognosis of GC. Statistical analysis was performed using the Statistica 10.0 software.

RESULTS

H. pylori was detected in the adjacent to the tumor GM in 84.5% of cases, of which a high degree of contamination was noted in 50.4% of the samples. Coccoid forms of *H. pylori* were detected in 93.4% of infected patients, and only coccoid-in 68.9%.

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It was found that a high degree of GM contamination by the coccoid forms of *H. pylori* was observed significantly more often in diffuse type of GC (P = 0.024), in poorly differentiated GC (P = 0.011), in stage T3-4 (P = 0.04) and in N1 (P = 0.011). In cases of moderate and marked concentrations of *H. pylori* in GM, a decrease in 10-year relapse free and overall survival from 55.6% to 26.3% was observed (P = 0.02 and P = 0.07, respectively). The relationship between the severity of the GM contamination by the spiral-shaped forms of *H. pylori* and the clinical and morphological characteristics and prognosis of GC was not revealed.

CONCLUSION

The data obtained indicates that *H. pylori* may be associated not only with induction but also with the progression of GC.

Key Words: Gastric cancer; *Helicobacter pylori*; Coccoid and spiral forms of bacteria; Rapid urease test; Relapse free survival; Overall survival

Core Tip: The role of *Helicobacter pylori* (*H. pylori*) in the progression of gastric cancer (GC) is not well understood. Our results indicate that in GC, a high degree of gastric mucosa contamination by coccoid forms of *H. pylori* is associated with advanced stages of the disease and deterioration of long-term results of treatment.

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INTRODUCTION

Gastric cancer (GC) continues to be one of the most common malignant diseases in the world[1,2]. Despite a decreasing trend in the incidence of GC in most countries of the world, the treatment results of this pathology cannot be considered satisfactory. In the structure of mortality from malignant neoplasms, this pathology firmly occupies 2nd place in most developed countries of the world, and the 5-year survival rate of radically operated patients does not exceed 15%-30%[3,4].

It is important to note that it is impossible to improve the long-term results of malignant neoplasms treatment without knowledge of the mechanisms associated with their progression[3]. Clinical studies in recent years indicate that inflammatory infiltration of the tumor stroma and surrounding tissues can have an important prognostic value and affect the long-term results of malignant neoplasm treatment[5-9]. A number of studies have shown that inflammatory infiltration of the tumor stroma is associated with the body's adequate immune response to the tumor, and may be a favorable prognosis factor in various malignant neoplasms[10,11], including GC[12,13]. At the same time, the data obtained by other researchers indicates that pronounced inflammatory infiltration of the tumor stroma, especially T-reg lymphocytes and macrophages, may be a factor contributing to the progression of malignant neoplasms[14,15]. There was a decrease in the overall survival (OS) and relapse-free survival (RFS) of GC patients with a high content of Foxp3 + T-reg in the tumor stroma and regional metastases[16-18] and macrophages[16,19]. It is believed that inflammatory infiltration of the tumor stroma can contribute to tumor progression by activating the mechanisms of angiogenesis, expression of E- and L-selectins, formation of the products of lipid peroxidation and free radicals, destruction of connective tissue matrix and basement membranes of epithelia by proteolytic enzymes, and activation of epithelial-mesenchymal transformation[20-23].

When studying the role of inflammation in the progression of GC, it is impossible to ignore the problem of *Helicobacter pylori* (*H. pylori*) infection. *H. pylori* is a gram-negative, spiral-shaped bacterium, the habitat of which is the gastric mucosa (GM) and

duodenum. *H. pylori* differs from other bacteria in a set of properties that make it possible to colonize the GM and persist for a long time under conditions that are unfavorable for other microorganisms[24,25]. These include: (1) The ability to produce a special enzyme-urease; (2) Synthesis of lytic enzymes that cause the depolymerization and dissolution of gastric mucus, consisting mainly of mucin; (3) The mobility of the bacterium, which is ensured by the presence of 5-6 flagella; (4) The high adhesiveness of bacteria to GM epithelial cells of the GM and elements of connective tissue due to the interaction of bacterial ligands with the corresponding cells receptors; (5) Production of various exotoxins (VacA, CagA, and others); (6) Instability of the *H. pylori* genome; (7) The presence of vegetative and coccoid forms of bacteria; and (8) possibility of intracellular persistence and translocation outside the GM [26-29].

It should be noted that despite the huge number of studies devoted to *H. pylori*, it is still not clear whether *H. pylori* is involved only in the initiation of the tumor process in the stomach, or whether it can affect the mechanisms of tumor progression. The relationship between the severity of *H. pylori* infection and the clinical and morphological characteristics of GC and long-term results of this pathology treatment remains poorly studied, and in this connection, the question of the expediency of anti-Helicobacter therapy in patients with invasive GC remains open.

Objective

To assess the features of *H. pylori* infection in patients with Stage I-IIIB GC and their correlation with the clinical and morphological characteristics of the disease, the presence of antibiotic therapy (AT) before surgery, and long-term treatment results.

MATERIALS AND METHODS

The patients

One hundred and nine patients with GC who had undergone radical surgery (R0) between May 2007 and March 2010 at the Orenburg Regional Clinical Oncology Center, were included in this prospective cohort pilot study. Study inclusion criteria were: Histologically proven invasive GC; no evidence of distant metastases; radical surgery (R0); no prior gastric surgery; no previous chemotherapy or radiotherapy. The study did not include patients with decompensation of cardiovascular and renal diseases, exacerbation of chronic inflammatory processes, severe allergic processes, or who received glucocorticoids, antihistamines, and non-steroidal anti-inflammatory drugs. The study was performed in accordance with the Helsinki Declaration and internationally recognized guidelines, and the privacy of patients was protected by decoding the data according to the privacy regulations of the Orenburg Regional Clinical Oncologic Center (Russia, Orenburg). All patients provided written informed consent. The protocol was approved by the Institutional Review Board of the Orenburg State Medical University (Russia, Orenburg).

Clinical and pathological data including age, tumor localization, stage, type of surgery, histology, the presence of AT before surgery, postoperative therapy, and long-term results of treatment were retrieved from the routine reports for analyses. The distribution of patients according to the clinical and pathological characteristics of GC is presented in Table 1.

When interviewing patients, it was found that 45 patients received AT before admission to the clinic due to a preliminary wrong diagnosis of gastric ulcer or chronic gastritis. The following combinations of antibacterial drugs were most commonly ordered: Amoxicillin + clarithromycin (34 patients), amoxicillin + clarithromycin + metronidazole (6 patients), amoxicillin + metronidazole (3 patients), other drugs (2 patients). Information about the antibacterial drugs, the timing and duration of their intake was entered into the primary patient documentation and then taken into account in the analysis. We considered only those patients who underwent AT in the period from 1 to 1.5 mo before the operation, lasting at least seven days, and using two or more antibacterial drugs.

The long-term results of treatment were assessed for the period from May 12, 2007 to April 12, 2021. The median follow-up period was 86.2 mo. As of April 12, 2021, 26 (24.5%) patients were alive, 54 (50.9%) had died from the progression of GC, 20 (18.9%) had died from causes other than GC, and six (5.6%) left the region at different follow-up periods. Malignant tumors of other localizations were diagnosed in 8 patients at different times after the operation: Non-Hodgkin's lymphomas-in three, prostate cancer-in one, lung cancer-in two, laryngeal cancer-in one, and breast cancer-in one patient. With the exception of one patient with non-Hodgkin's lymphoma, the other

Table 1 The distribution of patients according to the clinical and pathological characteristics of gastric cancer

Characteristics of gastric cancer	*n*	Percent
Age	61.7 ± 1.03 years (from 24 to 81 years, the median–was 61 years)	
Sex		
Men	72	66.1
Women	37	33.9
Tumor localization		
Upper third	18	16.5
Middle third	32	29.4
Lower third	57	52.3
Total gastric cancer	2	1.8
T status		
T1	12	11
T2	30	27.5
T3	62	56.9
T4	5	4.6
N status		
N0	57	52.3
N1	20	18.3
N2	32	29.3
TNM		
T1-2N0M0	40	36.7
T3N0M0	17	15.6
T3-4N1M0	20	18.3
T3-4N2M0	32	29.3
Types of GC		
Intestinal type	50	45.9
Diffuse-types	59	54.1
Grade (histolology)		
G1	31	28.4
G2	19	17.4
G3	33	30.3
Signet ring cell carcinoma	26	23.9
Type sugery		
Subtotal distal resection	85	78
Subtotal proximal resection	18	16.5
Gastrectomy	7	6.4

GC: Gastric cancer.

patients died from the progression of these diseases. The causes of death were not associated with malignant tumors for the other patients. During the period 2020-2021, seven patients contracted coronavirus disease-19, one of whom died from the disease, but the rest are alive.

Detection H. pylori infection

H. pylori in the GM and tumor was determined by rapid urease test (RUT) and by immunohistochemically (IHC) using the antibody to *H. pylori*.

RUT

After removal of the stomach (within 30 min) a greater curvature of the organ was opened and biopsy samples were taken from the tumor and the adjacent macroscopically non-tumorous GM at a distance of 3-5 cm from the tumor margin. The samples were placed on test strips (HELPIL–test, "AMA", Russia) for three minutes. The presence and the severity of the infection were evaluated by the color change of the indicator from yellow to blue.

According to the intensity and the time of the appearance of a blue color, we distinguished three degrees of infection: 3+: Marked (+++)-bright staining in the first minute of the study; 2+: Moderate (++)–for an average intensity of staining for 2 min and 1+ mild (+)-weak staining for three minutes. If the color of the indicator did not change, or became dirty gray, and if after repeated research the same result was received, the test was evaluated as negative. The same samples were later used for histological analysis and IGH.

Immunohistochemistry

The presence and features of *H. pylori* infection were studied in samples of the GM adjacent to the tumor, in the tumor tissue, in the omentum, and regional lymph nodes of 46 patients, by immunohistochemistry.

The sections for IGH were dewaxed and rehydrated by sequential immersion in xylene and graded ethanol and water. For antigen retrieval, the sections boiling for 10 min in citrate buffer (pH 6) and endogenous peroxidase activity was blocked with 30 mL/L hydrogen peroxide solution. Slides were incubated at room temperature with the anti-*H. pylori* (RB-9070, Thermo Fisher Scientific, the immunogen is purified *H. pylori*) rabbit polyclonal antibodies in diluted at 1:1000 for 30 min.

The visualization system included DAB (UltraVision LP Detection System HRP Polymer & DAB Plus Chromogen) and hematoxylin counterstaining. For negative control sections, the primary antibody was replaced with phosphate-buffered saline and processed in the same manner.

The concentration of *H. pylori* in the GM detected by IGH was graded as 1+ for mild, 2+ for moderate, and 3+ for marked according to the Sydney system[30]. The presence of point inclusions giving a positive reaction with antibodies to *H. pylori* in the cytoplasm of epithelial cells of deep gastric glands and of the lymphoid cells of the lamina propria of GM as well as in omentum and lymph node, were taken into account.

All sections were carefully and completely scanned by two of the authors (MS and OT) without knowledge of the clinical and pathological data.

The data obtained was compared with clinical features of GC: Stage, localization, histology, the presence of AT before surgery, and 10-year OS and RFS.

Statistics

Statistical analysis was performed using the Statistica 10.0 software. The correlations between different data were evaluated using the nonparametric Spearman's rank correlation or gamma correlation. Chi-square tests were carried out to analyze the difference of distribution among the categorized data. Mann–Whitney U nonparametric test was used to compare the value of the quantitative data. The survival was analyzed using the Kaplan-Meier method. The log-rank test was used to compare survival curves between subgroups of patients. A value of $P < 0.05$ was considered statistically significant.

RESULTS

The features of H. pylori infection in GC

Of 93 patients (84.5%) demonstrated positive RUT. According to RUT the concentration of *H. pylori* in GM was mild (1+) in 38 (34.8%) patients, moderate (2+)-in 37 (33.9%) and marked (3+)–in 8 (16.5%). RUT was negative in 16 patients (14.7%).

It was found that urease activity 2+ and 3+ were significantly more frequent in GM than in tumors (in 55.5% and 13.3% of cases, respectively, $P = 0.01$). In more than half the cases (53.3%), the urease activity in the tumor was lower than in the adjacent GM.

Forty-six samples of GM were stained by IHC. The coccoid forms of *H. pylori* were found to prevail in GM adjacent to the tumor (Figure 1A). Coccoid forms of *H. pylori* only were identified in 31 (63.1%) patients, and coccoid and spiral in 12 (30.4%) patients (Figure 1B). No signs of infection were found in 3 (6.5%) patients. The concentration of coccoid and spiral forms of *H. pylori* in GM according to IGH was 1+ in 24 (52.2%) and 1 (19.6%) patients, 2+-in 11 (23.9%) and 4 (8.7%), and 3+-in 8 (17.4%) and 1 (2.2%) patients. A positive correlation between the concentration of coccoid and spiral forms of *H. pylori* (gamma = 0.642, $P < 0.0001$) was noted.

The localization of *H. pylori* in GM was observed not only in the surface mucous gel layer of the stomach (Figure 1C) but also within the cytoplasm of the gastric superficial-foveolar epithelium (Figure 1D). The point inclusions giving a positive reaction with antibodies to *H. pylori* were also revealed in the cytoplasm of the epithelial cells of deep gastric glands (in 41.3% samples) and of the immune cells (Figure 1E) of the lamina propria of GM (in 43.5% samples), as well as of the intraepithelial lymphocytes (Figure 1F). The close relationship between the presence of point inclusions in immune cells and epithelial cells (gamma = 0.642, $P < 0.0001$) was noted. The individual cocci or their small clusters, and sometimes the short rod bacterium, were also often detected in the lamina propria of the GM. We also found bacteria in the omentum and lymph nodes. In the omentum the bacteria were presented predominantly by cocci between 0.5 and 1 μm in diameter (Figure 2A). Cocci were arranged most commonly by the small compact groups up to 10-15 cells in the immediate vicinity of the LN capsule. The bacteria were located mainly between the adipocytes. However, it was not clear whether bacteria were outside of the cells or in a narrow rim of cytoplasm of the fat cells.

In the tissue of lymph nodes we usually observed the small accumulations of cocci between 10 and 15 cells (Figure 2B). Bacteria were located between lymphocytes of the paracortical area, but not so compact, as in the omentum. Quite often the concentration of bacteria was observed around the nuclei of cells that can testify to their intracellular localization.

Correlations of clinical and pathological characteristics of GC with the severity of H. pylori infection according to RUT data

The gamma correlation coefficient test (gamma) showed that the severity of *H. pylori* in GM according to RUT positively correlated with the T status (gamma = 0.537, $P < 0.00001$), N status (gamma = 0.371, $P = 0.0007$) and stage (gamma = 0.520, $P < 0.00001$), and negatively correlated with the presence of AT in anamnesis (gamma = -0.418, $P = 0.003$). The marked (+++) and moderate (++) degrees of *H. pylori* infection were more often observed in Grade 2 and Grade 3, in T3-4 status, in N1 status, in the T3-4N1-2 stage, and in the absence of AT in anamnesis (Table 2). Correlations of *H. pylori* concentration in GM according to RUT with 10-year OS and RFS of GC patients were not determined.

It is important to note that the presence in AT 1-1.5 mo before surgery was associated with a significant improvement in RFS and OS (Figure 3), however, this applied only to patients with local GC (T1-3N0). In advanced GC (T3-4N1 and T3-4N2) there were no significant differences in patient survival (Figure 4).

Correlations of clinical and pathological characteristics of GC with the concentration of spiral and coccoid forms of H. pylori in the GM

It is important to note that correlations between the clinical and pathological characteristics of GC and the concentration of *H. pylori* spiral forms in GM were not found. However, the concentration of *H. pylori* coccoid forms correlated with age ($\rho = -0.502$, $P = 0.0006$), histology (gamma = 0.550, $P = 0.0004$), T status (gamma = 0.709, $P = 0.0001$), N status (gamma = 0.509, $P = 0.002$) stage (gamma = 0.636, $P = 0.0002$), and 10-year RFS (gamma = -0.521, $P = 0.008$) and OS (gamma = -0.500, $P = 0.044$). In cases with a moderate and marked concentration (2+ or 3+) of *H. pylori* coccoid forms in GM compared to cases with a low concentration (1+ or without infection) the patients were younger (57.9 ± 2.5 years *vs* 66.2 ± 1.4 years, respectively, $P = 0.004$) and the diffuse type of GC, poorly differentiated tumors (G3), T3-4 stage and N1 stage of GC were more often observed (Table 3). In cases of moderate and marked concentrations of *H. pylori* in GM, a decrease in 10-year progression-free survival (PFS) and OS survival from 55.6% to 26.3% was observed ($P = 0.02$ and $P = 0.07$, respectively). PFS and OS curves, depending on the concentration of coccoid forms of *H. pylori* in GM, are presented in Figure 5.

Table 2 Clinical and pathological characteristics of gastric cancer depending on the data rapid urease test

Characteristics of gastric cancer	Degrees of infection according to RUT test				*P* value
	No or mild, *n* (%)		Moderate or marked, *n* (%)		
Age	63.8 ± 10.1		58.8 ± 11.2		0.008
T status					
T1	9	75	3	25	0.01
T2	20	66.7	10	33.3	
T3	24	38.7	38	61.3	
T4	1	20	4	80	
N status					
N0	37	64.9	20	35.1	0.0001
N1	2	10	18	90	
N2	15	46.9	17	53.1	
TNM					
T1-2N0M0	28	70	12	30	0.002
T3N0M0	9	52.9	8	47.1	
T3-4N1-2	17	32.7	35	67.3	
Types of GC					
Intestinal type	27	54	23	46	0.39
Diffuse-types	27	45.8	32	54.2	
Grade (histolology)					
G1	22	70	9	30	0.005
G2	5	26.3	14	73.7	
G3	12	36.4	21	63.6	
Signet ring cell carcinoma	15	57.6	11	42.3	
Antibiotic therapy before surgery					
No	25	41.2	37	68.5	0.025
Presence	28	52.8	17	31.5	

RUT: Rapid urease test; GC: Gastric cancer.

There were no correlations between the presence of point inclusions in the cytoplasm of epithelial cells of deep gastric glands, in the stroma immune cells, and in the intraepithelial lymphocytes with the clinical and pathological characteristics of GC.

DISCUSSION

A large amount of clinical and experimental data testifies to the important role of *H. pylori* in the occurrence of GC[31-34], but, there is less research into the features of *H. pylori* infection in patients with GC and its role in tumor progression, and the results are quite contradictory. These contradictions relate to many aspects, such as: (1) The frequency of infection in patients with GC. This data varies widely and ranges from 36% to 100%[35-40]; (2) The relation of infection with GC prognosis. Some researchers have noted an improvement in the long-term results of GC treatment in infected patients[41,42], while others, on the contrary, found that the presence of H. pylori infection was associated with a decrease in patient survival[43,44]; and (3) The connection between the infection and a histologic type of GC. In some studies, it was noted that patients with an intestinal type of GC are more often infected with *H. pylori* than patients with the diffuse type of GC[45,46]. Other researchers did not find a

Table 3 Clinical and pathological characteristics of gastric cancer depending on the concentration of *Helicobacter pylori* coccoid forms in the gastric mucosa

Characteristics of gastric cancer	Degrees of infection according to RUT test				*P* value
	No or mild, *n* (%)		Moderate or marked, *n* (%)		
Age	66.2 ± 1.4		57.9 ± 2.5		0.004
T status					
T1	6	85.7	1	14.3	0.04
T2	9	81.8	2	18.2	
T3	12	44.4	15	55.6	
T4	0	0	1	100	
N status					
N0	18	78.3	5	21.7	0.01
N1	1	16.7	5	83.8	
N2	8	47.1	9	52.9	
TNM					
T1-2N0M0	14	82.3	3	17.6	0.02
T3N0M0	4	66.7	2	33.3	
T3-4N1-2	9	39.1	14	60.9	
Types of GC					
WIntestinal type	18	75	6	25	0.02
Diffuse-types	9	40.9	13	59.1	
Grade (histolology)					
G1	14	93.3	1	6.7	0.008
G2	4	44.4	5	55.6	
G3	2	28.3	5	71.4	
Signet ring cell carcinoma	7	46.7	8	53.3	
Antibiotic therapy before surgery					
No	16	64.5	10	38.5	0.655
Presence	11	55	9	45	

RUT: Rapid urease test; GC: Gastric cancer.

difference in *H. pylori* infection in patients with different histological types of tumors [47].

It is believed that the differences noted are associated with the fact that to reveal the infection the authors used the methods that were significantly different in their sensitivity, and primarily, to coccoid forms of bacteria. Most of the studies were carried out without considering coccoid forms of *H. pylori* and the concentration of bacteria in GM. The use of the biochemical method for the detection of urease activity and immunohistochemistry for visualization of bacteria in our study allowed us not only to assess the presence of infection in patients with GC, but also to mark some of its features associated with the localization of bacteria in the stomach, with a ratio of cocci and spiral forms, and the degree of bacterial contamination of GM.

Our data for the Orenburg region recorded a high rate of *H. pylori* infection in patients with GC (84.5%). The coccoid forms of *H. pylori*, preserving a high degree of urease activity, dominated in GM in patients with GC. They were found in 93.4% of infected patients, with only coccoid forms of *H. pylori*-in 68.9%.

It is known that the coccoid forms of *H. pylo*ri can arise in response to unfavorable environmental factors, such as AT [47,48]. These forms are resistant to AT[49,50] and are able to form biofilms[51] and avoid the immune system[50]. They express a higher rate of cagE mRNA than their spiral counterparts[52], and by increasing the synthesis

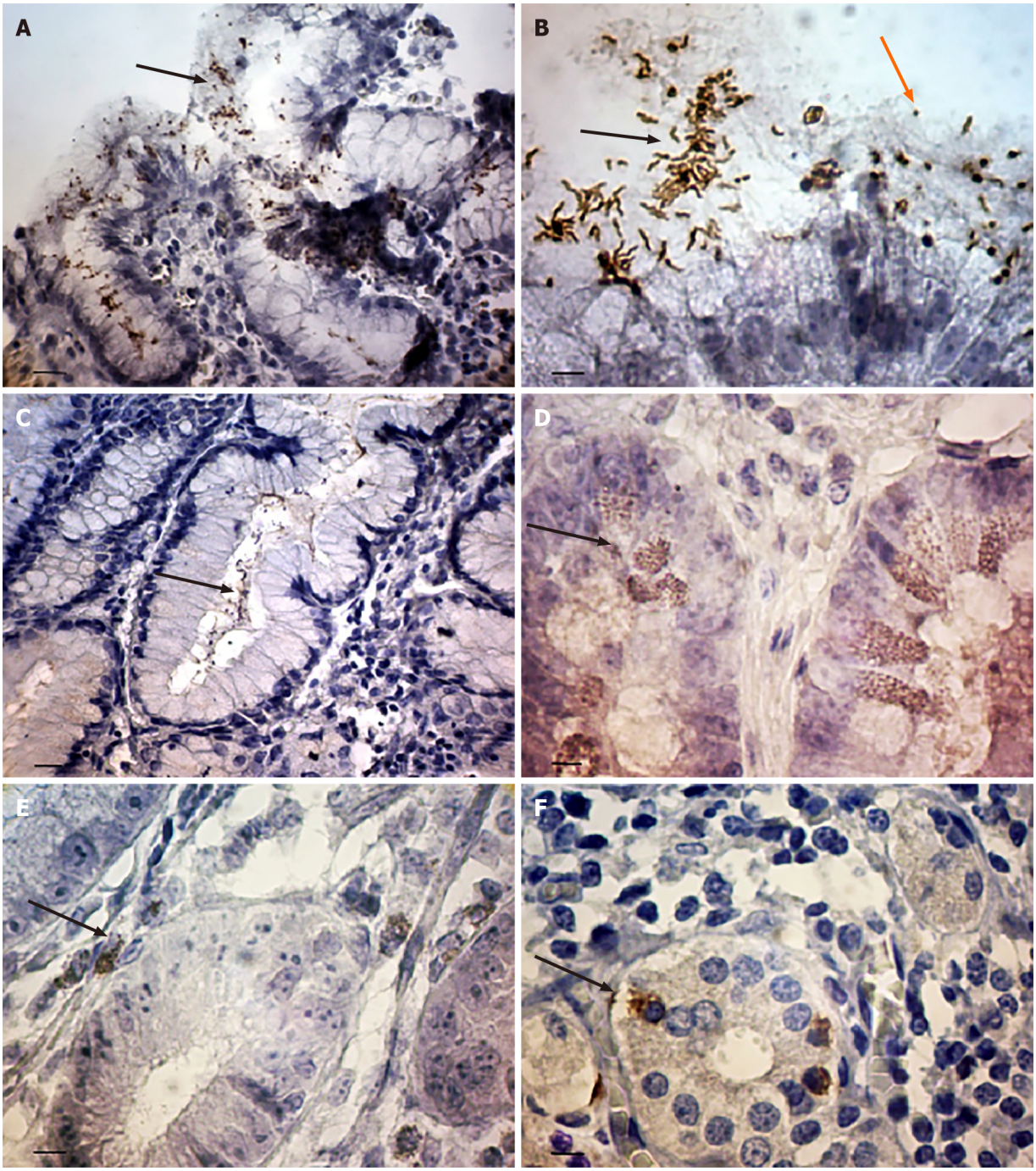

Figure 1 The features of *Helicobacter pylori* localization in gastric mucosa in patients with gastric cancer. A: Coccoid forms of *Helicobacter pylori* (*H. pylori*) in the gastric pit. The some bacteria within the cytoplasm of epithelium cells (arrows); B: The spiral (black arrows) and coccoid (orange arrows) forms of *H. pylori* on the surface of superficial-foveolar gastric epithelium; C: The bacteria in the surface mucous gel layer of stomach (arrows); D: The point inclusions giving a positive reaction with antibodies to *H. pylori* within the cytoplasm of epithelial cells of deep gastric glands (arrows); E: The point inclusions giving a positive reaction with antibodies to *H. pylori* within the cytoplasm of the immune cells of the lamina propria of gastric mucosa (arrows); F: The point inclusions giving a positive reaction with antibodies to *H. pylori* within the cytoplasm of intraepithelial lymphocytes (arrows). Immunoperoxidase staining with anti-*H. pylori* antibody, immersion. Bars: A: 20 μm; B: 10 μm; C: 20 μm; D-F: 10 μm.

of tumor necrosis factor-alpha (TNF-α)-inducing protein (Hps), which is introduced into the cytosol and cell nuclei, they can activate nuclear factor-kappaB (NF-κB) and the expression of TNF-α and other cytokines involved in carcinogenesis[53,54]. The effect on the proliferation of gastric epithelial cells in the *H. pylori* coccoid forms is also stronger than in the helical forms[55], and they can induce the expression of VEGF-A and transforming growth factor-β[56]. The ability to transform into coccoid forms was also found to be characteristic of the most virulent strains of *H. pylori*[50,54].

It should be noted that the higher infection by coccoid forms of *H. pylori* in patients with GC, compared to patients with gastritis or gastric ulcer, had been mentioned by

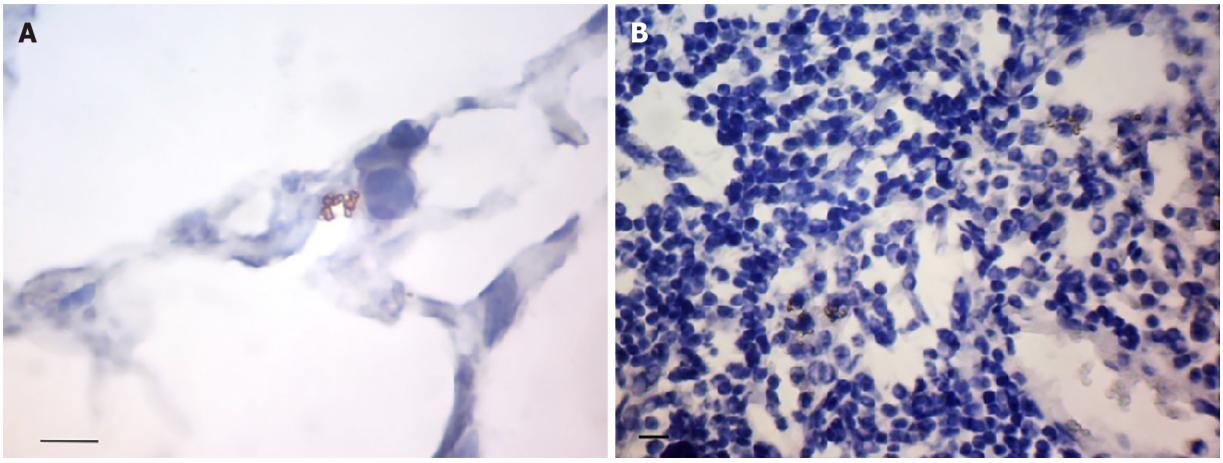

Figure 2 The features of *Helicobacter pylori* localization in omentum and lymph node in patients with gastric cancer. A: The small group of cocci located in the central part of the omentum adipocyte; B: The congestions of bacteria around the nucleus of lymphocytes in the paracortical area of lymph node. Immunoperoxidase staining with anti-*Helicobacter pylori* antibody, immersion, Bars: 10 μm.

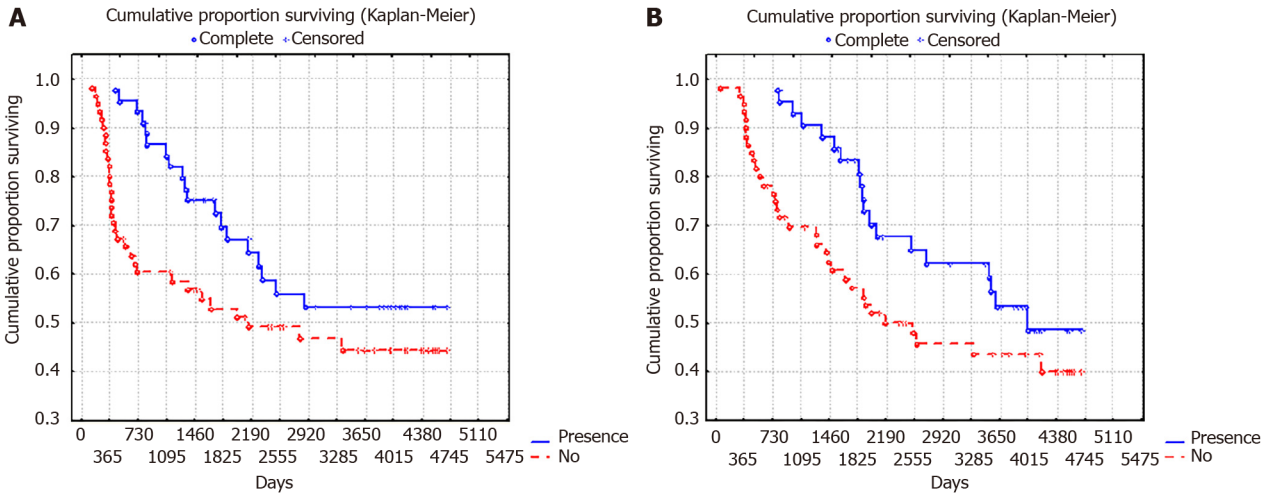

Figure 3 10-year overall surviving and relapse-free surviving of patients with gastric cancer depending on the presence of antibiotic therapy 1-1.5 mo before surgery (*P* = 0.02). A: 10-year overall surviving; B: Relapse-free surviving.

other researchers[57]. A number of studies have shown that coccoid forms of *H. pylori* retained urease activity[58] and the expression of such antigens as CagA, UreA, porin, components of the Cag type IV secretion system (TFSS), antigen-binding adhesin of the blood group BabA and others[59,60].

The use of immunohistochemistry in this study made it possible to detect the bacteria not only in the gastric mucus and on the surface of epithelial cells, but also within the cytoplasm of epithelial and immune cells of GM. Such intracellular expression was characterized by point inclusions giving a specific reaction with antibodies to *H. pylori*.

The intracellular persistence of *H. pylori* has been demonstrated by many investigators. They found *H. pylori* in the cytoplasm of epithelial cells, intercellular spaces, in the lamina propria of GM, and in the lumen of small vessels[61-63]. We assume that the point inclusions in the cytoplasm of epithelial and immune cells giving a positive reaction with antibodies to *H. pylori* is similar to those particle-rich cytoplasmic structure (PaCS) described earlier in the human superficial-foveolar epithelium and its metaplastic or dysplastic foci[64]. The authors found that the PaCS are a colocalization of VacA, CagA, urease, and outer membrane proteins with NOD1 receptor, ubiquitin-activating enzyme E1, polyubiquitinated proteins, proteasome components, and potentially oncogenic proteins like SHP2 and ERKs[64]. They believe that PaCS is a novel, proteasome-enriched structure arising in ribosome-rich cytoplasm at sites of *H. pylori* product accumulation.

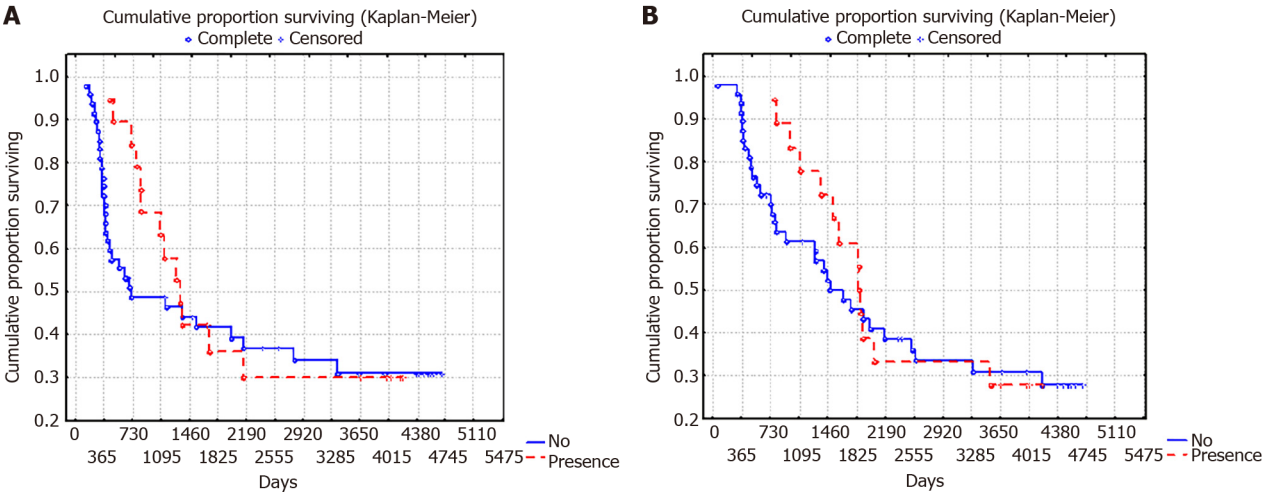

Figure 4 10-year overall surviving and relapse-free surviving of patients with T3-4N1-2M0 stages of gastric cancer depending on the presence of antibiotic therapy 1-1.5 mo before surgery (*P* = 0.78). A: 10-year overall surviving; B: Relapse-free surviving.

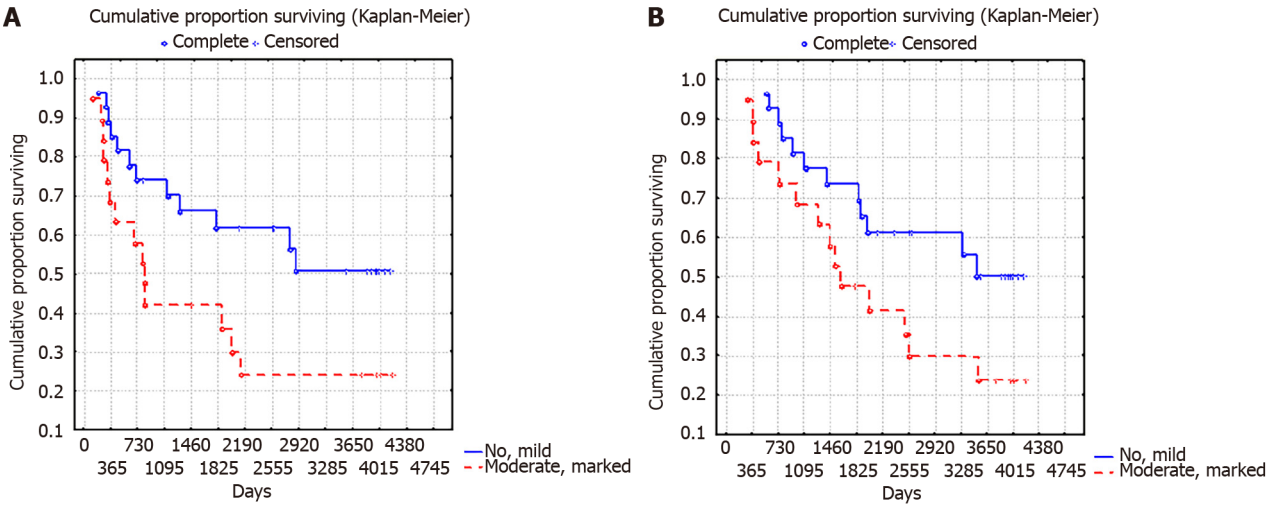

Figure 5 10-year overall surviving and relapse-free surviving of patients with gastric cancer depending on the concentration of *Helicobacter pylori* coccoid forms in the gastric mucosa (*P* = 0.02). A: 10-year overall surviving; B: Relapse-free surviving.

We believe that the immune cells with point inclusions in the lamina propria of GM are likely to be macrophages. The data obtained by several researchers suggests this conclusion[65,66]. It is noted that even the absorbed bacteria retains their viability in macrophages, which may be associated with the violation of the phagosome maturation[66-68]. The use of confocal microscopy enabled the localization of the bacteria within the cells to be associated with the endosomal and lysosomal markers, and found that *H. pylori* could use the vesicles of autophagosomes (autophagic vesicles) for its own replication[63,69].

The study found that the concentration of *H. pylori* coccoid forms in GM was the most significant clinical factor. This factor was associated with the tumor histology, T status, N status, stage, 10-year PFS, and OS. The moderate and marked concentrations of coccoid forms of *H. pylori* were more often found in the diffuse type of GC (P = 0.024) and T3-4 (P = 0.04) stage. Interestingly, the high concentration of *H. pylori* is more frequent in Stage N1 than in N2 (at 90.0% and 53.1%, respectively, P = 0.024).

The moderate and marked concentrations of coccoid forms of *H. pylori* represented a prognostic factor associated with the decrease of 10-year RFS and OS from 55.6% to 26.3% (P = 0.02 and P = 0.07, respectively).

It should be noted that the results of this study do not allow us to unambiguously judge the effect of *H. pylori* on GC progression. A decrease in OS and DFS in patients with moderate and marked concentrations of *H. pylori* coccoid forms in the GM may be due to the fact that these patients had more advanced stages and more aggressive

forms of GC. Meanwhile, there are more and more studies showing that *H. pylori* infection can promote GC progression by activating the NF-κB signaling pathway and induction of interleukin-8 secretion[70], the activation of epithelial-mesenchymal transformation[71-74] and angiogenesis[75,76], as well as increasing the invasive properties of tumor cells[77]. It can be assumed that the administration of AT before surgery contributes to the reduction of the inflammatory process activity and normalization of the adhesive properties of tumour cells, which in turn decreases metastasis risk and improves the long-term results of the treatment of GC. The data literature on the improvement of the long-term results of malignant tumours treatment when using antibacterial drugs testify in favour of this hypothesis[78-80].

CONCLUSION

The data obtained indicates that *H. pylori* may be associated not only with induction but also with the progression of GC. It can be assumed that the prevalence of coccoid forms of bacteria and their intracellular persistence can affect the mechanisms of tumor progression. Further appropriate studies regarding the role of *H. pylori* in the progression of GC are obviously advisable.

ARTICLE HIGHLIGHTS

Research background

Gastric cancer (GC) continues to be one of the most common malignant diseases in the world. It is known that *Helicobacter pylori* (*H. pylori*) infection, initiating the development of a chronic inflammatory process in the gastric mucosa (GM), is the leading risk factor for GC. At the same time, clinical studies indicate that inflammatory infiltration of the tumor stroma and surrounding tissues can have an important prognostic value and affect the long-term results of malignant neoplasm treatment.

Research motivation

It is still not clear whether *H. pylori* is involved only in the initiation of the tumor process in the stomach, or whether it can affect the mechanisms of tumor progression.

Research objectives

The aim of this study was to establish the features of *H. pylori* infection in patients with GC and their correlations with clinical and morphological characteristics of diseases and long-term results of treatment.

Research methods

In this prospective observational study, we included all patients with GC who had undergone radical surgery (R0) between May 2007 and March 2010 at the Orenburg Regional Clinical Oncology Center. Features of the *H. pylori* infection and its severity was determined by rapid urease test and by immunohistochemically using the antibody to *H. pylori*. The data obtained we compared with clinical features of GC: Stage, localization, histology, the presence of antibiotic therapy (AT) before surgery, and 10-year overall and disease-free survival.

Research results

We found *H. pylori* infection in the adjacent to the tumor GM in 84.5% of cases. We have established that the coccoid forms of *H. pylori* predominate in the GM of patients with GC. A high rate of infection by coccoid forms of *H. pylori* has been associated with more aggressive type of GC, advanced stage, and decline of a 10-year overall and disease-free survival. The presence of AT 1-1.5 mo before the operation was associated with an improvement in the 10-year survival rate of patients with local (T1-3N0M0), but not advanced (T3-4N1-2M0) stages of GC.

Research conclusions

These results indicate that *H. pylori* may be associated not only with induction but also with the progression of GC.

Research perspectives

The results obtained do not allow one to draw unambiguous conclusions about the role of *H. pylori* in the progression of GC. Further appropriate prospective studies regarding the role of *H. pylori* in the progression of GC are obviously advisable.

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SYSTEMATIC REVIEWS

Determination of gluten immunogenic peptides for the management of the treatment adherence of celiac disease: A systematic review

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Abstract

BACKGROUND

Gluten is a complex mixture of proteins with immunogenic peptide sequences triggering the autoimmune activity in patients with celiac disease (CeD). Gluten immunogenic peptides (GIP) are resistant to gastrointestinal digestion and are then excreted *via* the stool and urine. Most common detection methods applied in the follow-up visits for CeD patients such as serology tests, dietetic interviews, questionnaires, and duodenal biopsy have been proved to be inefficient, invasive, or inaccurate for evaluating gluten-free diet (GFD) compliance. Determination of excreted GIP in stool and urine has been developed as a non-invasive, direct, and specific test for GFD monitoring.

AIM

To summarize published literature about the clinical utility of GIP determination in comparison to the tools employed for GFD monitoring.

METHODS

PubMed and Web of Science searches were performed using the keywords "gluten immunogenic peptides" or "gluten immunogenic peptide" and a

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combination of the previous terms with "feces", "stools", "urine", "celiac disease", "gluten-free diet", and "adherence" to identify relevant clinical studies published in English and Spanish between 2012 to January 2021. Reference lists from the articles were reviewed to identify additional pertinent articles. Published articles and abstracts reporting the clinical use of GIP determination in stool and/or urine for the follow-up of patients with CeD in comparison with other tools in use were included. Case reports, commentaries, reviews, conference papers, letters, and publications that did not focus on the aims of this review were excluded.

RESULTS

Total of 15 publications were found that involved the use of GIP determination in stool and/or urine to monitor the adherence to the GFD in comparison to other tools. Studies included both children and adults diagnosed with CeD and healthy volunteers. Overall, these preliminary studies indicated that this novel technique was highly sensitive for the detection of GFD transgressions and therefore could facilitate the follow-up of patients with CeD. Tools identified in this work included the CeD-specific serology, dietetic questionnaires, symptomatology, and the duodenal biopsy. Review of the literature revealed that the rates of GFD adherence may vary between 30%-93% using either stool or urine GIP determination, 49%-96% by the serology, 59%-94% using the dietetic questionnaires, 56%-95% by the reported symptoms and 44%-76% with the duodenal biopsy. In addition, the association between the different methods and histological abnormalities (Marsh II-III) was found to be 33%-100% for GIP determination (stool and urine), 25%-39% for CeD-specific serology, 3%-50% for dietetic questionnaires, and 22%-28% for the symptomatology.

CONCLUSION

Excreted GIP detection is the precise approach for determining voluntary or involuntary gluten consumption in CeD patients preventing future complications arising from gluten exposure.

Key Words: Celiac disease; Gluten-free diet; Gluten immunogenic peptides; Immunoassays; Stool; Urine

Core Tip: A strict gluten-free diet (GFD) is the only available treatment for celiac disease. However, treatment adherence is difficult due to the ubiquitous nature of gluten, hurting patients' quality of life. Despite several tests to evaluate GFD compliance, it has been proven to be invasive or inefficient. The determination of gluten immunogenic peptides (GIP) in stool and urine has been developed as a non-invasive, direct, and specific test for GFD monitoring. We herein summarized the current available literature meeting the clinical utility of GIP determination compared to the available tools in use.

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INTRODUCTION

Gluten is a heterologous polymorphic mixture of proteins called prolamins. Wheat prolamins are termed gliadins and glutenins. Prolamins provide food products with special functional properties such as elasticity as well as extensibility and are characterized by high proline and glutamine content, allowing for the more efficiently

packing of proteins but also complicating the enzyme-mediated hydrolysis of their tight structures[1]. Consequently, many of these proteins are insufficiently degraded by gastric and pancreatic enzymes in the gastrointestinal tract. Therefore, after the ingestion of gluten-containing foods, some gluten peptides can enter the intestinal epithelium and trigger an immune response in genetically predisposed individuals suffering from celiac disease (CeD)[2-4].

Evidence suggests that gluten can cross the intestinal barrier *via* the transcellular pathway perpetuating intestinal inflammation in the context of gluten intolerance[5, 6]. The α-gliadin 33-mer peptide has been described as one of the most immunodominant gluten peptides, harboring several T cell epitopes[2]. Another three gluten peptides have been shown to trigger high immunogenicity observed in most CeD patients[7]. Thus, most of the immune response against gluten may be accounted for by a limited group of gluten epitopes[7]. The anti- α-gliadin 33-mer antibodies A1 and G12 could specifically and sensitively detect excreted gluten immunogenic peptides (GIP) in stool and urine[8,9], confirming the resistance of GIP to human gastrointestinal digestion as well as their absorption into the bloodstream.

CeD is a systemic disease, involving well-known key immune factors, including the human leukocyte antigen (HLA-DQ2 and HLA-DQ8), the anti-tissue transglutaminase (anti-tTG) antibodies, and gluten[10]. As a systemic disease, there are intestinal and extraintestinal symptoms that can be presented individually or in combination. In addition, patients may also be completely asymptomatic[11]. Intestinal presentation of CeD is common in both the pediatric and adult patient population and is characterized by diarrhea, loss of appetite, abdominal distention, bloating, pain, constipation, or weight loss[12,13]. The most common extraintestinal symptoms are iron deficiency anemia, osteopenia or osteoporosis, hypertransaminasemia, neurological afflictions, or changes in reproductive function[10]. Nevertheless, a relevant proportion of patients present atypical symptoms or remain asymptomatic despite damage to the intestinal mucosa and elevated CeD-specific serum antibodies, which may delay diagnosis or even be undiagnosed[14].

Following CeD diagnosis, patients must follow a strict, life-long gluten-free diet (GFD), the only treatment currently available, which not only reduces disease symptoms but also allows for the healing of the intestinal epithelia and prevents long-term complications[15-17]. However, following a strict GFD is challenging and requires substantial daily effort, hurting the quality of life in addition to psychological problems and fear of involuntary gluten intake, even in patients considering themselves to be strictly adherent[15,18-20]. Gluten-free food availability, inadequate food labeling regulations, cost, and safety are the main barriers related to GFD[18,21, 22]. Therefore, the frequency of voluntary and involuntary transgressions is high. It was reported that at least 50% of adult patients are not fully adherent to the GFD in a daily or weekly period of observation[8,9,23,24]. In addition, 36%-55% of patients who expressed their complete adherence to a GFD did not achieve histological remission, potentially from inadvertent lapses in their daily gluten intake[24-27]. Inadvertent gluten ingestion was suggested to be more frequent than intentional intake, not only when eating out, but also at home[28-30]. A systematic review recently reported adherence rates ranging from 23% to 98% in the pediatric population, determined by using all available methods for evaluating adherence[31].

Although continuous patient monitoring is expected to improve GFD adherence, there is no consensus on how frequent and which tools to use in the patient follow-up [32-35]. All available guidelines recommended clinical and dietary evaluation as well as serology tests at least once a year or every two years to confirm the GFD adherence in addition to detecting possible complications[14]. However, monitoring approaches have not been comprehensively assessed with a clear lack of a gold standard for comparisons[36-38]. Thus, various questions often arise among caregivers and patients regarding the best approach for detecting gluten exposure. GFD follow-up through the detection of excreted GIP has the benefits of being non-invasive, objective, and specific, highlighting its potential as a complementary technique for monitoring CeD treatment[8,9].

Despite the increasing number of studies on the use of GIP excretion determination for the assessment of GFD compliance, there are limited reference guidelines on the detection of GIP in stool and urine for monitoring the treatment in patients diagnosed with CeD. The purpose of this systematic review is to compile insights from studies that tackled the practical issues related to the clinical utility of the available methods for GIP determination compared to current GFD adherence monitoring methods.

MATERIALS AND METHODS

Literature search strategy

PubMed and Web of Science searches were performed using the search terms "gluten immunogenic peptides" or "gluten immunogenic peptide" and a combination of the previous terms with "feces", "stools", "urine", "celiac disease", "gluten-free diet", and "adherence" using the Boolean AND operator, which allows the establishments of logical relations among concepts. References of included full-text articles were scrutinized for additional studies.

Inclusion and exclusion criteria

The eligibility criteria were based on the PICOS (Participants, Intervention, Comparison, Outcomes, Study design) acronym. Articles eligible to be included in this review were required to meet the following criteria: (1) The study reported both children and adult patients diagnosed with CeD, since it would be more useful for readers as the evidence available about the use of this tool is limited; (2) The intervention was the use of GIP determination in stool and/or urine as a tool for monitoring the rate of adherence to the GFD in comparison with the current tools in use, like the serology, dietetic questionnaires, symptomatology, and duodenal biopsy; (3) It was a prospective cohort/cross-sectional/case-control study; (4) The article was written in English or Spanish language; and (5) The article was published between 2012 and January 2021.

We excluded publications that did not focus on the aim of this review. If a full-text paper could not be obtained, but the abstract presented sufficient data, the publication was included. Case reports, commentaries, reviews, conference papers, and letters were excluded.

Retrieved manuscripts were reviewed by the authors, and the data were extracted and described.

RESULTS

Through the literature review, our searches yielded 67 results after the removal of duplicates. In total, 52 entries were excluded for different reasons: 23 did not respond to the aim of this publication, 28 were reviews, case reports, or conference papers, and one had not abstract or full-text available. After screening, 15 publications were eligible for further study. Of these, 14 had a full text in English and one had an English abstract. Two publications reported complementary data about the same study (Figure 1).

The 15 publications included both children and adults diagnosed with CeD. Healthy adult and pediatric volunteers were also included in many of the studies. The articles were published between 2016 and 2021. The characteristics of included studies are summarized in Table 1[8,9,22-24,29,30,39-46].

DISCUSSION

Methods for the determination of excreted GIP

Analytical methods require a standard analyte for quantitative determination. The main issue is the selection of a standard gluten peptide in the huge heterogeneity of the gluten proteins and the countless number of gluten hydrolyzed fragments. The a-gliadin 33-mer peptide (LQLQPFPQPQLPYPQPQLPYPQPQLPYPQPQPF) is resistant to hydrolysis *via* gastric, pancreatic, and intestinal brush-border membrane proteases, which allows for its detection in human excretions. This peptide is considered one of the most immunodominant gluten peptides for CeD patients, as it contains three overlapping T cell epitopes, repetitions of p57-68[2,47,48]. Monoclonal antibodies A1 and G12 were generated against the α-2-gliadin 33-mer peptide specifically and sensitively detecting significant amounts of excreted GIP in stool and urine[8,9]. The reactive epitope profile may thus play an important role in the detection of excreted GIP. Even though there are a variety of antibodies on the market that allow for gluten detection in food, such as R5, 401.21, α20, 14G11, and 13F6, only G12 and/or A1 are useful for gluten peptide sensitive detection in stool and urine[8,9,49-52].

Urine samples are highly heterogeneous matrices with low protein content, making complicating the development of immunoassays for biomarkers detection. Urine

Table 1 Studies that have used gluten immunogenic peptides determination in stool and/or urine for gluten-free diet monitoring

Ref.	Design	Study population	Intervention	Main results
Comino *et al* [8]	Prospective, multicenter, observational study	184 adult and pediatric CeD patients	Fecal GIP ELISA, serology, questionnaires, and symptoms to evaluate adherence to the GFD	GIP-positive results were found in 12%-28% of children < 12 years-old, 30% in > 13 years-old females and 60% in > 13 years-old males. Low correlation of anti-tTG and anti-DGP markers and poor adherence to the GFD
Moreno *et al* [9]	Randomized controlled study	58 adult and pediatric CeD patients and 76 healthy controls	Urine GIP LFIA test, serology, and duodenal biopsy to evaluate adherence to the GFD	About 50% CeD patients were GIP-positive. High correlation of GIP quantifiable concentration in urine with persistent villus atrophy in treated CeD patients (n = 25). No correlation between serology and mucosal damage
Gerasimidis *et al*[39]	Cross-sectional study cohort for a subgroup	63 pediatric CeD patients	Fecal ELISA GIP test, serology, and questionnaires to evaluate gluten intake during diagnosis and adherence to the GFD after diagnosis	GIP-positive results in 95% of de novo patients with CeD during diagnosis. GIP-positive results were found in 17% and 27% of patients after 6 and 12 months of the beginning of the GFD, respectively. GIP-positive results were found in 16%, 16%, and 14% of patients considered compliant according to the Biagi score, tTG, and clinical assessment, respectively
Comino *et al* [40]	Prospective, multicenter, observational study	64 pediatric CeD patients	Fecal GIP ELISA, serology, questionnaires, and symptoms to evaluate adherence to the GFD after diagnosis	Most children (97%) were GIP-positive at diagnosis. A decrease of GIP detection was observed on a GFD, but the rate of GIP-positive results increased from 13% at 6 months to 25% at 24 months. Anti-tTG antibody levels showed low sensitivity to identify patients with GIP-positive results. Dietitian assessment was only moderately correlated with GIP detection
Costa *et al*[41]	Cross-sectional study and prospective cohort	44 adult CeD patients	Fecal GIP ELISA, stool and urine LFIA GIP tests, serology, questionnaires, and symptoms to evaluate adherence to the GFD	25% of patients had at least one GIP-positive test, 32% in asymptomatic patients and 15.8% in symptomatic patients. Dietary assessment estimated gluten intake in only 50% of GIP-positive samples. Anti-tTG and anti-DGP positive results in 3/12 and 6/12 of GIP-positive cases, respectively
Silvester *et al* [29,30]	Prospective longitudinal study	18 adult CeD patients	Monitoring GFD adherence by collection of daily food, stool, and urine samples for the analysis of GIP content, and relationship with duodenal biopsy, serology, questionnaires, and symptoms	GIP were detected in 66,7% patients. No significant correlation was found between gluten ingestion and non-invasive measures of GFD adherence. Most patients with normal anti-tTG had ≥ 1 GIP-positive sample (64%), 2/3 of these had persistent villous atrophy (Marsh 3a) and 2/3 of those with all GIP-negative samples had normal villous architecture (Marsh 0-1) but 4/6 with Marsh 0 had detectable gluten in ≥ 1 sample
Ruiz-Carnicer *et al* [23]	Prospective observational study	22 newly diagnosed CeD patients, 77 CeD patients following a GFD and 13 healthy volunteers	Urine LFIA GIP test to evaluate adherence to the GFD and comparison with serology, clinical manifestations, dietary questionnaire, and histological results	Mucosal damage (Marsh II-III) was found in 24% of CeD patients, 94%of these had ≥ 1 GIP urine sample. 60-80% of these were asymptomatic, had negative serologic results and were compliant with treatment regarding the dietary questionnaire. GIP-negative results were found in 97% of the patients without mucosal damage
Fernandez-Miaja *et al*[22]	Cross-sectional study	80 pediatric CeD patients	Relationship of fecal LFIA GIP for GFD monitoring GFD with CDAT, serology and sociodemographic and clinical data	Acceptable agreement was found between GIP detection and CDAT questionnaire (92.5% and 86.3% adherence rate, respectively). Most patients (83.3%) with GIP-positive results had negative anti-tTG antibodies
Porcelli *et al* [42]	Cross-sectional study	25 CeD patients	Assessment of compliance with the GFD using Fecal GIP ELISA testing, the Biagi questionnaire, evaluation of symptoms and serology	GIP-positive results were found in 4 patients, 2 of these complied with the GFD according to the Biagi questionnaire. All GIP-negative patients were asymptomatic. Levels of anti-tTG antibodies were significantly higher in GIP-positive patients than in GIP-negative patients
Roca *et al*[43]	Prospective, cross-sectional study	43 pediatric CeD patients at follow-up (Group 1) and 18 at diagnosis (Group 2)	Fecal GIP ELISA and LFIA analysis to monitor in real life the adherence to GFD Comparison to food record questionnaire and serology	Group 1: GIP-positive results were found in of 34.9% patients by ELISA (46,7% also by LFIA). 48.8% of patients had positive anti-tTG antibodies (4 reported symptoms) and 10 of these had GIP-positive results by ELISA (70% also by LFIA) (2 reported symptoms). All the transgressions detected by food record were also detected with GIP
Porcelli *et al* [44]	Cross-sectional study	55 CeD patients: 27 adults and 28 children	Assessment of compliance with the GFD using Fecal GIP ELISA, the Biagi questionnaire, evaluation of symptoms and serology	GIP-positive results were found in 8 patients, 71.4% of these were asymptomatic and 37.5% had raised anti-tTG antibodies. A significant association was found between the Biagi score and GIP-positive results but according to the Biagi score, 57.1% of GIP-positive patients followed the diet strictly and 5.4% of GIP-negative subjects did not comply with the diet

Laserna-Mendieta *et al* [45]	Prospective observational study	97 adolescent and adult CeD patients	Evaluation of the sensitivity and specificity of fecal GIP LFIA test to detect duodenal lesions in CeD patients on a GFD and comparison to serology and questionnaires	Compared to the duodenal histology, GIP LFIA test showed similar sensitivity (33%) and specificity (81%) to anti-tTG antibodies. No relationship was found between GIP and questionnaires but an association between GIP and patients' self-reported gluten consumption was seen
Stefanolo *et al* [24]	Prospective observational study	53 adult CeD patients	Fecal GIP ELISA and urine LFIA GIP test, anti-tTG, anti-DGP, and questionnaires to evaluate adherence to the GFD in symptomatic and asymptomatic patients	At least one GIP-positive result in 88.7% of patients for the 4 wk period. Patients who had symptoms had elevated GIP levels for more weeks than patients who did not have these symptoms ($P < 0.05$). Correlation was found between GIP and anti-DGP antibodies but not with levels of anti-tTG antibodies
Fernández-Bañares *et al* [46]	Multicenter prospective observational study	76 adult CeD patients	Fecal GIP ELISA, anti-tTG, questionnaires and symptomatology to evaluate villous atrophy persistence after 2 years on a GFD	Persistent villous atrophy was present in 53% of patients at follow-up, 72% of these were asymptomatic and 75% had negative anti-tTG antibodies. Most patients were adherent to the GFD according to the dietary evaluation. In contrast, GIP-positive results were found in ≥ 1 fecal sample of 77% of patients with villous atrophy and in 60% of patients with mucosal recovery

GIP: Gluten immunogenic peptides; LFIA: Lateral flow immunoassay; GFD: Gluten-free diet; CeD: Celiac disease; anti-tTG: Anti-tissue transglutaminase; anti-DGP: Anti-deamidated gluten peptide; CDAT: Celiac disease adherence test.

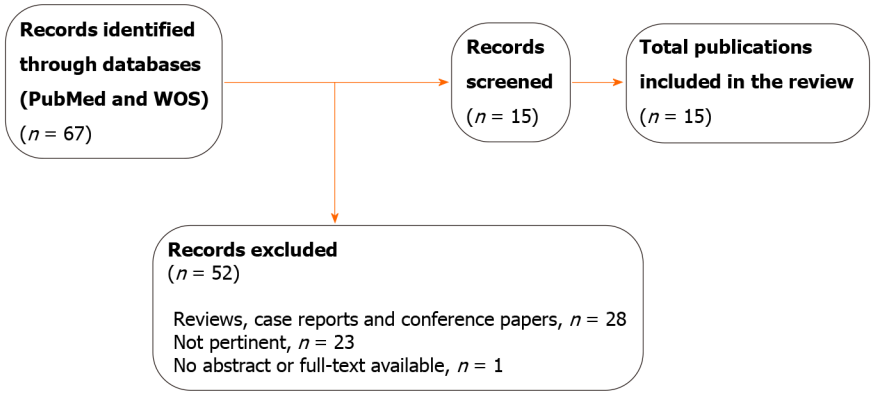

Figure 1 Flow-chart of the publications search and selection.

contains organic molecules such as urea, creatinine, and uric acid, inorganic ions such as K^+, Na^+, Cl^-, and Ca^{2+}, cells, as well as peptides of more than 1500 proteins[53]. The concentration of these compounds and the pH usually exhibit considerable variability not only among individuals but also between different urine samples taken from the same individual[54]. The complex composition of these samples and its variability, in addition to the high frequency of matrix interferences, complicate the reproducibility and robustness of urine immunoassays. Chatziharalambous *et al*[53] evaluated 11 commercial ELISA assays for the detection of urine biomarkers, reporting that only three of them met the requirements of FDA validation guidelines[55].

The currently available tests for GIP detection in stool and/or urine (Table 2) are immunoassays adapted from those used for gluten detection in foods to maximize sensitivity. Lateral flow immunoassay (LFIA) tests can detect GIP from concentrations of 0.15 μg GIP/g in stool and 2.2 ng GIP/mL in urine after less than 30 minutes, showing both tests a high sensitivity (98.5% and 97%, respectively) and specificity of 100%[9,56]. While these tests provide qualitative data, semiquantitative results could also be obtained in urine samples using the LFIA coupled with a lateral flow reader [9]. A quantitative G12-based sandwich ELISA test for stools was developed to increase the sensitivity and quantitative determinations. The analytical sensitivity of the assay was 0.16 μg GIP/g stool (limit of quantification), and it was validated in a multicenter clinical study, showing a diagnostic sensitivity and specificity of 98.5% and 100%, respectively[8].

Estimation of the time and amount of ingested gluten

Although a significant correlation between gluten intake and excreted GIP concentration was reported, high variability is usually observed among subjects[9,29,30,43]. Interindividual diversity (weight, sex, age, gut microbiota, *etc.*), the type of gluten-

Table 2 Available immunomethods to detect gluten immunogenic peptides in stool and urine

Technique	Antibodies	Sample	Analytical sensitivity	Diagnostic sensitivity	Diagnostic specificity	Ref.
ELISA	G12/G12	Stool	0.16 μg/g	0.98	1	[8]
LFIA	G12/A1	Stool	0.15 μg/g	0.97	1	[56]
LFIA	G12/A1	Urine	2.2 ng/mL (LOD), 6.25 ng/mL (LOQ)	0.91	0.99	[9]

ELISA: Enzyme-like immunosorbent assay; LFIA: Lateral flow immunoassay; LOD: Limit of detection; LOQ: Limit of quantification.

containing food (beer, pasta, bread, cookies, *etc.*), the amount of daily liquid intake, and the accompanying diet may have a considerable impact on the resulting GIP concentration as well as the excretion time in urine and stool samples, especially when the gluten exposure is not regular[9,29,30,43].

It appears difficult to predict the specific time and amount of gluten intake with high accuracy based on excreted GIP concentration and even considering the time of sample collection. However, some constraints regarding the expected time for GIP detection and the limits of detection could have been determined in different studies (Table 3).

It was observed that after consuming normal gluten containing diet, a negative result in both urine and stool samples was rarely observed in the following 6-12 h and 3-5 days, respectively, after the last gluten-containing meal[9,43]. Moreover, in individuals with multiple regular transgressions, the interval between gluten ingestion and urine sample collection for maximal sensitivity of detection was generally consistent between 4 and 24 h[29,30].

Despite the variability of GIP concentrations found among individuals in most studies, a significantly low GIP content was typically observed in patients with CeD compared to healthy individuals with no diet restrictions[8,9].

Clinical utility of GIP determination in urine or stools

Fecal immunoassays were first suggested as a novel method for the detection of gluten intake during diagnosis and GFD monitoring[57]. Two different methods could be used to estimate the amount of ingested gluten depending on the situation: LFIA test for either clinical laboratories or point-of-care settings due to its simplicity, while ELISA would be more suitable when a quantitative analysis and/or high throughput are convenient[58,59]. In a prospective, nonrandomized, partially-blinded, multicenter study GFD compliance of CeD children and adults was examined by measuring fecal GIP (determined by ELISA), a dietary questionnaire, celiac serology, and clinical response, as markers of diet adherence[8]. Their results revealed detectable amounts of GIP in the stools of about 30% of the analyzed patients on a GFD for at least one year in comparison to the 18% found when assessing by dietary questionnaire or by determination of anti-tTG antibodies in the serum alone. Indeed, less GIP-positive results were found in those declared non-compliant by the food questionnaire, while 70% of patients who did not declare any gluten intake had positive levels of GIP in stools[8].

Then, Moreno *et al*[9] demonstrated that urine samples could be used to monitor GFD compliance *via* an LFIA test. These tests revealed a high level of GFD infringement in patients on long-term treatment (48% and 45% in adults and children, respectively)[9]. Thus, several studies have compared GIP detection results in stool and urine for GFD monitoring, with information obtained *via* CeD-specific serology, dietary questionnaires, symptomatology, and duodenal biopsies, revealing the limitations of all these methods used to evaluate GFD transgressions and the concordance between them (Table 4).

A branch of the first study with newly diagnosed pediatric CeD patients (n = 64) showed that the percentage of diet adherence decreased on follow-up at 6, 12, and 24 mo, as the rate of GIP-positive stools increased from 13% to 25%[40]. Meanwhile, anti-deamidated gliadin peptide (anti-DGP) antibodies normalized by 24 mo, and anti-tTG antibodies were elevated in 20% of the patients. Some children, particularly the older ones, were reported to have a propensity for GFD non-compliance, and 46% of non-adherent participants had at least two GIP-positive stools during follow-up[40]. Similar data were reported by other groups[22,44]. Patients with higher levels of gluten exposure exhibited prolonged ascension of anti-tTG antibodies (P < 0.05) than those with GIP-negative results.

Table 3 Parametrical features of the gluten immunogenic peptides excretion using lateral flow immunoassay and enzyme-like immunosorbent assay methods

Specifications for the determination of GIP excretion after gluten intake	Sample	Time ranges (h)	Gluten source (amount)	Method
Shortest time to detect GIP	Urine	3-9	GCD (> 2 g)	LFIA [9,29,30]
	Stool→	< 24	GCD (> 2 g)	LFIA; ELISA[43]
Longest time to detect GIP	Urine	36	GCD (> 2 g)	LFIA[9,29,30]
	Stool	> 72	GCD (> 2 g)	LFIA; ELISA[29,30,43]
Minimal gluten intake to detect excreted GIP	Urine		> 40-500 mg/d; 25-50 mg	LFIA; SPE + LFIA[9,41]
	Stool		> 40 mg/d	ELISA, LFIA[41,43]

GIP: Gluten immunogenic peptides; GCD: Gluten containing diet including different food types; LFIA: Lateral flow immunoassay; ELISA: Enzyme-like immunosorbent assay; SPE: Solid phase extraction.

Table 4 Determination of gluten-free diet non-adherence using different tools, *n* (%)

Ref.	Stool GIP+	Urine GIP+	anti-tTG+	anti-DGP+	Questionnaires[1]	Symptoms	Duodenal biopsy (Marsh II/III)
Comino *et al*[8]	56 (30)	-	32 (18)	11 (6)	25 (18)	9 (5)	-
Moreno *et al*[9]	-	12 (48)	4 (16)		-	-	7 (28)
Gerasimidis *et al*[39]	11 (19)	-	12 (20)	-	4 (6)	-	-
Comino *et al*[40]	6 (25)	-	7 (20)	0 (0)	-	-	-
Costa *et al*[41]	11 (25)	3 (7)	9 (21)	18 (45)	18 (41)	19 (43)	-
Silvester *et al*[29,30]	5 (28)	8 (44)	7 (39)	-	4 (22)	8 (44)	10 (56)
Ruiz-Carnicer *et al*[23]	-	44 (58)	9 (12)	-	14 (23)	18 (23)	18 (24)
Fernández-Miaja *et al*[22]	6 (8)	-	3 (4)	-	10 (13)	-	-
Roca *et al*[43]	15 (35)	-	22 (51)	-	4 (9)	4 (9)	-
Porcelli *et al*[44]	8 (15)	-	3 (6)	-	5 (11)	16 (34)	-
Laserna-Mendieta *et al*[45]	22 (23)	-	11 (12)	-	17 (18)	-	6 (28)
Stefanolo *et al*[24]	33 (62)	37 (70)	22 (42)	25 (47)	-	18 (34)	-
Fernández-Bañares *et al*[46]	53 (70)	-	17 (22)	-	6 (8)	15 (20)	40 (53)

[1]Questionnaires used were Celiac Disease Adherence Test (CDAT), Biagi Score and standard food records evaluated by dietitians.
GFD: Gluten-free diet; GIP: Gluten immunogenic peptides; anti-tTG: Anti-tissue transglutaminase; anti-DGP: Anti-deamidated gluten peptide; -: Not available.

In agreement with these data, Gerasimidis *et al*[39] found that most patients following a GFD had GIP-negative results, while 18% exhibited recent gluten exposure, which was not detected by using anti-tTG antibodies and the Biagi score. These findings confirmed the limitations of dietary evaluation and serology in adult patients with CeD on a GFD. A quarter of patients considered adherent by those methods had detectable GIP, using both LFIA and ELISA tests, in at least one of two independent tests during the 2 wk of the study. A 65,9% of concordance was observed between dietary reports and GIP results. Only four patients had high serum antibody values and two of them confirmed dietary non-compliance, in agreement with GIP results[41].

Recent research demonstrated that both ELISA and LFIA methods confirmed suspected and unsuspected dietary exposure in stool samples of CeD children and adolescent patients on a long-term GFD. However, no significant association was found between longer GFD duration, and the amount of GIP recovered in stool[43].

GIP-positive results were obtained from 35% of patients by ELISA (47% of these were also confirmed by LFIA). However, based on the dietary questionnaire, 90.7% of patients were compliant with the treatment. All the patients revealed as non-adherent by the questionnaire had GIP-positive stools. Furthermore, anti-tTG antibodies were detected in five patients, three of whom were also GIP-positive. However, the authors suggested that these elevated levels may be related to the short length of treatment (< 12 mo) and a lack of GFD adherence, as a patient with negative serology and GIP-negative stools was identified.

Differences in diet compliance rates based on a GIP LIFA test in stools and a validated adherence questionnaire (CDAT) (92.5% *vs* 86.3%, respectively) were observed among 80 CeD children and adolescent patients[22]. Nevertheless, the methods exhibited acceptable concordance (Kappa: 0.31, $P = 0.004$). Of those patients with good adherence using CDAT ($n = 66$), three had GIP-positive results. Porcelli *et al* [44] also found a significant association between strict diet adherence estimated by the Biagi score and fecal GIP detection. According to the Biagi score, 94.6% of GIP-negative patients exhibited good adherence. However, the questionnaire failed in the identification of 57.1% of GIP-positive patients, while GIP detection did not recognize 5.4% of gluten exposures declared *via* the questionnaire. In this study, 62.5% of GIP-positive patients exhibited negative anti-tTG antibodies levels.

The results from the DOGGIE BAG study[29,30] also confirmed that diet transgressions in patients with CeD were frequent despite efforts made to strictly follow the GFD. In this prospective longitudinal study, the food consumed by patients, in addition to their urine and stool samples, were analyzed for gluten intake and GIP excretion, respectively, throughout 10 days before a biopsy at 24 mo after the diagnosis. Gluten was found in the food of 9/18 participants at concentrations as high as 200 ppm, and GIP-positive results were obtained in 12/18 participants reporting good adherence to the GFD (8 of these had positive urine samples, and five had positive stool samples). No correlation was observed between gluten exposure and commonly used non-invasive measures of GFD adherence. Most of the participants (73%) who suspected a gluten exposure had at least one positive food, stool, or urine sample. Among the remaining participants who did not suspect any gluten intake, four of them resulted positive for GIP[29,30]. Other authors also found a significant association between patient self-reported gluten consumption and GIP detection. GIP was detected in 52.9% of patients who suspected gluten intake in the last month in comparison to 16.3% for those who were not aware of any gluten exposure[45].

On the other hand, several studies examined the association between the available tools for GFD monitoring and the duodenal biopsy, currently considered the gold standard (Table 5). Moreno *et al*[9] assessed the correlation between duodenal biopsies and GIP concentration in urine samples. Analysis of duodenal biopsies revealed that all the adult patients with small intestine damage (Marsh II/III) had GIP-positive urine samples ($n = 7$). In addition, there was a significant correlation between the absence of GIP in urine and the absence of villus atrophy in the gut intestinal epithelium. In agreement with other publications[60-62], this study confirmed the poor correlation of serological tests with mucosal healing as well as the limitations of dietary history questionnaires in the assessment of GFD adherence.

In addition, a comprehensive study with a cohort of 77 participants under treatment with a GFD for ≥ 24 mo revealed that 58% had detectable GIP in their urine in at least one sample of three collected during the week[23]. Among the patients with GIP-negative results, 97% of them did not present histological abnormalities (Marsh 0–I), while among patients with GIP-positive results, 17 of 44 (39%) had histological damage in the intestinal epithelial (Marsh II-III), with only 16% presenting positive serological results. Significant differences were found in GIP concentrations between participants with Marsh II–III and Marsh 0–I. The highest sensitivity was observed when at least one of three urine samples tested GIP-positive (94.4%). However, this combination exhibited low specificity (53.4%). In contrast, the optimal specificity was obtained when a single urine sample was collected on the visit day (84.2%). Then, it was expected that one GIP-positive urine sample on the day of the visit would reveal a regular habit of GFD non-compliance not-restrained by the medical supervision. The authors did not observe concordance between gluten exposure measured by GIP and serology, or through a CDAT questionnaire and symptomatology, all of which exhibited low sensitivity for mucosal damage (38.9%, 22.2%, and 42.9%, respectively).

About the association of duodenal biopsy and GIP in fecal samples, Fernández-Bañares *et al*[46] found that most of CeD patients (77%) with persistent villous atrophy obtained a GIP-positive result in at least 1 stool sample after 2 years under GFD. In contrast, dietary evaluation failed to detect most of these gluten exposures, considering these cases as excellent or good adherent to the diet. In addition, 72.5% of

Table 5 Rate of transgressions in the gluten-free diet using different tools in presence/absence of mucosal atrophy by duodenal biopsy, *n* (%)

Ref.	GIP+ (Stool and/or urine)	Serology+	Questionnaires[1]	Symptoms
Duodenal biopsy (Marsh II/III)				
Moreno *et al*[9]	7 (100)	2 (29)	-	-
Silvester *et al*[29,30]	8 (80)	-	-	-
Ruiz-Carnicer *et al*[23]	17 (94)	7 (39)	6 (43)	4 (22)
Laserna-Mendieta *et al*[45]	2 (33)	2 (33)	3 (50)	-
Fernández-Bañares *et al*[46]	31 (78)	10 (25)	1 (3)	11 (28)
Duodenal biopsy (Marsh 0/I)				
Moreno *et al*[9]	5 (28)	2 (11)	-	-
Silvester *et al*[29,30]	4 (50)	-	-	-
Ruiz-Carnicer *et al*[23]	27 (47)	2 (3)	8 (17)	14 (24)
Laserna-Mendieta *et al*[45]	20 (22)	9 (10)	77 (85)	-
Fernández-Bañares *et al*[46]	22 (61)	7 (19)	5 (14)	4 (11)

[1]Questionnaires used were Celiac Disease Adherence Test (CDAT), Biagi Score and standard food records evaluated by dietitians.
GFD: Gluten-free diet; GIP: Gluten immunogenic peptides; -: Not available.

those patients were asymptomatic, and 75% of them obtained negative anti-tTG antibodies. Despite GIP-positive results were also found in 60% of patients with mucosal recovery, no significant differences were found in the amount of GIP between patients with persistent villous atrophy and recovered patients[46].

Other authors reported a weaker association between evident gluten exposure, as measured by GIP detection in urine and stool, and persistent villous atrophy[29,30,45]. In the DOGGIE BAG study referred to above, at least one GIP-positive sample was found in 7 of 11 patients with normal serology, and 2/3 of them had persistent villous atrophy (Marsh IIIa) after 24 mo of follow-up. Further, 2/3 of patients with GIP-negative results for all samples were Marsh 0-I, showing significant mucosal recovery [29,30]. A possible explanation for the discordance described before is that occasional or low gluten exposure may be sufficient for GIP detection but not enough to induce mucosal damage in some patients with CeD[23,29,30,45]. A delay of intestinal mucosal recovery[29,30,45] and improved compliance of participants during the study was also considered[29,30]. Besides, Laserna-Mendieta *et al*[45] reported a low sensitivity but acceptable specificity of GIP for the detection of mucosal damage, however for the calculation they included in the same group Marsh 1 patients (n = 21), who do not present mucosal damage but lymphocytic infiltration, and Marsh 2 and 3 patients (n = 6). Moreover, in this study, weak GIP-positive results were discarded for analysis and duodenal biopsies were performed in two hospitals with different pathologists, entailing a risk of interobserver variability[63,64]. Hence, with these results, the introduction of GIP detection as a non-invasive and sensitive assessment approach for GFD adherence may reduce the need for endoscopy and could identify potential intestinal mucosal damage not detectable *via* serological tests or dietary questionnaires. In addition, intestinal mucosal recovery could be predicted based on recurring negative GIP tests.

Regarding the relationship between symptoms and GIP excretion, only a small percentage of CeD patients reported symptoms in the studies with the highest number of celiac volunteers, although a score system for symptomatology was not used[8]. Roca *et al*[43] detected GIP in samples from 22.7% of asymptomatic pediatric patients negative for anti-tTG antibodies. In contrast, Costa *et al*[41] reported that most adult patients with gluten transgressions determined *via* GIP detection were asymptomatic, although the difference was not statistically significant due to the low number of cases enrolled. Similar data were published by Porcelli *et al*[44] wherein the rate of asymptomatic patients with GIP-positive results was 71.4%.

A recent publication[24] studied the relationship of stool and urine GIP results with gastrointestinal symptoms in CeD adult patients for 4 wk to represent a real-life scenario. 62% of the patients were found to have at least one GIP-positive stool test,

and 69.9% had positive urine samples. The results suggested that symptoms in CeD patients under a GFD are the consequence of gluten exposure. A significantly higher rate of GIP-positive stool samples was observed in symptomatic compared to asymptomatic patients (77.8% *vs* 54.3%, respectively). However, a group of asymptomatic patients also had the highest number of GIP-positive urine samples per patient. This observation highlights the importance of distinguishing asymptomatic patients due to no gluten consumption from those patients consuming gluten because they were asymptomatic.

Considering that avoiding symptoms is the main motivation for adhering to a GFD, GIP content could be a useful indicator for the identification of asymptomatic patients with considerable immunogenic gluten exposure preventing gut mucosal recovery. Furthermore, GIP detection may help in symptomatic patients with CeD to determine whether non-responsive celiac disease or refractory celiac disease occurs due to recurrent gluten exposure or due to additional factors inducing persistent symptoms, such as the consumption of FODMAPs or intestinal dysbiosis[40,41,65,66].

CONCLUSION

Excreted GIP detection in either stool or urine is a precise approach for determining voluntary or involuntary gluten consumption. However, isolated GIP measurements might not identify intermittent compliance, unless punctual transgressions took place close to the moment of sample collection. It appears that the use of multiple samples (at least two) contributes to higher sensitivity and specificity of GIP detection[23,24]. In a considerable number of studies, biopsies and recurrent excretion of GIP indicated that serological tests, symptoms, and dietary questionnaires were not sufficient indicators of GFD adherence[9,23,29-31]. However, anti-tTG antibodies and clinical dietary assessment have been recently suggested as complementary tools in the evaluation of GFD adherence[44,45]. In this context, Porcelli *et al*[44] proposed the use of the Biagi score in combination with fecal GIP tests using a binary logistic regression model. In contrast, other groups did not find anti-tTG levels to be a relevant marker of adherence even though anti-DGP antibodies levels have been shown to significantly correlate with the quantity and frequency of GIP excretion in stools[24,41].

Regarding which method is more appropriate, Roca *et al*[43,67] proposed GIP testing of fecal samples as a non-invasive method that allows patient empowerment for self-managing the disease. ELISA is to be used for the laboratory quantification of GIP, while LFIA strips should be used for patient self-control following suspected involuntary infringements. Even though GIP evaluation may represent an extra cost for CeD patients monitoring, its inclusion in the pursuing of strict GFD adherence would reduce health expenditure by preventing future complications arising from gluten exposure in overlooked asymptomatic patients and would improve the self-esteem or "peace of mind" of patients adhering to the GFD, decreasing the need for additional assessment and endoscopy in non-responsive celiac disease or refractory celiac disease, especially in children.

Some authors have already proposed algorithms for the application of the determination of GIP in stools[39] and urine[23] for the follow-up of patients with CeD. It is expected that the application of those protocols with some variations will be progressively introduced in the guidelines to assess the adherence of patients to the GFD. Future studies may also determine the utility of GIP excretion analysis for other applications, such as the confirmation that sufficient gluten has been ingested before diagnosis or to discover unknown aspects of gluten protein metabolism.

ARTICLE HIGHLIGHTS

Research background

A lifelong strict gluten-free diet (GFD) is the only available treatment for celiac disease (CeD), which reduces symptoms and allows the healing of the intestinal mucosa, preventing long-term complications. However, total exclusion of gluten is difficult to achieve in practice and voluntary and involuntary transgressions are highly frequent.

Research motivation

Gluten immunogenic peptides (GIP) detection in stool and urine is becoming increasingly apparent as a noninvasive and reliable marker for close and efficient GFD

monitoring in patients with CeD.

Research objectives

The authors aimed to summarize published data regarding the performance of GIP determination in stool and urine in comparison to other available tools in assessing GFD adherence in patients with CeD.

Research methods

The authors conducted a systematic review searching in PubMed and Web of Science clinical studies that reported the performance of GIP determination in stool and/or urine with other biomarkers for the evaluation of treatment adherence in adult and pediatric patients with CeD.

Research results

The authors screened 67 articles and 15 articles were included for full-text analysis. In the selected publications GIP determination in stool and/or urine were compared with at least one of the following markers: levels of CeD-specific serology, results from CeD-specific non-specific questionnaires, symptomatology, and duodenal biopsy. Fecal determination by ELISA was the most investigated GIP marker, followed by urine rapid test and stool rapid test. Variability was seen in the concordance between the different tools among the studies reviewed due to the differences found in the study design, the target population, and the methods used. One of the main outcome measures reviewed was the diagnostic accuracy of these tools in assessing mucosal healing. An association of mucosal status and GIP detection was observed in some publications, where serology, questionnaires, and symptomatology showed lower sensitivity. Furthermore, GIP detection may help in symptomatic patients to determine whether non-responsive CeD or refractory CeD occurs due to recurrent gluten exposure or due to additional factors.

Research conclusions

The introduction of GIP detection in stool and/or urine as a non-invasive marker for GFD monitoring in patients with CeD may reduce health expenditure by preventing future complications arising from gluten exposure not detectable by other tools in use. Stool and urine rapid tests may be an option for disease self-managing by the patient, while fecal ELISA test could be used for GIP quantification in the laboratory.

Research perspectives

The introduction of algorithms for GIP determination within the follow-up of patients with CeD in guidelines will allow its routine use in clinical practice. In addition, future studies may also determine their utility for other applications such as the confirmation of gluten consumption during the diagnosis process or the investigation of gluten metabolism.

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CASE REPORT

Pancreatic paraganglioma diagnosed by endoscopic ultrasound-guided fine needle aspiration: A case report and review of literature

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Author contributions: Lanke G composed and drafted the paper; Stewart JM provided path images, revised, and edited the draft; Lee JH conceptualized, designed, revised, and edited the draft.

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Abstract

BACKGROUND

Pancreatic paragangliomas (PPGL) are rare benign neuroendocrine neoplasms but malignancy can occur. PPGL are often misdiagnosed as pancreatic neuroendocrine tumor or pancreatic adenocarcinoma.

CASE SUMMARY

We reviewed 47 case reports of PPGL published in PubMed to date. Fifteen patients (15/47) with PPGL underwent endoscopic ultrasound-guided fine needle aspiration (EUS-FNA). Only six (6/15) were correctly diagnosed as PPGL. All patients with PPGL underwent surgical resection except three (one patient surgery was aborted because of hypertensive crisis, two patients had metastasis or involvement of major vessels). Our patient remained on close surveillance as she was asymptomatic.

CONCLUSION

Accurate preoperative diagnosis of PPGL can be safely achieved by EUS-FNA with immunohistochemistry. Multidisciplinary team approach should be considered to bring the optimal results in the management of PPGL.

Key Words: Pancreatic paraganglioma; Endoscopic ultrasound-guided fine needle aspiration; Meta-iodobenzylguanidine scan; Metanephrines; GATA-3; Immunohistochemistry; Case report

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Core Tip: The morphologic overlap between pancreatic paraganglioma and neuroendocrine tumor is significant. An accurate diagnosis by endoscopic ultrasound-guided fine needle aspiration requires firstly that the possibility of paraganglioma is considered and secondly that a cell block is available for immunohistochemical stains. A patient-centered approach supported by a multidisciplinary team of radiologists, advanced endoscopists, endocrinologists, pathologists, oncologists, and surgeons is paramount in the management of pancreatic paraganglioma.

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INTRODUCTION

Paragangliomas are rare neuroendocrine neoplasms arising from the sympathetic and parasympathetic paraganglia. This tumor is called pheochromocytoma in the adrenal medulla and elsewhere is known as extra-adrenal paraganglioma or simply as paraganglioma. The malignant potential of these tumors is difficult to predict. Most behave in a benign manner, but metastasis, which best defines malignant paraganglioma, may occur in 15%-20%[1]. When found in or around the pancreas this tumor is often misdiagnosed as pancreatic neuroendocrine tumor (PNET) or even pancreatic adenocarcinoma. In this study, we report a case of pancreatic paraganglioma diagnosed by endoscopic ultrasound-guided fine needle aspiration (EUS-FNA) and review of the literature on pancreatic paraganglioma.

CASE PRESENTATION

Chief complaints

A 73-year-old female presented with a chief complaint for evaluation of an incidental finding of peripancreatic lymph node.

History of present illness

She underwent computed tomography (CT) of the abdomen and pelvis as part of her routine surveillance for extranodal marginal zone B-cell lymphoma of mucosa-associated lymphoid tissue (MALT-lymphoma) of the lung and was found to have peripancreatic lymph node. She denied any abdominal pain, change in bowel habit, weight loss, nausea, or vomiting.

History of past illness

Her medical history was significant for MALT-lymphoma, invasive lobular breast carcinoma, hypertension, atrial fibrillation, mitral valve prolapse, mitral valve stenosis, and actinic keratosis. Her surgical history included a mastectomy with sentinel lymph node dissection, laparoscopic cholecystectomy, tonsillectomy, left knee replacement, and bilateral carpal tunnel repair.

Personal and family history

Her family history was significant for colon cancer in maternal grandmother at the age of 65 years, prostate cancer in brother at the age of 63 years, and melanoma in mother. She had no history of alcohol or tobacco abuse. She has 2 children and attained menopause at the age of 52 years. Her medications included aspirin, furosemide, carvedilol, rosuvastatin, amiodarone, digoxin, anastrozole, and Eliquis.

Physical examination

Her physical examination was unremarkable, and her abdomen was soft nontender, nondistended with no palpable mass.

Laboratory examinations

Laboratory exam including fractionated metanephrines, chromogranin, and gastrin were negative.

Imaging examinations

CT of the abdomen and pelvis showed 2 cm × 1.1 cm lymph node adjacent to the pancreatic head (Figure 1A).

Endoscopy

EUS showed a 19 mm × 11.5 mm hypoechoic lesion near the pancreatic head (Figure 1B). Two FNA passes using a 25-gauge needle were performed *via* trans-duodenal approach (Figure 1C).

Pathology

Direct FNA smears showed tumor with neuroendocrine features. Initial immunoperoxidase stains performed on cell block sections were positive for synaptophysin and chromogranin, which seemed to confirm the morphologic impression of PNET. The pathologist was subsequently informed about the peripancreatic location and lack of a definite pancreatic lesion.

FINAL DIAGNOSIS

After additional testing showed the tumor to be positive for GATA-3 and negative for keratin with low expression of Ki-67 (less than 1%), the FNA diagnosis was revised to paraganglioma.

TREATMENT

Our patient was referred to endocrine surgery team after the FNA diagnosis of paraganglioma. After a thorough discussion with the patient on the benefits and risks of surgical resection, the patient elected to remain on close surveillance since she was asymptomatic with a 2-cm, nonfunctioning paraganglioma.

OUTCOME AND FOLLOW-UP

After a 1-year follow up, patient was found to have stable asymptomatic peripancreatic paraganglioma with no increase in size.

DISCUSSION

Paragangliomas are non-epithelial neuroendocrine neoplasms arising in close association with components of the parasympathetic and sympathetic nervous systems [2]. Most parasympathetic paragangliomas are nonfunctional and located along the glossopharyngeal and vagal nerves in the neck and base of the skull[3]. Sympathetic paraganglia secrete catecholamines (functional) and they are commonly located in the paravertebral ganglia of thorax, abdomen, and pelvis[3]. The incidence of extra-adrenal paraganglioma is unclear as these are often described with pheochromocytoma. In the United States, approximately 500-1600 cases are diagnosed every year and the combined annual incidence of pheochromocytoma/paraganglioma is approximately 0.8 per 100000 person-years[4,5]. Pancreatic paragangliomas are more common in women than men (2:1) and the mean age of incidence is 52 years (19-85 years)[6].

Patients with functional paragangliomas can experience hypertension, headache, sweating, and palpitations due to the excessive secretion of catecholamines[7]. Nonsecretory paragangliomas may present with abdominal mass with or without abdominal pain, but most are found incidentally on imaging studies[8,9]. CT has a sensitivity of approximately 90% in the identification of extra-adrenal paragangliomas, which frequently appear as highly vascular structures with areas of intralesional hemorrhage and necrosis[8,10]. The CT findings of pancreatic paragangliomas differ from those of pancreatic ductal adenocarcinoma by their location at the pancreatic

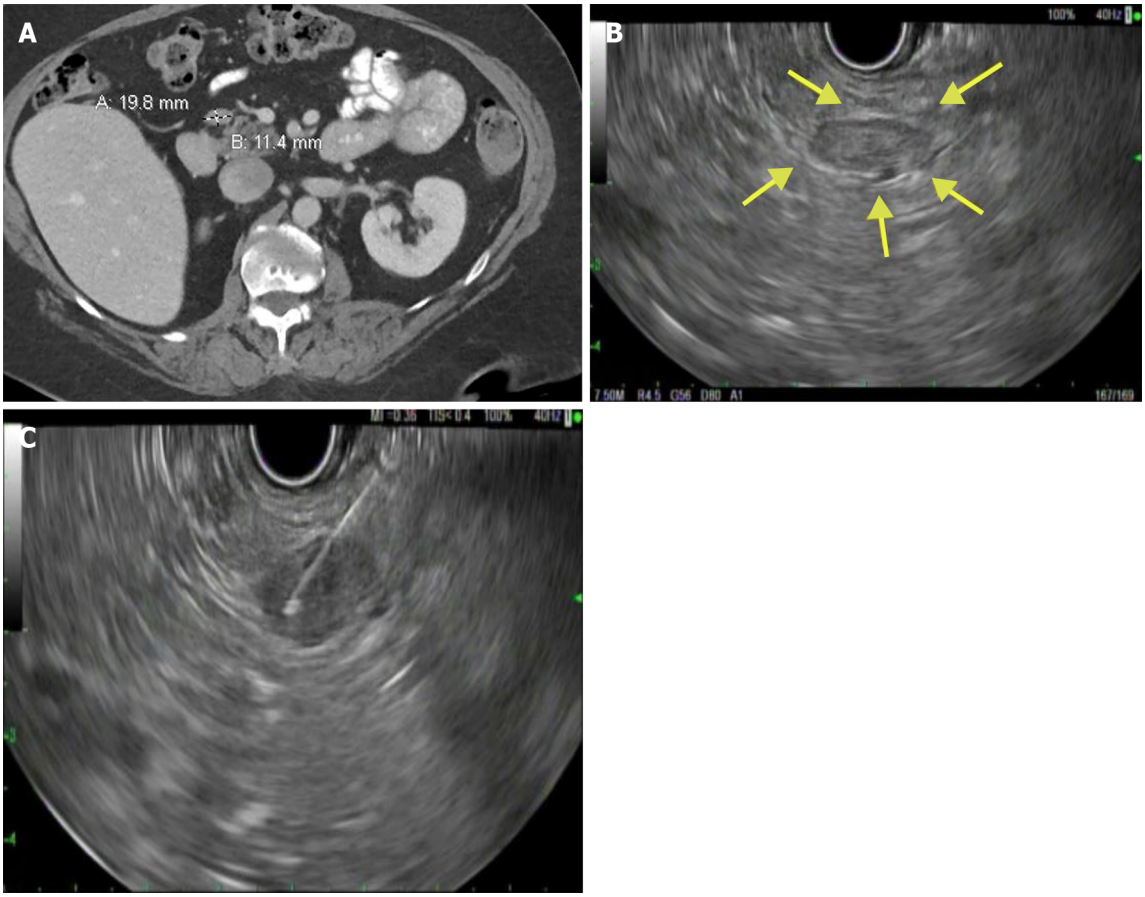

Figure 1 Computed tomography and endoscopy examinations. A: Computed tomography of abdomen pelvis showing a peripancreatic lymph node adjacent to the pancreatic head; B: Endoscopic ultrasonography showing a hypoechoic lesion near the pancreatic head; C: Endoscopic ultrasound-guided fine needle aspiration of the peripancreatic lesion.

head and absence of biliary dilation, although mild pancreatic duct dilatation is sometimes seen[11]. Paragangliomas are also differentiated from nonfunctioning islet cell tumor of the pancreas by observation of early contrast filling of the prominent draining veins of the tumor and the portal vein[12]. Magnetic resonance imaging (MRI) provides tissue characterization superior to CT without radiation[13]. Working synergistically, meta-iodobenzylguanidine (MIBG, I^{123} or I^{131}) scan is useful in differentiating functional from nonfunctional paragangliomas as well as in the detection of tumors in unusual locations, multiple primary tumors, and metastasis[13]. MIBG scan has a sensitivity of 85% and specificity of 95%-100% in the detection of extra-adrenal paragangliomas. Plasma or urinary metanephrines can be used to further establish the diagnosis of functional paragangliomas[13,14].

While most paragangliomas are solitary and sporadic, they can be multicentric and hereditary. Genetic testing should be considered in all patients diagnosed with paraganglioma as nearly 40% (pheochromocytoma and paraganglioma) carry germline mutations. Genetic testing allows for the identification of simultaneous cancers in hereditary syndromes and assists with screening family members at high risk[15]. The most common genetic mutations associated with paragangliomas are *RET* gene in multiple endocrine neoplasia type 2A and 2B, *VHL* in von Hippel-Lindau disease, *NF1* in neurofibromatosis type 1, and succinate dehydrogenase (*SDH*) B, D, C genes[15].

One of the most valuable tools that can assist in establishing the diagnosis of paraganglioma is EUS, which both enables localization of the mass and acquisition of tissue samples for cytology *via* FNA. When not considered in the differential diagnosis, pancreatic paragangliomas can be easily misdiagnosed on EUS-FNA cytology as pancreatic neuroendocrine tumor (NET)[16,17]. Some authors suggest that EUS-FNA should not be done in functional paragangliomas as it can trigger the secretion of catecholamines[18]. In our case, the diagnosis was not established before EUS-FNA and there were no complications during and after the procedure.

On cytology, the cells of paragangliomas are relatively uniform in size, epithelioid in appearance with round to oval nuclei, and arranged in loosely cohesive clusters

Table 1 Reported cases of pancreatic paraganglioma in the literature

No.	Ref.	Age	Gender	Size	Location	EUS-FNA	Preop-diagnosis	Surgery	Postop diagnosis
1	Fujino *et al* [21]	61	Male	2.5 cm	Uncinate process	No	PNET	Pancreaticoduodenectomy	PPGL
2	Ohkawara *et al*[33]	72	Female	4 cm	Head	No	NET	Surgical resection of head	PPGL
3	Perrot *et al* [34]	41	Female	4.3 cm	Tail	No	PPGL	Tumor resection	PPGL
4	Tsukada *et al* [35]	51	Male	2.5 cm	Uncinate	No	PNET	Surgical resection	PPGL
5	Kim *et al*[12]	57	Female	7 cm	Head	No	Non-functioning islet cell tumor	Pancreaticoduodenectomy	PPGL
6	Paik[36]	70	Female	4.2 cm	Tail	No	None	Distal pancreatectomy	PPGL
7	He *et al*[37]	40	Female	4.5 cm	Uncinate	No	None	Surgical resection	PPGL
8	Higa and Kapur[38]	65	Female	2.1 cm	Uncinate	No	None	Pancreaticoduodenectomy	PPGL
9	Al-jiffry *et al* [39]	19	Female	9.5 cm	Head and neck	No	Sarcoma	Pancreaticoduodenectomy	PPGL
10	Zhang *et al* [27]	50	Female	6 cm	Head	Yes	Functional PPGL	Chemotherapy	PPGL
11	Zhang *et al* [27]	63	Female	4 cm	Head	No	Functional PPGL	Surgical resection	PPGL
12	Borgohain *et al*[40]	55	Female	19 cm	Tail	No	Pancreatic cancer	Surgical resection	PPGL
13	Straka *et al* [41]	53	Female	Not mentioned	Head	No	None	Surgical resection	PPGL
14	Meng *et al* [11]	54	Female	3 cm	Head	No	None	Surgical resection	PPGL
15	Meng *et al* [11]	41	Female	6.2 cm	Head	No	None	Surgical resection	PPGL
16	Misumi *et al* [42]	47	Female	1.5 cm	Head	EUS only	PNET	Pancreaticoduodenectomy	PPGL
17	Bartley *et al* [43]	75	Female	15 cm	Tail	No	Pancreatic cyst	Not available	PPGL
18	Bartley *et al* [43]	70	Female	3 cm	Head	No	Pancreatic cyst	Not available	PPGL
19	Cope *et al* [44]	72	Female	14 cm	Head	No	Cystadenoma	Not available	PPGL
20	Zamir *et al* [45]	47	Male	10 cm	Body	No	Pancreatic cyst	Not available	PPGL
21	Parithivel *et al*[46]	85	Male	6 cm	Head	No	NET	Surgical resection	PPGL
22	Wang *et al* [32]	30	Female	6.4 cm	Tail	No	None	No surgery	PPGL
23	Ganc *et al*[18]	37	Female	4.8 cm	Head	Yes	NET	Pancreatico duodenectomy	PPGL
24	Tumuluru *et al*[47]	62	Female	2.9 cm	Body	Yes	NET	Distal pancreatectomy/splenectomy	PPGL
25	Ginesu *et al* [48]	55	Male	2.5 cm	Uncinate	No	NET	pancreaticoduodenectomy	PPGL
26	Liang and Xu[6]	41	Male	6.4 cm	Uncinate	No	NET	pancreaticoduodenectomy	PPGL
27	Lin *et al*[3]	42	Female	6.3 cm	Body	No	NET	Middlesegment pancreatectomy	PPGL

28	Nguyen *et al* [23]	70	Female	5.8 cm	Tail	Yes	PPGL	Surgical resection	PPGL
29	Zeng *et al*[19]	58	Female	6.5 cm	Head	Yes	NET	Surgical resection	PPGL
30	Zeng *et al*[19]	53	Female	2.5 cm	Head	Yes	NET	Surgical resecion	PPGL
31	Singhi *et al* [16]	61	Female	14 cm	Tail	Yes	Pseudocyst	Surgical resection	PPGL
32	Singhi *et al* [16]	52	Female	14 cm	Body	Yes	PPGL	Not performed	PPGL
33	Singhi *et al* [16]	54	Female	6.5 cm	Head	Yes	PPGL	Surgical resection	PPGL
34	Singhi *et al* [16]	40	Male	5.1 cm	Body	Yes	NET	Surgical resection	PPGL
35	Singhi *et al* [16]	78	Female	17 cm	Body	Yes	Spindle cell neoplasm	Surgical resection	PPGL
36	Singhi *et al* [16]	44	Male	5.5 cm	Head	Yes	PPGL	Surgical resection	PPGL
37	Singhi *et al* [16]	38	Male	15 cm	Body	No	None	Surgical resection	PPGL
38	Singhi *et al* [16]	47	Male	7.5 cm	Body	No	NET	Surgical resection	PPGL
39	Singhi *et al* [16]	37	Female	5.7 cm	Tail	No	NET	Surgical resection	PPGL
40	Fite and Maleki[49]	40	Male	5.1 cm	Peripancreatic	No	NET	Surgical resection	PPGL
41	Fite and Maleki[49]	23	Female	7.0 cm	Peripancreatic	No	NET	Surgical resection	PPGL
42	Malthouse *et al*[50]	58	Male	8 cm	Head	No	NET	Not available	PPGL
43	Malthouse *et al*[50]	45	Female	8 cm	Head	No	Retro peritoneal tumor	Not available	PPGL
44	Sangster *et al* [10]	50	Male	Not available	Head	Yes	Poorly differentiatedcarcinoma	Radiation treatment	PPGL
45	Lightfoot *et al*[51]	66	Male	6 cm	Head/uncinate	No	None	Pancreaticoduodenectomy	PPGL
46	Abbasi *et al* [52]	61	Female	7.2 cm	Head/uncinate	Yes	NET	Pancreaticoduodenenctomy	PPGL
47	Present case	73	Female	2 cm	Head	Yes	PPGL	No surgery	PPGL

PPGL: Pancreatic paraganglioma; PNET: Pancreatic Neuroendocrine tumor; EUS-FNA: Endoscopic ultrasound-guided fine needle aspiration.

[19]. Morphological patterns like acinar/glandular architecture and rosette-like arrangements can be observed in paragangliomas[20]. In histologic sections, the tumor is typically composed of nests of cells separated by a highly vascularized network[21].

Although the morphologic overlap between paraganglioma and NET is significant, the distinction can be confidently made with immunoperoxidase stains, which require a cell block preparation. Both pancreatic paragangliomas and NETs readily express neuroendocrine markers like synaptophysin and chromogranin[19]. While most NETs are immunoreactive to pancytokeratins (AE1/AE3 and CAM 5.2) but not vimentin, paragangliomas show the opposite profile[19]. GATA-3 and PAX-8 can also be used to distinguish paragangliomas from NETs. Paragangliomas from any anatomic site are immunoreactive to GATA-3 in approximately 55% of the cases, but NETs are always nonreactive[22]. Of note, GATA-3 can be positive in cells of breast, urothelial, and pancreatic origin[23]. PAX-8 has a sensitivity of 88% and specificity of 74% for primary pancreatic NETs, but paragangliomas have weak or negative immunoreactivity to PAX -8[24-26]. In our case, FNA smears with Papanicolaou stain showed abundant, tangled cellular processes and relatively uniform nuclei with finely granular chromatin and indistinct nucleoli (Figure 2). FNA cell block with hematoxylin and eosin stain (Figure 3A), diffuse cytoplasmic staining with chromogranin (Figure 3B),

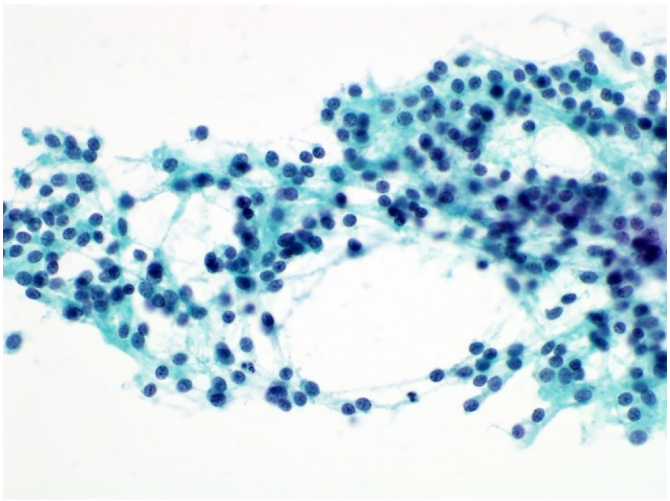

Figure 2 Fine needle aspiration direct smear. Papanicolaou stain, × 400.

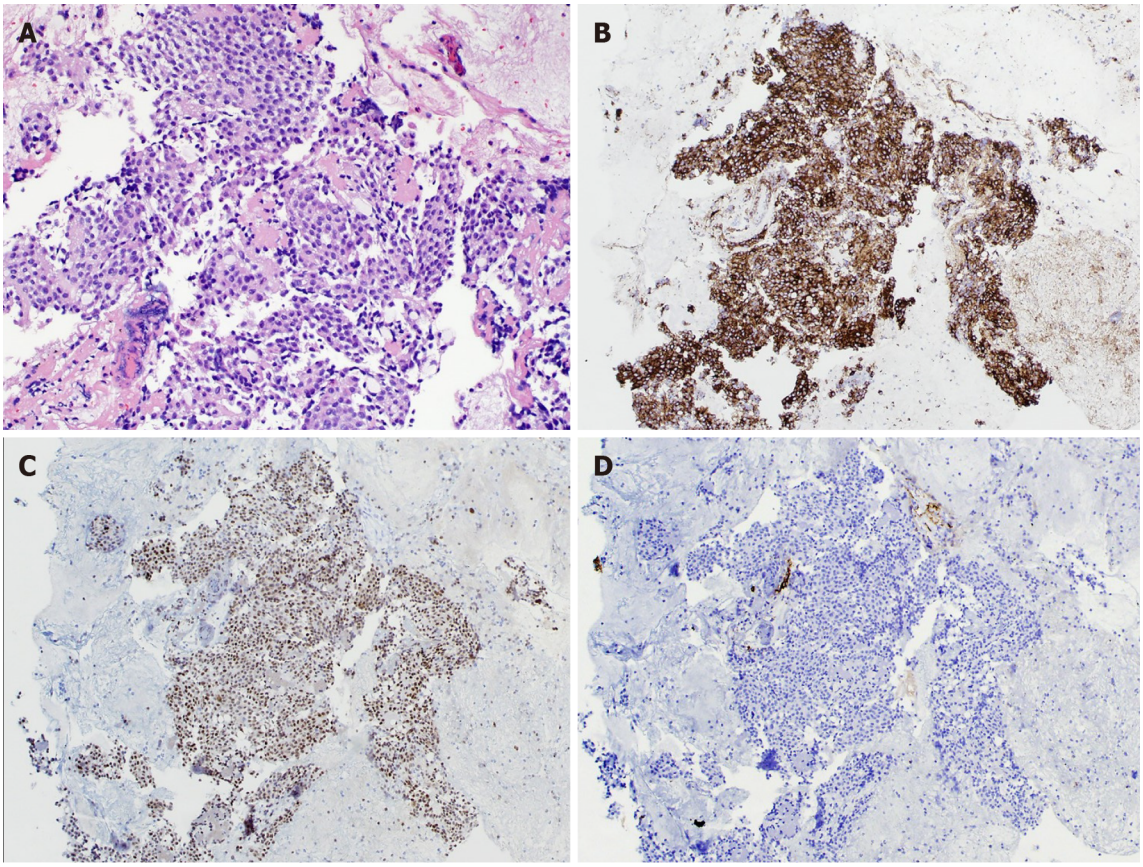

Figure 3 Fine needle aspiration cell block. A: Hematoxylin and eosin stain, × 200; B: Diffuse cytoplasmic staining, chromogranin (× 100); C: Diffuse nuclear staining, GATA-3, (× 100); D: No staining, keratin cocktail (× 100).

diffuse nuclear staining with GATA-3 (Figure 3C), and no staining with keratin cocktail (Figure 3D).

Since there are no definitive criteria for the diagnosis of malignancy in paraganglioma apart from metastasis, the treatment of choice for paraganglioma is surgical resection. For functional paragangliomas, preoperative administration of α-adrenergic receptor blocker can help prevent a hypertensive crisis during the surgery[27]. The most common sites of metastasis include the regional lymph nodes, bone, lung, and liver, and the dissemination usually occurs through blood or lymph nodes[28]. When surgery is not feasible, radiation therapy can be considered[29]. For malignant paragangliomas, treatment with I^{131} MIBG or combination chemotherapy (cyclophos-

phamide, vincristine, and dacarbazine) is effective[30]. Octreotide is also useful in inoperable paragangliomas[31].

Our review of the literature in the English language found 47 case reports of pancreatic paragangliomas published in PubMed to date (Table 1). Fifteen patients with pancreatic paragangliomas underwent EUS-FNA; six were correctly diagnosed as paraganglioma; six were misdiagnosed as NET; one had no diagnosis; one was diagnosed as spindle cell neoplasm; and one was diagnosed as pseudocyst. All patients with a pancreatic paraganglioma underwent surgery except three: one patient who developed a hypertensive crisis during the surgery (thus surgery was aborted) [32]; and two patients with metastasis or involvement of the major vessels[10,16].

Our case illustrates that accurate preoperative diagnosis of paraganglioma can be safely made by EUS-FNA. When paragangliomas are small and asymptomatic, it would be reasonable to follow them with periodic imaging studies.

CONCLUSION

Pancreatic paragangliomas are rare and EUS-FNA is a valuable tool in establishing the diagnosis. When assessing a lesion in the pancreas, paraganglioma should be included in the differential diagnoses along with PNET and pancreatic ductal adenocarcinoma. As EUS-FNA can trigger a hypertensive crisis in functional pancreatic paragangliomas, pre-procedure use of alpha-adrenergic blocker should be considered. To bring the optimal result in the management of paraganglioma, it is imperative to have a multidisciplinary team approach involving radiologists, advanced endoscopists, endocrinologists, pathologists, oncologists, and surgeons.

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CASE REPORT

Abdominal cocoon in children: A case report and review of literature

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Abstract

BACKGROUND

Abdominal cocoon or "encapsulating peritoneal sclerosis" (EPS) is an uncommon and rare cause of intestinal obstruction. Only a few cases have been reported in paediatric patients. Typically, EPS is described as the primary form in young adolescent girls from tropical and subtropical countries because of viral peritonitis due to retrograde menstruation or a history of peritoneal dialysis. Most patients are asymptomatic or present with abdominal pain, which is likely to occur secondary to subacute bowel obstruction. Findings at imaging, such as ultrasound, computed tomography, and magnetic resonance imaging, are often nonspecific. When diagnosed, EPS is characterized by total or partial encasement of the bowel within a thick fibrocollagenous membrane that envelopes the small intestine in the form of a cocoon because of chronic intraabdominal fibroinflammatory processes. The membrane forms a fibrous tissue sheet that covers, fixes, and finely constricts the gut, compromising its motility.

CASE SUMMARY

We present a case of EPS in a 12-year-old boy 8 wk after primary surgery for resection of symptomatic jejunal angiodysplasia. There was no history of peritoneal dialysis or drug intake.

CONCLUSION

In this report, we sought to highlight the diagnostic, surgical, and histopathological characteristics and review the current literature on EPS in paediatric patients.

Key Words: Abdominal cocoon; Peritoneal encapsulation; Encapsulating peritoneal sclerosis; Intestinal obstruction; Children; Case report

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Core Tip: Abdominal cocoon syndrome is rare in children. Cases with no history of previous peritoneal dialysis are practically nonexistent. Our report of a 12-year-old boy reveals that abdominal surgery can be a trigger for the development of encapsulating peritoneal sclerosis (EPS). Indications for surgery for EPS are usually due to mechanical ileus, and the final diagnosis is made intraoperatively. Surgical therapy for EPS is the first choice, and total intestinal enterolysis of EPS seems to be the best approach. The long-term prognosis for children is good.

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INTRODUCTION

Encapsulating bowel disease is a very rare but serious condition. The differentiation between congenital peritoneal encapsulation (CPE) and encapsulating peritoneal sclerosis (EPS) is obvious. CPE, reported for the first time in 1868 by Cleland[1], is the result of an embryological intestinal malformation that occurs when the yolk sac returns to the abdominal cavity, creating an accessory membrane covering the bowel. This is to be distinguished from EPS, which is far more common than CPE and was first described in 1907 by Owtschinnikow[2] with the term "peritonitis chronica fibrosa incapsulata". In 1987, Foo *et al*[3] used the term "abdominal cocoon" for primary or idiopathic EPS in cases where no established cause could be identified.

EPS has been recognized to occur in various geographically and ethnically diverse locations. In addition to various terms, such as "peritoneal sclerosis, peritoneal fibrosis, sclerosing peritonitis, sclerotic thickening of the peritoneal membrane, sclerosing obstructive peritonitis or encapsulating peritonitis", EPS is the most common name[4]. Because inflammatory features are not always apparent, the use of the word peritonitis, as in the general terminology, is not preferred. ESP has been proposed as the appropriate term by the International Society for Peritoneal Dialysis when peritoneal dialysis is the reason for the ubiquitous inflammatory processes that cause cocoon formations. Today, most authors use the term abdominal cocoon not only for the primary or idiopathic form but also for the secondary form of EPS[5-8] (Figure 1). The primary type is classically described in young adolescent girls from tropical and subtropical countries, which is mostly idiopathic in nature. A few of the etiopathogenesis proposed for the primary form are viral peritonitis, retrograde menstruation with superimposed viral infection, and gynecological infection-inducing cell-mediated immunological tissue damage. However, these theories may not explain the etiopathogenesis in all patients, as this condition is also seen in men, premenstrual women, and children. The secondary form can occur secondary to multiple predisposing factors in addition to peritoneal dialysis, *e.g.*, peritoneum inflammation with fibroblastic proliferation, systemic lupus erythematosus, liver cirrhosis, endometriotic cyst, abdominal trauma/surgery, abdominal tuberculosis, or malignancy[9,10].

EPS is characterized by total or partial encasement of the bowel within a thick fibrocollagenous membrane that envelopes the small intestine in the form of a cocoon as a result of chronic intraabdominal fibroinflammatory processes[11]. The result is the formation of fibrous tissue sheets that cover, fix, and ultimately constrict the gut, compromising its motility. Most patients are asymptomatic or present with (recurrent) abdominal pain, which is likely to occur secondary to partial/complete bowel obstruction. Usually, patients have had prior episodes with similar symptoms that have resolved spontaneously. With the development of complete sclerosis due to the formation of a cocoon, there are overt signs of mechanical ileus.

Clinically, the abdomen is often soft at palpation. A soft nontender mass may be palpable in the central part of the abdomen, which represents clumped-up bowel loops. Imaging findings seen on ultrasound (US), computed tomography (CT), and magnetic resonance imaging (MRI) are often nonspecific[7]. Other imaging modalities, such as barium studies or positron emission tomography studies, have been discussed and have not revealed any appreciable advantage in the diagnosis of EPS. Therefore,

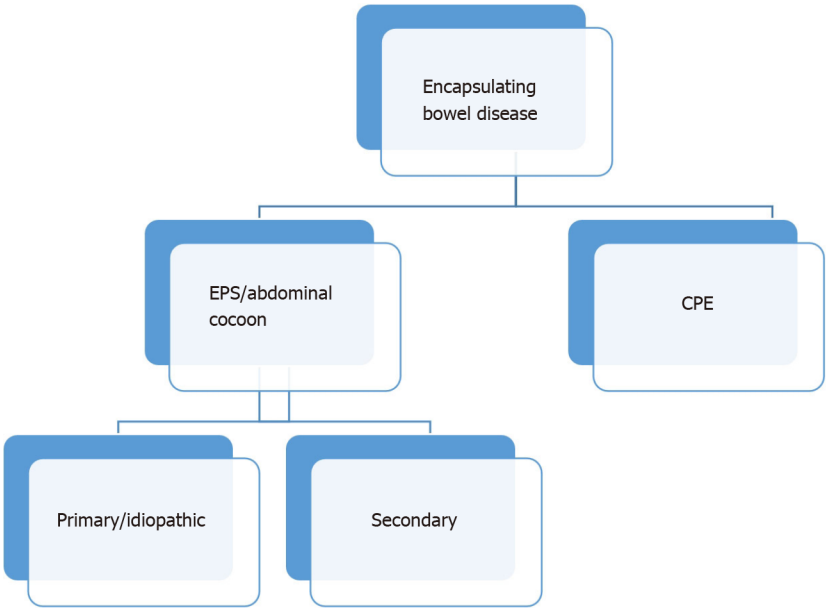

Figure 1 Flow chart and classification for encapsulating bowel diseases. EPS: Encapsulating peritoneal sclerosis; CPE: Congenital peritoneal encapsulation.

this condition is often only diagnosed intraoperatively, followed by histological analysis[12].

EPS has a clearly different histology than CPE. The thin membrane on the visceral peritoneum contributes to the formation of the intestinal encapsulation of EPS. Histologically, the membrane is comprised mainly of organized fibrin, probably derived from plasma exudation from the peritoneal microvasculature. Peritoneal fibroblasts appear swollen and exhibit an increased level of cellularity, accompanied by the expression of various activation and proliferation markers. Persistent inflammatory changes are also predictive of the onset of EPS[4].

The development of EPS has been divided, taking into account clinical and pathological findings in four stages, as proposed by Nakamoto[13]. The pre-EPS stage is characterized by the presence of ultrafiltration failure and/or altered solute transport status; this is followed by inflammatory, encapsulating, and obstructive phases, the last of which is characterized by intestinal obstruction and the formation of an "abdominal cocoon". A staging system has been proposed in the peritoneal catheter (PD)-associated EPS literature based on a combination of clinical, laboratory, and radiographic findings. Nakamoto[13] categorized patients with EPS into Stage 1 (pre-EPS), Stage 2 (inflammatory), Stage 3 (encapsulating), and Stage 4 (chronic) based on abdominal symptoms, inflammation, encapsulation, and intestinal findings (Figure 2). Different conservative therapeutic approaches have been proposed depending on the stage of the disease, which include nutritional support, immunosuppression with corticosteroids as the best studied, colchicine, azathioprine, mycophenolate mofetil, cyclosporine, and mammalian target of rapamycin. Furthermore, antifibrotics such as tamoxifen can increase the efficiency of immunosuppressive drugs.

The division into four disease stages enables stage-appropriate therapy for EPS. Table 1 shows the stage-dependent therapy of EPS according to Nakamoto[13]. For a long time, surgical treatment of EPS was not considered appropriate due to the high perioperative mortality rate of up to 70%. On the other hand, several studies showed that, independent of the therapy procedure, the overall mortality in EPS patients was 67%. In a recent study, the mortality in surgically treated patients was only 42%. This reduction in mortality can only be achieved by timely and adequate surgical therapy. However, to date, there is neither a uniform surgical therapy concept nor standardized perioperative management.

To our knowledge, there has been no prior definitive, systematic review of EPS in children without a history of PD. Due to the assumption that younger patients are at greater risk for EPS, it is important to highlight the age groups of the patients.

In the following, we present a case of a 12-year-old boy with EPS 8 wk after primary laparotomy. We report his medical history, clinical presentation at admission, laboratory values, preoperative diagnostic and intraoperative findings, histological results, and days until discharge (short-term outcomes). The main objective of the

Table 1 Summary of therapy options of encapsulating peritoneal sclerosis according to the staging system

Stage	Therapy options
Stage 1/pre-EPS	Discontinuation of pertioneal dialysis
	("Sedation" of the peritoneum)
	Peritoneal lavage
	Glucocorticoids
Stage 2	Glucocorticoids
Stage 3	Glucocorticoids
	Total parenteral nutrition
Stage 4	Operation

EPS: Encapsulating peritoneal sclerosis.

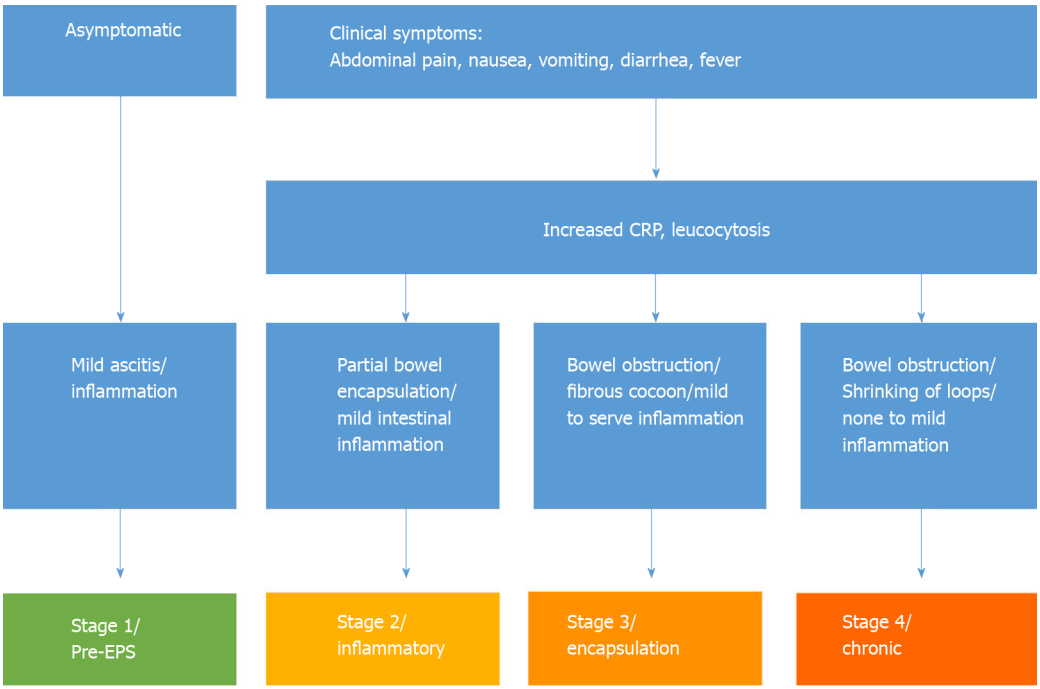

Figure 2 Stages of encapsulating peritoneal sclerosis with associated clinical, serologic, and intestinal findings. EPS: Encapsulating peritoneal sclerosis; CRP: C-reactive protein.

present study was to perform a systematic review of the literature to identify studies on EPS/abdominal cocoons in children. For review, we scanned PubMed, Medline, and Google Scholar search engines for articles containing the terms "EPS", "abdominal cocoon", "abdominal cocoon syndrome", "encapsulating bowel disease", and "SEP", children and paediatric" published from January 1990 to January 2020. Terms were entered alone or in combinations.

We included all articles reporting case series or case reports in the English and German languages. All series and case reports reporting patients younger than 18 years were included (quantitative screening). Further eligibility criteria were a report of the clinical presentation, sex and age, operation as the treatment, and histopathological findings (qualitative screening). Data for all clinical, diagnostic, therapeutic, and etiologic findings were collected from the different databases. We included primary (idiopathic) and secondary forms [abdominal surgery, abdominal trauma, abdominal tuberculosis, peritoneal shunts, liver transplantation, recurrent peritonitis, beta-blocker treatment (practolol or propranolol), chemotherapy, gastrointestinal malignancy, systemic lupus erythaematosus, or parasitic infection] of EPS with the exception of EPS due to PD. We excluded PD-associated EPS because, as mentioned above, in these cases, ESP is the appropriate term with inflammatory processes in the

cocoon, such as formation of the bowel. We then created a PRISMA flow chart showing the results of the literature search (Figure 3).

CASE PRESENTATION

Chief complaints

A 12-year-old boy with Xq24 deletion syndrome presented with symptoms of intermittent intestinal obstruction 8 wk after primary surgery.

History of present illness

The child was symptomatic with a distended abdomen, refusal of feeds, and episodes of abdominal pain. Furthermore, he presented with large gastric residuals that became bilious in the next week.

History of past illness

Eight weeks previously, he presented with suspected intestinal bleeding without further symptoms. US, abdominal MRI, endoscopy, and laparoscopy did not reveal the cause of the chronic bleeding. Specialized angiographic MRI was suspicious for a small intraluminal vascular tumour in the ileum. Limited laparotomy and meticulous palpation of the small bowel enabled the identification of the tumour in the lower ileum. Limited small bowel resection and primary anastomosis were performed. Further clinical investigation of the whole small and large bowel did not reveal any further pathological findings. Histopathological examination showed a venous malformation (2.5 cm × 2.0 cm) at the transition of the jejunum to the ileum. The following clinical course was uneventful. The boy was discharged on the 5th postoperative day.

Personal and family history

Except for Xq24 deletion syndrome, there was no other personal or family history of acute or chronic disease.

Physical examination

On physical examination, the patient showed a distended abdomen and abdominal pain. The examination of the heart, lungs, and further organ status showed no abnormalities. Due to his Xq24 deletion syndrome, he showed psychomotor delay, intellectual disability, and a characteristic craniofacial dysmorphism.

Laboratory examinations

Laboratory findings showed slightly elevated C-reactive protein (0.3 mg/dL) and normal findings across the rest of the blood panel.

Imaging examinations

The abdominal US showed pendulum peristalsis and free fluid in the abdominal cavity, and a plain abdominal X-ray showed some air fluid levels (Figure 4A). A gastrointestinal contrast study was performed, which revealed distended small bowel loops (maximum diameter of 50 mm) and narrow segments of the large intestine. There was appropriate intestinal transit, with the presence of contrast in the jejunal and ileal loops after 1 h of administration. The contrast agent arrived at the terminal ileum and caecum at 2 h of intestinal transit. Due to the inconclusive results, we performed MRI, which revealed a distended duodenum and small bowel loops with multiple intraluminal air fluid levels and thickened walls but with no signs of obstruction (Figure 4B).

FINAL DIAGNOSIS

Due to the clinical symptoms of obstructive bowel disease and inconclusive radiological signs, the patient underwent relaparotomy. Intraoperatively, we found a completely encapsulated small bowel (Figure 5). The massive, fibroconnective membrane encapsulated the bowel from the ligament of Treitz to the ileocecal junction. The thick, firm, and white membrane was not attached to the parietal peritoneum. Microscopic findings showed partially mesothelium-covered, predom-

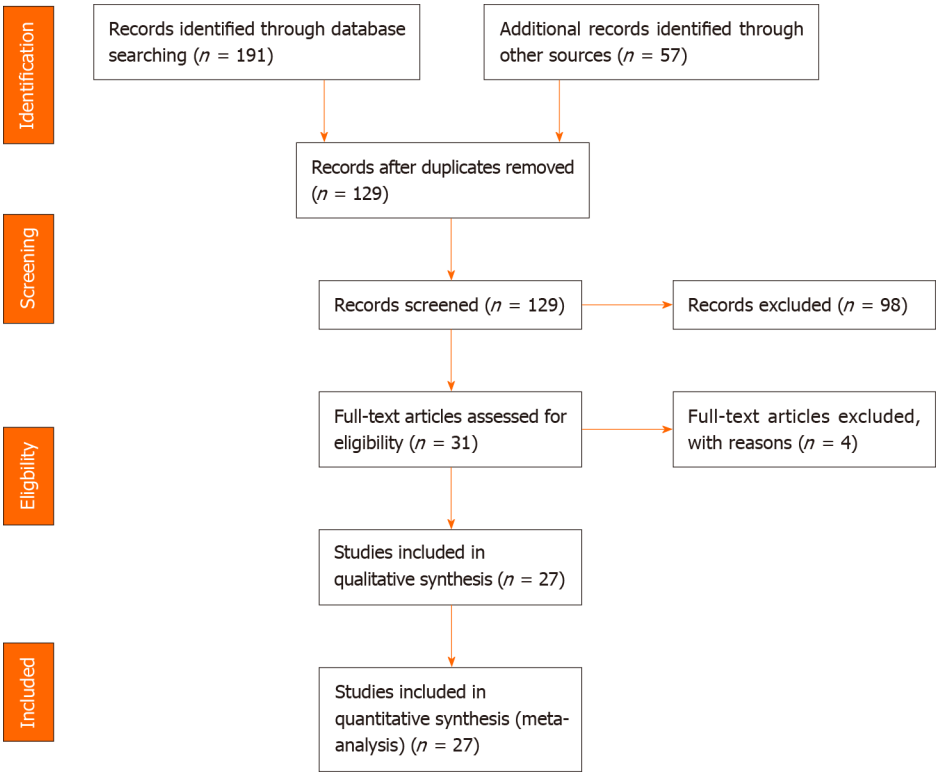

Figure 3 PRSIMA Flow chart showing the results of the literature search.

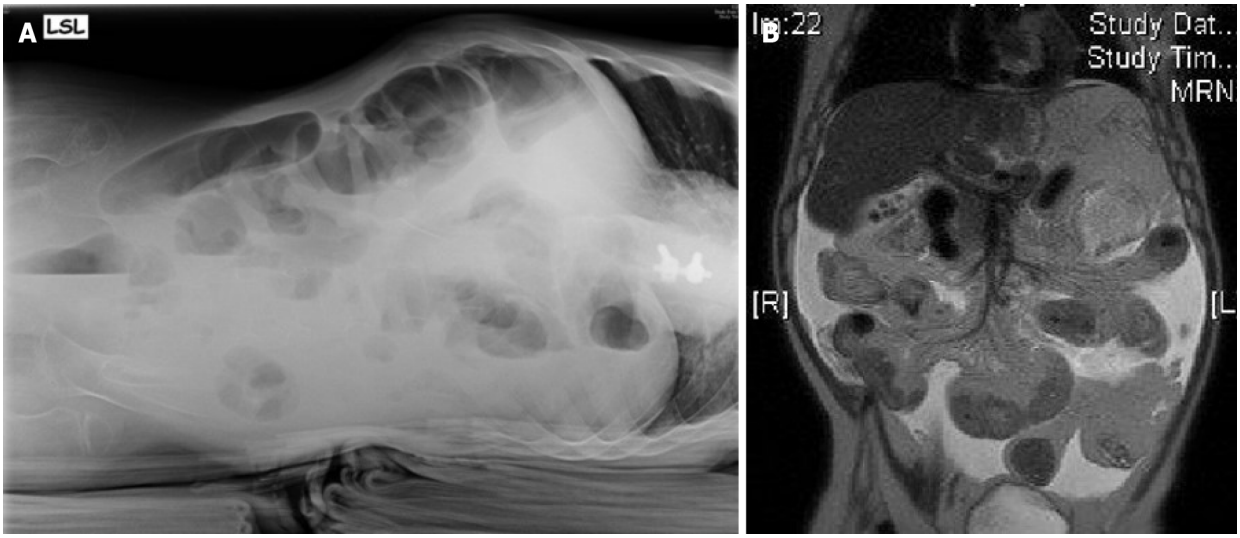

Figure 4 Diagnostic imaging. A: X-ray with lateral abdominal view showing some air fluid levels; B: Magnetic resonance imaging in coronal plane with distended duodenum and small bowel loops with multiple intraluminal air fluid levels and thickened walls.

inantly edematous loosened fibre-rich connective tissue with prominent, predominantly small vessels with a prominent endothelial lining with hyperchromatic oval nuclei, mostly positive for CD31 and CD34. No evidence of malignancy was found (Figure 6).

TREATMENT

We performed extensive adhesiolysis by peeling the fibrous pseudocapsulae from the small bowel. This resulted in freeing of the small bowel but also in multiple serosal injuries with diffuse bleeding. No bowel perforation occurred.

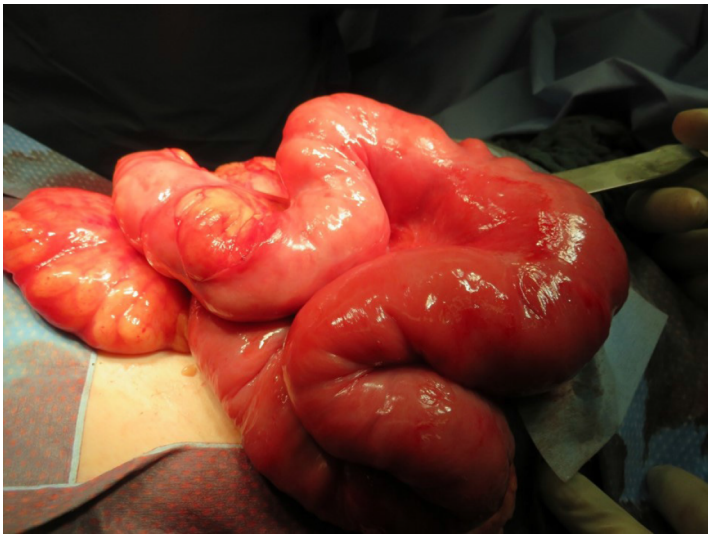

Figure 5 The small bowel is shortened, considerably thickened, and covered with a fibrous membrane, which separates and constricts the entire small intestine from approximately 10 cm distal to Treitz's ligament to approximately 5 cm proximal to the ileocecal junction.

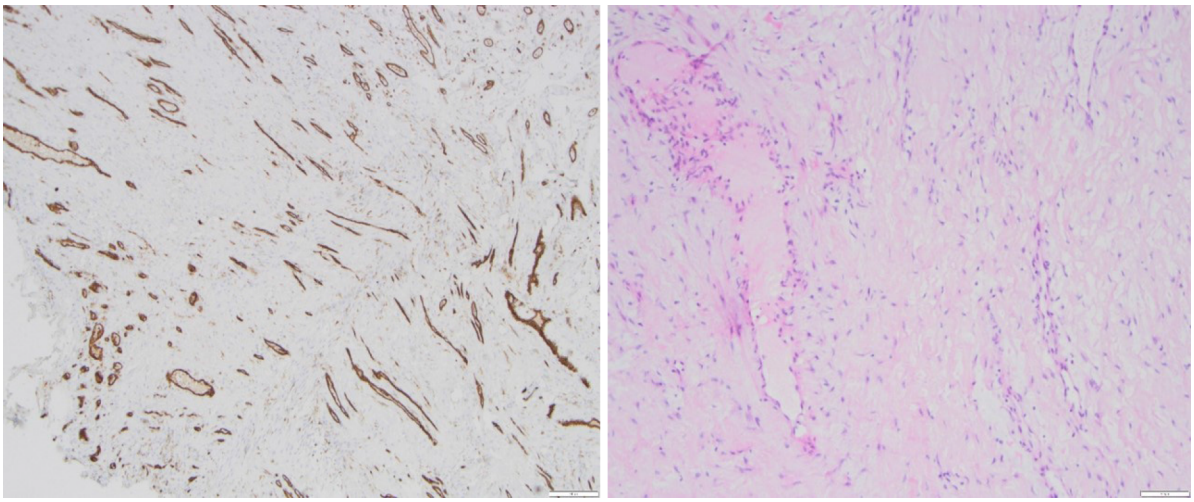

Figure 6 Microscopic findings showed partially mesothelium-covered, predominantly edematous loosened fiber-rich connective tissue with prominent, predominantly small vessels with prominent endothelial lining with hyperchromatic oval nuclei.

OUTCOME AND FOLLOW-UP

The following clinical course was prolonged due to peritonitis, massive ascites, and paralytic ileus. Eventually, 2 wk after surgery, the first feeds could be established, and the child was discharged on the 23rd postoperative day. The follow-up is now 12 mo and uneventful. A summary of the most relevant aspects of the case is listed in Table 2.

DISCUSSION

Literature review

Articles identified after reviewing and applying the inclusion criteria are listed in Table 3. No comparable case could be found in children presenting with EPS after surgery. Sahoo *et al*[14] reported EPS as an idiopathic form in a case series of 4 patients (3 boys, 1 girl) between 6 and 8 years of age, and Mehta *et al*[15] and Bassiouny *et al*[16] reported EPS in a 7-year-old boy without underlying disease. Ahmad *et al*[17] mentioned a case report of EPS in a newborn as a consequence of meconium peritonitis. In total, 27 articles could be found reporting 33 patients (Table 3). Among these patients, 24 were girls and 9 were boys. Their ages ranged from 2 d to 17 years.

Table 2 Summery of our patient data

Age	Clinical symptoms	Labatory findings	Intraoperive findings	Histology
11 yr	Abdominal pain; Distended abdomen; Vomiting (bilious)	CRP: 0.3 mg/dL	Complete; encapsulation of the small bowel	Fibrous connective tissue with prominent vas-cularization, predomi-nantly from the venous type

CRP: C-reactive protein.

Especially in older patients (≥ 10 years of age), EPS was detected more often in girls (f:m = 22:2). Various radiological modalities for diagnosis (singly or in addition) were reported, such as abdominal X-rays (n = 21), US (n = 18), contrast studies (n = 8), and CT scans (n = 11). In three cases, no diagnostic tool was mentioned. Thirty-two patients underwent surgery (laparotomy), while 1 patient underwent conservative treatment with antitubercular medications. When laparotomy was performed, adhesiolysis and excision was the treatment of the choice (n = 32). Two patients underwent appendectomy simultaneously, and two patients underwent temporary enterostomy due to pronounced bowel inflammation. In 3 patients, limited bowel resection, including the ileocecal region, was performed since it was too difficult to incise and release the envelope without injuring the intestines. In all reported cases with surgical treatment, the operation and postoperative period were uneventful.

Discussion

The clinical picture of EPS is largely unknown in the medical world[18]. The embryonal condition is characterized by an accessory peritoneal sac derived from the peritoneum of the yolk sac as it is withdrawn into the abdominal cavity during the 12th week of gestation[3]. Only a few risk factors are known[9]. This disease can occur as a late consequence of long-term peritoneal dialysis[19-23]. However, even this is not necessarily always the case, since cases are known in which EPS is already visible shortly after initiation of PD therapy[11]. In these cases, the mortality depends on the duration of PD, ranging between 30% and 60%. In other cases where EPS is not associated with PD, various reports have reported beta blockers as the cause of EPS [10]. In animal models, practolol inhibits the release of surfactant from type II pneumocytes, resulting in damage to the abdominal serosa[24]. Tumours with peritoneal dissemination and infectious peritonitis are other frequently mentioned risk factors for EPS, supported by epidemiological data. Pathogens significantly increase the risk, highlighting *Staphylococcus aureus*, fungi, *Pseudomonas* spp., and *Haemophilus influenza*[3,4,10]. Fowler *et al*[25] suggested echovirus infection as an etiologic factor, and Foo *et al*[3] thought tuberculosis could be involved.

Several theories have been formulated about the pathogenesis of idiopathic EPS [26], for example, retrograde menstruation due to virus infection or pelvic peritonitis with tissue damage incurred by cell-mediated immunity[3,5,11]. Retrograde menstruation explains the relatively high number of adolescent girls with idiopathic abdominal cocoons (Table 3). Moreover, several tumour entities and/or abdominal operations may lead to adhesions and peritoneal fibrosis[27-29]. Therefore, overall, the most common theory is developing a membrane in the form of sclerosis secondary to peritonitis[17,18]. Prospectively controlled randomized studies are lacking, and most studies on EPS present small patient collections over a long period of time[10-13].

The presented case is very unusual because of the child's young age, the atypical geographical region, and a very rare cause (secondary to abdominal surgery). Most of the other publications with paediatric patient collections are secondary forms after tuberculosis infection or after prolonged PD. The short time interval to the primary laparotomy in our patient is a special feature that has not been described in the literature thus far. EPS after kidney transplantation (as an abdominal surgical intervention) has been reported in case reports; however, these patients also had a history of PD or chronic kidney diseases[30]. In many cases of idiopathic EPS, no correlation can be detected with any potential risk factor[4,31-33]. Nevertheless, two considerations are important: First, in these forms, peritoneal disorders are often associated with general connective tissue damage, particularly of the serous membranes, such as abdominal trauma; this indicates a major role of immunopathogenesis in EPS. The second factor can be a genetic predisposition, which is suggested due to the high frequency of EPS in women from subtropical regions and familial forms such as multifocal family fibrosclerosis.

Table 3 Systematic literature review-patient data

Ref.	Age/sex	Clinical presantation	Diagnostic	Etiology	Treatment
Ahmad *et al*[17], 2013	2 d/m	Polyhydramnion, vomiting, abdominal distension, failure to pass meconium	US, X-ray	Meconium peritonitis	Surgery
Sahoo *et al*[14] 1996	6 yr/m	Abdominal pain, bilious vomiting	US, X-ray, BMFT	Idiopathic	Surgery
Mehta *et al*[15], 1994	7 yr/m	Colicky periumbilical pain, vomiting, constipation	US, X-ray	Idiopathic	Surgery
Bassiouny *et al*[16], 2011	7 yr/m	Crampy abdominal pain	X-ray, contrast enema	Idiopathic	Surgery
Sahoo *et al*[14], 1996	6 yr/m	Constipation, on-and-off vomiting	US, X-ray, BMFT	Idiopathic	Surgery
Tolstrup *et al*[35], 2017	8 yr/f	Signs of enteritis	CT	Idiopathic	Surgery
Sahoo *et al*[14], 1996	8 yr/f	Intermittent bilious vomiting, abdominal distension	BMFT	Idiopathic	Surgery
Sahoo *et al*[14], 1996	8 yr/m	Colicky abdominal pain, vomiting, constipation	X-ray	Idiopathic	Surgery
Kaushik *et al*[10], 2006	9 yr/m	Abdominal pain, vomiting, constipation	-	Abdominal tuberculosis	Surgery (with temp. enterostomy)
Okobia *et al*[37], 2001	10 yr/f	Abdominal pain, abdominal distension, weight loss	X-ray	Idiopathic	Surgery (with appendectomy)
Bassiouny *et al*[16], 2011	12 yr/f	Colicky abdominal pain, vomiting	US, X-ray	Idiopathic	Surgery
Okobia *et al*[37], 2001	12 yr/f	Colicky abdominal pain, abdominal distension	US, X-ray	Idiopathic	Surgery (with appendectomy)
Dehn *et al*[29], 1985	12 yr/f	Abdominal pain, bilious vomiting	US, X-ray	Idiopathic	Surgery
Kayastha *et al*[23], 2012	13 yr/f	Abdominal pain in tright iliac region, vomiting	US	Idiopathic	Surgery
Sreevathsa *et al*[33], 2013	13 yr/f	Colicky central abdominal pain	US, X-ray, BMFT	Idiopathic	Surgery (with ileocaecal resection)
Pillai *et al*[26], 2006	13 yr/f	Abdominal pain, vomiting	US, X-ray, CT	Idiopathic	Surgery
Chatura *et al*[40], 2012	14 yr/f	Colicky abdominal pain	US	Idiopathic	Surgery (with ileocolectomy)
Ibrahim *et al*[41], 2009	14 yr/m	Colicky abdominal pain, vomiting, constipation	X-ray	Idiopathic	Surgery (with appendectomy)
Calvo *et al*[42], 2015	14 yr/f	Colicky abdominal pain, bilious vomiting, weight loss	US, CT	Idiopathic	Surgery
Shah *et al*[43], 2013	14 yr/f	Colicky abdominal pain, vomiting	CT / BMFT	Idiopathic	Surgery
Sreevathsa *et al*[33], 2013	14 yr/f	Colicky central abdominal pain	US, X-ray, BMFT	Idiopathic	Surgery (with ileocaecal resection)
Kumar *et al*[44], 2012	14 yr/f	Colicky abdominal pain, vomiting	US, CT	Idiopathic	Conservatively
Yucel *et al*[53], 2011	15 yr/f	Abdominal pain	X-ray, CT	Idiopathic	Surgery
Raju[45], 2004	15 yr/f	Recurrent abdominal pain	X-ray, contrast study	Idiopathic	Surgery
Ndiaye *et al*[46], 2012	15 yr/f	Subocclusive obstruction, loss of weight (11kg)	CT	Idiopathic	Surgery
Choudhury *et al*[47], 2009	15 yr/f	Crampy abdominal pain right abdomen, weight loss	-	Idiopathic	Surgery (with partial ileocolic resection)
Mordehai *et al*[48], 2001	15 yr/f	Crampy abdominal pain	US, X-ray	Idiopathic	Surgery
Mohanty *et al*[49], 2009	15 yr/f	Colicky abdominal pain, vomiting	US, X-ray, CT	Idiopathic	Surgery
Kumar *et al*[36], 2009	16 yr/f	Abdominal pain in the left lower abdomen, vomiting	US, X-ray, CT	Idiopathic	Surgery
Jayant *et al*[50], 2011	16 yr/f	Small bowel obstruction	CT	Idiopathic	Surgery

Kaushik *et al*[10], 2006	17 yr/m	Abdominal pain, vomiting, constipation	-	Abdominal tuberculosis	Surgery (with temp. enterostomy)
Naniwadekar *et al* [51], 2014	17 yr/f	Abdominal pain, vomiting, constipation	US, X-ray, GI scopy	Idiopathic	Surgery
Kaur *et al*[52], 2012	17 yr/f	Recurrent abdominal pain	US, X-ray, CT	Idiopathic	Surgery

BMFT: Barium meal follow-through; CT: Computer tomography; f: Female; GI: Gastrointestinal; m: Male; US: Ultrasound.

In reports including patients with long-term PD, pathogenesis is often described with the "two-hit"-hypothesis[3,4] when disruption of normal peritoneal/mesothelial physiology is a result of extensive PD over a period of years and predisposes the individual to a second hit that triggers the process. This second hit may take the form of an episode of peritonitis, discontinuing PD, or an acute intra-abdominal event. In our case, this hypothesis is unlikely because he was a non-PD patient, and there was a very short period between the two events. Therefore, the reason for the development of EPS in our patient must be found in the previous operation with resection of lymphangioma as an abdominal trauma and trigger for sclerosis. Another reason for developing EPS could be cystic lymphangioma of the small bowel itself as a benign vascular tumour with peritoneal dissemination. Garosi[19] and Célicout *et al*[34] described different tumour entities in connection with EPS in patients without PD, including histiocytic lymphoma, gastric, ovarian, pancreatic and renal cell carcinoma, and oedematous polyposis of the colon. Cystic lymphangioma as a trigger for EPS could not be found in the literature thus far. Because the lymphangioma was resected in total during the first operation, we believe that primary abdominal surgery was the trigger for EPS in our patient.

Diagnostic modalities

Because the early clinical features are often nonspecific[35,36], it is difficult to make a definite preoperative diagnosis of EPS. There are no specific serological markers for EPS. Ultrasonography may show dilated small bowel loops, ascites with or without parietal loculations, and a membrane covering the small bowel loops with occasional formation of a mass of bowel loops and adhesions[37]. Abdominal X-rays have a low sensitivity, showing mostly dilated small bowel loops with multiple air fluid levels. Peritoneal and bowel wall calcification has also been reported. Abdominal CT has a higher sensitivity (70%-90%) to detect the etiology of high-grade small bowel obstruction but should be used cautiously due to radiation risks in young patients. In our search of the literature, MRI has not been reported in paediatric patients with abdominal cocoons. Nevertheless, MRI has been recently reported as another useful modality in diagnosing EPS and may be a preferred technique in children.

Surgical procedures

In the international literature, two surgical therapy approaches for EPS have been described but were only reported in studies within adult patient collections. In 1998, C élicout *et al*[34] published a study in which 3 of 32 patients had dialysis-associated EPS. In the remaining patients, the EPS was the result of a malignancy (14 of 32) or due to b-blocker intake (5 of 32). The indication for the operation was, in all cases, the ultima ratio after long-lasting ileus disease. Japanese reports are based on studies that only included PD patients[38]. The intraoperative approach in Japan includes removal of the interenteric adhesions and the detachment of the encapsulating small intestinal membranes. The occlusive membranes on the intestine are dissected through longitudinal incisions. However, peritoneal sclerosis makes it difficult to identify the "right" layer, so special attention must be paid to serosa lesions. These are insidious because they can lead to occult perforations postoperatively with an increase in small bowel motility and the resulting increased pressure. In case of doubt, any altered segments of the small intestine are therefore resected. In Japan, in suitable cases, solitary stenoses are dilated with a Miller-Abott probe from the intraluminal side. Thus, even tandem stenoses can be corrected without resection. The balloon is deflated after completion of the dilatation and left *in situ* for splinting of the intestine. Usually, the probe is removed after 1 wk. In the opinion of the Japanese authors, the Noble plicature can prevent reobstruction by kinking as well as the reoccurrence of adhesions or entrapment of the small intestine in the Douglas cavity or in the excavation rectovesicalis, respectively.

The use of probes for intestinal splinting and the methods of plication have been discussed very controversially in Germany and have only historical significance. In our opinion, these methods, which carry their own risk, are not reliable enough to prevent the recurrence of ileus disease. In an international comparison, Japan has the most experience with EPS.

In addition to total intestinal enterolysis, which was performed in all cases with surgical treatment, deserosation, and decapsulation, peritonectomies, fibrin membranectomy, and omentectomies were performed in accordance with the findings. Additionally, an opening of the bursa omentalis for the decompression of fluid or for complete unleashing of the stomach may be essential.

There are hardly any reports from the Anglo-American region regarding surgical therapy for EPS. Studies from Australia[39] and Canada[8] mostly refer to conservative therapy for the disease. The surgical procedure is only mentioned as laparotomy and adhesiolysis. Therefore, our strategy was like the international approach with the avocation of extensive enterolysis and detachment of the encapsulating small intestinal membranes.

Postoperative complications

In work published in 1998 by Célicout *et al*[34], the authors showed a mortality rate of 19% and an anastomosis failure rate of 12%. The total complication rate of 38% was the lowest at that time in the literature but was still considered too high[21]. Ten years later, Kawaguchi *et al*[5] published a retrospective study that included 130 surgically treated EPS patients. The reported mortality was 6.9%. However, the recurrence rate was very high (25.4%). Of the 130 patients, 33 had to be reoperated on for recurrence or postoperative ileus, and some patients underwent reoperations up to six times. In our reported case, as well as in the reports with paediatric patients, there were no complications after the operation, and no recurrence was described.

Histological examination

A few histological features, such as fibrin deposits, fibroblast swelling, angiogenesis, and mononuclear inflammatory infiltrate, differentiate peritoneal fibrosis from actual EPS. Since this means that even fewer predictive histological features are available, the German Peritoneal Biopsy Register was established at the initiative of the German Society of Nephrology. Here, we present prospective histomorphology characteristics of peritoneal biopsies with the aim of analysing the pathogenesis of the disease and producing reproducible algorithms for histological diagnosis confirmation. Therefore, the histological evaluation of EPS can now be increasingly based on standardized criteria. The Institute of Pathology at the Robert Bosch Hospital has become the reference centre for the histomorphology evaluation of peritoneal biopsies of the "European EPS Working Group". Our histological results showed fibrous connective tissue with prominent vascularization, predominantly from the venous type, matching an early form of EPS.

CONCLUSION

The diagnosis of EPS is mainly made intraoperatively, and surgery is often indicated due to symptomatic ileus. A history of abdominal trauma, surgery, or abdominal tumour can be a trigger for developing EPS. Surgery, including adhesiolysis and excision, is the first choice of treatment. Total intestinal enterolysis of EPS is the best surgical approach. The long-term prognosis for children is good.

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LETTER TO THE EDITOR

Gastrointestinal symptoms in patients with COVID-19: Is there a relationship with mortality and new variations of SARS-CoV-2?

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Abstract

Coronavirus disease 2019 (COVID-19) is caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). Although, respiratory symptoms are typical the digestive system is also a susceptible target with gastrointestinal symptoms present even in the absence of respiratory symptoms. The gastrointestinal symptoms of COVID-19 include diarrhea, abdominal pain, anorexia, and nausea among other symptoms. Some questions that remain to be answered include: Do patients with gastrointestinal symptoms have a higher mortality? SARS-CoV-2 variants are already a global reality: Do these variants present with a greater prevalence of gastrointestinal symptoms? Do patients with these symptoms warrant more intensive care unit care?

Key Words: COVID-19; SARS-CoV-2; Gastrointestinal symptoms; Intensive care unit; Variant

Core Tip: With the emergence of new variants of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and its clinical manifestations, the following questions have arisen: are gastrointestinal symptoms and complications the same as the other variants or are they different? And are these related to severity of the disease? In this letter to the editor, we discuss the relationship between the new SARS-CoV-2 variants with gastrointestinal manifestations and the severity of disease with the new variants.

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TO THE EDITOR

We read with interest the article by Cao *et al*[1] titled "Coronavirus disease 2019 (COVID-19) and its effects on the digestive system."

In 2020, our team conducted a cohort study[2] evaluating 400 patients diagnosed with COVID-19 that, even though it is the largest study in Latin America, unfortunately, was not included in the author's review. In our study, 33.25% of patients reported one or more gastrointestinal symptoms, with diarrhea being the most common, representing approximately 17% of the total. This data corroborates the author's review. It was also found that when these patients had gastrointestinal symptoms, they had a greater tendency to have other concomitant symptoms, especially myalgia and fatigue ($P < 0.05$).

It was also observed that patients with chronic kidney disease ($P < 0.05$), using chronic immunosuppressants, or chronic use of angiotensin receptor blockers or angiotensin-converting enzyme inhibitors had a higher prevalence of gastrointestinal symptoms. Admission to the intensive care unit (ICU), need for mechanical ventilation, length of stay in the ICU, length of hospital stay, need for vasopressor support, laboratory results, and hospital mortality did not differ based on the presence of gastrointestinal symptoms ($P > 0.05$). Regression analyzes showed that immunosuppression [odds ratio (OR): 2.60 (95% confidence interval (CI): 1.20-5.63)], male sex [OR: 1.94 (95%CI: 1.12-3.36)], and older age [OR: 1.04 (95%CI: 1.02-1.06)] were associated with increased mortality.

As described above, our study[2] demonstrated, before the emergence of the new variants of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2)[3,4], that gastrointestinal symptoms were not associated with a greater need for ICU admission or the severity of the disease in patients in Latin America.

This is a very controversial topic; some studies suggest a less severe clinical evolution in patients with gastrointestinal symptoms[5,6], whereas in the study by Redd *et al*[7], there was no statistical difference.

The study by Leal *et al*[8], evaluating 234 European patients with digestive symptoms, also had a more favorable and non-severe prognosis.

In a review carried out by Wang *et al*[9], it was observed that pancreatic damage or mesenteric ischemia/thrombosis could increase mortality.

In the pediatric population, in the study by de Paula *et al*[10], logistic regression analysis identified that laboratory-confirmed COVID-19 pediatric patients with gastrointestinal symptoms had an increased risk of cardiac abnormalities confirmed by echocardiogram (OR: 6316; 95%CI: 1.717-79043; $P = 0.012$).

We emphasize that in all these studies there was no distinction regarding the variety of SARS-CoV-2 studied.

It is currently known that the high expression of angiotensin-converting enzyme 2 in the lung and the intestinal tract makes the small bowel and colon highly susceptible to SARS-CoV-2 infection, which offers a potential explanation for diarrhea observed in many COVID-19 patients. Since tryptophan absorption requires angiotensin-converting enzyme 2, its deficiency can alter the intestinal microbiota and cause intestinal inflammation[11].

Given the above, we believe a more realistic discussion to be made, and we ask the authors the following: (1) Do patients with gastrointestinal symptoms have more severe disease outcomes or not? (2) Do SARS-CoV-2 variants have a greater gastrointestinal involvement? (3) Do SARS-CoV-2 variants have a relationship between gastrointestinal symptoms and/or disease severity? and (4) Do these patients have a greater need for admission to ICUs?

Finally, we would like to congratulate and thank the authors on the level of shared evidence.

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