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## AIMS AND SCOPE

The primary aim of the *World Journal of Gastrointestinal Pathophysiology* (*WJGP*, *World J Gastrointest Pathophysiol*) is to provide scholars and readers from various fields of gastrointestinal pathophysiology with a platform to publish high-quality basic and clinical research articles and communicate their research findings online.

*WJGP* mainly publishes articles reporting research results and findings obtained in the field of gastrointestinal pathophysiology and covering a wide range of topics including disorders of the esophagus, stomach and duodenum, small intestines, pancreas, biliary system, and liver.

## INDEXING/ABSTRACTING

The *WJGP* is now abstracted and indexed in Emerging Sources Citation Index (Web of Science), PubMed, PubMed Central, China National Knowledge Infrastructure (CNKI), and Superstar Journals Database.

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FRONTIER

# Molecular and genetic markers in hepatocellular carcinoma: *In silico* analysis to clinical validation (current limitations and future promises)

Sarah El-Nakeep

**ORCID number:** Sarah El-Nakeep 0000-0003-2830-5052.

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**Sarah El-Nakeep,** Gastroenterology and Hepatology Unit, Department of Internal Medicine, Faculty of Medicine, Ain Shams University, Cairo 11591, Egypt

**Corresponding author:** Sarah El-Nakeep, MD, Associate Professor, Gastroenterology and Hepatology Unit, Department of Internal Medicine, Faculty of Medicine, Ain Shams University, Ramsees street, Cairo 11591, Egypt. sarahnakeep@gmail.com

## Abstract

Hepatocellular carcinoma (HCC) is the second cause of cancer-related mortality. The diagnosis of HCC depends mainly on α-fetoprotein, which is limited in its diagnostic and screening capabilities. There is an urgent need for a biomarker that detects early HCC to give the patients a chance for curative treatment. New targets of therapy could enhance survival and create future alternative curative methods. *In silico* analysis provides both; discovery of biomarkers, and understanding of the molecular pathways, to pave the way for treatment development. This review discusses the role *of in silico* analysis in the discovery of biomarkers, molecular pathways, and the role the author has contributed to this area of research. It also discusses future aspirations and current limitations. A literature review was conducted on the topic using various databases (PubMed, Science Direct, and Wiley Online Library), searching in various reviews, and editorials on the topic, with overviewing the author's own published and unpublished work. This review discussed the steps of the validation process from *in silico* analysis to *in vivo* validation, to incorporation into clinical practice guidelines. In addition, reviewing the recent lines of research of bioinformatic studies related to HCC. In conclusion, the genetic, molecular and epigenetic markers discoveries are hot areas for HCC research. Bioinformatics will enhance our ability to accomplish this understanding in the near future. We face certain limitations that we need to overcome.

**Key Words:** Hepatocellular carcinoma; *In silico* analysis; Bioinformatics; Biomarkers; Molecular pathways; Genetics; Epigenetics

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**Core tip:** Hepatocellular carcinoma (HCC) is the second cause of cancer-related mortality. The importance of having an early detecting biomarker is to allow for curative measures to be applicable, and prognostic biomarkers to detect survival, in dealing with the disease. *In silico* analyses allow us to discover new genetic and epigenetic biomarkers, along with establishing the coexpression patterns, which impact HCC survival. Also, it allows for understanding the molecular pathways for HCC pathogenesis, and the discovery of potential therapeutic options. In this article, I review the current discoveries and limitations that face researchers to reach their ultimate goal of establishing clinical practice guidelines. I give an overview of the future potential that could benefit integrated research on HCC and discuss my own research related to the topic.

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## INTRODUCTION

Hepatocellular carcinoma (HCC) is the most common primary liver cancer. HCC is the sixth most prevalent cancer, and the second leading cause of cancer mortality[1]. The incidence of HCC in different countries is related mostly to the geographic prevalence of certain risk factors such as chronic hepatitis B and C, aflatoxins, and alcoholism[2,3]. HCC causes annual morality exceeding 700 000 cases[4], and high recurrence rate after treatment, with overall 5-year survival (< 50% of cases)[5], and even lower numbers reach the endpoint of 10-year survival (< 10% of cases), despite aggressive treatment[6].

Cirrhosis can proceed to HCC in 5%–30% of patients after an average duration of 5 years[7]. Most HCC cases arguably occur on top of cirrhosis, but we cannot ignore the 20% of cases that occur without any preceding cirrhosis. Thus, cirrhotic and noncirrhotic causes of HCC are explained by different pathogenic mechanisms[8,9].

A debate has arisen about the relation of increased incidence of HCC among patients receiving new direct-acting antivirals (DAAs) for hepatitis C treatment, but recent studies have shown that the risk of *de novo* and recurrent HCC with DAAs is actually lower than without, although not completely abolished[10,11].

Many HCC studies use bioinformatics as a method to determine the molecular pathways affected by HCC, along with the genetic and epigenetic control of those pathways. These proteomic and genomic studies are the future of personalized medicine, where precision therapy could offer patients a management plan specified for their individual mutations, with high curative capabilities. We have visualized how intercepting a molecular pathway as in sorafenib, a multikinase inhibitor that enhances apoptosis, could increase the survival of advanced HCC by several months, but unfortunately, recent studies have shown that resistance to the drug is evolving [12,13].

The Cancer Genome Atlas Research Network has conducted a study project on 33 cancers with poor prognosis, provided that there were suitable available tissue samples for experimental validation through antibodies' multiplex analysis, and they included HCC among other cancers. The HCC study included 363 cases for whole-exome sequencing and 196 for further proteomic, epigenetic and DNA-methylation analyses. They found that the molecular pathways most affected in HCC are those that deal with the following: cell proliferation, differentiation, growth, apoptosis, and immortalization (through telomerase)[14].

In this review, I explore the steps for validation of molecular markers, with the limitations encountered to validate a novel biomarker, the research in molecular pathways related biomarkers, the role of bioinformatics in the discovery of those pathways, and future aspirations.

## IMPORTANT QUALITIES IN DIAGNOSTIC AND PROGNOSTIC MARKERS THAT HAVE TO BE MET

The early diagnosis of HCC is a crucial issue, as all of the available curative measures are only effective in early stages of cancer (liver resection, liver transplantation, radiofrequency ablation). They are curative in early the Barcelona Clinic Liver Cancer stages (0 and A), where the size of the tumor does not exceed 5 cm in its largest diameter in one nodule, or the size does not exceed 3 cm in three nodules (Stage A) [15]. Thus, early screening of HCC is an effective tool for both early detection and treatment, which increases overall survival and yields better prognosis. Unfortunately, the screening process of HCC suffers a huge limitation, which is the low sensitivity of its most accepted biomarker, α-fetoprotein (AFP).

So, the current European Association for the Study of the Liver (EASL), and the American Association of Study of Liver Disease (AASLD) guidelines recommend the following; due to cost-effectiveness, abdominal ultrasound should only be used in the screening process, while AFP is limited to the diagnosis or screening of at high-risk populations. Where we find AFP sensitivity reaching as low as 20% positivity in early stages of cancer, with fluctuating levels of the biomarker in cirrhotic patients due to other reasons, causing further confusion in reaching the diagnosis[16-18], AFP is removed from the screening assessment guidelines altogether, as mentioned above.

It is important to note that, in Japan, the at-risk populations for HCC are still screened by 3-mo abdominal ultrasound, and AFP, in addition to another two biomarkers, *lens culinaris*-agglutinin-reactive fraction of AFP, and PIVKA-II (protein-induced by vitamin K absence or antagonist-II). All these are included in the Japanese insurance plan of at-risk populations[19]. Other markers considered for HCC diagnosis are: Dickkopf-1, which is a good biomarker for HCC with negative AFP[20], and des--carboxy prothrombin, which is directly correlated with tumor size and has higher sensitivity than AFP, so can be used in screening more effectively[21]. Unfortunately, none of the aforementioned biomarkers reaches the final acceptance to be added to any of the clinical practice guidelines for HCC due to cost-effectiveness, difficult availability, or high variability across studies.

I shared in the research work of determining some of the cost-effective biomarkers that are both cheap and effective, for establishing the diagnosis and staging of HCC, including a study on Golgi protein (GP)73, where the combined sensitivity of both AFP and GP73 was 84.4% and specificity of 95.6%[22]. Our results were similar to the meta-analysis of the diagnostic accuracy of GP73 in HCC, where combined GP73 and AFP had pooled sensitivity of 87% and pooled specificity of 85%[23].

AFP is the only widely validated biomarker for HCC diagnosis, and prognosis in most clinical guidelines despite its limitations. To overcome its limitations we are still searching for a new biomarker. This is an ongoing process, requiring computational, experimental, and clinical validation. Figure 1 shows the most important factors that are required in an effective diagnostic and prognostic biomarker.

## STEPS FOR VALIDATION FOR A NEW BIOMARKER

The only approved biomarker for diagnosis by both the American and European guidelines is AFP. Other biomarkers are approved in Japanese and Korean guidelines as mentioned earlier. AFP, the biomarker that stood the test of time, has its own problems as low sensitivity making it weak as a surveillance method, which caused the ASLD and EASL to remove it from the screening of HCC except for high-risk populations[24,25]. Searching for a new biomarker is an ongoing process, which needs computational, experimental, and clinical validation, as shown in Figure 2.

The novel biomarkers discovered through bioinformatics analysis usually pass through different steps of validation. First, computational validation *(in silico* validation), through assessing correlated genes, then by statistical analysis of different genetic expressions[26]. Hence, the most statistically significant biomarkers, with a plausible molecular pathogenic background, will pass to the next stage. Experimental validation on the HCC tissues on resected tumor from patients or experimental laboratory cells as HeLa cells (*in vitro* studies). Later, clinical validation in the sera of patients with established diagnosis to determine the actual *in vivo* predictive diagnostic and prognostic capabilities of the biomarker. In this stage, we calculate the diagnostic test accuracy of the biomarker through identifying its specificity, sensitivity, and area under the curve (AUC), along with other important related parameters.

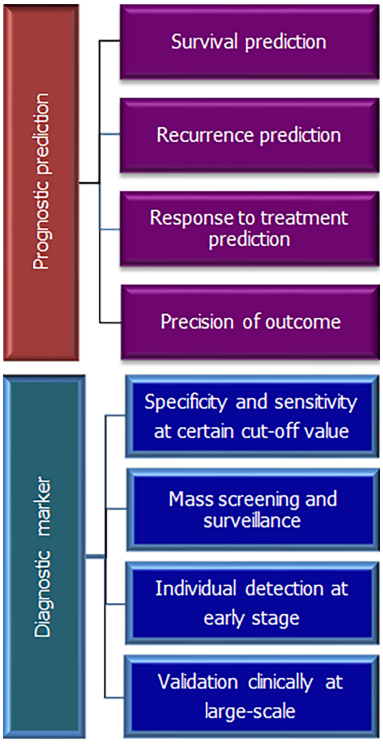


**Figure 1 Showing important features of prognostic and diagnostic markers.**

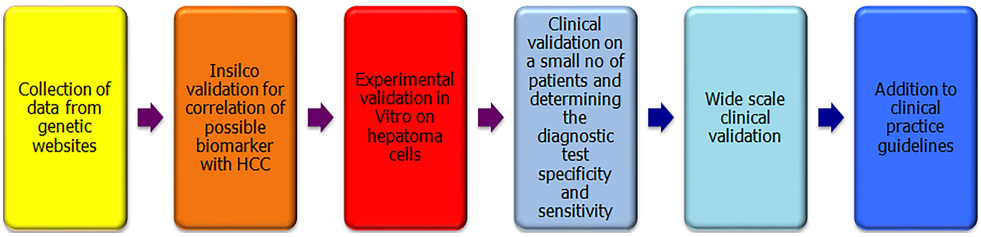


**Figure 2 Pathway for validation of the biomarkers in hepatocellular carcinoma.**

Diagnostic biomarkers should have a correlation with a clinical endpoint to be used in clinical trials, whether to help assessing this clinical endpoint or relate to it, as a surrogate marker. Then, external validation is examined through wide-scale studies, for the most acceptable biomarker in sensitivity and specificity. These studies must be done on variable and random populations (different ethnicity, gender, age groups, stages of the disease, *etc.*). When the biomarker reaches this stage of diagnostic accuracy validation, and proves to be cost-effective, then it can be added to clinical practice according to the level of evidence provided (the type of studies conducted *i.e.*, cohort, randomized controlled trial, case–control, *etc.*, and the size of the population examined)[27-29].

The steps for validation of a miRNA biomarker are[30]: (1) Data processing and screening of differentially expressed miRNAs; (2) Construction of the miRNA signature; (3) Confirmation of the miRNA signature; (4) miRNA signature validation using the OncomiR database and Gene Expression Omnibus (GEO) dataset; (5) Functional analysis; (6) *In vitro* analysis; (7) Testing on patients sera for diagnostic test accuracy; (8) Wide-scale clinical validation; and (9) Adding as a biomarker to the HCC diagnosis guidelines according to the level of evidence.

## DISCOVERING NEW BIOMARKERS: MOLECULAR PATHWAYS DISCOVERY AND DETERMINING OF THE GENETIC-EPIGENETIC-PHENOTYPIC LINKAGE THROUGH CLINICAL STUDIES

HCC is a cancer of poor prognosis, especially when discovered in late stage, which is usually the case, due to lack of early detection by biomarkers, lack of effective chemotherapeutic treatment, and limited molecular target treatment. Understanding the molecular and genetic pathways is vital to overcome these obstacles, and reach better prognostic outcomes. Recently, an accumulation of data regarding genetic and epigenetic biomarkers became available, for both *in vitro* laboratory analysis and *in silico* analysis[31].

Many pathways have been critically assessed as the key element of early diagnosis of HCC or as the key predictive outcome (whether metastasis, relapse or complete recovery) after curative interventions. These pathways include cellular effects, such as: cell proliferation, growth, differentiation and immortality. Moreover, disturbance or mutation affects functions such as: vascular angiogenesis, inflammatory response, programmed cell death and autophagy. Formulation of drugs that could intercept these molecular pathways to establish a treatment plan with good prognostic capabilities is under investigation[32].

### *Autophagy pathway in HCC: published and unpublished work of the author*

Autophagy is defined as the degradation of cellular components by lysosomal fusing with autophagosomes, and forming autolysosomes, as a homeostatic regulation for aging, stress, immunological response, or anticancer response. The role of autophagy in HCC is a complicated one. Whereas basal autophagy is responsible for anticancer protection of the organ, as carcinoma progress to a late stage, autophagy helps the cancerous tissues' survival and growth. Autophagy genes and their regulatory proteins linked to HCC include, *Beclin-1*, *ATG5* and *ATG7*. They control many molecular pathways such as: phosphatidylinositol-4,5-bisphosphate 3-kinase PI3K/AKT/mTOR, ERK/mitogenactivated protein kinase (MAPK), and apoptosis p53 pathways among others[33-36].

Our research on this pathway, linking the autophagy control of *ATG-4B* mRNA expression through noncoding miRNA-661 through bioinformatics methods proved to be of a clinical value after clinical validation. We found that combination of both biomarkers had specificity of 82.1% and sensitivity of 100%, especially in early HCC. The prognosis in the form of tumor-free survival was improved with the decline in the serum level of the two biomarkers as proved by multivariate analysis[37].

### *Hepatitis B and C associated HCC and the molecular pathways discovered: published work*

Hepatitis C virus (HCV) and hepatitis B virus (HBV) are the most common risk factors associated with HCC[38]. HBV is responsible for about half of HCC cases worldwide, in addition to most of the childhood associated HCC[7].

In a recent study, the researchers performed bioinformatics analyses using data from GEO database, to show the possible molecular pathways, which cause HBV to induce HCC. They formed heatmaps of the top 50 downregulated genes, and the top 50 upregulated genes associated with HCC occurrence. They found that there are six genes most significantly controlling the following pathways: carbon, certain amino acids, and retinol metabolism. They presented the molecular and cellular cycle pathways through the protein–protein interaction networks[39]. Furthermore, HBV-related HCC is linked to mutation of the *TP53* gene, along with viral genetic integration with the host DNA[14].

As for HCC associated with HCV, while using hierarchical clustering of the hub genes[40], the authors found overexpression of three genes: cyclin B1 coding gene, kinesin family member 20A coding gene, and hyaluronan mediated motility receptor coding gene. These were associated with decreased survival in patients with HCV-associated HCC[41].

Similarly, our team conducted a study about the relation of *IL-28* genetic polymorphism and HCC associated with HCV, in the era of interferon treatment of HCV infection. We found that the T allele was higher in both chronic liver disease (CLD) and HCC groups, with prevalence of 50% and 70%, respectively. As compared to the C allele, where the prevalence in CLD *versus* HCC groups was 30% and 50%, respectively, but the differences between the groups were not significant[42]. Our results were similar to a recent study conducted on the Chinese population, which

found that the T allele was associated with a higher risk of HCV-related HCC[43]. A recent meta-analysis found a strong correlation between *IL-28B* genetic polymorphism and HCC association with HBV or HCV infection, where CC and TT genotypes of certain single nucleotide polymorphisms (SNPs) of *IL-28B* were protective against HCC occurrence[44].

In addition, a bioinformatics study found that the *IL-28B* gene has a relation to HCC recurrence through gene expression profiles on 20 HCC *versus* 91 CLD samples, further researched *in silico* by gene set enrichment analysis and one-way hierarchical clustering for microarray analysis. They found on subsequent clinical validation in 183 HCC patients that certain *IL-28B* locus SNPs are associated with HCC recurrence [45].

### Role of noncoding RNAs as epigenetic biomarkers for HCC: including published work

Both long noncoding RNAs and miRNAs are considered noncoding chromosomal regions, originating in the introns of the chromosomal DNA. They are responsible mainly for the control of the exons' stimulation–inhibition process[46]. Exons are the chromosomal blocks of DNA responsible for encoding proteins. The noncoding RNAs have many functions including: controlling protein metabolism, and maintaining chromosomal structure, besides segregation through telomerase generation[46,47].

As an example, the role of miRNA-20a in controlling the mRNA of *CyclinD1* (a proto-oncogene) was studied using bioinformatics prediction methods through matching the seed region of the miRNA with the chosen sequence, to predict related miRNA targets. This oncogene is responsible for controlling progression of G1 to S phase, and hepatocellular growth, through regulation of the Wnt signaling pathway. Later, this was confirmed by experimental validation in HepG2 cells[48]. Table 1 shows the molecular pathways.

Also, miR-1180-3p is upregulated in HCC and associated with increased tumor proliferation, resulting in poor survival. Functional computational analysis and KEGG pathways maps showed that this epigenetic marker is linked to MAPK pathway regulation, in addition to control of cellular proliferation, apoptosis and differentiation [49]. Table 1 shows the molecular pathways.

Our team has published work on the relation between different miRNAs and oncogenes and HCC, especially those associated with HCV, and comparing their diagnostic efficacy to that of the established marker AFP. For example, autophagy genetic markers are correlated with miRNA-661, as mentioned earlier. lncRNA-UCA1 (urothelial carcinoma associated 1), c-JUN (cellular jun proto-oncogene), miR-143 and miR-550a were studied in the serum of HCV-infected HCC patients[37,50,51]. lncRNA-UCA1 had a sensitivity of 91.4% and specificity of 88.6%, while c-JUN had a sensitivity of 91.4% and specificity of 91.4% with AUC of 91%[51]. Also, miR-550a had an inverse relation with miR-550a with sensitivity of 91.89% and specificity of 90.24%, while miR-143 did not show any relation to HCC occurrence[50].

### Role of telomeres in HCC initiation and prognosis: work in progress

Telomeres function mainly in capping the chromosomal end to protect it from damage. They consist of nucleoprotein repeats. Telomeres can be transcribed into long noncoding RNAs, thus having an epigenetic control on the telomere homeostasis and telomerase enzymatic activity. Telomeres that bear such functions are called telomeric repeat-containing RNA (TERRA)[52,53]. The role of TERRA in HCC prognosis has been recently studied; it is downregulated in HCC and causes poor prognosis due to metastasis and cell growth, as studied *in vivo* and *in vitro*[54]. Through bioinformatics analyses, several regulatory protein motifs (regulating TERRA) at the end of chromosomes were identified and confirmed through experimental siRNA transcription on HeLa cells, when transfected[55]. Determination of the mechanisms of control of telomere homeostasis and telomerase enzyme will enable researchers to discover drugs that could modify this pathway, in order to cure cancer proliferation, and metastasis (Figure 3). I am involved in ongoing research in this area.

### Other important molecular pathways

Other important molecular pathways that are studied in HCC are shown in Table 1. (1) Proliferation pathway is enhanced through the inhibition of various transcription factors (TFs). TFs present a form of differentiation therapy, which decreases cancer growth[56]. (2) Cellular growth in HCC: growth factor dysregulation causes disturbance in hepatocyte growth, and is considered as a treatment option for HCC[57]. (3) Angiogenesis in HCC: diagnosis and prognosis of HCC have different associations with various growth factors, including vascular endothelial growth factor (VEGF),

**Table 1 Molecular pathways affected in hepatocellular carcinoma and their related protein-coding genes[14,33,34] and KEGG pathways database[35]**

| Function | Pathways and genetic regulators |
|---|---|
| Proliferation | Wnt pathway: MYC, FGF19, APC, AXINMAPK/ERK signaling pathway: mTOR, ERK 1/2 |
| Cell growth and angiogenesis | RTK/RAS/PI(3)K pathway: PIK3CA, VEGF, EGF, MET, KRAS, PTEN, AKT1/2, FGFR1, NF1, TSC1/2, TGF-β pathway: SMAD2/3, SMAD4 |
| Apoptosis | TP53 signaling pathway: MDM4, MDM2, CDKN2A, RPS6KA3 |
| Cell immortality | Telomerase production: TERT |
| Cell cycle progression | RB1, CCND1, CDK4, CCNE1 |
| Cell differentiation | HNF1A |
| Autophagy | RAS/RAF/MEK/ERK pathway, PI3K-AKT (AKT kinase)-mTOR pathway, and Wnt/β-catenin signaling pathway: Becilin-1, ATG3, ATG5, ATG7 |
| Inflammatory response | IL-6 stimulation: STAT3, HNF1, IL6ST, GNAS |
| Chromatin modifiers | BAP1, ARID1A/B, IDH1/2, SMACA4, KMT2D |

*Wnt:* Wingless and Int-1 (combined word); FGF19: Fibroblast growth factor 19 coding gene; MAPK: Mitogen-activated protein kinase; ERK: Extracellular signal-regulated kinase; mTOR: The mechanistic target of rapamycin; VEGF: Vascular endothelial growth factor; EGF: Epidermal growth factor; KRAS: K-Ras coding gene; PTEN: Phosphatase and tensin homolog coding gene; FGFR1: Fibroblast growth factor receptor 1 coding gene; SMAD family: Signal transducers for receptors of the transforming growth factor beta coding genes; Tp53: Tumor protein P53; MDM2: E3 ubiquitin ligase to degrade p53 coding gene; *RPS6KA3*: Ribosomal Protein S6 Kinase A3 coding gene; TERT: Telomerase reverse transcriptase coding gene; CCND1: Cyclin D1 Coding gene; CDK4: Cyclin-dependent kinase 4; CCNE1: Cyclin E1 coding gene; HNF1A: HNF1 Homeobox A coding gene; IL: Interleukin; ATG: Autophagy Related coding gene; *STAT3*: Signal transducer and activator of transcription 3; GNAS: Guanine nucleotide binding protein; BAP1: BRCA1 associated protein-1; ARID1A/B: AT-rich interactive domain-containing protein 1A/B; IDH1/2: Isocitrate dehydrogenase 1/2; KMT2D: Lysine Methyltransferase 2D coding gene.

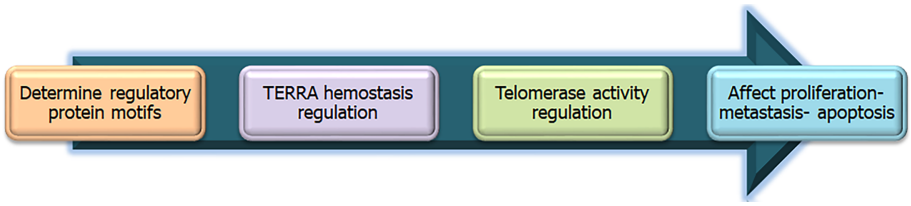


**Figure 3 Proposed mechanism of drug development using bioinformatics and molecular knowledge about telomere homeostasis.**

epidermal growth factor, transforming growth factor, *etc*. (4) Inflammatory response: for example, the effect of the interleukin pathway, and chronic inflammatory response in chronic hepatitis or steatohepatitis could result in activation of this pathway. And (5) Cell cycle progression: as mentioned earlier in control of cyclin D1, this could also form a suitable drug target.

## COMPUTATIONAL METHODS USED IN MOLECULAR PATHWAY DISCOVERY

### *Interactive networks formed by data mining*

Genetic networks formed through data mining are formed of two types: supervised learning, which mainly investigates data through statistical analysis of the patterns of coexpression presented in different genes; and unsupervised learning, which mainly deals with the discovery of genetic signatures to predict occurrence of certain diseases [58]. Both are considered methods of artificial intelligence and machine learning. Identifying the coexpression of genetic patterns for diagnosis and prognosis, through machine learning, could help formulate personalized therapeutic targets and advance precision medicine[59]. Figure 4 shows the pathways of bioinformatics analysis, and the general directions aimed in using *in silico* analysis.

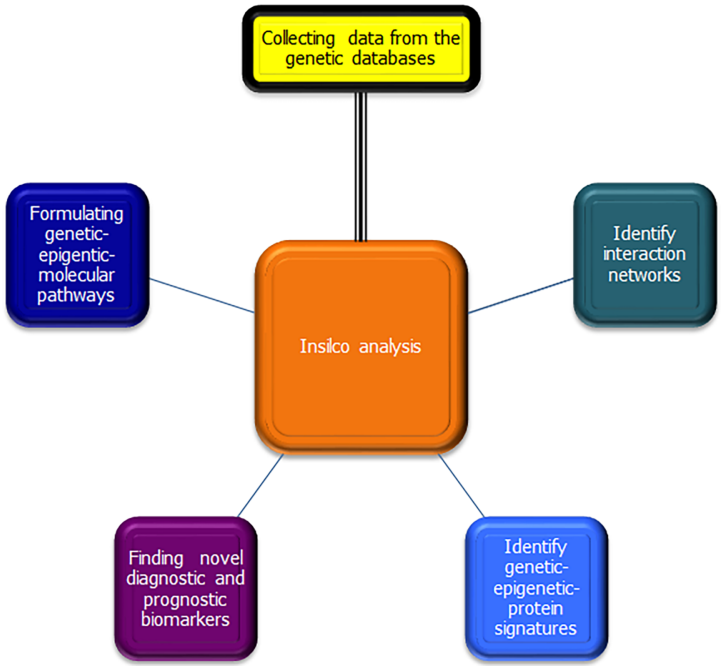


**Figure 4 Pathways and aims of bioinformatics analysis.**

### *Forming a miRNA signature*

miRNA signature is a group of miRNAs that act collectively as one diagnostic or prognostic biomarker for a certain disease. By using the most relevant and lowest number of miRNAs to achieve the highest possible sensitivity and specificity of this biomarker in diagnosis or prognosis, we can create a relevant signature. Recently a group of scientists used support-vector-machine-based technology to assess the relation of miRNA signatures with clinical staging of HCC. The results showed 23 miRNAs with collective high sensitivity and specificity in differentiating early from late HCC, while seven miRNAs helped to determine the prognosis and survival in HCC patients[60].

### *Forming of prognostic biomarker coexpression signatures in HCC*

Genetic or protein signatures formed by alignment of different sequences, preferably through multiple sequence alignment, could provide information about the "most conserved" sequence in a protein or gene or miRNA, through comparison between genes inherited by different species with a common ancestor (homologs), including similar genes in different species (orthologs), or different genes in the same species (paralogs). Coexpression signatures might help to categorize proteins or genes in different familial sets to predict their prognostic effect. There are different types of coexpression signatures including patterns, fingerprints and profiles[61-63].

A group of researchers collected different known HCC prognostic genes from various genetic and oncological databases, then through Lasso-Cox modeling a single prognostic signature, composed of the five genes *CCNB2*, *DYNC1LI1*, *KIF11*, *SPC25* and *KIF18A*, was tested in HCC tissues from patients by immunohistochemistry against HCC survival[64]. Another group of researchers found a single 14-gene signature for the prediction of HCC outcome[65].

A recent proteomic study used data mining to examine a new prognostic predictor protein signature. They found that four proteins, proliferating cell nuclear antigen, MutS homolog, cyclin-dependent kinase 1 and asparagine synthetase, were expressed in HCC tissue, and formed a single protein signature that predicted HCC survival. Most studies have used clinical proteomic databases including Clinical Proteomic Tumor Analysis Consortium (CPTAC) and Cancer Proteome Atlas (TCPA) as the source for genetic data collection[66].

### *Forming a gene coexpression network*

A research group used 389 differentially expressed genes (retrieved from the GEO database) to build a gene coexpression network using the Robust Rank Aggregation method to aggregate ranked genes, and found that 40 hub genes (*i.e.*, functionally

significant in the module formed) were linked to HCC diagnosis, including 30 hub genes that were linked to HCC prognosis. Subsequent clinical validation of those most significant (only three novel biomarkers), was done on 32 HCC patients, showing upregulation in all three biomarkers, and their upregulation was associated with advanced tumor staging and worse prognosis. Those three novel biomarkers had not been assessed before in HCC, and all were linked to the regulation of cellular methylation process[67].

### *Bioinformatic analysis for HCC-therapy drug candidates (through molecular pathways or drug docking)*

An important area in the discovery of novel drug candidates is drug-docking analysis. This is the first line of drug discovery in the era of bioinformatics, and has provided us with research on new applications of existing drugs and discovery of novel ones. This area, despite being interesting, is strictly used by pharmacology and biology specialists, and it is only during clinical validation that clinicians become aware of it during assessment of new drugs in clinical trials.

Following *in silico* validation, the first step is *in vitro* validation on hepatocellular culture, and later *in vivo* through animal or preclinical trials.

The first human trial is considered Phase 0 and is conducted only on healthy humans, as an exploratory phase prior to examining the treatment on affected patients. Later in Phase I/II, we establish primarily the safety and secondarily the efficacy, while Phase III concentrates mainly on establishing the efficacy of the drug. Finally, the post-marketing phase (Phase IV) determines the effectiveness of the drug in real life settings. Both Phase III and IV also ascertain the occurrence of adverse events (*i.e.*, safety) in real life settings[68]. In case of known and established drugs already in use for other illnesses, drugs discovered through molecular docking can bypass the animal trials and Phase 0, and go straight to phase I/II clinical trials (Figure 5).

In HCC, many studies considered molecular docking as a way to discover new drug targets. Different pharmacological compounds were considered as drug targets, for example, berberine, which affects the PI3K/AKT signaling pathway[69], or phytoconstituents of *Cocculus hirsutus* (coclaurine, haiderine and lirioresinol), which affects the VEGF receptor pathway[70]. A recent study used both molecular docking and genetic networks to design anti-HCC drugs from an ancient traditional Chinese medicine SiNiSan (SNS). SNS affects primarily the p53 pathway, thus regulating apoptosis[71].

Drug docking requires knowledge and experience in computational programs and algorithms. An easier and more approachable way to search for drug candidates is through selecting pharmacogenic compounds achieved by bioinformatic analysis of different molecular pathways, then proceed to *in vitro* analysis in cellular culture, followed by *in vivo* analysis in animal trials, then all through the aforementioned steps of validation. Our team has just published a paper on this topic, where cyan was used as an antioxidant for the inhibition of HCC proliferation, through modulation of the cell cycle in Wistar rats. The drug effect resulted in lower levels of expression of long noncoding RNA MALAT1, and tubulin 1 mRNA, and higher levels of expression of miR-125b. We chose this drug target through *a priori* bioinformatic analysis, followed by laboratory confirmation, and later by *in vivo* animal trials[72].

Other areas of bioinformatics research include whole-exome sequencing, transcriptome sequencing, and cistrome analysis[73].

## LIMITATIONS TO USING *IN SILICO* ANALYSIS AND CLINICAL VALIDATION

### *Cost-effectiveness*

Data mining is an option to examine the association between genetic material and clinical diseases. Meanwhile, the data collection cost is high. Moreover, most genetic and epigenetic biomarkers incur a high cost for laboratory assessment, mostly supplemented by grants or national or international funding.

Cost-effectiveness of applying those novel biomarkers for general clinical practice has yet to be determined. This needs large-scale population studies, and specifically designed cost-effective models[74-76].

Generally speaking, a novel biomarker should be cost-effective to be applicable in clinical practice guidelines after its wide-scale validation. Ultrasound has proven to be cost-effective in screening, with or without the addition of AFP, as a part of the two-stage biomarker–ultrasound screening[77]. This is a critical issue, not only in develo-

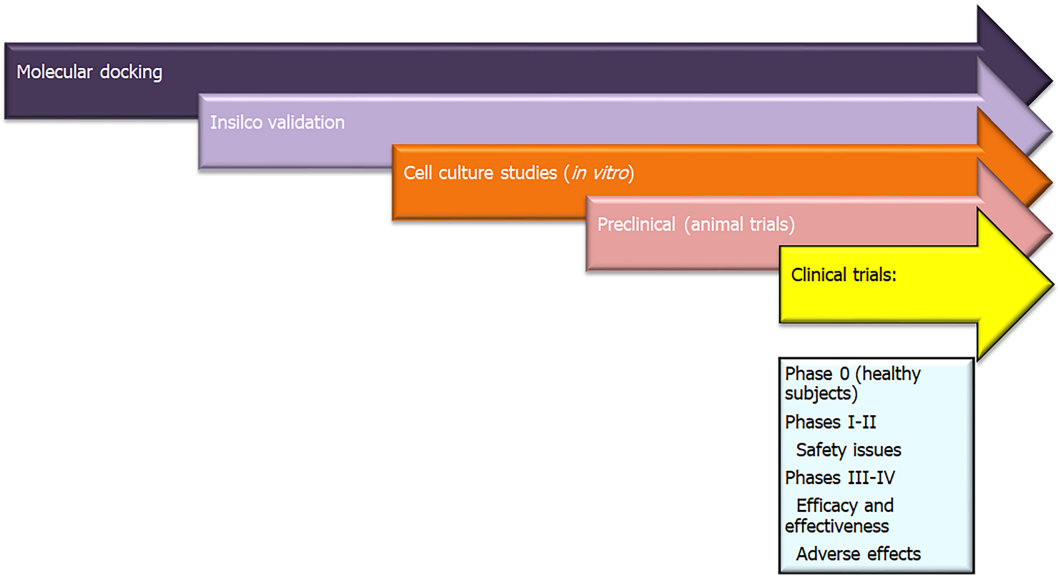


**Figure 5 Role of molecular docking from drug discovery to clinical use.**

ping and low-income countries but also in developed countries, while designing their clinical guidelines by healthcare system authorities.

Another problem faced is that laboratory analyses mostly require highly specialized researchers to handle the genetically fragile materials efficiently and without contamination or destruction. Preferably, this kind of research is conducted in highly specialized research laboratories for genetic analysis. Bioinformatics laboratories should always be constructed as a part of these physical laboratories.

## CONCLUSION

The future holds out hope for personalized medicine, where we can treat HCC on an individual level, through assessing the genetic and epigenetic background of the patient, and then planning a specified management, considering the highest benefit and the lowest risk to the patient. The future also offers the promise of early detection of HCC, which has been the main obstacle in achieving our goal of cure, as most of the cases are diagnosed beyond the reach of curative methods, in late clinical stages.

Moreover, we can offer the chance for prognostic prediction of overall survival and tumor recurrence in HCC patients.

Proteomics, genomic, epigenomic and transcriptomic analyses provide massive data on the expression profiles of HCC; however, we are still unclear of their exact role or underlying mechanisms of action. Future studies are needed to integrate these data to provide a clear picture of the disease[66]. For example, S100A9 and granulin protein affect the progression of tumor and metastasis[78], and the inclusion complex of curcumin/β-cyclodextrin polymer prohibitsgrowth of HepG2 cell line[79]. These examples provide diagnostic and prognostic biomarkers for HCC severity and clinical progression, and further research on the affected molecular pathways as possible therapeutic targets specific to each patient, i.e., precision medicine[80,81].

Personalized medicine and individual planning for the management of patients with HCC are the future of medicine. To achieve this we need a multidisciplinary team of hepatologists, oncologists, clinical pharmacists, hepatic surgeons, interventional radiologists, nursing teams, psychiatrists, and social workers. All this should take place in specialized facilities, such as tertiary or specialized hospitals, which deal with these special types of cases. These facilities must include data storage access to a genetic bank, a blood and tissue bank, along with the required bioinformatics specialists to enter, retrieve and analyze data when needed. Finally, supportive teams of social workers, supporting family members and friends, while having effective communication with the medical team, are all essential in procuring the best possible outcome for the patient.

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*REVIEW*

# Current treatment strategies and future perspectives for gastrointestinal stromal tumors

Yoichi Sugiyama, Masaru Sasaki, Mohei Kouyama, Tatsuya Tazaki, Shinya Takahashi, Atsushi Nakamitsu

**ORCID number:** Sugiyama Yoichi 0000-0002-8063-3961; Masaru Sassaki 0000-0002-9115-3381; Mohei Kouyama 0000-0002-7774-9541; Tatsuya Tazaki 0000-0002-9327-9749; Shinya Takahashi 0000-0002-5339-6534; Atsushi Nakamitsu 0000-0003-3538-6129.

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Grade E (Poor): 0

**Yoichi Sugiyama, Masaru Sasaki, Mohei Kouyama, Tatsuya Tazaki, Atsushi Nakamitsu,** Department of Gastrointestinal Surgery, JA Hiroshima General Hospital, Hatsukaichi 738-8503, Hiroshima, Japan

**Shinya Takahashi,** Department of Surgery, Institute of Biomedical and Health Sciences, Hiroshima University, Hiroshima 734-8551, Japan

**Corresponding author:** Yoichi Sugiyama, MD, PhD, Doctor, Surgeon, Surgical Oncologist, Department of Gastrointestinal Surgery, JA Hiroshima General Hospital, Jigozen 1-3-3, Hatsukaichi 738-8503, Hiroshima, Japan. sugiyama0113@gmail.com

## Abstract

Gastrointestinal stromal tumors (GISTs) are mesenchymal tumors that originate from the gastrointestinal tract, mostly from the stomach. GISTs are derived from the myenteric interstitial cells of Cajal and are caused by several mutations in the c-kit and platelet-derived growth factor receptor genes. Clinically, GISTs are detected by endoscopic and imaging findings and are diagnosed by immunostaining. Surgery is the first line of treatment, and if the tumor is relatively small, minimally invasive surgery such as laparoscopy is performed. In recent years, neoadjuvant therapy has been administered to patients with GISTs that are suspected of having a large size or infiltration to other organs. Postoperative adjuvant imatinib is the standard therapy for high-risk GISTs. It is important to assess the risk of recurrence after GIST resection. However, the effect of tyrosine kinase inhibitor use will vary by the mutation of c-kit genes and the site of mutation. Furthermore, information regarding gene mutation is indispensable when considering the treatment policy for recurrent GISTs. This article reviews the clinicopathological characteristics of GISTs along with the minimally invasive and multidisciplinary treatment options available for these tumors. The future perspectives for diagnostic and treatment approaches for these tumors have also been discussed.

**Key Words:** Gastrointestinal stromal tumor; Minimally invasive surgery; Laparoscopic surgery; Imatinib; Neoadjuvant therapy; Risk assessment

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**Core Tip:** Radical resection is the most effective treatment for gastrointestinal stromal tumors, but there are other options including minimally invasive surgery and multidisciplinary treatment, which involves the use of neoadjuvant therapy in consideration of tumor size and location. Combination with tyrosine kinase inhibitors is important for maximizing the therapeutic effect of surgery. To predict the effect, it is important to examine the presence of tumor mutations, including type, location of the mutation, and molecular subtype. We herein discuss the current treatment strategies for gastrointestinal stromal tumors and promising treatments based on clinical trials.

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## INTRODUCTION

Gastrointestinal stromal tumors (GISTs) are rare tumors that account for 3% of all gastrointestinal tumors. GISTs originate from spindle-shaped cells known as Cajal cells, which behave as pacemakers and are normally found in the proximal muscles surrounding the intermuscular plexus of the gastrointestinal tract[1]. Hirota *et al*[2] reported that receptor tyrosine kinase KIT expression was observed in most GISTs; they also suggested that GISTs usually exhibit gain-of-function mutations in the c-kit gene encoding KIT and may be caused by a specific genetic abnormality[2].

The standard treatment for GISTs is radical resection; for tumors classified as high-risk, the standard treatment includes the administration of adjuvant imatinib for at least 3 years post-surgery[3]. This is because it is difficult to determine whether a GIST is benign or malignant even by pathological examination. For adjuvant therapy, the risk of GIST recurrence has been stratified by assessing the mitotic index, tumor size, and tumor location.

In addition, surgical approaches have been diversified according to the size and location of the tumor. Less invasive surgical procedures such as laparoscopy and laparoscopic and endoscopic cooperative surgery (LECS) have been performed for small GISTs, while preoperative chemotherapy is used to improve the probability of complete resection and prognosis for giant GISTs. Furthermore, when considering the selection of preoperative and postoperative drug treatment, genetic analyses have made it possible to predict, to some extent, the therapeutic effect, recurrence risk, and prognosis.

The purpose of this review is to provide an overview of the clinical features, its diverse treatment modalities, and strategies for genetically informed drug therapy of GISTs.

## MANAGEMENT OF GIST

The National Comprehensive Cancer Network (NCCN) Guidelines Version 1.2021 was published on October 30, 2020, with the aim of describing basic treatment strategies for GIST[4]. If GISTs are suspected on endoscopy, imaging, and endoscopic ultrasound (EUS), then an EUS-guided puncture can be performed to confirm the diagnosis. An abdominal/pelvic contrast computed tomography (CT) or abdominal/pelvic contrast magnetic resonance imaging is recommended for every patient. In the case of submucosal tumors (SMTs) measuring less than 2 cm, the clinician should consider performing periodic endoscopic and radiographic surveillance. If there is a trend towards increase or high-risk features on EUS (unclear borders, cystic degeneration, ulceration, hemorrhage, and heterogeneity), curative surgery must be considered whenever possible[5]. For SMTs measuring over 2 cm, surgery is recommended if findings on imaging are suspicious of GIST, if there is a trend towards increase, or high-risk features. For SMTs measuring over 5 cm or if symptoms are observed (bleeding and pain, among others), surgery is recommended even if the diagnosis is

not confirmed.

When GIST is suspected, the treatment strategy differs depending on whether complete resection is possible. For resectable tumors with minimum morbidity, surgery is recommended; resection should be accomplished with histologically negative margins. For tumors that are not resectable without significant morbidity, administration of neoadjuvant therapy is the appropriate approach. In these cases, a biopsy is needed for confirming the diagnosis of GIST and for genetic examination. If the tumor is unresectable or if there is metastatic disease, tyrosine kinase inhibitor (TKI) treatment should be initiated.

## SURGERY

For primary, non-metastatic GISTs, radical resection is the main treatment. Securing a margin at the time of excision is critical, as clean margins will affect the prognosis. For GISTs that have invaded or adhered to surrounding organs or viscera, *en bloc* resection including surrounding tissues is necessary to achieve R0 resection.

However, due to anatomical constraints, especially when the tumor is located in the esophagus, duodenum, and rectum, invasive surgery is often required; high complication rates are a problem. Conversely, when minimally invasive local resections are performed, surgical margins and long-term outcomes are questionable. Wei *et al*[6] retrospectively evaluated the outcomes of pancreaticoduodenectomy (PD) versus local resection in duodenum GISTs. The short time results were better in the local resection group, and there was no difference in prognosis based on the surgical procedure. They reported that tumor size and location were independent prognostic factors[6]. Therefore, for GISTs located in the mesenteric side of the second portion of the duodenum, PD is generally recommended; however, enucleation is recommended if the tumor is less than 5 cm in size. Wang *et al*[7] reported that in rectal GISTs, local excision provided a higher rate of anorectal preservation, shorter operative times, and fewer postoperative complications than radical resection, and that the long term results were similar in terms of recurrence-free survival (RFS). Based on these results, local resection and minimally invasive surgery are recommended whenever possible for GISTs that occur in anatomically complex regions.

Since GISTs rarely metastasize to the lymph nodes, routine lymphadenectomy is not necessary unless the lymph nodes are enlarged. However, caution is required in the case of wild-type GISTs. Most GISTs that occur in adults are caused by mutations in the *KIT* or platelet derived growth factor receptor (*PDGFRA*) genes, but 10%-15% of GISTs in adults and 85% in children are wild-type GISTs. Wild-type GISTs primarily affect young females; the main site is generally gastric, and they are multifocal yet indolent[8]. The pathogenic mechanism of wild-type GISTs is unknown, but one possible cause is dysfunction of the succinate dehydrogenase (SDH) complex in tumor cells. Along with paragangliomas, this type of GIST, is a component of the Carney-Stratakis syndrome, characterized by germline mutations of the SDH subunit[9]. Wild-type GISTs are also associated with pediatric GISTs and non-familial tumors; this is known as the Carney triad (wild-type GISTs, paraganglioma, and pulmonary chondroma) that is not associated with *SDH* germline mutations[10]. In SDH-mutant GISTs, lymph node metastases are frequently observed. Boikos *et al*[11] reported that in SDH-mutant GISTs, the incidence of nodal lesions was as high as 65%; half of them had lymph node metastasis. Therefore, resection of enlarged lymph nodes should be considered in patients with SDH-mutant GIST.

### *Resectable GISTs with minimal morbidity*

**Laparoscopic and LECS:** Laparoscopic surgery is considered for selected GISTs of small size located in easily accessible locations. Especially for tumors less than 5 cm, laparoscopic resection is acceptable[12]. In a systemic review and meta-analysis, laparoscopic surgery was recognized to be safe and feasible due to less intraoperative blood loss, early postoperative recovery, shortened hospital stay, and a lower rate of postoperative complications[13]. However, when performing laparoscopic resection, it is essential to obtain negative resection margins for complete resection of the localized tumor; in addition, great care should be taken to avoid capsule damage to prevent tumor spillage[14].

When the tumor is located near the cardia, partial gastrectomy should be considered instead of proximal gastrectomy. However, if the tumor is of luminal-growth type and close to the cardia, an extensive resection of the margins is often required. Minimum resection margins can be challenging and will often result in a proximal gastrectomy.

In such cases, the lesion can be resected to the minimum necessary extent by observing the tumor from the lumen with an endoscope and determining the excision line. Hiki *et al*[15] first established a technique for performing minimally invasive local excision using a laparoscope and an endoscope; this was the first report on LECS in 2008. Since then, many facilities have introduced LECS in Japan, and evidence on its usefulness has been reported. A method based on a similar concept attracted attention in the 2000s; it involved completion of endoscopic treatment with laparoscopic assistance as part of the Natural Orifice Transluminal Endoscopic Surgery (NOTES) and was reported as hybrid NOTES[16]. Notably, intraoperative endoscopy is becoming increasingly popular for laparoscopic GIST resection, especially when the tumor is less than 3 cm or the location is difficult to access[17]. In Japan, gastric GISTs are often found to be relatively small; many LECS procedures have therefore been performed. To date, five representative LECS techniques have been developed.

Classical LECS is an extremely efficient method, because each step is simple and clear, technically easy, and surgery can be completed in a relatively short time. In addition, since the lesion is collected *via* the abdominal wall, there is no restriction on the size of the tumor; this is one of the merits of this procedure. However, this procedure requires opening of the stomach wall; there is therefore a potential risk of leakage of gastric contents or tumor into the abdominal cavity. Thus, this procedure should be applied with caution in tumors where the mucosal surface is exposed, such as in SMT with ulcers. In such cases, the non-open technique described below should be selected.

Inverted LECS[18] is a technique that prevents the contents of the stomach from leaking into the abdominal cavity. The edge of the resected gastric wall is first stitched and lifted, and the tumor is inverted into the stomach cavity. After the tumor is dropped into the stomach and removed orally using an endoscope, the stomach dissection line is temporarily closed by hand suturing and completely closed with stapling. Inverted LECS can prevent gastric juice from leaking to some extent, but it may not be applicable for all sites such as posterior wall lesions, among others, as it is not an entirely non-open technique. Therefore, completely non-open techniques were developed, such as non-exposed endoscopic wall-inversion surgery (NEWS)[19-21], closed-LECS[22], and a combination of laparoscopic and endoscopic approaches to neoplasia with a non-exposure technique (CLEAN-NET)[23,24].

NEWS was first devised as a way to resect early gastric cancer without opening the stomach wall[19]. The first step is to place an incision in the seromuscular layer around the tumor using a laparoscope; after pushing the tumor into the luminal side of the stomach, the seromuscular layer is continuously sutured. The next step involves making an endoscopic incision in the submucosa surrounding the intruded tumor. The lesion is then dissected and retrieved orally. The advantage of this technique is that the incision can be made under direct visual observation with an endoscope or laparoscope, while the tumor resection is completely closed.

Kikuchi *et al*[22] reported on a similar closed LECS technique. After local injection of the submucosal layer, a mucosal incision is made with an endoscope; this is followed by suturing of the serosal muscular layer while inverting the lesion with a spacer. The seromuscular layer is incised again *via* an endoscope. The tumor is then retrieved orally, and the mucosal edge is closed using the same procedure as in NEWS. These procedures are excellent, especially for intraluminal GISTs; this is because they allow for an appropriate resection line. These techniques are very useful for small GISTs. However, one limitation is that the diameter of the tumor can only be up to 3 cm, because the resected tumor needs to be removed orally.

CLEAN-NET was developed by Inoue *et al*[23]; it is a non-exposed excision technique that involves incision of the serosa and muscularis, while preserving the continuity of the mucosa[23]. Unlike a normal laparoscopic local resection, this procedure allows for minimal local excision by first incising the serosa and muscularis, stretching the mucosa, and then pulling the lesion outward. The tumor is collected trans-abdominally, allowing for a relatively large GIST of up to 5 cm to be retrieved. However, this method tends to provide a slightly larger margin, because all sections are performed from the abdominal cavity. It is therefore not suitable for areas where a large surgical margin cannot be obtained, such as near the cardia.

The features of each LECS are summarized in Table 1. The choice of each technique depends on the size, location, and growth pattern of GISTs. Especially for ulcerated GISTs, the non-open techniques of NEWS, closed LECS, and CLEAN-NET are good options. In addition, NEWS and closed LECS are good alternatives for intraluminal type GIST and closed LECS for the extraluminal type[25,26].

**Table 1 Various laparoscopic and endoscopic cooperative surgery procedures for gastrointestinal stromal tumors**

| Procedure | Yr | Author | Indication | Non-exposure | First approach | Preferred type and location | Extraction site | Suturing |
|---|---|---|---|---|---|---|---|---|
| Classical LECS | 2008 | Hiki | < 5 cm ulcer (-) | No | Endoscopic | Intraluminal > extraluminal; Anterior wall | Trans abdominal | Hand or mechanical |
| Inverted LECS | 2012 | Nunobe | < 5 cm ulcer (±) | No | Endoscopic | Intraluminal > extraluminal; Anterior wall | Either site | Hand or mechanical |
| Closed-LECS | 2017 | Kikuchi | < 3 cm ulcer (+) | Yes[1] | Endoscopic | Intraluminal < extraluminal; Anterior wall | Trans oral | Hand |
| NEWS | 2011 | Goto | < 3 cm ulcer (+) | Yes | Laparoscopic | Intraluminal < extraluminal; Anterior wall | Trans oral | Hand |
| CLEAN-NET | 2012 | Inoue | < 3 cm ulcer (+) | Yes | Laparoscopic | Intraluminal < extraluminal; Anterior wall | Trans abdominal | Mechanical |
| PEIGS | 1995 | Ohashi | < 3 cm ulcer (+) | No | Laparoscopic | Intraluminal > extraluminal; Posterior wall | Either site | Hand or mechanical |

[1]Open the gastric wall.

LECS: Laparoscopic endoscopic cooperative surgery; NEWS: Non-exposed endoscopic wall-inversion surgery; CLEAN-NET: Combination of laparoscopic and endoscopic approaches to neoplasia with a non-exposure technique; PEIGS: Percutaneous endoscopic intragastric surgery.

**Percutaneous endoscopic intragastric surgery:** The percutaneous endoscopic intragastric surgery (PEIGS) technique was first reported by Ohashi *et al*[27]. A method using three indwelling intragastric ports had been devised; since then, intragastric surgery by various methods such as single incision and needlescopic PEIGS has been reported [25]. PEIGS is a surgical procedure in which an endoscope and forceps are inserted into the stomach lumen through the abdominal and anterior gastric walls. This procedure is useful for intraluminal gastric SMT. In this case, determining an adequate resection margin is not easy because of the difficulty in confirming the tumor location from outside the gastric wall. Especially for lesions on the posterior wall of the cardia, the laparoscopic approach is complicated and relatively time-consuming. In contrast, intragastric surgery can obtain an easy approach and good operative view; PEIGS is therefore suitable for such cases. The problem with this procedure is the risk of surgical site infection secondary to pseudo-perforation. However, Kanehira *et al*[28] reported the incidence of surgical site infection to be approximately 2%, which was well within the acceptable range.

**Endoscopic resection:** There are various reports on the removal of intraluminal SMTs with an endoscope alone[29-31]. In these procedures, endoscopic full-thickness resection may be performed for intraluminal SMT originating in the muscularis propria (MP) layer. This procedure involves incising the MP layer around the SMT first; the serosal layer is then incised to generate perforation. The SMT with surrounding tissue is then removed using a snare, and the perforated gastric wall is closed using an endoscopic clip and an endloop[31]. However, this procedure involves the risk of leakage of the gastric contents due to pseudo perforation. To solve this problem, over-the-scope-clip and snaring are being developed as a full-layer suture device[32]. In this procedure, the over-the-scope-clip is first placed in the lesion, and the base of the lesion is completely resected by the snare to prevent pseudo-perforation. This technique is especially useful for small SMTs of 2 cm or less.

Newer therapies, such as endoscopic ultrasound alcohol ablation, have shown promising results. EUS-guided injection of 1.5 mL of 95% ethanol was performed for primary or metastatic GISTs without technical incidents[33]. While long-term follow-up is required to ascertain its efficacy and safety, it may be considered for high-risk patients.

### *Resectable GISTs with significant morbidity*

**Neoadjuvant therapy:** Surgical resection is the mainstream for GIST treatment, and

complete resection without damage caused by pseudo-capsule resection is essential. If the tumor is large and is suspected to have infiltrated to other organs, the complete resection rate may decrease and the recurrence rate due to intraoperative tumor rupture may be higher. Additionally, even if complete resection of a larger tumor is achieved, the risk of recurrence increases with tumor size[34]. For such cases, the rate of extensive surgery is increased; this is associated with significant morbidity. Preoperative treatment with imatinib is therefore attempted in such cases, as tumor shrinkage is essential for ensuring a negative surgical margin and avoiding the risk of rupture from subsequent surgical procedures.

Function-preserving surgery is another aim of preoperative administration of imatinib. When considering function preservation by avoiding extended surgery, the effect of neoadjuvant therapy is greatly influenced by the location of the tumor. Tumor shrinkage at the esophagogastric junction can convert a total gastrectomy into a local resection. In duodenal GISTs close to the pancreatic head, PD may be avoided by neoadjuvant therapy. Neoadjuvant imatinib allows for preservation of the anal sphincter in certain rectal GISTs. Indeed, neoadjuvant imatinib has been commonly administered in retrospective series for GISTs located in such locations.

Based on two large-scale clinical databases, the BFR14 trial[35] and the European Organization for Research and Treatment of Cancer (EORTC) Soft Tissue and Bone Sarcoma Group[36] from four Dutch institutions[37], several studies have reported on neoadjuvant imatinib for GISTs. Tielen *et al*[37] performed a cohort study on preoperative imatinib for locally advanced GISTs. All tumors were over 5 cm or ill-located for surgery. The response rate (RR) to preoperative treatment was 83%, and the R0 resection rate was 84%, with no tumor perforation occurring during the operation. The 5-year progression-free survival (PFS) and overall survival (OS) were 77% and 88%, respectively. The PFS tended to be better in the neoadjuvant imatinib group, but statistical significance was not detected. Among reports on neoadjuvant imatinib, the EORTG Soft Tissue and Bone Sarcoma Group study is the largest; the results of preoperative administration of imatinib at a dose of 400 mg for locally advanced GISTs have been reported. The average duration of imatinib administration was 40 wk. In this report, the RR was 80%, and the R0 resection rate was 83%. Five-year disease-free survival and disease-specific survival were 65% and 95%, respectively. The postoperative complication rate was 15%, although surgical re-intervention was required in only 3%. The authors concluded that preoperative imatinib administration appears safe, and it is a promising treatment for patients with locally advanced or marginally resectable primary GISTs.

The contribution of preoperative imatinib therapy varies depending on the location of the tumor and is considered particularly effective in the esophagogastric junction, duodenum, and rectum. Jakob *et al* showed that those who received neoadjuvant imatinib for rectal GISTs had a significantly higher rate of negative surgical margins than those who did not receive treatment. All patients with positive resection margins and postoperative recurrence had not received preoperative treatment. In patients undergoing preoperative imatinib therapy for locally advanced rectal GISTs, a complete resection rate was obtained in 77%, which is higher than that of patients not treated preoperatively[38]. These results suggest that preoperative imatinib was associated with an increased R0 resection rate and also allowed for surgery in anatomically difficult areas.

Three prospective multicenter phase II trials have evaluated the efficacy of neoadjuvant imatinib in locally advanced GISTs[39-42]. RTOG 0132 was the first trial and reported short- and long-term results. Eligible patients had GIST with primary disease greater than 5 cm or metastatic/recurrent disease greater than 2 cm. Thirty-one of the 53 patients had primary GIST and were evaluated as the preoperative imatinib group. Preoperative imatinib was administered at a dose of 600 mg for 8–12 wk until surgery, and postoperative adjuvant therapy was planned for 2 years. In this report, the RR was 7%, and R0 resection rate was 68%. The lower RR compared to other reports was attributed to the shorter duration of neoadjuvant imatinib therapy. The 5-year PFS and OS were 57% and 77%, respectively. This trial has proved to be feasible and was not associated with significant postoperative complications.

The results of a phase II trial on preoperative imatinib therapy for large gastric GISTs in Japan and South Korea have been reported recently. For patients with large gastric GIST (> 10 cm), imatinib was administered at a dose of 400 mg for 6-9 mo until surgery. The primary endpoint was the R0 resection rate, and the secondary end points were RR, PFS, and OS. The RR was 62%, and R0 resection rate was 91%. At a median follow-up of 32 mo, the 2-year PFS was 89% and OS was 98%. These results suggest that neoadjuvant imatinib administered at a dose of 400 mg for 6-9 mo would be a promising treatment for patients with high-risk GISTs. Long-term follow-up is

expected to prove the contribution of neoadjuvant imatinib to survival in high-risk GISTs.

These advanced treatments are expected to improve the prognosis, and many studies have reported such results (Table 2). Neoadjuvant therapy is expected to preserve organ function, avoid tumor rupture, reduce complications, and ultimately prolong overall survival; however, the evidence of efficacy remains to be established.

**Important aspects for neoadjuvant therapy:** The NCCN and European Society for Medical Oncology guidelines recommend that GIST must be diagnosed pathologically if neoadjuvant therapy is to be considered[4,43]. Tissue sampling can be obtained by endoscopic or bowling biopsy, but sometimes this is not sufficient for confirming the diagnosis. Percutaneous biopsy and tissue collection by laparotomy are contraindicated due to the risk of peritoneal dissemination. However, Eriksson *et al*[44] reported that percutaneous biopsy of GISTs collects sufficient tissue and does not increase the risk of recurrence in patients who receive imatinib preoperatively[44]. In addition to GIST diagnosis, it is recommended to check for genetic mutations before starting preoperative treatment to ascertain whether the treatment is likely to be effective. KIT exon 11 and 9 mutants will respond to imatinib, but higher doses of imatinib are required for response in cases of KIT 9 mutations[45].

Nilotinib is a selective TKI with a potency similar to that of imatinib[46]. A randomized phase III trial on the efficacy and safety of nilotinib as a first-line treatment was conducted[47]. In this study, the PFS was higher with imatinib in the KIT exon 9 group but similar in the KIT 11 group. Thus, for patients with KIT exon 11 mutations who cannot receive imatinib, nilotinib is a promising preoperative agent.

It is also known imatinib has no therapeutic effect on GIST with the PDGFRA exon 18 D842V mutation, which has a poor prognosis. The NAVIGATOR study was a phase I trial to assess the efficacy and safety of avapritinib administration for unresectable GISTs patients, who tested positive for the PDGFRA exon 18 D842 V mutation[48]. In patients with PDGFRA exon 18 D842 V-mutant GIST, 88% had a response; 9% had complete responses, and 79% had partial responses. Based on the results of this trial, the Food and Drug Administration approved the use of avapritinib in adult patients with unresectable or metastatic GIST who have PDGFRA exon 18 mutations, including D842V mutations. Therefore, in patients with resectable GISTs associated with significant morbidity, and those having PDGFRA exon 18 mutations including the D842 mutation, neoadjuvant avapritinib is considered.

**Evaluation of the response and treatment period:** CT is the most used imaging modality to determine the effect of neoadjuvant imatinib; however, depending on the conditions, magnetic resonance imaging may be more useful for patients who are allergic to CT contrast media, those who have tumors located at specific sites such as the rectum, or those who require evaluation for liver metastases. CT can assess the change in both, tumor size and tumor viability. If imatinib has a therapeutic effect, the inside of the tumor is necrotic and degenerative, although the tumor size does not change at first. Evaluating metabolic rather than morphologic changes may therefore be more reliable for early treatment assessment. Therapeutic effect determination by the Response Evaluation Criteria In Solid Tumor criteria may also underestimate the response. The Choi Criteria[49], however, evaluates the size of the tumor and its density; it is therefore useful for evaluating the therapeutic effect of TKI. However, in order to measure changes in vascularization and to measure tumor density, CT should be obtained in arterial and portal phases[50]. Positron emission tomography (PET)/CT is highly sensitive for GISTs and can evaluate the effect of treatment earlier than tumor size changes. Previous studies have shown that PET/CT can predict imatinib response within 1-8 d[51]. Therefore international guidelines recommend early evaluation of response by PET/CT (within 2-4 wk) when neoadjuvant treatment with imatinib is administered, and rapid readout of activity is necessary[4].

The optimal duration of preoperative administration of imatinib is still unclear, but the most suitable timing for maximum effect is before secondary resistance is acquired. The pharmacological effect of imatinib is rapid, but this drug acts as a cytostatic agent; tumor shrinkage therefore takes time. In unresectable GISTs it takes an average of 3 mo for the tumor to shrink with imatinib; a plateau is reached at 6 mo[52]. In a study on patients with metastatic or unresectable GISTs, the median time to tumor progression was 12 mo; tumor progression occurred in half of the patients within 2 years of starting imatinib[53]. Tirumani *et al*[54] reported that in a cohort receiving neoadjuvant with imatinib, best response was achieved at wk 28; thereafter, a plateau response continued until wk 34[54]. These results suggest that the appropriate duration of preoperative imatinib may be for 6-9 mo.

**Table 2 Studies on neoadjuvant imatinib therapy for gastrointestinal stromal tumors**

| Ref. | Clinical trial | Yr | Design | Endpoint | Cases | Agent/Dose | Patients | Duration | RR | R0 rate | Adjuvant imatinib | PFS | OS |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Prospective study | | | | | | | | | | | | | |
| Eisenberg *et al* [39] | RTOG0132 trial | 2009 | Phase II | RFS | 30 (all; 52) | Imatinib/600 mg | GIST (> 5 cm) | 8-12 wk | 7% | 77% | 24 mo | 2-yr PFS; 83% | 2-yr OS; 93% |
| Wang *et al*[40] | RTOG0132 (long follow up) | 2012 | | | 31 (all; 53) | | | | | | | 5-yr PFS; 57% | 5-yr OS; 77% |
| Doyon *et al*[41] | | 2012 | Phase II | RR | 14 | Imatinib/400 mg | Locally advanced GIST | 6 mo | 43% | 79% | 12 mo | 4-yr DFS; 64% | 4-yr OS; 100% |
| Kurokawa *et al* [42] | Asia | 2017 | Phase II | PFS | 53 | Imatinib/400 mg | Gastric GIST (> 10 cm) | 6-9 mo | 62% | 91% | 36 mo | 2-yr PFS; 89% | 2-yr OS; 98% |
| Retrospective study | | | | | | | | | | | | | |
| Blesius *et al*[35] | BFR14 trial | 2011 | Subset phase III | - | 25 | Imatinib/400 mg | Locally advanced GIST | 4.2 mo (median) | 60% | 32% | 13-24 mo | 3-yr PFS; 67% | 3-yr OS; 89% |
| Rutkowski *et al* [36] | EORTC | 2012 | Database | - | 161 | Imatinib/400 mg | Locally advanced GIST | 40 wk (median) | 80% | 83% | At least 1 yr (56%) | 5-yr DFS; 65% | 5-yr DSS; 95% |
| Tielen *et al*[37] | | 2013 | Database | PFS/OS | 57 | Imatinib/400 mg | GIST (> 5 cm) and/or ill-located for surgery | 8 mo (median) | 83% | 84% | 1, 2 yr or lifelong (58%) | 5-yr PFS; 77% | 5-yr OS; 88% |

RFS: Relapse-free survival; RR: Response rate; PFS: Progression-free survival; OS: Overall survival; DSS: Disease-specific survival.

**Postoperative therapy:** In GIST classified as high-risk after curative surgery, adjuvant imatinib therapy is standard treatment; the recommended period of therapy is at least 3 years[55]. However, there is no consensus on postoperative adjuvant therapy for patients treated with neoadjuvant imatinib. In the RTOG0132 trial; the 5-year disease-free survival in patients who received adjuvant imatinib was better than that in patients who did not receive the drug. However, recurrence occurred within 2 years of completion of adjuvant imatinib. Therefore, for patients treated with neoadjuvant imatinib, postoperative adjuvant therapy is required for 3 years, similar to the period of adjuvant therapy required for high-risk GIST.

### *Surgical intervention for metastatic GIST*

The treatment of unresectable, advanced, and recurrent GISTs is mainly based on TKI administration; however, surgical intervention may be possible in some cases. If the response to imatinib is good and the disease is controlled, surgery may be indicated. This includes cases of initially unresectable GIST that has responded well to imatinib

and become resectable, locally progressed GIST due to secondary resistance, low-volume stage IV disease, or cases requiring palliative surgery for symptoms such as bleeding or obstruction. If complete resection can be achieved, surgical intervention in combination with imatinib is more effective[56,57]. A retrospective study reported that GIST patients who respond to imatinib therapy have significantly higher complete resection rates and better PFS and OS than those who do not respond to imatinib. Additionally, two randomized controlled trials evaluated the efficacy of multidisciplinary treatment combining imatinib with surgical intervention for recurrent or metastatic GISTs[56,58]. Xia *et al*[56] investigated the efficacy of surgery and pre-and post-operative imatinib administration for advanced GISTs with liver metastasis and reported that the OS was better with surgical intervention. Furthermore, surgical resection offered better OS in GIST patients who had a poor response to imatinib therapy in the 6 mo prior to surgery. These findings suggest that in some cases, patients with liver metastases from GIST may have a better prognosis with surgical intervention than with imatinib alone. However, the indication for and optimal timing of surgery are still unclear, and future consideration is awaited.

Surgery after second line treatment such as sunitinib is considerably rare; however, certain retrospective studies report on its efficacy. Yeh *et al*[59] reported on the benefit of surgical intervention in metastatic GIST with local progression while receiving sunitinib. They reported fewer complications (15.3%) and significantly prolonged PFS and OS. Surgery may contribute to the suppression of events such as bleeding and ileus caused by the growth of tumors that have acquired secondary resistance to sunitinib; it may also improve disease control by removing resistant lesions. The results of cytoreductive surgery for GIST with local progression during regorafenib treatment in the third line have also been reported[60]. Although there is a bias in the retrospective study, the PFS and OS were 12.9 mo and 32.2 mo, respectively; these were better than those of patients who did not undergo cytoreductive surgery. However, it is important to note that the complication rate was as high as 33%, although the surgery was performed on relatively young patients with good performance status.

Based on the above findings, cytoreductive surgery for selected GISTs that have acquired resistance in the second and third line may provide local control, serve as a bridge to drug therapy, and ultimately improve prognosis.

## RISK ASSESSMENT AND ADJUVANT THERAPY

The tumor size and mitotic index are important in assessing the risk of recurrence of GISTs, but it is difficult to assess whether a tumor is a benign or malignant based on these features alone. Miettinen *et al*[61] reported that in GISTs with a diameter of more than 10 cm and a mitotic index of ≤ 5 mitoses/50 high power field, the recurrence rate of small intestinal GIST is considerably higher than that of gastric GIST[61]. Therefore, in addition to tumor size and mitotic index, tumor site is also included in their classification. The Joenssu classification, that includes tumor location and considers tumor capsule rupture cases where recurrence is almost inevitable, is useful in that it efficiently selects only the group at high risk of recurrence[62].

As described previously, tumor size, mitotic count, and primary location are important in assessing the risk of GIST recurrence; however, measuring mitotic count on a slide is highly individualized and depends on the ability to distinguish the cells from other cells such as apoptotic bodies and pyknotic cells, among others. In SDH-deficient GISTs, mitotic count does not predict tumor behavior[63]. Therefore, at the basic research level, an attempt has been made to predict the risk of GIST recurrence by measuring gene expression related to DNA methylation; this has been shown to be an effective predictor[64].

Imatinib is administered as adjuvant therapy for the high-risk group after surgery, as GISTs generally harbor an imatinib-sensitive mutation. The most frequent *KIT* exon 11 mutations are sensitive to imatinib, whereas the PDGFRA exon 18 D842 V-mutation is considered to be imatinib-resistant. Tumor mutation analysis is important for estimating the therapeutic effect of imatinib; however, whether evaluation of tumor mutations is more useful than the above risk assessment is controversial. Under the circumstances, a study examined the indications for adjuvant therapy by gene mutation analysis. In GIST patients with *PDGFRA* mutations and *KIT* exon 11 duplication, mutation, or deletion of one codon, good RFS has been achieved with surgery alone. Therefore, this type of genetic variation is an independent factor that affects RFS beyond recurrence risk classification numbers. These results suggest that

adjuvant therapy is not necessary for these genetic mutations.

Three randomized phase III trials have reported on the efficacy of postoperative adjuvant imatinib (Table 3). The first trial was the ACOSOG Z9001 study by the American College of Surgeons Oncology Group. The major eligibility criterion was complete resection of the primary GIST, tumor diameter more than 3 cm, and positivity for KIT on immunostaining. In this study, imatinib administration for 1 year conferred significantly better RFS than placebo [98% *vs* 83%, hazard ratio (HR) = 0.35, $P < 0.0001$]. In the largest phase III trial, EORTC 62024 patients were randomly assigned to receive imatinib at a dose of 400 mg for 2 years or surgery alone. The high or intermediate-risk group with R0 or R1 surgical margins was eligible for inclusion. The 5-year imatinib failure-free survival was 87% in the imatinib administered group and 84% in the control group (HR = 0.79, $P = 0.21$); the primary endpoint was therefore not significant. However, when the high-risk subgroup was analyzed, there was a trend towards better imatinib failure-free survival in the imatinib group (79% *vs* 73%, $P = 0.087$). The results of these studies revealed that adjuvant imatinib improved RFS when administered to patients with operable GIST; however, its influence on OS remains uncertain.

In the open-label, multicenter, randomized phase III SSGXVIII/AIO trial, patients with GIST who underwent radical surgery but were at high-risk were enrolled; they received adjuvant imatinib therapy for 1 or 3 years after surgery. The primary endpoint was RFS; the secondary endpoints were OS and safety. The 5-year and 10-year RFS were 71.4% and 52.5%, respectively, in the 3-year group, and 53.0% and 41.8% in the 1-year group (HR = 0.66, $P = 0.003$). The 5-year and 10-year OS rates for the 3-year group were 92.0% and 79.0%, respectively; for the 1-year group, they were 85.5% and 65.3%, respectively. The difference was statistically significant (HR = 0.55, $P = 0.004$). Therefore, administration of adjuvant imatinib for at least three years is the standard treatment for patients in the high-risk group[3,55]. The cited article reported that approximately 50% of deaths may be avoided during the first 10 years after surgery with longer adjuvant imatinib treatment.

A study on long-term administration (5 years or more) has been reported in the phase II PERSIST trial[65]. The 5-year RFS was 90%, while the OS rate was 95%. Six of 7 patients who developed recurrence did so after completing adjuvant imatinib. Furthermore, among the patients with an imatinib-sensitive KIT exon 11 mutations, only 1 experienced recurrence, which occurred after imatinib was discontinued. This indicates that long-term imatinib administration in patients with imatinib-sensitive mutations is effective in preventing the recurrence of GIST. Two randomized trials on the effects of long-term adjuvant imatinib therapy, namely, sSGXXII and IMADGIST, are ongoing and their results are awaited.

## SYSTEMIC THERAPY

### *Gene analysis*

**KIT mutations:** Imatinib is expected to be more than 80% effective in patients with unresectable, advanced, and recurrent GIST; the median OS after treatment with imatinib is 50 mo[66]. However, the therapeutic effect depends on the sensitivity of the GIST to imatinib; this can be predicted to some extent by identifying gene mutations. The most frequent gene mutation is that of KIT exon 11 (65%), followed by that of exon 9 (10%). Approximately 90% of KIT exon 11 and 50% of KIT exon 9 mutation GISTs respond to imatinib; however, the therapeutic effect is different to a certain extent. In the EORTC study, GISTs with exon 11 mutations showed high efficiency to imatinib and increased PFS and OS than those with exon 9 mutations. However, the relationship between imatinib doses and therapeutic effects also differs by gene mutations. In GIST patients with KIT exon 9 mutations, increasing the dose of imatinib (800 mg/d) prolonged PFS. Conversely, in patients with KIT exon 11 mutations or wild-type GIST, imatinib dose escalation did not improve PFS. However, the contribution of imatinib dose increase to OS in exon 9 mutation cases was not clear even on meta-analysis; the finding has therefore remained controversial[45].

Mutations in exon 13 and 17 are very rare; compared to other mutations, they occur more frequently in the small intestine. Genetic mutations in secondary resistant GISTs are often found in exons 13 and 17; secondary mutations occur mostly in exon 13, which constitutes the adenosine triphosphate (ATP) binding domain, and in exon 17, which constitutes the activation loop[67]. Many secondary mutations in the ATP binding domain are sensitive to sunitinib even after imatinib resistance; however, most of the secondary mutations in the activation loop are resistant to both, imatinib and

**Table 3 Clinical studies on adjuvant imatinib**

| Trial | ACOSOG Z9001 | SSG XVIII/AIO | EORTC 62024 | PERSIST-5 |
|---|---|---|---|---|
| Study/yr | Phase III/2009 | Phase III/2012, 2020 | Phase III/2015 | Phase II/2018 |
| Number | 359 (total: 713) | 397 (199 *vs* 198) | 454 (total: 908) | 91 |
| Eligible criteria | Tumor size ≥ 3 cm | High risk group | Intermediate and high-risk group | Intermediate and high-risk group |
| Treatment dose | 400 mg/d | 400 mg/d | 400 mg/d | 400 mg/d |
| Duration | 1 yr *vs* placebo | 1 yr *vs* 3 yr | 2 yr *vs* placebo | 5 yr |
| Risk classification | | | | |
| High risk | NA | 178 (89%) | 266 (58.6%) | 67 (74%) |
| Intermediate risk | | 15 (8%) | 186 (41%) | 24 (26%) |
| *Etc.* | | 6 (3%) | 2 (0.4%) | |
| Residual tumor | | | | |
| R0 | 325 (90.5%) | 169 (85%) | 381 (83.9%) | 90 (99%) |
| R1,2 | 34 (9.5%) | 30 (15%) | 73 (16.1%) | 0 (0%) 1; unknown |
| Tumor rupture | | | | |
| No | NA | 164 (82%) | 404 (89%) | NA |
| Yes | | 35 (18%) | 50 (11%) | |
| End point | | | | |
| Primary endpoint | RFS | RFS | IFFS | RFS |
| Secondary endpoint | | OS, safety | RFS, OS, safety | OS |
| Results | 1-yr RFS; 98% *vs* 83% (HR = 0.35, $P < 0.0001$); OS: Not significant | 5-yr RFS; 71% *vs* 53% (HR = 0.66, $P = 0.003$); 5-yr OS; 92% *vs* 86%; 10-yr OS; 79% *vs* 65% | 5-yr IFFS; 87% *vs* 84% (HR = 0.79, $P = 0.21$); 3-yr RFS; 84% *vs* 66%; 5-yr RFS; 69% *vs* 63% | 5-yr RFS; 90%; 5-yr OS; 95%; 45 (49%) pts early discontinuation of imatinib |

NA: Not associated; RFS: Relapse-free survival; OS: Overall survival; IFFS: Imatinib failure-free survival.

sunitinib.

Mutations in exon 8 are even rarer, with only a few cases reported in the past and an estimated frequency of approximately 0.3%. The most common genotype of exon 8 mutations is Del-Asp419; the others known are ThrTyrAsp (417-419) Tyr. In pediatric mastocytosis, the reported type of c-kit mutation in exon 8 is Del-Asp419. Hartmann *et al*[68] reported that GIST patients with Del-Asp419 mutations had mastocytosis as well as multiple GISTs, suggesting an association. The most common sites are the small intestine and duodenum, and it appears to arise from extragastric sites. Many GISTs with exon 8 mutations have metastases at the time of diagnosis or are classified in the high risk group; this indicates the possibility of aggressive behavior. Sensitivity to imatinib has been demonstrated *in vitro*. In clinical practice, it has been administered as adjuvant therapy to the intermediate to high-risk group, with no observed recurrence for 24 mo[69].

**PDGFRA mutations:** Mutations in the PDGFRA gene account for 5%-10% of all GISTs and are found mostly in the stomach. Mutations are present in exons 12, 14, and 18, with mutations in exon 18 being the most common; the most common genotype was D842V. D842V mutations are resistant to imatinib, but sensitive to avapritinib. In D842V mutant GISTs, avapritinib was found to be highly effective, with a response rate of 90% and a mean response duration of 34 mo[70]. Hence, the NCCN guidelines recommended avapritinib as first-line therapy for PDGFR D842V-mutant GIST. Among exon 12 and 14 mutants, V561D and N659K are the most common mutations, respectively; both types are sensitive to imatinib. Most GISTs with this mutation are of epithelioid morphology and have relatively good prognosis[71].

**Wild-type GISTs:** KIT/PDGFRA wild-type GISTs account for approximately 10%-15% of all GISTs. The pathogenesis of wild-type GISTs is unknown, but inactivation of neurofibromatosis type 1 (NF1) and SDH and gain-of-function mutations in genes downstream of KIT and PDGFRA (RAS and BRAF) have been suggested as a possible cause. Mutations in this alternate signaling pathway may lead to primary resistance to imatinib. SDH-deficient GISTs have a higher probability of responding to sunitinib and regorafenib[72], and are considered to have a good prognosis. NF1-related GISTs are multiple and most often located in the small intestine. Histologically, they are of the spindle cell type, contain many stained filamentous fibers and S100-positive cells, have few mitotic figures, and have a relatively good prognosis. NF1-related GISTs may result from related syndromes; up to 25% of NF-1 patients may develop GISTs over their lifetime[73].

BRAF mutations, which are mainly found in melanoma, thyroid papillary carcinoma, and colorectal carcinoma, are localized in exon 15, with valine at position 600 replaced by aspartic acid (V600E). V600E BRAF mutations destabilize the inactive conformation of the BRAF kinase; activated BRAF stimulates the activation of the mitogen-activated protein kinase pathway to induce atypical cell proliferation. This mutation accounts for 4%-13% of GISTs, and are found most frequently in the small intestine, followed by the stomach. The prognosis is relatively good, although they are not highly sensitive to imatinib. The growth of tumors with mutations in BRAF is inhibited by the use of BRAF inhibitors such as dabrafenib, which blocks kinase activity. Dabrafenib has also been reported to have a good therapeutic effect in GIST [74]. Conversely, reports suggest that approximately 50% of patients develop resistance to BRAF inhibitors within 6 mo of initiation of treatment with a single agent[75]. The mechanism of resistance to single-agent BRAF inhibitors is believed reactivation of mitogen-activated protein kinase kinase and extracellular signal-regulated kinase through a bypass pathway, that does not involve BRAF[76]. In malignant melanoma, the combination of BRAF and mitogen-activated protein kinase kinase inhibitors is believed to potently inhibit tumor growth and delay the development of resistance; the same therapeutic effect is expected for GISTs with BRAF mutations.

The impact of KIT, PDGFRA, and BRAF mutational status on the natural history of localized GISTs has been reviewed by Rossi *et al*[77]. They found that GIST patients with KIT mutations had a poorer prognosis than those with PDGFRA mutations or with triple negative (KIT, PDGFRA, and BRAF wild-type) tumors. In addition, they classified GISTs into three molecular risk groups using multivariable Cox regression models. Group I, including KIT exon 13, PDGFRA exon 12, and BRAF mutated GISTs, had the best prognosis. Group II, including KIT exon 17, PDGFRA exon 18 D842V, and PDGFRA exon 14 mutants and triple-negative mutation GISTs, had intermediate prognosis. Group III, including KIT exon 9, exon 11, and PDGFRA exon 18 mutations apart from D842V, had the worst prognosis. These results suggest that genetic mutations have prognostic value and that grouping by mutation is useful in determining the indications of adjuvant therapy; it also complements clinicopathological risk stratification. The features of KIT mutation types are shown in Table 4.

### *Liquid biopsy*

To confirm genetic mutations, and especially secondary mutations, it is necessary to collect tumor tissue. However, if the tumor is located deep in the abdominal cavity owing to recurrence after surgery or bone metastasis, obtaining tumor tissue is challenging. To solve this problem, a liquid biopsy method has been developed for detecting mutations in tumor-related genes using tumor-derived DNA (circulating tumor DNA: ctDNA)[78]. There is a risk of complications from tissue biopsy; in addition, even if a biopsy specimen is used, it is difficult to evaluate the fission image and MIB-1 labeling index of the entire tumor, as the tissue of GIST is not necessarily homogeneous. Liquid biopsy for detecting ctDNA is noninvasive and safe and provides a highly sensitive biomarker. Kang *et al*[79] reported a simple method for detecting primary and secondary mutations in ctDNA from liquid biopsy samples obtained from GIST patients. Additionally, they suggested that these gene mutations could serve as predictive markers for drug resistance. By identifying resistance mutations from plasma DNA, it is possible to increase the dose of imatinib or quickly switch to another drug. In order to apply this method clinically in the future, technical aspects such as reliability and detection sensitivity need to be established.

### *Drugs other than imatinib*

In GIST patients who experience disease progression during imatinib administration, develop secondary resistance, or cannot tolerate imatinib administration, sunitinib and

**Table 4 Clinical features of various molecular subtypes of gastrointestinal stromal tumors**

| Gene mutation | Exon | Proportion | Common mutation | Treatment | Characteristics |
|---|---|---|---|---|---|
| KIT | 11 | 70% | Del-inc557/558 | Sensitive to imatinib, secondary mutation resistant to sunitinib, some effect for regorafenib | High risk of recurrence |
| | | | p.W557_K558 del | | Adverse prognosis effect in stomach |
| | | | SNSs and Dup | | Relatively good prognosis |
| | 9 | 10% | A502-'503 Dup | Need high dose of imatinib, effective for sunitinib | Mainly in small intestinal, worse prognosis |
| | 13 | 1% | Lys642Glu | Secondary mutation resistant to imatinib | Mainly in small intestinal |
| | 17 | 1% | Asn822Lys | Secondary mutation resistant to imatinib and sunitinib, but responding to regorafenib | Mainly in small intestinal |
| | 8 | 0.30% | Del-Asp419 | Sensitive to imatinib | Extragastric, metastatic prone nature |
| PDGFRA | 18 | 5% | Asp842Val (D842V) | Responds to avapritinib, resistance to imatinib | Mainly in gastric and favorable prognosis |
| | 14 | 1% | Apn659Lys | Sensitive to imatinib | Relatively good prognosis |
| | 12 | | V561D | Sensitive to imatinib | Relatively good prognosis |
| Wild-type GIST | | 10%-15% | SDH-deficient | Not sensitive to imatinib, response to sunitinib, regorafenib | Overall indolent disease |
| | | | NF1 | Not sensitive to imatinib, response to sunitinib | Mainly in the small intestine and good prognosis |
| | 15 | 1% | BRAF | Not sensitive to imatinib, response to dabrafenib | Relatively good prognosis |
| | | | K-RAS | Not sensitive to imatinib | |

PDGFRA: Platelet derived growth factor receptor; SNSs: Single-nucleotide substitutions; Dup: Duplication; SDH: Succinate dehydrogenase; NF1: Neurofibromatosis type 1.

regorafenib are recommended for second and third-line treatment, respectively. Sunitinib is a multi-targeted TKI inhibitor that targets c-kit, PDGFRA, and vascular endothelial growth factor receptor, thereby inhibiting angiogenesis and cell proliferation. The issue of secondary resistance as well as primary mutations should be taken into account when considering second-line treatment. Sunitinib is active in KIT exon 11 mutations but less effective against GISTs having secondary resistance after imatinib; it is more effective in treating GISTs with exon 9 mutations or of the wild-type. However, sunitinib shows high inhibitory activity against mutations in the ATP-binding site (exon 13); however, its activity is reduced by mutations in the activation-loop region (exons 17 and 18).

Regorafenib is also a multikinase inhibitor for vascular endothelial growth factor receptor 1/2, PDGFR, Kit, BRAF, and RAF and includes mediators that act on angiogenesis and the tumor microenvironment to promote tumor growth. Gene mutations have also been reported to impact the therapeutic effect of regorafenib, which in a genetic search of the primary tumor was found to be particularly effective in patients with metastatic GIST with KIT exon 11 mutations or SDH deficiency[80]. In another study, GIST patients with KIT exon 17 mutations, who had been previously treated with TKI, showed particularly good response to treatment and prolonged PFS[81].

Ripretinib has been recently included in the NCCN guidelines as a fourth-line drug for patients with GIST, whose disease has progressed on imatinib, sunitinib, and regorafenib. This drug is a KIT and PDGFRA inhibitor that blocks initiating KIT mutations 13, 14, 17 and 18; they include KIT D816V and PDGFRA D842V and are expected to show considerable therapeutic effect. Recently, a double-blind randomized placebo-controlled study was conducted in GIST patients with progression on at least imatinib, sunitinib, and regorafenib. In this trial, PFS improved significantly in the group administered ripretinib compared with placebo (6.3 *vs* 1.0 mo, HR = 0.15, $P < 0.0001$); the safety was acceptable[82].

Although TKIs are useful drugs for GIST, their expected effect may not be obtained due to the issue of primary and secondary resistance. Research is therefore ongoing to find new drugs. In recent years, immunotherapy for cancer is gaining popularity, and its therapeutic effect has been clinically proven. Immune checkpoint inhibitors, such as programmed death protein 1 and cytotoxic T-lymphocyte-associated antigen 4, block the transmission of inhibitory signals to maintain T-cell activation and restore anti-tumor effects. Basic research suggests that GISTs with the D842V mutation show immune cells with increased cytolytic activity, and more tumor cells express programmed death protein 1 and programmed death ligand 1[83]. In addition, regulatory T cells and CD8+ T-cells are overexpressed, while the proportion of CD4+ T-cells is low. These data imply that immunotherapy is effective for patients with GIST, especially for those with D842V mutant tumors. The results of several ongoing clinical trials, especially those evaluating combination therapy with other immune therapeutic agents and TKIs are awaited.

## CONCLUSION

Laparoscopic surgery and LECS have not only made it possible to ensure complete curative resection in GIST but have also made it possible to perform less invasive surgery aimed at functional preservation. There is also a wider range of available surgical techniques, which may be selected depending on the location and growth pattern of the tumor. It is expected that multimodal treatment with TKIs and surgery will be an option for progressive GISTs and the results of several clinical trials are awaited. Treatment based on genetic information has been established; in the future, novel treatment strategies with newly developed TKIs, molecularly targeted drugs, and immunotherapy may therefore play important roles in the treatment of GIST.

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ORIGINAL ARTICLE

Basic Study

# Combined antrum and corpus biopsy protocol improves *Helicobacter pylori* culture success

Denise E Brennan, Colm O'Morain, Deirdre McNamara, Sinead M Smith

**ORCID number:** Denise E Brennan 0000-0001-8200-3181; Colm O'Morain 0000-0002-1847-6782; Deirdre McNamara 0000-0003-2324-3382; Sinead M Smith 0000-0003-3460-3590.

**Author contributions:** McNamara D conceived the study; Brennan DE and Smith SM performed experiments, acquired and analysed data; O'Morain C and McNamara D recruited patients and collected samples; Smith SM prepared the manuscript; all authors critically reviewed the manuscript and approved the final version; Smith SM and McNamara D contributed equally.

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**Denise E Brennan, Colm O'Morain, Deirdre McNamara, Sinead M Smith,** Department of Clinical Medicine, Trinity College Dublin, Trinity Centre, Tallaght University Hospital, Dublin D24, Ireland

**Corresponding author:** Sinead M Smith, BSc, PhD, Assistant Professor, Department of Clinical Medicine, Trinity College Dublin, Trinity Centre, Tallaght University Hospital, Tallaght, Dublin D24, Ireland. smithsi@tcd.ie

## Abstract

### *BACKGROUND*

*Helicobacter pylori* (*H. pylori*) causes chronic gastritis, peptic ulcer disease, gastric adenocarcinoma and mucosa-associated lymphoid tissue lymphoma. Eradication rates have fallen, mainly due to antimicrobial resistance. Consensus guidelines recommend that first-line treatment is based on the local prevalence of antimicrobial resistance and that rescue therapies are guided by antimicrobial susceptibility testing (AST). However, *H. pylori* culture is challenging and culture-based AST is not routinely performed in the majority of hospitals. Optimisation of *H. pylori* culture from clinical specimens will enable more widespread AST to determine the most appropriate antimicrobials for *H. pylori* eradication.

### *AIM*

To determine whether dual antrum and corpus biopsy sampling is superior to single antrum biopsy sampling for *H. pylori* culture.

### *METHODS*

The study received ethical approval from the joint research ethics committee of Tallaght University Hospital and St. James's Hospital. Patients referred for upper gastrointestinal endoscopy were invited to participate. Biopsies were collected in tubes containing Dent's transport medium and patient demographics were recorded. Biopsies were used to inoculate Colombia blood agar plates. Plates were incubated under microaerobic conditions and evaluated for the presence of *H. pylori*. Statistical analyses were performed using Graphpad PRISM. Continuous variables were compared using the two-tailed independent *t*-test. Categorical variables were compared using the two-tailed Fisher exact test. In all cases, a *P* value less than 0.05 was considered significant.

### *RESULTS*

In all, samples from 219 *H. pylori*-infected patients were analysed in the study. The

and hepatology

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mean age of recruited patients was 48 ± 14.9 years and 50.7% ($n$ = 111) were male. The most common endoscopic finding was gastritis (58.9%; $n$ = 129). Gastric ulcer was diagnosed in 4.6% ($n$ = 10) of patients, while duodenal ulcer was diagnosed in 2.7% ($n$ = 6). Single antrum biopsies were collected from 73 patients, whereas combined antrum and corpus biopsies were collected from 146 patients. There was no significant difference in age, sex or endoscopic findings between the two groups. *H. pylori* was successfully cultured in a significantly higher number of cases when combined antrum and corpus biopsies were used compared to a single antrum biopsy [64.4% ($n$ = 94/146) *vs* 49.3% (36/73); $P$ = 0.04)].

***CONCLUSION***

Combined corpus and antrum biopsy sampling improves *H. pylori* culture success compared to single antrum biopsy sampling.

**Key Words:** *Helicobacter pylori*; Culture; Antimicrobial susceptibility testing; Antimicrobial; Antrum; Corpus



**Core Tip:** *Helicobacter pylori (H. pylori)* antimicrobial susceptibility testing is critical to accurately detect antimicrobial resistance, thereby influencing appropriate treatment choices, promoting antimicrobial stewardship and increasing *H. pylori* eradication rates. However, *H. pylori* culture represents a challenge and is limited to a small number of specialized centres and reference laboratories. Increasing biopsy sample number has been suggested to improve culture success, but data directly comparing dual biopsy *vs* single biopsy sample collection for *H. pylori* culture are lacking. Here we show that combined corpus and antrum biopsy sampling improves *H. pylori* culture success compared to single antrum biopsy sampling.

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## INTRODUCTION

*Helicobacter pylori* (*H. pylori*) causes one of the most common bacterial infections globally, colonising the stomach of approximately half of the world's population. This bacterium is of interest clinically as the causative agent of chronic gastritis, peptic ulcer disease, gastric adenocarcinoma and mucosa-associated lymphoid tissue lymphoma. *H. pylori* has been designated a class I carcinogen by the World Health Organisation (WHO)[1]. Treatment usually involves stomach acid suppression using a proton pump inhibitor (PPI) together with 2-3 antimicrobials. However, treatment success has been impacted in recent years, largely due to the emergence of antimicrobial-resistant *H. pylori*. Indeed, the WHO has included *H. pylori* on their priority list of antibiotic-resistant microorganisms[2].

Primary resistance rates for clarithromycin, metronidazole and levofloxacin are 15% or higher in nearly all WHO regions[3]. Recent data on *H. pylori* antimicrobial resistance in European countries revealed overall primary resistance rates of 21.4%, 15.8% and 38.9% for clarithromycin, levofloxacin and metronidazole, respectively[4]. As resistance rates vary from region to region[3-5], consensus guidelines[6-11] recommend that first-line treatment for *H. pylori* is based on primary resistance rates in a given population. If the prevalence of primary clarithromycin resistance is unknown, it is recommended to perform clarithromycin antimicrobial susceptibility testing (AST) before using clarithromycin-based first-line triple therapy. *H. pylori* AST is also recommended to guide rescue therapy following 2 treatment failures[8]. Thus, methods to detect antimicrobial resistance are of great importance both for surveying resistance rates in different regions and for personalising *H. pylori* treatment.

Traditionally, *H. pylori* AST has been performed by culturing the bacteria from stomach tissue biopsies taken during endoscopic examination and determining the minimum inhibitory concentration of an antimicrobial agent required to inhibit bacterial growth[12]. But *H. pylori* is a fastidious organism and culture is challenging and time-consuming with reported success rates varying from 55%-93%[13,14]. Culture success is influenced by many factors, including PPI use, tissue sampling site, choice of transport medium and *H. pylori* growth conditions[4,15]. This study aimed to determine whether a dual antrum and corpus biopsy sampling protocol was superior to a single antrum biopsy protocol for the successful culture of *H. pylori*.

## MATERIALS AND METHODS

### Study design and ethics

The study was carried out at Tallaght University Hospital, Dublin, Ireland, which is affiliated with Trinity College Dublin. The study received ethical approval from the joint research ethics committee of Tallaght University Hospital and St. James's Hospital. Patients referred for upper gastrointestinal endoscopy were invited to participate. Patients were prospectively recruited to determine the culture success rate when combined antrum and corpus biopsies were used. The culture success rate when single antrum biopsies were used was determined retrospectively.

### Study population

Inclusion criteria were (1) Ability and willingness to participate in the study and to provide informed consent; and (2) Confirmed *H. pylori* infection as indicated by a positive rapid urease test (TRI-MED Distributors, PTY LTD, Washington, United States) at 30 min and by histology. Exclusion criteria were (1) Age less than 18 years; (2) Pregnancy or lactation; (3) Severe intercurrent illness; (4) Recent antimicrobial use (within 4 wk); and (5) Bleeding problems or use of blood thinning drugs.

### Sample collection

At endoscopy, biopsy samples from each patient were placed directly into collection tubes containing Dent's transport medium [brain heart infusion broth containing 2.5% (w/v) yeast extract, 5% sterile horse serum and *H. pylori* Selective Supplement (Oxoid, Basingstoke, United Kingdom)]. When both antrum and corpus biopsies were collected from a patient, the two tissue samples were placed into the same collection tube. Biopsy samples were processed for culture as soon as possible following endoscopy, usually within 6 h. If processing was delayed, samples were refrigerated at 4 °C and used to inoculate plates within 24 h.

### H. pylori culture

The tissue samples were inoculated onto Columbia blood agar plates containing 5% laked horse blood (VWR International, Lutterworth, Leicestershire, United Kingdom) and incubated at 37 °C under microaerobic conditions generated using the CampyGen 2.5 L Atmosphere Generation System (Oxoid). When both antrum and corpus biopsies were collected, they were inoculated onto the same plate. Plates were examined for the presence of *H. pylori* for up to 7 d. *H. pylori* was identified by visual inspection of the colonies, a positive urease test and by polymerase chain reaction.

### Statistical analysis

Statistical analysis was performed using GraphPad Prism (GraphPad Software Inc., CA, United States). Continuous variables are presented as arithmetic mean and standard deviation. Continuous variables were compared using the two-tailed independent *t* test. Categorical variables are presented as percentages and their 95% confidence intervals (95%CI). Categorical variables were compared using the two-tailed Fisher exact test. In all cases, a *P* value less than 0.05 was considered significant.

## RESULTS

In all, samples from 219 *H. pylori*-infected patients were analysed. The mean age of recruited patients was 48 ± 14.9 years and 50.7% were male (Table 1). The most common endoscopic finding was gastritis (58.9%; *n* = 129). The rates of more serious *H. pylori*-associated diseases, such as gastric ulcer, duodenal ulcer and intestinal

**Table 1 Patient demographics**

| | Total, *n* = 219 | Single, *n* = 73 | Combined, *n* = 146 | *P* value[1] |
|---|---|---|---|---|
| Mean age (yr) | 48 ± 14.9 | 49 ± 15.9 | 48 ± 14.5 | 0.43 |
| **Sex** | | | | 0.32 |
| Male | *n* = 111 (50.7%) | *n* = 41 (56.2%) | *n* = 70 (47.9%) | |
| Female | *n* = 108 (49.3%) | *n* = 32 (43.8%) | *n* = 76 (52.1%) | |
| **Endoscopy findings** | | | | |
| Normal | 18 (8.2%) | 5 (6.8%) | 13 (8.9%) | 0.80 |
| Gastritis | 129 (58.9%) | 40 (54.8%) | 89 (61.0%) | 0.57 |
| Gastric ulcer | 10 (4.6%) | 3 (4.1%) | 7 (4.8%) | 1.00 |
| Duodenal ulcer | 6 (2.7%) | 2 (2.7%) | 4 (2.7%) | 1.00 |
| Intestinal metaplasia | 1 (0.5%) | 1 (1.4%) | 0 (0%) | 0.33 |
| Duodenitis | 11 (5.0%) | 3 (4.1%) | 8 (5.5%) | 0.76 |
| Oesophagitis | 4 (1.8%) | 3 (4.1%) | 1 (0.7%) | 0.12 |
| Barrett's oesophagus | 5 (2.3%) | 3 (4.1%) | 2 (1.4%) | 0.34 |
| Hiatus hernia | 9 (4.1%) | 3 (4.1%) | 6 (4.1%) | 1.00 |
| Telangiectasia | 1 (0.5%) | 0 (0%) | 1 (0.7%) | 1.00 |
| Portal hypertensive gastropathy | 1 (0.5%) | 1 (1.4%) | 0 (0%) | 0.33 |
| No data | 24 (11.0%) | 9 (12.3%) | 15 (10.3%) | 0.65 |

[1]Single versus combined.

metaplasia were low in the study cohort at 4.6% (*n* = 10), 2.7% (*n* = 6) and 0.5% (*n* = 1), respectively (Table 1).

Single antrum biopsies were collected from 73 patients, whereas combined antrum and corpus biopsies were collected from 146 patients. There was no significant difference in age, sex or endoscopic findings between the two groups (Table 1). *H. pylori* was successfully cultured in a significantly higher number of cases when combined antrum and corpus biopsies were used compared to a single antrum biopsy [64.4% (*n* = 94/146) *vs* 49.3% (36/73); *P* = 0.04) (Table 2)].

## DISCUSSION

*H. pylori* AST is critical to accurately detect antimicrobial resistance, thereby influencing appropriate treatment choices, promoting antimicrobial stewardship and increasing *H. pylori* eradication rates. While molecular AST methods are available, these are primarily limited to the detection of clarithromycin-and levofloxacin-associated DNA mutations. Culture-based AST remains the only method currently available to test all the antimicrobials potentially useful for *H. pylori* treatment[16]. Despite the importance of culture-based AST , *H. pylori* culture is not routinely performed in the majority of hospitals[5-7,11] either to survey resistance rates or to tailor therapies. From a microbiology perspective, *H. pylori* is challenging to culture. In this study, we report an increased culture success rate when a dual antrum and corpus biopsy protocol was used compared to using a single antrum biopsy (64.4% *vs* 49.3%; *P* = 0.04). While a significant improvement in culture success was observed, a rate of 64.4% is lower than some previous reports. PPI use is known to impact the diagnostic accuracy of *H. pylori* culture[8]. While patients attending for endoscopy at our centre are encouraged to refrain from PPI use 2 wk prior to their scheduled endoscopy, in practice many do not. Nonetheless, the 15% increase in culture success rate reported here provides a strong rationale for a combined biopsy approach.

It is not surprising that the more biopsy specimens used for culture, the higher the chance of recovering *H. pylori* and this practice has been suggested elsewhere[15,17]. However, recent guidelines on the management of *H. pylori*[6-8,11] do not include

**Table 2 Culture success rate of *Helicobacter pylori* using single antrum biopsies versus combined antrum and corpus biopsies**

| | Culture positivity rate | *P* value |
|---|---|---|
| Single biopsy | 49.3% (36/73; 95%CI: 38.2-60.5) | 0.04[a] |
| Combined biopsies | 64.4% (94/146; 95%CI: 56.3-71.7) | |

[a]*P* value < 0.05.

specific recommendations on biopsy sampling protocols for *H. pylori* culture and studies directly evaluating culture success using a single *vs* combined biopsy sampling protocol are lacking. The biopsy sampling location is important for a number of reasons. Firstly, collecting biopsies from both the antrum and the corpus takes into account patchy distribution of *H. pylori* in the stomach, which can occur with PPI use [15,18,19]. Furthermore, intragastric location-specific differences in the evolution of *H. pylori* have been reported across strains within the same individual[20]. In terms of AST, it is important to collect biopsies from both sites, as these differences extend to the antimicrobial susceptibility profiles between strains isolated from the corpus and those from the antrum of the same patient[21,22]. Thus, resistance to a given antimicrobial could be missed if biopsy samples from only one location are taken, potentially having a negative impact on treatment outcome.

A limitation of our study is that patients were recruited prospectively to the dual biopsy sampling group, while the single antrum biopsy culture success rate was analysed retrospectively. However, it should be noted that for the entire duration of the patient recruitment and sample collection phases of the study, we followed the standardized protocols of the European *H. pylori* Antimicrobial Susceptibility Testing Working Group[4]. Therefore, the sample transport protocols, microbiological media and culture conditions and methods were consistent throughout the entirety of the study, thereby limiting heterogeneity in this regard.

## CONCLUSION

In conclusion, combined corpus and antrum biopsy sampling improves *H. pylori* culture success compared to single antrum biopsy sampling.

## ARTICLE HIGHLIGHTS

### *Research background*

*Helicobacter pylori (H. pylori)* represents a public health issue as the causative agent of chronic gastritis, peptic ulcer disease, gastric adenocarcinoma and mucosa-associated lymphoid tissue lymphoma. Success rates for current therapies have fallen over the years, mainly due to antimicrobial resistance. International guidelines recommend that treatment choices are based on local antimicrobial resistance rates. However, *H. pylori* culture is challenging and culture-based antimicrobial susceptibility testing (AST) is not routinely performed in most healthcare facilities.

### *Research motivation*

Optimisation of *H. pylori* culture from clinical specimens will enable more widespread AST for *H. pylori*.

### *Research objectives*

This research aimed to evaluate biopsy sampling protocols to enhance *H. pylori* culture success, specifically to determine whether dual antrum and corpus biopsy sampling was superior to a single antrum biopsy sampling protocol.

### *Research methods*

Stomach tissue biopsies from rapid-urease test positive patients were collected in tubes containing Dent's transport medium. Biopsies were used to inoculate Colombia blood agar plates. Plates were incubated under microaerobic conditions and evaluated for the presence of *H. pylori*. Culture success rates when a single antrum biopsy was used

were compared to those when dual antrum and corpus biopsies were used.

### Research results

*H. pylori* was successfully cultured in a significantly higher number of cases when combined antrum and corpus biopsies were used compared to a single antrum biopsy sample.

### Research conclusions

A combined corpus and antrum biopsy sampling approach improves *H. pylori* culture success compared to a single antrum biopsy sampling protocol.

### Research perspectives

Optimisation of *H. pylori* culture methods will encourage more widespread AST. Antimicrobial resistance surveillance is the key to determining the most appropriate antimicrobials for *H. pylori* eradication.

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## MINIREVIEWS

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*MINIREVIEWS*

# Application of electron microscopy in gastroenterology

Masaya Iwamuro, Haruo Urata, Takehiro Tanaka, Hiroyuki Okada

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**Masaya Iwamuro, Hiroyuki Okada,** Department of Gastroenterology and Hepatology, Okayama University Graduate School of Medicine, Dentistry, and Pharmaceutical Sciences, Okayama 700-8558, Japan

**Haruo Urata,** Central Research Laboratory, Okayama University Medical School, Okayama 700-8558, Japan

**Takehiro Tanaka,** Department of Pathology, Okayama University Hospital, Okayama 700-8558, Japan

**Corresponding author:** Masaya Iwamuro, MD, PhD, Assistant Professor, Department of Gastroenterology and Hepatology, Okayama University Graduate School of Medicine, Dentistry, and Pharmaceutical Sciences, 2-5-1 Shikata-cho, Kita-ku, Okayama 700-8558, Japan. iwamuromasaya@yahoo.co.jp

## Abstract

Electron microscopy has long been used in research in the fields of life sciences and materials sciences. Transmission and scanning electron microscopy and energy-dispersive X-ray spectroscopy (EDX) analyses have also been performed in the field of gastroenterology. Electron microscopy and EDX enable (1) Observation of ultrastructural differences in esophageal epithelial cells in patients with gastroesophageal reflux and eosinophilic esophagitis; (2) Detection of lanthanum deposition in the stomach and duodenum; (3) Ultrastructural and elemental analyses of enteroliths and bezoars; (4) Detection and characterization of microorganisms in the gastrointestinal tract; (5) Diagnosis of gastrointestinal tumors with neuroendocrine differentiation; and (6) Analysis of gold nanoparticles potentially used in endoscopic photodynamic therapy. This review aims to foster a better understanding of electron microscopy applications by reviewing relevant clinical studies, basic research findings, and the state of current research carried out in gastroenterology science.

**Key Words:** Transmission electron microscopy; Scanning electron microscopy; Energy-dispersive X-ray spectrometry; Gastrointestinal disease, gastroesophageal reflux disease; Pathogens

**Core Tip:** This review provides an overview of transmission electron microscopy, scanning electron microscopy, and energy-dispersive X-ray spectrometry analyses used in the field of gastroenterology. Previously reported articles have been reviewed, with a focus on electron microscopy applications. The history and present trends in electron microscopy applications in patients and research associated with digestive system diseases are also summarized.

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## INTRODUCTION

In light microscopy, visible light is used to obtain magnified views of the object. As the resolution is related to the wavelength of light used to image a specimen, the resolution of an optical microscope is theoretically limited to approximately 200 nm. Thus, nanostructures cannot be observed using light microscopy. In contrast, electron beams are used in electron microscopy. As the wavelength of an electron beam is shorter than that of visible light, electron microscopy has extremely high resolution and provides sharp, finely detailed images of the surface or interior of biological and nonbiological specimens. In addition, energy-dispersive X-ray spectroscopy (EDX), which is a chemical microanalysis technique used in conjunction with electron microscopy, enables the analysis of elements or chemical characterization of a sample. Since the development of the first prototype in 1931, electron microscopes have been widely used in various fields, such as physics, chemistry, engineering, biology, and medicine [1]. Based on its versatility, electron microscopy analysis has been used in several studies covering various aspects of clinical samples obtained from patients with gastrointestinal diseases. This paper briefly discusses the fundamentals of electron microscopy and reviews the literature concerning the application of electron microscopy in gastroenterology science.

## ANALYTICAL METHODS IN ELECTRON MICROSCOPY

Analytical methods in electron microscopy can broadly be categorized into three types: Transmission electron microscopy, scanning electron microscopy (SEM), and EDX. The different types of electron microscopes used in these methods are related and often applied concurrently in the field of biology.

A transmission electron microscope irradiates a specimen with an electron beam. The object must be cut into very thin cross-sections because it is visualized through the spatial distribution of the transmitted electron beam. Although the use of transmission electron microscopy is limited to engineering science at the outset, it has been extensively used in the field of biology since the 1950s largely due to improvement of the microtome for ultrathin slice preparation using a diamond knife and the development of staining techniques based on heavy metals, such as osmium.

A scanning electron microscope produces an image using electrons reflected or generated from the surface of the specimen. The specimen is placed in a high vacuum state, and the surface is scanned with an electron beam focused by an electric or magnetic field. SEM produces a characteristic three-dimensional appearance that is useful for understanding the surface ultrastructure of a sample.

EDX is an X-ray system used to identify the elemental composition of a material. It has a semiconductor detector to detect the fluorescent X-rays generated when the primary X-ray beam illuminates the sample. The fluorescent X-rays emitted from the material have a spectrum of wavelengths characteristic of the types of atoms present in the specimen. EDX enables both qualitative and semiquantitative analyses of the elements based on the energy and number of generated electron-hole pairs. EDX is more suited for analyses of inorganic materials than organic materials.

In the field of gastroenterology, transmission and SEM and EDX analyses have been used to visualize cells (Figure 1) and pathogens, including parasites, bacteria, viruses, biofilms, and elements deposited in the gastrointestinal mucosa. Nonbiological materials, such as stents, powders, and bezoars, have also been analyzed at subnanometer resolution. In the following sections, we review examples of electron microscopy analyses in association with the pathophysiology of gastrointestinal disorders.

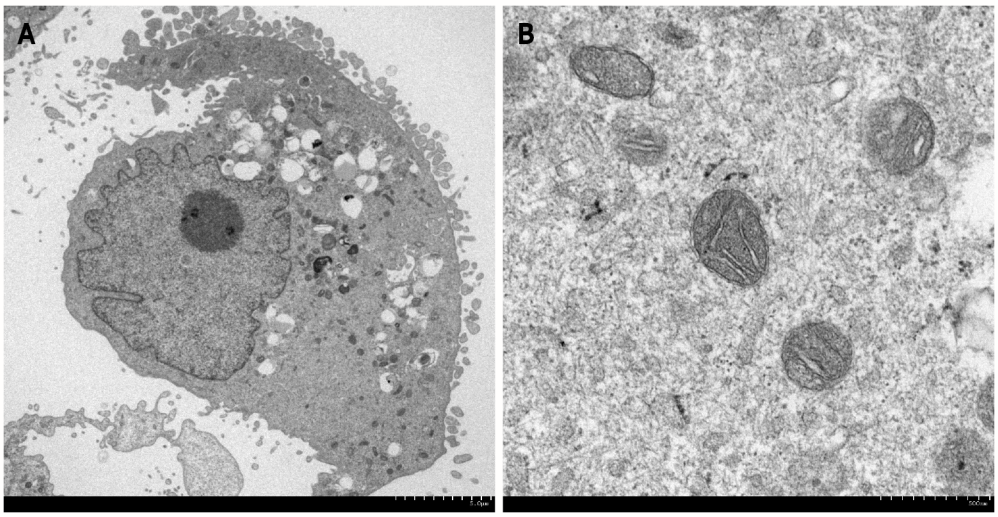


**Figure 1 Transmission electron microscopy image.** Transmission electron microscopy of the human cell line derived from gastric cancer (SH-10-TC) showing the morphology of cells and their organelles. A: Scale bars = 5 μm; B: Scale bars = 500 nm.

## EXAMPLES OF ELECTRON MICROSCOPY ANALYSES

### *Intercellular spaces of the esophageal epithelium*

The most typical example of electron microscopy analysis in gastroenterology is evaluation of the intracellular spaces of esophageal epithelial cells. Notably, some of the articles on this topic have been published in high-impact journals. Intercellular spaces in the esophageal epithelium are known to be dilated in patients with nonerosive reflux disease and in patients with esophagitis. Following several animal studies, endoscopic esophageal biopsy specimens taken from patients with ($n$ = 11) and without ($n$ = 13) recurrent heartburn were investigated in 1996 using transmission electron microscopy[2]. A dilated intercellular space diameter was observed in 8 of the 11 patients with heartburn, while none of the asymptomatic individuals exhibited this feature. Dilated intercellular space was also present in the normal-appearing, nonerosive mucosa of patients with symptomatic reflux disease. Other authors have provided further evidence that detached interepithelial cell junctions, which are observed as dilated intercellular spaces assessed by electron microscopy[3-5], correspond to early esophageal damage induced by acid reflux[6-8]. Dilatation of intercellular spaces in the esophageal epithelium is not observed in patients with functional heartburn, suggesting that this microscopic feature is specific to acid reflux[9]. Proton pump inhibitor therapy resulted in complete recovery of dilated intercellular spaces in > 90% of cases with nonerosive reflux disease and erosive esophagitis, indicating that the electron microscopy features are reversible[10,11].

Dilated intracellular spaces arise along the distal and proximal esophagus of patients with nonerosive reflux disease, suggesting that they may be an underlying mechanism accounting for the enhanced perception of proximal acid reflux[12]. Duodenal gastroesophageal reflux has also been reported to cause dilatation of intercellular spaces in the esophageal epithelium[3,13]. Similarly, in patients with laryngopharyngeal reflux and sore throat, this feature appears at the squamous basal and suprabasal levels in oropharyngeal biopsy specimens[14,15]. An investigation of patients with bronchial asthma[11, 16] and children with reflux-related cough[17] revealed that the intracellular spaces in the esophageal epithelium are significantly dilated compared with those in control patients, suggesting a pathophysiological correlation between gastroesophageal reflux and the development of these respiratory tract symptoms.

Although the width of the intracellular spaces can be measured using light microscopy[18], the sensitivity of light microscopy was 79.3%, and the specificity was 75.0%[19]. Owing to the inferior specificity of light microscopy analysis, electron microscopy seems to be more suitable for measuring intercellular spaces in the esophageal epithelium. Chu *et al*[20] reported the possible utility of in vivo confocal laser endomicroscopy to examine microalterations of the esophagus in patients with nonerosive reflux disease[20].

### *Eosinophilic esophagitis*

Eosinophilic esophagitis is a chronic, allergic inflammatory condition of the esophagus. Dilated intracellular spaces are evident in the esophageal epithelium of patients with eosinophilic esophagitis, which are significantly reduced after treatment[21]. Transmission electron microscopy revealed a significant decrease in the number of desmosomes[22] and an increased autophagic vesicle content[23] in active eosinophilic esophagitis compared with observations in normal individuals and inactive

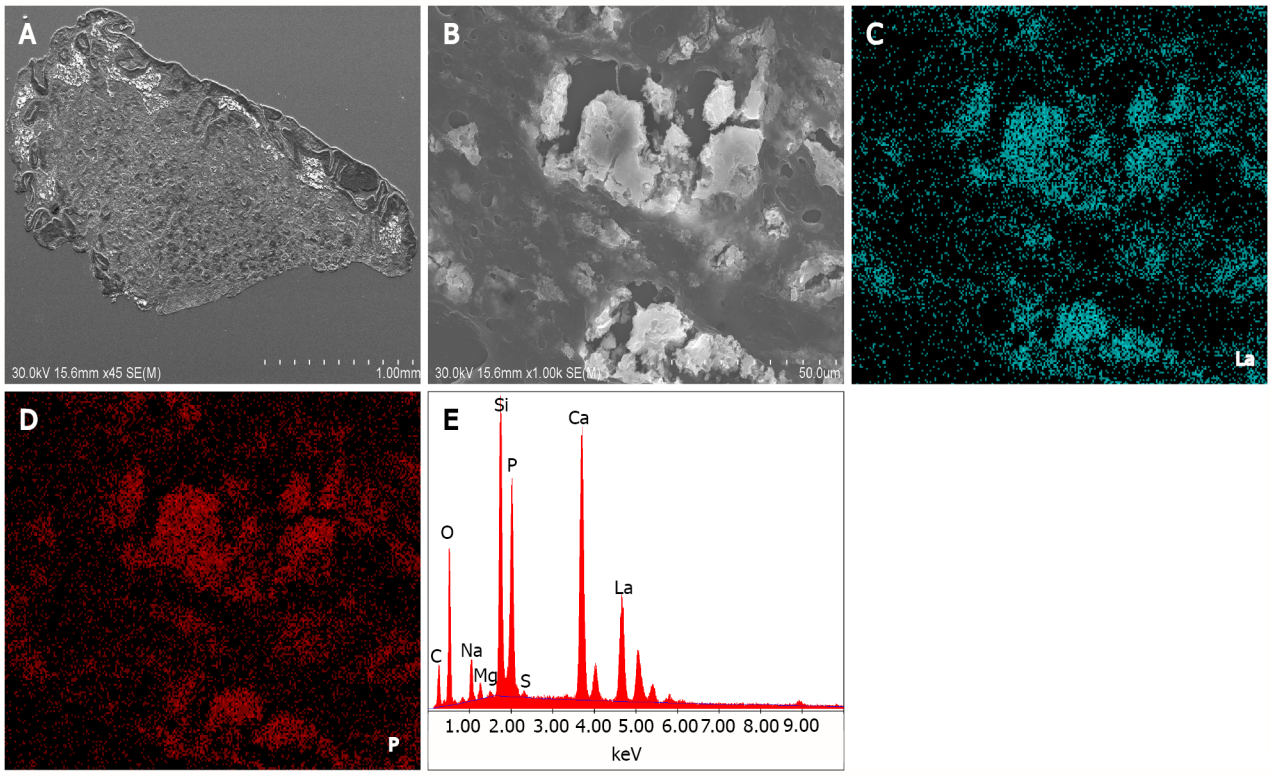


**Figure 2 Transmission electron microscopy images and spectra obtained by energy-dispersive X-ray spectrometry.** A: Lanthanum phosphate deposition in the gastric mucosa was diagnosed after analysis by scanning electron microscopy, which visualized deposited lanthanum as bright areas; B: Deposited lanthanum is composed of aggregates of particles; C and D: Elemental mapping showing the colocation of lanthanum (C) and phosphate (D); E: Energy-dispersive X-ray spectrometry.

eosinophilic esophagitis patients. Thus, electron microscopy may be useful for investigating the pathophysiology of eosinophilic esophagitis.

### *Lanthanum deposition*

Lanthanum carbonate is a phosphate binder taken orally and is commonly used in patients with chronic kidney disease. Although its tolerability and safety profile have been reported in hemodialysis patients, lanthanum deposition in the gastric and duodenal mucosa of these patients, in the form of lanthanum phosphate, has been reported in the literature[24-28]. On light microscopy examination of hematoxylin and eosin-stained specimens, deposited lanthanum is visible as a fine, amorphous, eosinophilic material. SEM revealed bright areas in the deposited lanthanum (Figure 2A). Images at higher magnification showed deposition as the accumulation of minute particles (Figure 2B). EDX analysis provided evidence directly related to the presence of lanthanum and phosphate (Figure 2C). Elemental mapping by EDX revealed that lanthanum (Figure 2D) and phosphate (Figure 2E) showed an identical location to that of the bright areas on SEM. Although lanthanum deposition in the gastrointestinal tract can be clinically diagnosed with conventional light microscopy observation of the fine, amorphous, eosinophilic material and medication information from the patient's current or past use of lanthanum carbonate, SEM has advantages in the detection of deposited lanthanum, as it is easily identified as a bright area.

### *Enteroliths and bezoars*

Enteroliths are calculi that occur in the intestines and include two types: "True" and "false" enteroliths. True enteroliths, for example, cholic acid and calcium stones, are generated from the sediments of substances found in enteric contents. False enteroliths, such as bezoars, gallstones, and foreign objects, are formed from indigestible substances stuck in the alimentary tract. Infrared spectroscopy is generally used to identify the chemical substances constituting enteroliths removed from patients. Electron microscopy and EDX have the advantages of imaging the microstructure and analyzing elements, allowing clarification of the nature of enteroliths.

Figure 3 shows examples of enteroliths and bezoars that we previously investigated. One patient had an enterolith in the stomach composed of bilirubin calcium, calcium carbonate, and fatty acid calcium [29] (Figure 3A and B). Another patient had a rare pharmacobezoar in the stomach, which was composed of magnesium oxide (Figure 3C–F)[30]. We also investigated the ultrastructure of the persimmon phytobezoar in the stomach (Figure 3G–I)[31]. Thus, electron microscopy and EDX analyses

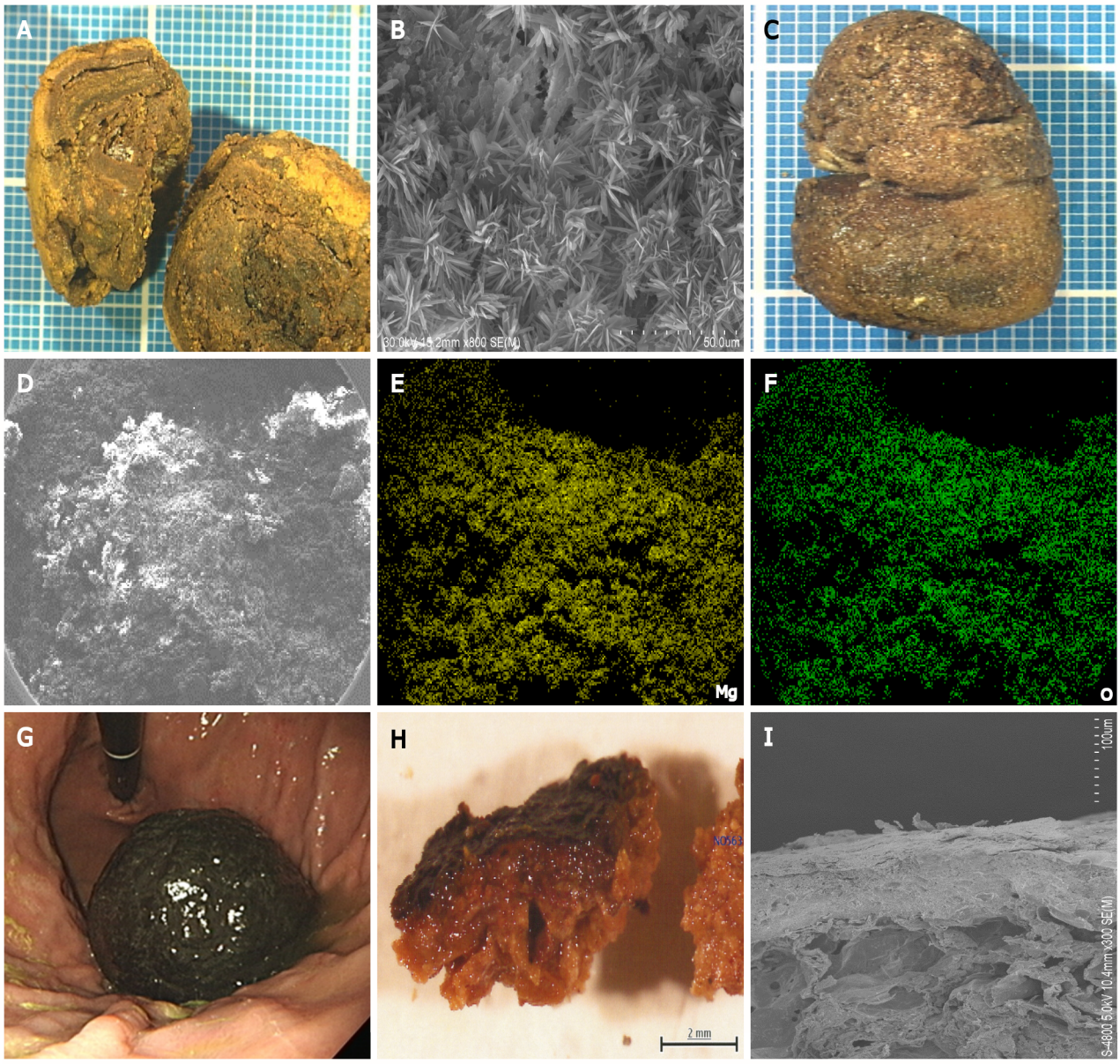


**Figure 3 Images of enteroliths.** A and B: An enterolith found in the stomach showing a laminar structure on the cut surface (A). Scanning electron microscopy demonstrating acicular crystals (B), suggesting epitaxial growth of the enterolith; C: Another patient had a pharmacobezoar composed of magnesium oxide in the stomach; D-F: Scanning electron microscopy showed a granular substance (D) and a diffuse distribution of magnesium (E) and oxide (F); G: A persimmon phytobezoar was observed in the stomach; H: The cut section shows that the color of the bezoar surface is black, and the interior is yellowish; I: On scanning electron microscopy, a high-density, continuous layer forming the exterior of the phytobezoar is evident on the cut surface, while sheet-like structures of curved or wiggly shapes constitute the inner part.

offer insights into the microstructure and elemental composition of enteroliths.

### *Pathogens including bacteria, parasites, and viruses in the gastrointestinal tract*

Electron microscopy has been widely used in microbiology to elucidate the number, distribution, and adherence of microorganisms in clinical samples. One of the typical applications of electron microscopy for pathogens in gastroenterology is the detection of *Helicobacter* species, such as *Helicobacter pylori*[32-36] and *Helicobacter heilmannii*[37]. These bacteria have a spiral form, which is a distinct difference from other bacteria. Another example is *Tropheryma whipplei*[38-41], which causes the rare systemic infectious disorder Whipple's disease. Electron microscopy revealed that *Tropheryma whipplei* shows a characteristic trilamellar plasma membrane. Other rare pathogens identified by electron microscopy include anisakiasis[42], amoebiasis[43], intestinal spirochetosis[44], *Sutterella wadsworthensis*[45], *Giardia intestinalis*[46], and *Brachyspira aalborgi*[47].

A biofilm is a thick layer formed by microorganisms attached to the surface of a solid material or liquid. SEM has been used to visualize the shape and localization of biofilms and the steps of the biofilm formation process. For instance, several authors have investigated the efficiency of the cleaning, disinfection, and sterilization processes of biofilm-contaminated endoscopes[48,49].

### *Gastrointestinal tumors with neuroendocrine differentiation*

Neuroendocrine and mixed neuroendocrine neoplasms can arise in most of the epithelial organs of the body and are not rare in the gastrointestinal tract. Transmission electron microscopy revealed that neuroendocrine tumor cells in the gastrointestinal tract contained numerous dense-core secretory granules of variable sizes and shapes in the cytoplasm. Because these neurosecretory granules are characteristic of neuroendocrine tumors, electron microscopy analysis has been used to support its diagnosis. For instance, neuroendocrine differentiation was assessed using electron microscopy images in cases of malignant peripheral nerve sheath tumors of the esophagus[50], gangliocytic paraganglioma in the duodenum[51], mixed acinar-endocrine carcinoma arising in the ampulla of Vater[52], combined adenocarcinoma and neuroendocrine tumors in the stomach[53], neuroendocrine carcinoma in the stomach[54], mixed acinar-endocrine neoplasm in the stomach[55], and large cell neuroendocrine carcinoma in the esophagogastric junction[56].

### *Gold nanoparticles potentially used in endoscopic photodynamic therapy*

Based on the properties of absorption and scattering of electromagnetic radiation, gold nanoparticles are emerging as promising agents and are of particular interest for applications in photothermal therapy, in addition to efficient drug carriers and diagnostic agents. For instance, endoscopic fluorescence-guided near-infrared photothermal therapy using gold nanoparticles is in development for the treatment of gastrointestinal tumors[57]. The size, morphology, and composition of synthesized gold nanoparticles and their location within tissue can be assessed using transmission electron microscopy and EDX analysis[58].

## CONCLUSION

Electron microscopy enables (1) Observation of ultrastructural differences in esophageal epithelial cells in patients with gastroesophageal reflux and eosinophilic esophagitis; (2) Detection of lanthanum deposition in the stomach and duodenum; (3) Ultrastructural and elemental analyses of enteroliths and bezoars; (4) Detection and characterization of microorganisms in the gastrointestinal tract; (5) Diagnosis of gastrointestinal tumors with neuroendocrine differentiation; and (6) Analysis of gold nanoparticles potentially used in endoscopic photodynamic therapy. Therefore, electron microscopy has had a profound impact on our knowledge and understanding of various digestive tract diseases. We hope that this article will help gastroenterologists widely utilize electron microscopy analysis for clinical diagnosis and basic research.

## FOOTNOTES

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**Country/Territory of origin:** Japan

**ORCID number:** Masaya Iwamuro 0000-0002-1757-5754; Haruo Urata 0000-0002-0268-6187; Takehiro Tanaka 0000-0002-1509-5706; Hiroyuki Okada 0000-0003-2814-7146.

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MINIREVIEWS

# Risk assessment of hepatitis E transmission through tissue allografts

Rafael Villalba, Vicente Mirabet

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**Rafael Villalba,** Center for Blood Transfusion, Tissues and Cells, Córdoba 14004, Spain

**Vicente Mirabet,** Cell and Tissue Bank, Centro de Transfusión de Valencia, Valencia 46014, Spain

**Corresponding author:** Vicente Mirabet, PhD, Senior Scientist, Cell and Tissue Bank, Centro de Transfusión de Valencia, Avenida del Cid, 65-A, Valencia 46014, Spain. mirabet_vic@gva.es

## Abstract

Hepatitis E virus (HEV) is a small non-enveloped single stranded RNA virus whose genotypes 3 and 4 have been associated with zoonotic transmission in industrialized countries. HEV infection is considered the main cause of acute hepatitis worldwide. In some cases, transfusion of blood components or organ transplantation have been reported as the source of infection. We have conducted a literature review on the risk of transmission through cell and tissue allografts. Although no case was found, measures to control this risk should be taken when donor profile (based upon geographical and behavioural data) recommended it. Issues to be considered in donor screening and tissue processing to assess and to reduce the risk of HEV transmission are approached.

**Key Words:** Hepatitis E; Tissue allograft; Risk assessment; Disease transmission; Donor screening; Bioburden reduction



**Core Tip:** This manuscript provide a novel perspective of the mode of transmission of hepatitis E virus (HEV). HEV is mainly transmitted *via* fecal-oral route, but in recent years other transmission routes have been reported, including blood-borne transmission. The processing of tissue allografts in duly accredited tissue banks provides safe and efficient products.

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## INTRODUCTION

There are several types of human tissues which are commonly used as allografts: Bone, tendon, cartilage, skin, cornea, amniotic membrane, stem cells, heart valve, blood vessel, *etc.* Almost all surgical disciplines benefit of its availability. Thus, millions of human tissue transplants are performed worldwide every year[1].

One of the drawbacks of these procedures is the potential for donor to recipient disease transmission. Although the real incidence of tissue allograft transmitted infection is unknown, some articles have published cases of viral, bacterial and fungal infections transmitted by tissues[2-5]. Regarding the different infectious agents, hepatotropic viruses have represented traditionally the real *workhorse* in maintaining the safety of tissues used for transplantation.

Hepatitis B virus (HBV) and hepatitis C virus (HCV) can cause acute and chronic hepatitis and potentially lead to the development of cirrhosis, liver cancer and death. In the European Union, estimated 4.7 million people have a chronic HBV infection, and 3.9 million people have chronic hepatitis C. Many of these infections may go undiagnosed as chronic infection is often asymptomatic and a hypothetical tissue donor could be a potential transmitter of the disease[6].

Risk factors for HBV and HCV infection are now clearly established[7-11]. In recent decades, various factors have contributed towards changes in HBV and HCV epidemiology, including improvements in donor tissue safety. A rigorous evaluation of clinical, behavioral, and personal risks is now performed as it may completely exclude a donor[12,13]. In addition to this, all potential tissue donors must be tested for both serological anti-HBc, HBsAg anti-HCV and for HCV-HBV by nucleic acid testing. Based on both criteria, the risk of HCV and HCV transmission is currently very low established in 1 in 34000 for HBV and 1 in 42000 for HCV[14].

Hepatitis E virus (HEV) infection is one of the main causes of acute hepatitis in both developed and developing countries. This infectious disease has a high prevalence and incidence in Europe and has a greater clinical impact in vulnerable populations, such as immunosuppressed patients, pregnant women, and patients with underlying liver disease[10,15,16].

To date, there are no specific recommendations for the screening of this disease in blood, tissue, or organ donors, which may cause this route to be an important source of disease transmission.

## INFORMATION RETRIEVAL SYSTEM

A search using the following search string: 'hepatitis E virus [Title/Abstract] OR HEV [Title/Abstract] NOT high endothelial venules [Title/Abstract]' was conducted. Applying these criteria on PubMed database (for articles published in last 20 years) 5485 records were recovered (Figure 1). This search was developed on 5$^{th}$ December 2020. Six hundred forty-three (11.7%) of them corresponded to reviews and 0.6% to systematic reviews (the first being published in 2009). When the search was restricted (using the Boolean operator *AND*) to the articles involving the word 'allograft', only 19 (0.3%) complied to the new condition. Seventy nine percent (15/19) of these last articles dealt only on organ transplantation, 2 on the transfusion of blood components (specially in relation to hematopoietic transplantation) and the other 2 were discarded because the reason for their recovery was the use of the acronym HEV (without description) to refer to high endothelial venules. Thus, to the best of our knowledge, the present paper is the first cross reference between HEV and tissue allografts.

## HEV

HEV is a small non-enveloped positive-sense, single-stranded RNA virus, encased within an icosahedral capsid of between 27 and 34 nm in size belonging to the family Hepeviridae within the genus Orthohepevirus. Seven different genotypes have been described for the HEV. Five of them (1-4 and 7) can infect humans and the other two (5, 6) are found only in animals (boar). Genotypes 1 and 2 (HEV-1, HEV-2) have been found only in humans while genotypes 3 and 4 circulate in several animals (including pigs, rabbit, cattle, sheep, horse, boar, deer, and shellfish) and genotype 7 in camel. Genotypes 1 and 2 are directly transmitted fecal-orally, or indirectly, mainly *via* contaminated water. Genotypes 3 and 4 (HEV-3, HEV-4) are zoonotic infections with an animal reservoir, being indirectly transmitted through food (when consumed raw or undercooked) or by direct contact with infected animals. Thus, professionals who work in contact with animals or their wastes and carcasses (farmers, veterinarians, workers attending animals, slaughterers, traders, and suppliers) could be in higher risk of HEV infection[14,15-18]. In an effort to avoid inconsistencies when the HEV subtypes are named, Smith *et al*[19] have proposed standardization for the assignation of HEV sequences to each subtype. Likewise, the World Health Organization promoted the development of international standards for diagnostic assays[15,20].

Additionally to the host and mode of transmission, HEV genotypes also vary in geographical distribution. Genotype 1 is prevalent in Africa and Asia, whereas HEV-2 can be found in México and West Africa. Thus, HEV-1 and HEV-2 are responsible for HEV outbreaks in developing countries, with

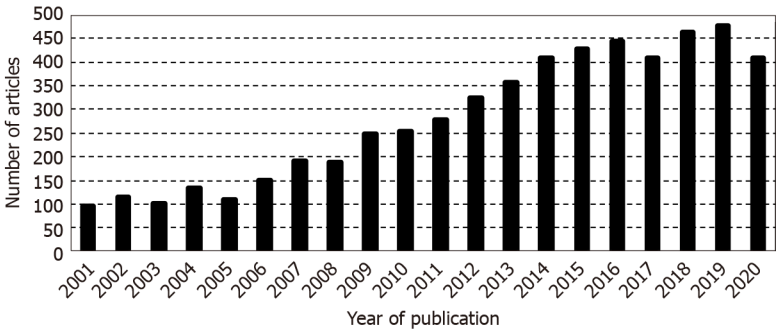


**Figure 1 PubMed timeline results *per* year on hepatitis E virus.**

limited sanitary conditions, due to contaminated drinking water. Genotypes 3 and 4 are associated with zoonotic transmission as autochthonous (locally acquired) infection in industrialized countries[21].

Clinical symptoms of HEV infection do not differ from other pathogens causing hepatitis. Therefore, diagnosis is performed by HEV RNA detection using real-time reverse transcription polymerase chain reaction with primers detecting all 4 genotypes affecting humans. Additionally, detection of HEV immunoglobulin (Ig) M and IgG antibodies is performed by enzyme linked immunosorbent assay. These data characterize HEV infection as acute (demonstration of specific IgM, rising levels of IgG, or detection of HEV RNA), passed, or chronic (positive results for HEV-RNA for more than 6 mo)[14,22]. Likewise, HEV antigen detection assay has been found to be used when HEV RNA testing is not available or time is limited[16]. Although HEV Ag shows low sensitivity with viral loads lower than 1000 copies/mL, it has shown good correlation with HEV-RNA, being useful in diagnosing infection in immunosuppressed patients[23,24]. Another issue to be considered, when Epstein-Barr virus and cytomegalovirus infection are present, is the risk of false positive results from anti-HEV IgM assays[25].

HEV transmission between persons by direct contact has proven very inefficient probably due to the high infective dose required[26]. Although it is associated with low mortality rates (< 1%) in the general population, this risk increases (approximately 20%) during pregnancy[14].

Although hepatitis viruses have been suggested to play a role of in the development of autoimmune hepatitis (AIH)[27], a large multicentre study did not find differences in the prevalence of anti-HEV IgG between AIH and healthy patients[28]. Additionally, they did not identify chronically HEV-infected patients within the AIH cohort.

HEV virions, as those of HAV (both hepatotropic virus but phylogenetically unrelated), are known to be non-enveloped in feces, but they circulate in the blood-stream coated in a lipid membrane. This kind of virus particles has been named quasi-enveloped virions[29].

The main risk factors on HEV infection to be considered for donor screening can be summarized in: Areas with limited access to essential services as water, sanitation, and health care facilities; Consumption of undercooked or raw foodstuffs from animals; Middle-aged and elderly men.

The severity of the consequences increases when these factors occur together with others related to the recipients, as pregnant women (because fulminant hepatitis occurs more frequently during pregnancy) or immunocompromised patients (as solid organ transplant recipients or patients receiving hematopoietic progenitor cell transplantation).

## RISK ASSESSMENT OF HEPATITIS E TRANSMISSION THROUGH SUBSTANCES OF HUMAN ORIGIN

HEV is considered to be the most common cause of acute hepatitis worldwide[30]. Its infection typically follows a fairly routine clinical course with an incubation period of 2 wk to 6 wk, followed by a detectable viraemia in serum along to symptoms such as abdominal pain, vomiting, jaundice, *etc.* Usually, the disease course is self-limiting. As said before, some individual profiles can lead to a more severe hepatic complication.

Whereas HEV-3 infection in healthy humans is mostly asymptomatic, HEV 3 can induce chronic infection in immunocompromised individuals and acute on chronic liver failure in patients with underlying liver diseases. Recent data suggest that the number of reported cases of HEV infections in Europe increased significantly during recent years[31].

Although HEV is not routinely screened during blood donation in most countries, there have been prospective studies that have been conducted searching for markers of HEV infection in serum samples from potential blood donors to assess the local risk for transfusion related HEV[30,32]. The prevalence of detectable anti-HEV IgG positivity among blood donors varies among countries (Table 1). Nevertheless, data can also vary among geographical regions of the same country[40]. Moreover,

**Table 1 Rates of anti-hepatitis E virus immunoglobulin G positivity in blood donors by country**

| Country | IgG positive rate (%) | Ref. |
|---|---|---|
| Argentina | 11.3 | Di Lello *et al*[33] |
| Austria | 13.5 | Fischer *et al*[34] |
| Bolivia | 16.2 | Konomi *et al*[35] |
| Brazil | 7 | Tengan *et al*[36] |
| China | 30 | Zhang *et al*[37] |
| Croatia | 20.2 | Miletić *et al*[38] |
| England | 10 | Beale *et al*[39] |
| France | 22.4 | Mansuy *et al*[40] |
| India | 17.7 | Tripathy *et al*[41] |
| Iran | 8.1 | Hesamizadeh *et al*[42] |
| Italy | 8.7 | Spada *et al*[43] |
| New Zealand | 9.7 | Hewitt *et al*[44] |
| Norway | 14 | Lange *et al*[45] |
| Poland | 43.5 | Grabarczyk *et al*[46] |
| Scotland | 9.3 | Thom *et al*[47] |
| Serbia | 15 | Petrović *et al*[48] |
| South Africa | 42.8 | Maponga *et al*[49] |
| Switzerland | 20.4 | Niederhauser *et al*[50] |
| Thailand | 29.7 | Jupattanasin *et al*[51] |
| The Netherlands | 24 | Alberts *et al*[52] |
| Uruguay | 10 | Bangueses *et al*[53] |
| United States | 9.5 | Stramer *et al*[54] |

IgG: Immunoglobulin G.

differences can also be observed depending on the type of diagnostic assay used for the seroprevalence assessment[38,55].

A few cases of HEV infection have been reported to be transmitted by blood transfusion[56]. Since the first reported case of transmission human to human in Japan, some other cases have been reported in many countries[31]. In all of these, the HEV genomic sequence from blood donor and patient matched identically, confirming that the origin of the HEV infection was from the blood and had been transmitted to the patient by transfusion.

There are few data regarding the prevalence of HEV in organ transplant patients. HEV transmission through solid organ transplant have been reported after liver, heart, lung and kidney transplantation [57-60], although to date the risk of HEV infection transmitted by transplantation is unknown.

We did not find data regarding HEV transmission by tissue allografts.

## RISK ASSESSMENT OF HEPATITIS E TRANSMISSION THROUGH TISSUE ALLOGRAFTS

Damaged or absent tissues can be replaced by biological (autografts and allografts) or artificial substitutes. Nowadays, tissue banks offer great availability of different kind of human tissues to be used as allografts, with high standards of safety and efficiency. Therefore, studies analyzing the prevalence of HEV among tissue donors would be needed, in addition to other studies carried out in tissue recipients that could reveal its potential infectivity.

The drawbacks of these studies must be taken into account since many recipients of bone, valves or skin are also recipients of blood components. It is therefore important in a risk assessment procedure to know the degree of imputability that human tissues could have at the implants for HEV transmission. Additionally, these studies could also provide data to evaluate the probability of transmission. The Netherlands provided a definition for both transfusion-associated hepatitis E and transplant-associated

infection (Euro CDC). Based in that criteria, tissue transplant-associated HEV infection can be defined as "an acute hepatitis E within 6-8 wk after tissue transplantation (detected by HEV-RNA), where the donor was HEV-RNA positive and at least HEV ORF1/ORF2 hypervariable regions of donor and recipient strains are identical by sequencing".

It would be important to know the possible medium-long-term side effects for HEV regardless of the implant results. These studies could be obtained by the knowledge about their severity, in order to complete the risk assessment.

There are tissues which can be sterilized since cell viability is not relevant for their clinical efficiency or their biomechanical properties are not significantly altered by the procedure. Likewise, the avascular character of some tissues (as cornea) carries lower risk than vascularized ones (as heart valves).

As very simple forms of life (small size and absence of free water) viruses can be preserved by freezing, not requiring controlled cooling or use of cryoprotectants, as glycerol, dimethyl sulphoxide or polyethylene glycol (the only presence of albumin in the storage solution could be effective for virus cryoprotection). Although virus infectivity can be compromised with long term storage at -20 °C, temperatures ≤ -80 °C allow virus to survive. Additionally, virus can survive to several cycles of freezing/thawing[61]. Conversely, the process of drying and storing at room temperature (conditions associated to lyophilization), could lead to the collapse of the lipid membrane[62].

The storage in liquid nitrogen vs. vapour nitrogen has been related to higher risk of cross-contamination due to faulty seal, leak, or breakage of the containers (bags, cryovials, straws), by acting the liquid environment as vehicle for infectious agent diffusion[63,64].

It is mandatory for tissue banks that provide sterile tissue allograft to follow several steps as donor screening, microbiological testing, aseptic harvesting and processing, disinfection, and, finally, terminal sterilization. According with the standards of the International Atomic Energy Agency (IAEA)[65], sterilization is defined as a validated process to destroy, inactivate, or reduce microorganisms to a sterility assurance level (SAL) of $10^{-6}$. Achieving this SAL by a validated process allows labelling of terminally sterilized allografts as sterile[66]. Validation refers to establishing documentary evidence that provides a high degree of assurance that a specific process will consistently produce a product meeting its predetermined specifications and quality attributes, and shall include the following elements[65]: (1) Qualification of the tissue allografts and their packaging for sterilization; (2) Qualification of the irradiation facility; (3) Process qualification using a specified tissue allograft or simulated products in qualified equipment; (4) A certification procedure to review and approve documentation of (1)-(3); and (5) Activities performed to support maintenance of validation.

A validated procedure for the sterilization of tissue allografts must demonstrate efficacy against all classes of microorganisms, throughout the tissue volume and, additionally, must not adversely affect the biological and biomechanical properties which are critical for its clinical use. The inclusion of a terminal inactivation step provides safety against not usually tested viruses in donor screening, such as HEV.

Both enveloped and non-enveloped viruses containing either DNA or RNA have been inactivated by low dose gamma irradiation of musculoskeletal tissues[67]. Both directly (by ionizing radiation) and indirectly (due to aqueous free radicals as intermediaries in the transfer of radiation energy to biological molecules) effects are involved in the inactivation of allografts bioburden[68].

Ethylene oxide inactivates all classes of microorganisms by alkylation of nucleic acids and proteins. However, concerns regarding its potential toxicity have led to a decrease of its use[69].

HEV retained infectivity at temperatures up to 60 °C[70], and heating for 1 min at 70 °C yielded a log reduction of 0.48, which was increased up to 3.67 at 95 °C[71]. Thus, virus heat inactivation at 71 °C for, at least, 20 min has been suggested[72]. Using a Lobator sd-2 system (telos, Marburg, Germany) validated to achieve a temperature of 82.5 °C the centre of femoral heads with a diameter of ≤ 56 mm, Pruss *et al*[73] obtained a titre reduction (4 $Log_{10}$ steps) of clinically relevant viruses.

Pruss *et al*[74] showed the treatment of spongiosa blocks with the peracetic acid-ethanol procedure as a methodology to sterilize bones (maximum thickness ≤ 15 mm). In this study, very slow inactivation kinetics for hepatitis A virus was observed. Thus, while a general reduction of virus titres by more than 4 $log_{10}$ was determined, only HAV showed a reduction below that threshold (2.87), with residual infectivity.

## CONCLUSION

Current evidence does not recommend to date the universal screening with HEV in tissue donors, although it could be advisable to include the revision of medical-social history about risk practices and in those cases be able to selectively screen for HEV.

## FOOTNOTES

**Author contributions:** Villalba R and Mirabet V contributed equally to this work.

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**Country/Territory of origin:** Spain

**ORCID number:** Rafael Villalba 0000-0001-5600-3276; Vicente Mirabet 0000-0003-1469-4210.

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## AIMS AND SCOPE

The primary aim of the *World Journal of Gastrointestinal Pathophysiology* (*WJGP*, *World J Gastrointest Pathophysiol*) is to provide scholars and readers from various fields of gastrointestinal pathophysiology with a platform to publish high-quality basic and clinical research articles and communicate their research findings online.

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*REVIEW*

# Gut microbiome: Linking together obesity, bariatric surgery and associated clinical outcomes under a single focus

Konstantinos Georgiou, Nikolay A Belev, Tilemachos Koutouratsas, Hector Katifelis, Maria Gazouli

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**Konstantinos Georgiou,** The First Propaedeutic Surgical Unit, Hippocrateion Athens General Hospital, Medical School, National and Kapodistrian University of Athens, Athens 11527, Greece

**Nikolay A Belev,** Medical Simulation Training Center, Research Institute of Medical University of Plovdiv, and UMPHAT "Eurohospital", Medical University of Plovdiv, Plovdiv 4002, Bulgaria

**Tilemachos Koutouratsas, Hector Katifelis, Maria Gazouli,** Basic Medical Sciences, Medical School, National and Kapodistrian University of Athens, Athens 11527, Greece

**Corresponding author:** Maria Gazouli, PhD, Professor, Basic Medical Sciences, Medical School, National and Kapodistrian University of Athens, Michalakopoulou 176, Athens 11527, Greece. mgazouli@med.uoa.gr

## Abstract

Obesity is increasingly prevalent in the post-industrial era, with increased mortality rates. The gut microbiota has a central role in immunological, nutritional and metabolism mediated functions, and due to its multiplexity, it is considered an independent organ. Modern high-throughput sequencing techniques have allowed phylogenetic exploration and quantitative analyses of gut microbiome and improved our current understanding of the gut microbiota in health and disease. Its role in obesity and its changes following bariatric surgery have been highlighted in several studies. According to current literature, obesity is linked to a particular microbiota profile that grants the host an augmented potential for calorie release, while limited diversity of gut microbiome has also been observed. Moreover, bariatric surgery procedures represent effective interventions for sustained weight loss and restore a healthier microbiota, contributing to the observed fat mass reduction and lean mass increase. However, newer evidence has shown that gut microbiota is only partially recovered following bariatric surgery. Moreover, several targets including FGF15/19 (a gut-derived peptide), could be responsible for the favorable metabolic changes of bariatric surgery. More randomized controlled trials and larger prospective studies that include well-defined cohorts are required to better identify associations between gut microbiota, obesity, and bariatric surgery.

**Key Words:** Bariatric surgery; Obesity; Gut microbiota; Micronutrient deficiency; Probiotics

**Core Tip:** Obesity represents a major cause of morbidity and mortality globally. Current knowledge suggests a connection between gut microbiota characteristics and obesity, while bariatric surgery has been shown to promote a healthier microbiota composition. However, the exact effects of these procedures remain unclear. In general, an increase in members of the phylum Bacteroidetes and Proteobacteria, and a decrease in members of the phylum Firmicutes is a common finding. This field of research can also inform clinicians' predictions of outcomes before and after bariatric surgery through analysis of patterns in gut microbiota.

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## INTRODUCTION

Obesity represents a huge health burden in society, and is linked with an increase in mortality rates[1]. Recent data suggest a crosstalk between gut microbiota (GM) and obesity, while obesity itself seems to be both a cause and a result of GM alterations[2]. In health, the GM is involved in energy intake, adjustment of glucose and lipid homeostasis, and micronutrient and vitamin composition[3]. This GM balance is disturbed in obesity presenting a series of pathological manifestations, including chronic inflammation, insulin resistance, and metabolic disturbance[2,3]. Moreover, obesity is linked with vitamin and mineral deficiencies, that aggravate GM synthesis and function[4,5].

Bariatric surgery (BS) is currently the sole long-term effective therapeutic option for morbid obesity [6]. A number of studies have identified important qualitative and quantitative changes in the GM after BS. Such treated patients have micronutrient deficiencies that may lead to deficiency-related syndromes [7,8], that include anemia (10%-74%) and neurological disorders (5%-9%)[7,9].

Given the presence of other coexisting factors that impair the postoperative nutritional status of these patients [energy-restricted higher protein intake and adequate nutritional supplementation diet, anatomical and physiological impairment of the gastrointestinal tract (GIT)][7,10], a consistent follow-up is essential.

The complicated interaction between obesity and GM phyla that includes gut microbiome modulations (and of their by-products) in obese subjects who undergo BS as treatment, are the aim of this review.

## OBESITY

Obesity represents the discrepancy between caloric intake and energy expenditure and is affected by genetic and environmental factors[11]. Obesity has been associated with type 2 diabetes mellitus (T2DM), increased arterial pressure, hypercholesterolemia, cardiovascular disease, apnea, musculo-skeletal disorders, cancer, impaired fertility, anxiety, and psychiatric disorders[12]. Currently, obesity results in more deaths than undernourishment and starvation together[13].

Worldwide, the term body mass index (BMI) is a tool for estimating obesity severity and is calculated by dividing the body weight (kg) by the square of height ($m^2$) of the individual. In adult subjects, a BMI between 18.5 to 25 kg $m^{-2}$ is considered normal; overweight is BMI 25 to 30, while obesity is defined as BMI over 30 kg $m^{-2}$. Obesity is classified by the World Health Organization into three categories; class I corresponds to a BMI of 30.00 to 34.99; class II between 35.00 and 39.99 and class III is a BMI that exceeds 40[14]. Additionally, a BMI > 50 kg $m^{-2}$ is termed superobesity. Regarding the treatment of obesity, it has been shown that in a time period of 2 years, most subjects reach or even exceed their initial weight[15].

## GUT MICROBIOTA IN HEALTHY SUBJECTS

### *Glossary of microbiota-related terms*

Microorganisms are present in the skin, respiratory system, the GIT, and the male and female genitourinary tracts[16].

The ecological community of symbiotic and pathogenic microbes composes the microbiota[17]. The term microbiota includes all species which form microbial communities, such as eubacteria, archeobacteria, fungi, and protists[18].

The term 'microbiome' refers to the microorganisms themselves. The study of all microbial DNA directly recovered from a sample such as from the gut is called metagenomics. The metagenome, refers to the complete genome of the microbiota[17], while the term 'shotgun metagenomics' describes the process of a sample's next-generation sequencing. This process produces primer-independent data that can then be analyzed with various reference-based and/or reference-free methods[16].

### *Gut microbiota under normal conditions*

In health, the microbial composition remains constant[19]. The largest microbe concentrations are found in the intestine, the skin, and the oral cavity[20]. Of these sites, the GIT is the most intensively colonized organ. In the past, it was widely shown that a healthy gut contains 1-1.5 kg of microbes a number that exceeds by about 10 times the number of the host's (human) cells[21]. However, more recent estimates suggest that the number of gut bacteria is of the same order as the number of human cells, weighing a total of 0.2 kg[22]. Approximately 1000 species colonize the gut, with microbial density increasing along the GIT from $10^1$ to $10^4$ microbes in the stomach to $10^{10}$ to $10^{12}$ cells per gram in the colon[17].

Due to the antimicrobial effects of hydrochloric acid and nitric oxide, microbes in the stomach and the small intestine are few[23,24]. However, the large intestine presents a better milieu for microbes, with better conditions to extract energy as well as essential nutrients[25,26]. The largest number of living microbes is located in the colon but due to the impermeable adherent mucus layer, there is no direct contact with the epithelium[27]. It is believed these bacterial species collectively yield 2 million genes (100 times the number of human genes. The number above agrees with the actual extent of microbial gene catalogs found in MetaHIT and the Human Microbiota Project[28].

### *Gut microbiota in obese subjects*

The GM along with the host's genotype and lifestyle, affect the pathophysiology of the disease and thus research interest in these associations has increased[2,29].

An important increase in adipose tissue of germ-free (GF) mice implanted with microbiota harvested from the cecum of ob/ob mice has been found, when compared to mice transplanted with a GM from lean rodents[30]. Transferring GM from genetically obese mice resulted in a 47% increase in fat mass, while the inoculation from lean mice increased adipose tissue mass by 26%[31].

Several factors contribute to how GM affects obesity, such as nutrient metabolism. For instance, hippurate, a microbial metabolite of dietary polyphenols, is reported to be associated with *Eubacterium dolichum* and visceral fat mass[32]. Additionally, it has been postulated that the circadian clock, which regulates diurnal oscillations of different biological processes such as feeding, can be influenced by the GM and therefore act as a contributor to diet-induced obesity[33].

Obesity also triggers low-grade chronic inflammation. A high-fat diet for 28 d, increased more than twice the systemic lipopolysaccharide (LPS) levels and the LPS-containing GM, thus presenting what is known as "metabolic endotoxemia". The increased LPS levels could trigger inflammation thus contributing to obesity and T2DM[34,35].

## BARIATRIC SURGERY

### *Bariatric surgery modalities*

When lifestyle and/or medication-based approaches are ineffective, BS is an option, as it is a highly effective therapeutic procedure for the treatment of obesity[36]. BS can be either restrictive or malabsorptive, by reducing food intake and promoting weight loss[37]. The available metabolic surgery procedures includes laparoscopic adjustable gastric band, vertical sleeve gastrectomy (VSG), Roux-en-Y gastric bypass (RYGB), biliopancreatic diversion (BPD), and BPD with duodenal switch (BPD/DS)[7,37].

### *Vertical banded gastroplasty*

This is a restrictive procedure. An incision is made on the lesser curvature of the stomach 6 cm from the esophagogastric junction. The lesser omentum is dissected followed by a 2 cm opening of the lesser sac. Dissection continues downward to 1 cm above the uppermost portion of the short gastric vessels. A calibrated transgastric window is created using a circular stapler creating a 20 mL gastric pouch volume. A polypropylene band is placed around the distal part of the gastric pouch[36,38,39].

### *Laparoscopic adjustable gastric band*

This is a restrictive procedure, more widely performed in the past, but its use has declined in popularity in the last 5 years[38]. A synthetic band is placed around the upper portion of the stomach, immediately after the gastroesophageal junction, thus creating a small gastric pouch of 20-30 mL. The band is inflated or deflated with saline to alter the level of constriction and to maintain a feeling of fullness with a smaller volume of food. At first, the early and prolonged satiety was attributed to the physically restricted meal volume and the delayed emptying of food from the pouch[40]. Today, it has been proved that most of the procedure's efficiency is due to the pressure applied on the intraganglionic laminar endings which convey afferent signals resulting in hunger reduction[41]. The average weight loss is about 45%-47% of the excess weight by 4-5 years postoperatively[42].

### *RYGB*

RYGB represents both a restrictive and malabsorptive procedure. Of note, apart from the mechanical restriction of caloric intake, RYGB impairs the absorption of nutrients. Of note, 15%-30% of the weight loss is maintained for at least 20 years after RYGB[43]. Moreover, after RYGB glycemic control improves in 90% of recipients[44].

### *VSG*

This is a restrictive procedure. VSG has increased in popularity as it is relatively easy to perform and a good clinical outcome is achieved[45]. In VSG, a vertical excision of approximately 75% of the stomach lengthwise with preservation of the pylorus is performed. It aims to make a small gastric pouch ("sleeve"), with a volume of approximately 100 mL, and to create a high-pressure chamber that easily produces sufficient pressure to overcome the tone of the pyloric sphincter, thus resulting in rapid gastric emptying[46]. This decreased gastric reservoir does not permit any distention and therefore provokes premature satiety, resulting in substantially reduced portion sizes.

Sleeve creation has an impact on hormone regulation, decreasing blood ghrelin levels and enhancing a state of satiety. The average weight loss is 60% excess body weight after two years postoperatively, along with an improvement in associated comorbidities[42]. Both short- and medium-term research reports showed that VSG is almost as effective as RYGB in reducing body weight and improving glycemic control[10,47].

### *BPD and BPD with duodenal switch (BPD and BPD/DS)*

This is a malabsorptive procedure. Being a quite radical procedure, it is only used occasionally. The BPD procedure involves a sleeve gastrectomy with the creation of a 200-500 mL gastric pouch. A Roux-en-Y gastroileostomy of 200 cm is formed with a common channel 50 cm from the ileocecal valve joining biliary and digestive enzymes. The weight loss achieved *via* BPD and/or BPD/DS is the greatest among any of the other bariatric procedures with excess weight loss of 70%-80% postoperatively[42,48].

Of all the aforementioned procedures, half of the bariatric procedures are VSG and approximately 40% are RYGB[49]. RYGB has been the primary choice for decades and thus millions of RYGB patients are present in the general population[13]. Table 1 shows the comparison between these bariatric approaches.

Today, BS is regarded as the only effective treatment for a pronounced and permanent weight loss [13]. The Swedish Obese Subject trial reported a weight loss following RYGB of 27% in 15 years, while non-operative approaches (lifestyle changes or pharmacological treatment) had no effect over this period. Controlled long-term studies (> 5 years) on the effects of VSG on weight loss are still scarce, but weight loss up to 5 years is similar to that of RYGB[13].

Lastly, branched-chain amino acids were significantly reduced after BS, a finding associated with alleviation of the "metabolic overload" observed in some tissues[50]. Trimethylamine-n-oxide, a metabolite proposed as a cardiovascular marker, was found to increase following BS. This increase was probably related to the GM changes observed after BS[50].

## THE MECHANISMS OF GASTRIC BYPASS

The gastric bypass procedure is an artificial condition in which the intestinal mucosal energy outflow is variable and capable of altering BMI and glucose levels.

The main reason behind weight reduction is a modified eating behavior that reduces energy intake. According to the foregut theory, food bypasses both the stomach and the duodenum, and the release of gut-derived hormones originating from these areas is altered, *e.g.*, the release of glucose-dependent insulinotropic peptide from the duodenum. A second theory known as the hindgut theory states that since the more distal parts of the intestine are now (following the procedure) exposed to nutrients and contact food sooner than normal, this provokes faster humoral responses.

RYGB also changes the circulating bile acid levels and those of the intestinal microbiota: Bile acids regulate glucose metabolism causing the release of GLP-1, provoking the synthesis and release of

**Table 1 Comparison of the two main bariatric surgery procedures**

| | Roux-en-Y gastric bypass | Vertical sleeve gastrectomy |
|---|---|---|
| **Technique** | (1) 15-30 mL gastric pouch; (2) Gastrojejunostomy (GJ); (3) Jejunojejunal anastomosis (Roux-en-Y); (4) 30-50 cm distal to the ligament of Treitz; and (5) Remnant disconnected but left *in situ* | (1) Excision of lateral 70%-80% of stomach along the greater curvature; and (3) Approximately 100 mL gastric reservoir (sleeve) |
| **Mechanism of action** | (1) Instantaneous food transfer to small intestine, altering: Gut hormones; Bile acids; Neural signaling; Gut microbiota; Gut-brain-endocrine; Adipocyte-brain axes; and (2) Results in reduced food intake, increased satiety and altered food preferences | (1) Alterations in: Gut hormones; Bile acids; Neural signaling; Gut microbiota; Gut-brain-endocrine; Adipocyte-brain axes; and (2) Results in reduced food intake, hunger, increased satiety and altered food preferences |
| **Advantages** | (1) Significant long-term weight loss; (2) Glycemic control improvement in 90% of cases; (3) Maintain percent EWL in the long term; (4) Hunger reduction and satiety; (5) Food preferences changes; and (6) Increases energy expenditure | (1) Significant long-term weight loss (approximately 10% less than RYGB); (2) Glycemic control as effective as RYBG; (3) Maintain percent EWL in the long-term; (4) Hunger reduction and satiety; (5) Food preferences changes; (6) No anatomical rerouting of food; (7) Short length of stay (< 2 d); (8) Technically simpler than RYGB; and (9) Lower complication rate than RYGB |
| **Disadvantages** | (1) Technically complex (two anastomoses) compared with AGB or VSG; (2) Higher complication rate than AGB or LSG; for example, anastomotic leak or dumping syndrome can occur; (3) Longer length of stay; (4) Long-term vitamin and/or mineral deficiencies (for example, vitamin B12, iron, calcium or folate); (5) Requires lifelong vitamin and/or mineral supplementation; (6) Lifelong dietary changes; (7) Increases alcohol addiction and suicide rates; and (8) postprandial hypoglycemia | (1) Anastomotic leak can be difficult to manage; (2) Susceptible to long-term vitamin and/or mineral deficiencies (less common than with RYGB); (3) Precautionary lifelong vitamin and/or mineral supplementation; (4) Lifelong dietary changes; (5) Irreversible; and (6) potential risk of Barrett esophagus |

EWL: excess weight loss; RYGB: Roux-en-Y gastric bypass.

fibroblast growth factor 19 which improves insulin sensitivity and glycemic control[51].

Circulating exosome microRNAs (miRNAs) constitute another mechanism that could explain bariatric surgery–associated outcomes[6]. Several studies have identified miRNAs that tend to increase or decrease in expression after bariatric surgery[52,53]. Of these, miRNA MiR-7, which has shown the most concrete post-surgical increase in studies, plays a role in the regulation of pancreatic beta-cell function in humans[53].

## SIDE EFFECTS OF BARIATRIC SURGERY

The 1-year mortality rate after BS is 1% and the 5-year mortality rate is 6%[54]. 4% of patients after BS experience surgical complications during the first month[55,56]. These include anastomotic leakage, hemorrhage, perforation, infection and inner herniation[55]. However, the latter is considerably decreased when the closure of any mesenteric defect became routine practice during the BS approach [57].

Chronic abdominal pain is a common side effect seen in patients after RYGB; half of RYGB patients experience abdominal pain and in a 5-year follow-up, a third of them still experienced pain[58]. It is important to clarify the underlying pathology following BS but its etiology remains obscure[59]. Furthermore, it is believed that 4% of patients who were not on opioids, became chronic users after BS [60] and therefore the attending physician of such patients who develops nausea and pain, must bear in mind the risk of iatrogenic opioid addiction.

Hypoglycemia in non-diabetic subjects appears in more than 64% of patients during the first 5 years after BS[61]. Several theories related to this have been proposed including enhanced B cell mass and function, lowered ghrelin levels, improved insulin sensitivity, and inadequate counter regulation[62]. Unfortunately, the side effects of hypoglycemia often persist for years and can decrease the patient's quality of life.

## GUT MICROBIOTA AFTER BARIATRIC SURGERY

A plethora of diseases are connected to GM changes including, atherosclerosis, non-alcoholic fatty liver disease, inflammatory bowel disease, and colorectal cancer[16]. BS plays a central role by affecting the abundance of many microbial species of the GM.

Most often, a decrease in *Firmicutes* and an increase in *Bacteroidetes* and *Proteobacteria*, abundance is observed after BS[63]. Both RYGB and vertical banded gastroplasty, have comparable long-term effects on GM function and composition. Moreover, feces from BS patients were transplanted in germ-free mice, and the mice gained less fat when compared to reciprocal mice transplanted with GM from obese subjects. These findings show a causal relationship between GM and BS-induced weight reduction[64].

Another study employed GM transplantation from mice that underwent RYGB to sham-surgery germ-free mice, which provoked weight loss compared to recipients of GM from non-operated mice[65].

The increase in pH (following BS) in the lumen and high levels of dissolved oxygen, affect the growth of aerobic microorganisms (such as *Proteobacteria*) and inhibit the growth of anaerobic bacteria[66].

In a recent systematic review, Davies *et al*[67] summarized 14 clinical studies involving 222 subjects (RYGB = 146, VSG = 25, biliointestinal bypass = 30, vertical banded gastroplasty = 7, and adjustable gastric band = 14). Major changes included a reduction in the abundance of *Faecalibacterium prausnitzii* and an increase in *E. coli*. Following VSG, a decrease in the abundance of *Firmicutes* was observed, while after RYBG an increase in *Bacteroidetes* and *Proteobacteria* was observed.

Their findings are summarized in Table 2. It was found that different types of BS result in dramatic changes in GM.

A systematic meta-analysis of 22 articles investigated the effect of BS on metabolic and GM profiles. Only two studies were randomized, while the rest were prospective studies[64,68,69]. The total sample size was 562; 411 patients underwent RYGB, and 97 underwent VSG[70].

As shown in Table 3, several microbes are affected by BS: some authors found increased *Bacteroides* while *Firmicutes* and *Bifidobacterium* had lower abundance in post-RYGB subjects[70,71].

In summary, it appears that BS reestablishes a healthier microbiota together with a slimmer metabolic profile, and possibly this microbiota readjustment contributes to a diminished fat mass and an increased lean mass. Nevertheless, the pathways through which the gut microbiota and their metabolites affect obesity are still obscure, and robust microbe manipulations that interfere with the host-bacteria interactions for the management of obesity still need to be developed[16].

## EFFECT OF BARIATRIC SURGERY ON SMALL INTESTINE BACTERIA

Obese subjects after BS can develop small intestine bacterial overgrowth (SIBO), which is defined as greater than $10^5$ colony-forming units per mL of proximal jejunal aspiration[72]. SIBO is a manifestation of obesity and a prospective study including 378 subjects with morbid obesity, reported that 15% of patients before undergoing RYGB had SIBO, and that this figure increased to 40% following the procedure[72].

SIBO diagnosis is made following a small intestine aspirate test. However, due to the invasive nature of this process the most acceptable detection technique is the "therapeutic trial", by empirically administering antibiotics due to the clinical complications associated with SIBO[73].

The malabsorption of vitamins A, D, E, and K (fat-soluble vitamins) is due to the bacterial deconjugation of bile acids by small intestine bacteria, while the formation of a toxic compound (lithocholic acid) further aggravates intestinal epithelial cell dysfunction and aggravates carbohydrate and protein malabsorption[74]. In contrast, in subjects with SIBO, vitamin K levels are within normal levels or increased as bacteria are capable of synthesizing menaquinone[75].

## EFFECT OF BARIATRIC SURGERY ON GUT HORMONES

Typically, food intake suppresses the hunger hormone ghrelin; however, in obese subjects, this mechanism might be disrupted. Thus, it has been reported that within days after BS, as a more quick release of nutrients to the distal small intestine starts to occur, increased production of gut satiety hormones such as PYY and GLP-1, and a reduced increase in ghrelin takes place[76].

After a meal, both PYY and GLP-1 are, proportional to the consumed calories, released from the L cells of the distal small intestine[77]. Following BS, the postprandial PYY levels are increased and the new levels are correlated with postoperative weight loss[78]. Also, the role of PYY in the regulation of feeding after RYGB has been assessed using octreotide, which blocks the secretion of most gut hormones and therefore increases food consumption[76].

Although the effects of PYY and GLP-1 on gastric emptying, glucagon secretion, and insulin release from the pancreas are well understood, the appetite change after BS seems to be a synergistic response of more than one gut hormone[79].

### *Gut microbiota signatures as predictors of long-term outcomes in bariatric surgery*

In a study by Gutiérrez-Repiso *et al*[80], fecal samples from 24 patients who had undergone bypass surgery at least two years previously were studied. The authors reported that patients who would go on to show greater rates of weight loss and low weight maintenance in the long-term tended to have a higher diversity of core microbiota in the mid-term. Furthermore, the bacterial genera *Sarcina*, *Butyrivibrio*, *Alkaliphilus*, *Lachnospira*, *Pseudoalteromonas*, and *Cetobacterium* were more abundant in stool samples in patients for whom gastric bypass surgery was more successful in the long-term[80]. Nevertheless, another study by Fouladi *et al*[81] failed to prove a significant difference in the microbiota between subjects with successful and poor BMI reduction after RYGB surgery[81]. In the same study,

**Table 2 Changes in human gut microbiota following bariatric surgery**

| ↑/↓ | RYGB | VSG |
|---|---|---|
| ↑ | *Akkermansia* (Verrucomicrobia) | *Bulleidia* (Firmicutes) |
| ↑ | *Escherichia* (Protobacteria) | *Roseburia intestinalis* (Firmicutes) |
| ↑ | *Klebsiella* (Protobacteria) | *Faecalibacterium prausnitzii* (Firmicutes) |
| ↓ | *Lactobacillus* (Firmicutes) | *Coprococcus comes* (Firmicutes) |
| ↓ | *Bifidobacterium* (Actinobacteria) | |
| ↓ | *Faecalibacterium prausnitzii* (Firmicutes) | |
| ↓ | *Coprococcus comes* (Firmicutes) | |

RYGB: Roux-en-Y gastric bypass; VSG: Vertical sleeve gastrectomy.

**Table 3 Literature findings on the postoperative changes of gut microbiota**

| Ref. | Postoperative GM changes | | |
|---|---|---|---|
| | Increased abundance | Decreased abundance | Comments |
| Graessler *et al*[71], 2013 | *Enterobacter, Citrobacter, Neurospora, Veillonella, Salmonella, Shigella, E. coli* tended to increase | *Faecalibacterium, Coprococcus, Helicobacter, Dictyostelium, Epidinium, Anaerostipes, Nakamurella, Methanospirillum, Thermomicrobium* | - |
| Kong *et al* [68], 2013 | *Bacteroides, Alistipes, Escherichia* | Firmicutes (*Lactobacillus, Dorea, Blautia*) *Bifidobacterium* | Increased richness of GM after RYGB |
| Palleja *et al* [50], 2016 | *Escherichia coli*, *Klebsiella pneumoniae*, 10 species belonging to the genus *Streptococcus*, 4 from *Veillonella*, 2 from *Alistipes*, *Bifidobacterium dentium*, *Enterococcus faecalis*, *F. nucleatum*, and *Akkermansia muciniphila* | *E. prausnitzii* | - |
| Tremaroli *et al*[64], 2015 | Gammaproteobacteria; Several Proteobacteria *(Escherichia, Klebsiella, Pseudomonas); E. coli* tended to increase but was not statistically significant | 3 species of Firmicutes; (*Clostridium difficile, Clostridium hiranonis, Gemella sanguinis*) | - |

GM: Gut microbiota; RYGB: Roux-en-Y gastric bypass.

Fouladi *et al*[81] transplanted fecal samples from patients with poor weight loss (PWL) and successful weight loss in antibiotic-treated mice, and reported that mice transplanted with PWL feces tended to gain more weight despite exhibiting similar feeding behaviors. Steinert *et al*[82] reported decreased mycobiotic diversity in fecal samples from patients before and after RYGB surgery.

## MICRONUTRIENT DEFICIENCIES AFTER BARIATRIC SURGERY

After BS, the micronutrient status of patients further deteriorates, which, in turn, affects the structure and composition of the GM[83]. Thus, after BS, more than 30% of patients develop nutritional deficiencies that may result in edema, hypoalbuminemia, anemia, and even peripheral neuropathy and Wernicke encephalopathy[83].

Unfortunately, these deficiencies persist despite vitamin and mineral supplementation. The deficiencies observed after BS are affected by eating behavior, decreased absorption, SIBO, or poor compliance to the suggested optimization of diet[84].

There is strong evidence that after RYGB and VSG, food intake restriction, reduced appetite, and gastrointestinal hormones changes are mechanisms for the observed weight loss[85]. VSG promotes gastric emptying, reduces gastroduodenal transit time, and decreases the release of hydrochloric acid and intrinsic factor. These effects, due to gastric fundus resection, affect gastrointestinal motility and therefore, the release and dissolution of several vitamins and minerals are diminished[86].

### *Vitamin $B_{12}$*

The anatomic alterations of the GIT due to BS lead to impaired release of both HCl and pepsin from the functional part of the remnant. In turn, this leads to diminished vitamin $B_{12}$ absorption, as well as to less interaction of gastric content with parietal cells, which produce the intrinsic factor, causing

malabsorption and deficiency of cobalamin[87,88]. It has also been shown that the deficiency of intrinsic factor is the main driver of post-surgical $B_{12}$ deficiency, although other molecules such as transcobalamin-1 may participate[89]. As expected, RYGB patients display a higher frequency of vitamin $B_{12}$ deficiency (37%-50%) than VSG patients (10%-20%)[90]. It has been reported that, despite adequate supplementation with physiological doses, $B_{12}$ levels are found to decrease within a few months following BS, and therefore, administration of high doses of $B_{12}$ is recommended right after BS[91].

### Folic acid

It is expected that after BS, folate absorption should be impaired due to hypochlorhydria and altered pH in the proximal jejunum[92]. However, it has been reported that folic acid may also be synthesized by bacteria in the colon. It seems that it is absorbed throughout the small intestine and even the colon, with a lowered rate of absorption. Therefore, following RYGB, the administration of usual doses of folate supplement is sufficient to prevent or correct folate deficiency, because a compensatory mechanism of intestinal absorptive capacity may be present[93].

### Vitamin $B_1$ (thiamine)

Thiamine deficiency symptoms rapidly develop after only 20 d of insufficient oral intake, faster than for any other vitamins[94]. Hyperemesis, a symptom rather common after BS surgery, impairs $B_1$ absorption and thus its deficiency can appear despite any oral supplementation. A large variety of pathologies are associated with thiamine deficiency, including beriberi, neuropathy, and Wernicke encephalopathy[95], which may present a medical emergency.

Bariatric patients may develop vitamin $B_1$ deficiency within six months following surgery. A study reported that in 118 cases of Wernicke encephalopathy detected postoperatively after either RYGB or VSG, almost 90% had hyperemesis[96]. A study reported that two years after RYGB, thiamine levels were deficient in 18% of patients[96]. In a recent retrospective study of VSG patients, 25.7% of subjects showed decreased thiamine levels within one year after VSG [97].

### Vitamin D and calcium

Following BS, bariatric patients have an increased risk of developing metabolic bone disease at any time during the rest of their lives. Furthermore, after BS, SIBO can also aggravate vitamin D deficiency[98]. As diminished acid secretion occurs after both RYGB and VSG, impaired dissolution and solubilization of nutrients can develop. Chronic vitamin D deficiency which subsequently leads to decreased bone mineral density has been observed three years after RYGB and VSG[99].

Following VSG, vitamin D malabsorption might be the effect of diminished exposure of nutrients to the digestive mucosa[100]. Although VSG does not involve intestinal anatomy, calcium uptake might be hampered through several possible mechanisms such as reduced calorie intake, hypochlorhydria, or the use of proton pump inhibitors[100]. In a large cohort study including 999 subjects, the prevalence of hypocalcemia postoperatively was 3.6%, with 15 patients (1.9%) undergoing RYGB, and 13 patients (9.3%) undergoing VSG. In the same study, the lowest calcium concentrations were found after approximately 3 years in the RYGB group, and after 239 d in the VSG group, respectively. The daily calcium intake administered was approximately 1750 mg[101].

### Iron

Following RYGB, 18%-53% of patients develop iron deficiency compared to 1%-53% of patients after VSG[102]. This is rather expected after RYGB, as the duodenum, which is the most efficient area for iron absorption, is bypassed. A study including 72 post-RYGB patients reported red meat intolerance in 49.2%, 42.2%, 46.4%, and 39% of subjects after 1, 2, 3, and 4 postoperative years, respectively[103]. Following VSG, iron deficiency is dominant and defined by malabsorption secondary to the amount of gastric resection which prevents reduction of $Fe^{3+}$ to $Fe^{2+}$.

Several mechanisms underlie the pathogenesis of postsurgical iron deficiency: After ingestion, the gastric acidic environment enhances iron absorption by favoring its ferrous form (2+), the only form of iron that can be absorbed[104]. Reduced HCl release in the gastric pouch and administration of $H_2$ blockers significantly impair iron absorption[105]. Also, iron-rich alimentation after BS is largely decreased due to caloric restriction and food aversions, especially to red meat[87].

## OTHER MICRONUTRIENT DEFICIENCIES

### Fat-soluble vitamins

After BS, some deficiencies of fat-soluble vitamin (vitamin A, E, and K) levels in plasma are observed due to malabsorption[7], but the frequency of these deficiencies is low with rarely reported clinical manifestations[106,107].

Vitamin A deficiency can be induced by diminished retinol and carotenoid intake due to calorie restriction. Additionally, the recommended low-fat diet following BS, contributes to poor absorption.

Interestingly, cirrhosis observed in BS subjects may impede vitamin A storage and synthesis[107]. Thus, the prevalence of vitamin A deficiency following RYGB is approximately 10%[108]. However, no changes in serum vitamin A concentration or optical function following RYGB or VSG were reported in a recent study[109].

### *Zinc, copper, and selenium*

A study analyzing micronutrient deficiencies after both RYGB and VSG during a follow-up of five years found reduced serum zinc concentrations in 25.7% and 12.5% of patients, respectively[110].

The prevalence of copper deficiency after RYGB is 10%. The development of symptomatic hypocupremia after BS is uncommon among subjects who adhere to the prescribed supplementation[111].

Selenium is a trace element and an important antioxidant (selenocysteine)[112]. Serum levels of zinc, selenium, and copper were stable following RYGB and VSG in subjects receiving supplementation[113].

## PROBIOTICS AND GUT MICROBIOTA: IMPLICATIONS FOR BARIATRIC PATIENTS

Probiotics are beneficial to the host even without inhabiting the gut or making major changes to GM [29]. The most common administered probiotics are *Lactobacillus, Bifidobacterium,* and *Sacharomyces genera*[114].

Although probiotic use is common postoperatively, studies on their efficacy after BS are scarce[115]. It is been reported that the high pH setting after RYGB, allows higher survival of probiotic bacteria during transition through the acidic milieu of the GI, thus making BS patients suitable candidates for probiotic therapy. Administration of probiotics appears to offer many beneficial effects to BS patients such as greater weight loss, decreased SIBO, improved vitamin synthesis and availability, and optimized micronutrient status[116].

## CONCLUSION

BS, the most effective operation for severe obesity, is continuously expanding its applications. However, the role of GM on the host's metabolism and digestion is also widely recognized. Nevertheless, current understanding of the mechanisms that link obesity and concurrent changes in GM remains unclear and current data suggest that BS can only partially restore the microbial imbalance.

The exact mechanisms that induce GM changes after BS remain unclear as different factors including diet, weight loss, and surgery are involved. Moreover, side effects that are triggered by the SIBO effect may also affect the weight loss process in patients who undergo BS.

The impact of BS is not well described, as microbiota alterations are not consistent, and they should be considered in the context of energy intake restriction and altered dietary quality. At the same time, no differences regarding GM modulation were observed among the two most common weight loss surgery techniques (RYGB and VSG). In general, an increase in members of the phylum *Bacteroidetes* and *Proteobacteria*, and a decrease in members of the phylum *Firmicutes* are the most consistently reported findings.

In brief, BS attempts to restore a healthier GM with a leaner metabolic profile, and this microbiota realignment could contribute to the observed reduced adipose tissue reduction, the increase in lean mass, and the reduction in obesity-related morbidity. However, the mechanisms by which microorganisms and their by-products restore the GM are poorly understood. Finally, the prognostic significance of microbiota patterns on long-term outcomes after BS require further elucidation.

## FOOTNOTES

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**Country/Territory of origin:** Greece

**ORCID number:** Konstantinos Georgiou 0000-0003-3615-2500; Nikolay V Belev 0000-0001-9248-8194; Tilemachos Koutouratsas 0000-0001-5161-7383; Hector Katifelis 0000-0001-5741-4288; Maria Gazouli 0000-0002-3295-6811.

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ORIGINAL ARTICLE

Basic Study

# Evaluating the regulation of transporter proteins and P-glycoprotein in rats with cholestasis and its implication for digoxin clearance

Parker Giroux, Patrick B Kyle, Chalet Tan, Joseph D Edwards, Michael J Nowicki, Hua Liu

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**Parker Giroux, Joseph D Edwards, Michael J Nowicki, Hua Liu,** Division of Pediatric Gastroenterology, Department of Pediatrics, University of Mississippi Medical Center, Jackson, MS 39216, United States

**Patrick B Kyle,** Department of Pathology, University of Mississippi Medical Center, Jackson, MS 39216, United States

**Chalet Tan,** Department of Pharmaceutics and Drug Delivery, University of Mississippi, Oxford, MS 38677, United States

**Corresponding author:** Hua Liu, MD, Assistant Professor, Division of Pediatric Gastroenterology, Department of Pediatrics, University of Mississippi Medical Center, 2500 North State Street, Jackson, MS 39216, United States. hliu@umc.edu

## Abstract

***BACKGROUND***

Cardiac and hepatic functionality are intertwined in a multifaceted relationship. Pathologic processes involving one may affect the other through a variety of mechanisms, including hemodynamic and membrane transport effects.

***AIM***

To better understand the effect of extrahepatic cholestasis on regulations of membrane transporters involving digoxin and its implication for digoxin clearance.

***METHODS***

Twelve adult rats were included in this study; baseline hepatic and renal laboratory values and digoxin pharmacokinetic (PK) studies were established before evenly dividing them into two groups to undergo bile duct ligation (BDL) or a sham procedure. After 7 d repeat digoxin PK studies were completed and tissue samples were taken to determine the expressions of cell membrane transport proteins by quantitative western blot and real-time polymerase chain reaction. Data were analyzed using SigmaStat 3.5. Means between pre-surgery and post-surgery in the same experimental group were compared by paired *t*-test, while independent *t*-test was employed to compare the means between sham and BDL groups.

***RESULTS***

Digoxin clearance was decreased and liver function, but not renal function, was impaired in BDL rats. BDL resulted in significant up-regulation of multidrug resistance 1 expression in the liver and kidney and its down-regulation in the small intestine. Organic anion transporting polypeptides (OATP)1A4 was up-regulated in the liver but down-regulated in intestine after BDL. OATP4C1 expression was markedly increased in the kidney following BDL.

***CONCLUSION***

The results suggest that cell membrane transporters of digoxin are regulated during extrahepatic cholestasis. These regulations are favorable for increasing digoxin excretion in the kidney and decreasing its absorption from the intestine to compensate for reduced digoxin clearance due to cholestasis.

**Key Words:** Cholestasis; Digoxin clearance; Organic anion transporting polypeptides; P-glycoproteins/multidrug resistance 1; Bile duct ligation



**Core Tip:** The heart, kidney and liver are inextricably linked by virtue of blood flow and metabolism of medications. Cholestasis induced by bile duct ligation resulted in liver functional injury and a decrease in digoxin clearance. Quantitative western blot and real-time polymerase chain reaction demonstrated the up or down regulation of membrane transporters multidrug resistance 1, organic anion transporting polypeptides (OATP)1A4, and OATP4C1 in the liver, kidney, and intestine. Cell digoxin transporters are regulated during cholestasis which is favorable for increasing digoxin excretion.

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## INTRODUCTION

The heart and liver are inextricably linked by virtue of blood flow and metabolism of medications, respectively. Chronic cardiac failure is characterized by cholestatic liver disease, manifested as elevation of gamma-glutamyl transferase and bilirubin[1]. Conversely, cholestatic liver disease can lead to cardiac dysfunction. Drugs with biliary elimination may have a decreased clearance in patients with cholestasis [2]. In an experimental model of cholestasis, bile duct ligation (BDL) in rats results in cardiomyopathy characterized by impaired basal cardiac contractility and reduced left ventricular pressure[3]. Furthermore, obstructive cholestasis results in impaired excretion of digoxin[4,5].

The identification of a number of organic anion transporting polypeptides (OATP) and P-glycoproteins also known as multidrug resistance 1 (MDR1) has revolutionized our understanding of the transport of biologic compounds and medications. To date, three transporters have been identified which are integral in digoxin clearance - MDR1, OATP1A4, and OATP4C1.

The main route of elimination of digoxin is renal excretion, which is closely correlated with the glomerular filtration rate and combined with tubular secretion and reabsorption. Smaller portion of digoxin is eliminated by bile duct with certain degree of enterohepatic recycling[6]. The movement of digoxin in to and out of cells is mediated by different cell membrane transporters. In the rat, OATP1A4 (also known as OATP2) is found on the basolateral membrane of hepatocytes and the membrane of enterocytes serving as an influx transporter[7-9]. Administration of the OATP1A4 inhibitor, amiodarone, resulted in increased plasma levels of intravenously administered digoxin secondary to decreased biliary excretion, liver distribution, and intestinal distribution of digoxin[10]. Administration of phenobarbital increased expression of *OATP1A4* mRNA and protein, resulting in a 4-fold increase in digoxin uptake[11].

The MDR1 transporter is found in the canaliculus of the liver, the apical membrane of mucosal cells in the intestine, and the apical membrane of proximal tubule epithelial cells in the kidney, and it has been shown as an efflux pump for digoxin[12,13]. In rodents MDR1 is coded for by 2 genes, *MDR1A* and *MDR1B*. *MDR1A* is highly expressed in the intestine, intermediately expressed in the brain, low expression in the kidney, and minimally expressed in the liver[14]. *MDR1B* is intermediately expressed in the kidney and has low expression in the brain and liver[14]. The ontogeny of *MDR1A* and *MDR1B*

expression in the kidney correlates with digoxin clearance[15]. MDR1 is important in the elimination of digoxin. It is located on the canalicular membrane of hepatocytes, where it transports digoxin into the canaliculus. In the intestine, MDR1 is found on the apical membrane of enterocytes, where it serves an effluxer role to inhibit absorption of digoxin. In the kidney, MDR1 is found on the apical membrane of the proximal tubule, where it transports digoxin into the urine[16]. OATP4C1 is found in the kidney, located on the basolateral membrane of proximal tubule epithelia cells[17]. The physiological role of OATP4C1 in the kidney has been shown to be coupled with MDR1 to promote the renal clearance of digoxin[17].

The distributions of cell membrane transporters vary in different tissues, and a transporter may function differently among the tissues[18]. This makes it difficult to explain the body's response to increased blood digoxin during cholestasis. Cholestasis results in increased expression of OATP1A4 and MDR1 in the liver which favors improved hepatobiliary excretion of digoxin[19-21]. The effect of cholestasis on OATP4C1 has not been studied to date.

We performed this study to determine the effect of cholestasis on the expression of transporters responsible for the uptake and excretion of digoxin in the liver, kidney, and intestine. The implications of the changes in the transporters for digoxin pharmacokinetics (PKs) are discussed.

## MATERIALS AND METHODS

### Chemicals

Unless otherwise stated, all chemicals used in this study were purchased from Sigma Chemical Co. (St. Louis, MO, United States). Digoxin injection solution was purchased from Baxter Healthcare Corporation (Deerfield, IL, United States). Antibodies for western blot were purchased as follows: Anti-MDR1 (Cat: ab170904; Lot: GR21757-38) and anti-OATP1A4 antibody (Cat: ab224610; Lot: GR319515-7) were purchased from abcam (Cambridge, MA, Unite States). Anti-OATP4C1 (Cat: 24584-1-AP) was purchased from Proteintech (Rosemont, IL, United States).

### Animals and treatment

Adult male Sprague Dawley rats (225-250 g, Harlan Sprague Dawley, Inc. Indianapolis, IN, United States) were used for the study. They were kept in plastic cages with free access to food and water with alternating 12-h periods of light and darkness. Rats were randomly divided into a sham group ($n$ = 6) and a BDL group ($n$ = 6).

BDL was performed as described in previous publications[22,23]. In brief, rats were anaesthetized with isoflurane, and a midline ventral incision was made through the linea alba and the bile duct was isolated. A ligature was placed to the proximal portion and another ligature to the distal portion of the bile duct and then the ligatures were tightened. The bile duct was divided between the ligatures. The abdomen was closed by double-layer running suture, and the animal was allowed to wake up on a heating pad. Sham-operated control rats underwent similar surgical procedures except the ligatures were withdrawn, leaving the bile duct intact. The animals were sacrificed post-surgery day 7 after a post-surgery PK study. Tissue samples (liver, small intestine, and kidney) were collected and saved at -80 °C and RNAlater solution (Ambion, Foster City, CA, United States). The study was approved by the Institutional Animal Care and Use Committee at the University of Mississippi Medical Center.

### PK Study for digoxin clearance

Digoxin clearance was examined by PK studies two days prior to BDL/sham surgery and seven days following the surgeries. In brief, digoxin 0.02 mg/kg was injected through penile vein. Blood samples were obtained *via* tail vein at 0, 2, 5, 10, 30, 60, 120, 240, and 360 min following administration of digoxin for the measurement of digoxin. A separate blood sample (250 μL) was collected from tail vein for the measurement of liver function and bilirubin. Biochemical measurements were performed using a Roche-cobas® c501 analyzer (Roche Diagnostics, Indianapolis, IN, United States) for serum digoxin, total protein, albumin, alanine transaminase (ALT), aspartate transaminase (AST), alkaline phosphatase (ALP), bilirubin, blood urea nitrogen (BUN), and creatinine.

### Real time-polymerase chain reaction for MDR1, OATP1A4, AND OATP4C1

RNA was isolated from the tissues (liver, small intestine, and kidney) using a PureLink RNA Mini Kit (Invitrogen, Waltham, MA, United States) following the manufacturer's protocol. First-strand cDNA was synthesized through reverse transcription of 0.5 μg of total RNA using iScript cDNA Synthesis system (Bio-Rad Hercules, CA, United States). Controls without reverse transcriptase were performed for each sample to ensure absence of genomic DNA. Real time polymerase chain reaction (RT-PCR) was carried out in a real time thermal cycler (iCycler, Bio-Rad) using iQ SYBR Green Supermix (Bio-Rad). Cycling conditions were 3 min at 95 °C, followed by 40 cycles of 15 s at 95 °C, 20 s at 60 °C, then 30 s at 72 °C. PCR specificity was tested *via* analysis of the melting curve and agarose gel electrophoresis. To semi-quantify input amounts of templates, standard curves were constructed with serial dilutions of

cDNA sample from a positive control (kidney cDNA for *MDR1* and *OATP4C1*, liver cDNA for *OATP1A4*). To standardize results, interpolated values for each sample were divided by the value of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase. Primers were designed with Primer 3 software[24] and checked for absence of cross-reactivity by BLAST search. The primer pairs used, product size, and positive controls are shown in Table 1.

### Quantitative western blotting for MDR1, OATP1A4 and OATP4C1

Cell membrane proteins were extracted from liver, intestine, and kidney tissues by using a Mem-PER Plus kit (Thermo Scientific, Rockford, IL, United States) following the manufacturer's protocol. Halt Protease & Phosphatase inhibitor cocktail (Thermo Scientific, Rockford, IL, United States) was added to the extracting buffer to avoid protein degradation during procedures. Sample protein concentration was determined by using a BCA Protein Assay kit (Thermo Scientific). The protein sample was prepared for western blot by a Pierce SDS-PAGE Sample Prep Kit (Thermo Scientific) for concentrating samples while removing interfering substances. After sample buffer treatment proteins were loaded and separated on a pre-casted 4%-20% gradient SDS-PAGE gel (Bio-Red, Hercules, CA, United States) and transferred to an Immobilon-FL PVDF membrane (Merck KGaA, Darmstadt, Germany). After transfer, membrane was stained with REVERT™ Total Protein Stain (LI-COR Biosciences, Lincoln, NE, United States) for 5 min at room temperature, and then the blot image was analyzed with the Odyssey CLx® infrared imaging system (LI-COR Biosciences, Lincoln, NE, United States). Following total protein stain, the membranes were incubated with Odyssey Blocking Buffer (Li-cor, Lincoln, NE, United States) for 1 h at room temperature for blocking nonspecific binding sites. Then membranes were incubated overnight at 4 °C with primary antibodies against MDR1 (1:1600, Cat: ab170904; Lot: GR21757-38, abcam Cambridge, MA, United States), anti-OATP1A4 antibody (1:1000, Cat: ab224610; Lot: GR319515-7, abcam)[25], and anti-OATP4C1 (1:600, Cat: 24584-1-AP, Proteintech, Rosemont, IL, United States). Following the primary antibody treatments, the membranes were incubated with secondary IR dye-800 conjugated anti-rabbit antibody (1:10000, IRDy 800CW, Li-cor, Lincoln, NE, United States) for 1 h at room temperature. Western blot images were captured with the Odyssey CLx® infrared imaging system (LI-COR Biosciences, Lincoln, NE, United States) and analyzed for fluorescence density using Odyssey 2.0 software. Validation tests for sample loading sizes of each tissue, primary antibodies and secondary antibody were performed before the measurements. MDR1, OATP1A4 and OATP4C1 signals were normalized to total protein of each sample.

### Statistical analysis

Data were analyzed by SigmaStat 3.5. The paired *t*-test was used to compare the means between pre-surgery and post-surgery in the same experimental group sham or BDL. The independent *t*-test was employed to compare the means between sham and BDL groups. The values from 6 rats in each group showed normal distributions. All tests were two-sided. The PKs of digoxin was analyzed by non-compartmental techniques. The area under the plasma area under the curve (AUC) was calculated. Values are expressed as mean ± SD. Statistical significance was considered at $P < 0.05$. The statistical methods of this study were reviewed by Dr. Lei Zhang, a biostatistician, at University of Mississippi Medical Center, Jackson, MS, United States.

## RESULTS

### Effect of BDL on PKs of digoxin in rats

Digoxin PK studies were performed 2 d prior to BDL or sham surgery; the results were compared with digoxin PK studies performed 7 d following surgery. As shown in Figure 1, there was no difference in digoxin PKs between BDL and sham group prior surgery (Figure 1A). Following surgery, digoxin clearance was reduced in the BDL group as compared to the sham group (Figure 1B).

AUC of the post-BDL rats was significantly increased compared to the AUC of the pre-BDL and the post-surgery sham group (Figure 1C). AUC of the post-surgery sham group was slightly higher than that of the pre-surgery sham group but did not reach statistical significance. The change of AUC in the sham group following surgery may result from stress, change of gastrointestinal motility, or other factors induced by the sham surgery.

### Biochemical parameters

Biochemical parameters including serum total protein, albumin, ALT, AST, ALP, total bilirubin, direct bilirubin, BUN, and creatinine are represented in Table 2. There was significant liver functional injury in BDL rats as indicated by decreased serum albumin and increased ALT, AST and ALP. Obstructive jaundice developed in the post-BDL group as shown by increased total and direct bilirubin. Sham surgery did not affect liver function or bilirubin levels as compared to pre-surgery sham rats. Kidney function as measured by BUN and creatinine was not altered by BDL or sham surgery.

**Table 1 Real time polymerase chain reaction primer sequences, product size and positive controls**

| Target gene | Primer sequences (5'-3') | Size (bp) | Positive control |
|---|---|---|---|
| *MDR1* | ATCAACTCGCAAAAGCATCC (F) | 116 | Kidney |
| | AATTCAACTTCAGGATCCGC (R) | | |
| *OATP1A4* | TGTGATGACCTGTGATAATTTTCCA (F) | 81 | Liver |
| | TTCTCCACATATAGTTGGTGCTGAA (R) | | |
| *OATP4C1* | TCAAGCTGGCAAAACTTCCC (F) | 239 | Kidney |
| | CCGCAAAGCTCGATGTCAAT (R) | | |
| *GAPDH* | AAGATGGTGAAGGTCGGTGT (F) | 98 | Liver |
| | GTTGATGGCAACAATGTCCACT (R) | | |

OATP: Organic anion transporting polypeptides; MDR1: Multidrug resistance 1; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

**Table 2 Liver panel, bilirubin, blood urea nitrogen and creatinine**

| | Sham | | BDL | |
|---|---|---|---|---|
| | Pre-surgery | Post-surgery | Pre-surgery | Post-surgery |
| Tot protein | 6.63 ± 0.27 | 6.53 ± 0.35 | 6.53 ± 0.42 | 6.75 ± 0.23 |
| Albumin | 4.08 ± 0.17 | 3.85 ± 0.34 | 4.05 ± 0.14 | 3.40 ± 0.13[a] |
| ALP | 137.8 ± 19.78 | 122.3 ± 14.45 | 141.5 ± 12.74 | 467.2 ± 59.79[a] |
| Bilirubin, D | 0.04 ± 0.01 | 0.03 ± 0.01 | 0.03 ± 0.01 | 6.62 ± 1.72[a] |
| Bilirubin, T | 0.04 ± 0.02 | 0.06 ± 0.01 | 0.05 ± 0.02 | 11.67 ± 1.82[a] |
| ALT | 36.00 ± 12.02 | 57.00 ± 10.47 | 24.83 ± 8.28 | 191.8 ± 42.29[a] |
| AST | 71.83 ± 11.53 | 82.17 ± 4.92 | 64.17 ± 7.57 | 525.8 ± 107.11[a] |
| BUN | 17.54 ± 2.71 | 16.17 ± 3.13 | 18.23 ± 4.21 | 19.00 ± 5.57 |
| Creatinine | 0.27 ± 0.03 | 0.25 ± 0.02 | 0.29 ± 0.03 | 0.28 ± 0.04 |

[a]$P < 0.05$ *vs* pre-surgery.

Values are expressed as means ± SD of 6 rats per group. BDL: Bile duct ligation; ALT: Alanine transaminase; AST: Aspartate transaminase; ALP: Alkaline phosphatase; BUN: Blood urea nitrogen.

### *Effect of BDL on protein expressions of MDR1, OATP1A4, and OATP4C1*

The expression of the organic anion transporters was analyzed by quantitative western blot as described in the methods. MDR1 was expressed in all the tissues examined: Liver, kidney, and small intestine (Figures 2A and 2B). BDL resulted in significant up-regulation of MDR1 expression in the liver and kidney and its down-regulation in the small intestine.

OATP1A4 protein was expressed in the liver and small intestine but it was not detectable in the kidney. OATP1A4 was significantly up-regulated by BDL in the liver and down-regulated in the small intestine (Figures 3A and 3B). The expression of the organic anion transporter OATP4C1 was tested in the kidney. BDL led to a significantly increased expression of OATP4C1 as compared with sham surgery rats (Figures 4A and 4B).

### *Effect of BDL on mRNA expressions of MDR1, OATP1A4, and OATP4C1*

Transcription levels of *MDR1*, *OATP1A4* and *OATP4C1* were examined by mRNA expressions *via* RT-PCR. *MDR1* mRNA was presented in all the tissues examined (Figure 5A). BDL markedly up-regulated *MDR1* expression in the liver and kidney, down-regulated it in the small intestine as compared with sham surgery rats. *OATP1A4* mRNA was expressed in the liver and small intestine (Figure 5B). A trace amount of *OATP1A4* mRNA was tested in the kidney tissue. *OATP1A4* mRNA was significantly up-regulated by BDL in the liver and down-regulated in the small intestine as compared with sham surgery rats. BDL did not alter *OATP1A4* mRNA expression in the kidney (Figure 5B). *OATP4C1* mRNA was expressed in the kidney and was significantly elevated after BDL surgery as compared with sham surgery rats (Figure 5C). A summary of the regulations of cell membrane transporters in kidney,

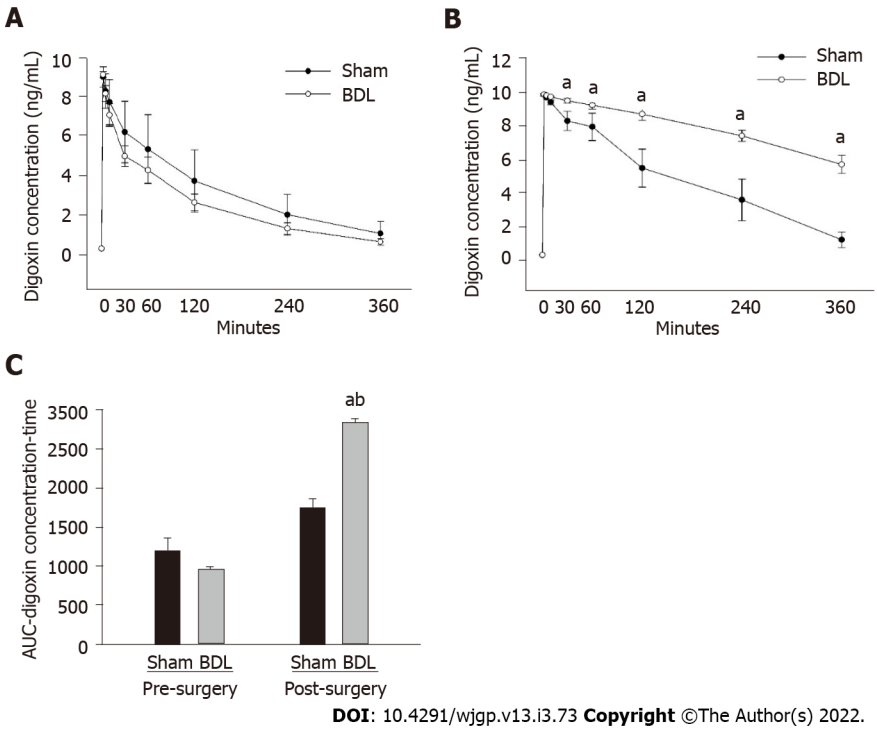


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**Figure 1 Effect of bile duct ligation on pharmacokinetics of digoxin in rats.** A: Pre-surgery digoxin pharmacokinetic studies was compared and presented as digoxin concentration-*versus*-time line curves; B: Post-surgery digoxin pharmacokinetic studies were compared and presented as digoxin concentration-*versus*-time line curves, C: Area under the curve, the area under the digoxin plasma concentration-*versus*-time. Values are expressed as means ± SD, n = 6; $^{a}P$ < 0.05 *vs* pre-surgery bile duct ligation group, $^{b}P$ < 0.05 *vs* post-surgery sham. BDL: Bile duct ligation; AUC: Area under the curve.

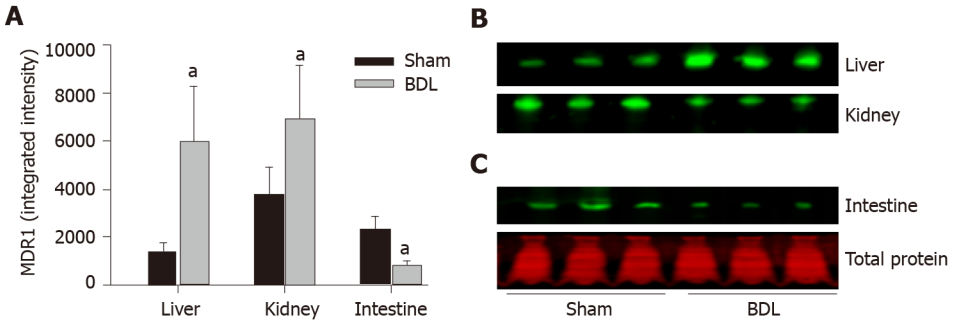


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**Figure 2 Effect of bile duct ligation on protein expressions of multidrug resistance 1.** Multidrug resistance 1 (MDR1) protein assay was performed by quantitative western blot. A: Fluorescence densities of the protein bands were measured and normalized to the relative total protein amount of each sample; B: The representative western blot images for the expression of MDR1 in the liver, kidney, small intestine; C: Representative western blot image for the total protein stain by REVERT™ Total Protein Stain kit. Values are depicted as means ± SD; *n* = 6; $^{a}P$ < 0.05 compared with sham surgery rats. BDL: Bile duct ligation; MDR1: Multidrug resistance 1.

intestine and liver, and potential effects on digoxin clearance are shown in Table 3.

## DISCUSSION

Digoxin remains an important medication for treatment of cardiac dysfunction, a condition known to predispose to hepatic injury resulting in cholestasis. Cholestasis predisposes to elevated serum levels of digoxin with increased risk of toxicity. Clearance of digoxin is a complex process with differences between humans and rodents. In the rat about 60%-70% of digoxin is metabolized and the remainder excreted by the kidney (about 20%-30%) and liver (about 10%)[26,27]. In normal conditions, renal excretion of digoxin is closely correlated with the glomerular filtration rate with certain degree of tubular secretion and reabsorption. A small portion of digoxin eliminated by the bile duct goes through enterohepatic cycling[6]. The trafficking of digoxin in and out of cells is mediated by different cell

**Table 3 Summary of the regulations of cell membrane transporters and potential effects on digoxin clearance**

| | Efflux | Influx | Effects |
|---|---|---|---|
| Kidney | MDR1: Up-regulated | OATP4C1: Up-regulated | Increase tubule exclusion |
| Intestine | MDR1: Down-regulated | OATP1A4: Down-regulated | Decrease intestinal absorption |
| Liver | MDR1: Up-regulated | OATP4C1: Up-regulated | Increase exclusion into bile duct |

OATP: Organic anion transporting polypeptides; MDR1: Multidrug resistance 1.

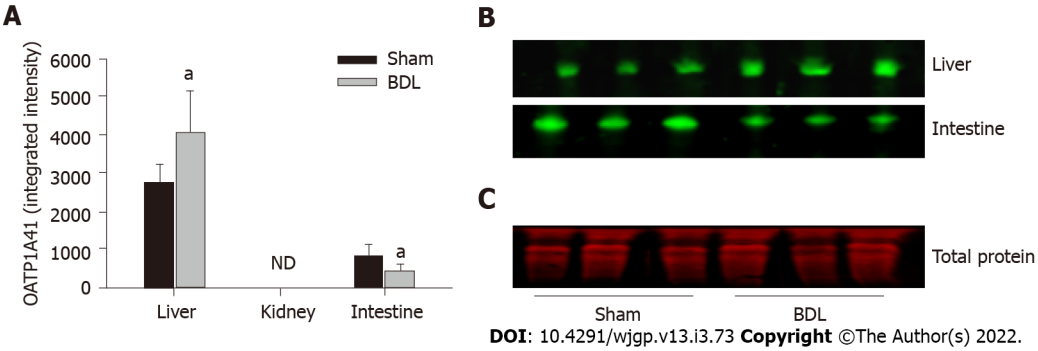



**Figure 3 Effect of bile duct ligation on protein expressions of organic anion transporting polypeptides 1A4.** Organic anion transporting polypeptides (OATP)1A4 protein assay was performed by quantitative western blot. A: Fluorescence densities of the protein bands were measured and normalized to the relative total protein amount of each sample; B: The representative western blot images for the expression of OATP1A4 protein in the liver, small intestine. OATP1A4 was not detected in the kidney by western blot; C: Representative western blot image for the total protein stain by REVERT™ Total Protein Stain kit. Values are expressed as means ± SD; $n = 6$; $^aP < 0.05$ compared with sham surgery rats. ND: Not detected; BDL: Bile duct ligation; OATP: Organic anion transporting polypeptides.

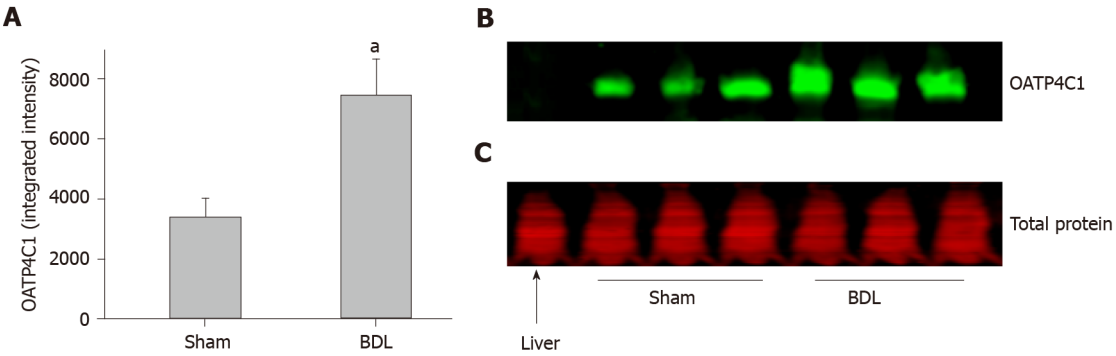



**Figure 4 Effect of bile duct ligation on protein expressions of organic anion transporting polypeptides 4C1 in the kidney.** Organic anion transporting polypeptides (OATP)4C1 protein assay was performed by quantitative western blot. A: Fluorescence densities of the protein bands were measured and normalized to the relative total protein amount of each sample; B: The representative western blot images for the expression of OATP4C1 protein in the kidney. Liver sample was loaded with kidney samples as negative control for OATP4C1; C: Representative western blot image for the total protein stain by REVERT™ Total Protein Stain kit. Values are expressed as means ± SD; $n = 6$; $^aP < 0.05$ compared with sham surgery rats. BDL: Bile duct ligation; OATP: Organic anion transporting polypeptides.

membrane transporters. Previous studies have demonstrated that uptake and efflux of digoxin are mediated by OATP1A4 and MDR1, respectively, in the liver and intestine[7-9], and by OATP4C1 and MDR1 in the kidney[17]. Cholestasis alters expression of MDR1 and OATP1A4 in a manner favorable for an increase in excretion of digoxin[19-21], while the effect of cholestasis on OATP4C1 in the kidney has not been studied to date. We undertook this study to determine changes in these digoxin transporters in a model of cholestasis and their implications for digoxin clearance.

Cholestasis was induced by BDL as evidenced by elevated serum transaminase and bilirubin levels. Digoxin clearance was decreased in the BDL group in keeping with prior studies in a rabbit model[4,5]. In the earliest study, BDL also resulted in elevation of serum creatinine prompting the authors to propose decreased renal excretion of orally administered digoxin as the major mechanism for decreased

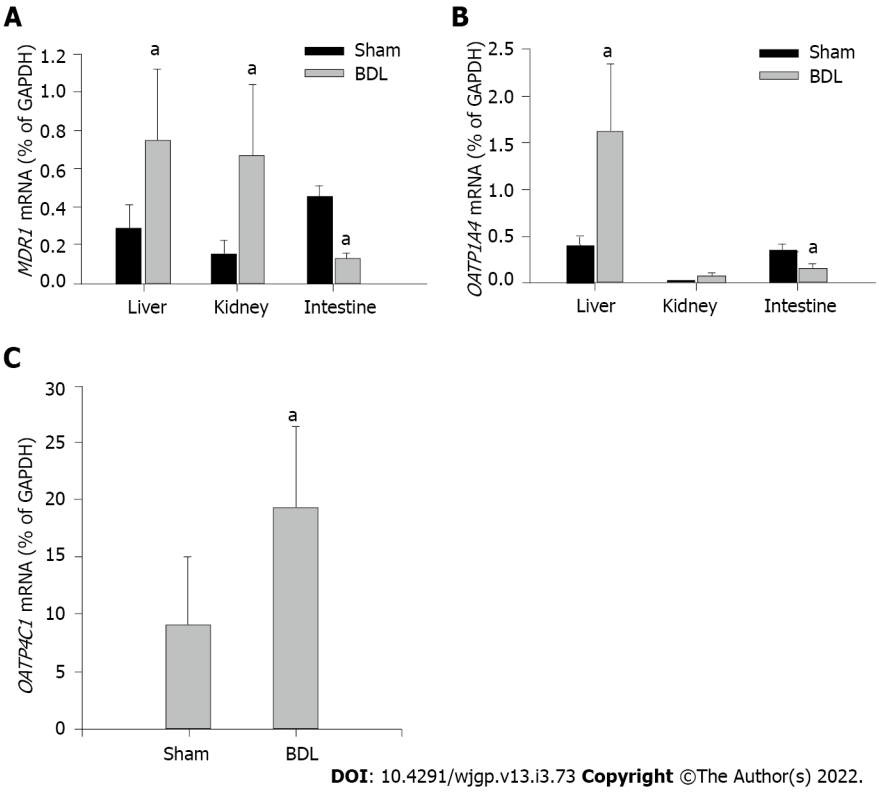


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**Figure 5 Effect of bile duct ligation on mRNA expressions of *multidrug resistance 1, organic anion transporting polypeptides 1a4* and *4C1*.** mRNA expression in each sample was standardized to its glyceraldehyde-3-phosphate dehydrogenase level. A: Expressions of *multidrug resistance 1* in the liver, kidney, small intestine, and the effect of bile duct ligation (BDL) on the mRNA expressions in each tissue; B: Expression of *organic anion transporting polypeptides* (*OATP*)*1A4* mRNA in the liver, kidney and small intestine, and the effect of BDL on *OATP1A4* mRNA expressions; C: Expression of *OATP4C1* mRNA in the kidney and the effect of BDL on its expression. Values are depicted as means ± SD; $n = 6$; $^{a}P < 0.05$ compared with sham surgery rats. BDL: Bile duct ligation; OATP: Organic anion transporting polypeptides; MDR1: Multidrug resistance 1; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

clearance with disruption of the enterohepatic circulation as a potential complicating factor[4]. In a follow-up study, BDL led to decreased clearance of intravenously administered digoxin, but with absence of elevated serum creatinine. The authors concluded that impaired hepatic function and interruption of the enterohepatic circulation impaired digoxin elimination[5]. Discovery of MDR1, OATP1A4, and OATP4C1 has allowed more in-depth investigation into the mechanisms of digoxin absorption and clearance.

MDR1 is found on the apical membranes of proximal tubule cells, enterocytes, and hepatocytes where it is responsible for efflux of digoxin. In rodents MDR1 is the product of the *MDR1* gene, which is made up of two forms, *MDR1A* and *MDR1B*[28]. Initial studies assessing the role of MDR1 in digoxin clearance focused on inhibiting the protein with quinidine, which inhibits intestinal excretion of digoxin [29]. To further study the role of MDR1 in digoxin clearance a knock-out model for MDR1A was created. In this model, fecal excretion of digoxin decreased and renal excretion increased compared to wild type animals, while there was no significant change in biliary excretion[30]. The authors concluded that the lower fecal excretion of digoxin was secondary to a decrease in drug excretion by the intestinal epithelium, rather than a decrease in biliary excretion. Increased renal excretion was surprising in the absence of MDR1A expression in the kidneys. The authors surmised that the increased renal clearance may be explained by other transporters (MDR1B) or increased glomerular filtration. They concluded that MDR1 contributes substantially to digoxin excretion *via* the intestinal epithelium and decreased re-uptake after biliary excretion[30].

Transport of digoxin in the liver is mediated by OATP1A4, responsible for uptake at the hepatocyte basolateral membrane, and MDR1, responsible for excretion into the bile at the apical membrane[7,14]. In the present study cholestasis/BDL led to increased expression of OATP1A4, increasing hepatic uptake of digoxin from the blood, and increased expression of MDR1, increasing biliary excretion of digoxin. Although these changes would predict increased clearance of digoxin through bile, ligation of the bile duct precludes this mode of clearance.

A carrier-mediated uptake of digoxin is responsible for its reabsorption of digoxin in intestine[31]. The carrier-mediated uptake was found to be sensitive to the OATP inhibitors BSP and apple juice, suggesting an OATP transporter as a likely candidate. Further support for an OATP transporter came from experiments using rat intestinal brush-border membrane vesicles which showed that an increased

digoxin uptake in the presence of proton and bicarbonate gradients and outwardly directed glutathione gradient[31]. Recent studies demonstrated that intestinal OATP1A4 is a carrier protein that transports drugs from gut into the portal circulation[8], and digoxin has been shown as a substrate of OATP1A4 [10]. Our result showed that BDL led to decreased expression of OATP1A4 in the intestine. Decreased expression of OATP1A4 in the intestine favors decreased absorption predicting improved drug clearance in the feces.

Although cholestasis results in changes in MDR1 and OATP1A4 favoring increased digoxin clearance, in the BDL model of cholestasis clearance of intravenously administered digoxin is limited to renal excretion. Although BDL led to changes that would predict increased clearance of digoxin through bile, ligation of the bile duct precludes this mode of clearance. Similarly, changes in the intestine following BDL favoring digoxin clearance in the feces are minimized by the study design. Digoxin administered intravenously would limit to amount of drug in the intestinal lumen. Further, BDL inhibits hepatic excretion of digoxin into the intestine.

In the kidney MDR1 is responsible for excretion of digoxin across the apical membrane of renal cells into urine[16]. Our result showed that OATP1A4 is not expressed in the kidney suggesting another transporter is responsible for transport of digoxin across the basolateral membrane into renal cells[17]. Mikkaichi *et al*[17] isolated an organic acid transporting peptide denoted OATP4C1 both in humans and rats. It is localized on the basolateral membrane of the proximal tubules of the kidney where it has been shown to be the primary transporter of digoxin into renal cells. MDR1 is co-localized with OATP4C1 in the proximal tubule. Renal failure leads to decreased expression in OATP4C1 but has no effect on expression of MDR1 suggesting that decreased digoxin clearance in renal failure is due to loss of OATP4C1 activity[17,32]. We have shown that cholestasis due to BDL results in increased expression in both MDR1 and OATP4C1 in the kidney favoring enhanced vectorial transport of digoxin from blood to urine by proximal tubule cells. To the best of our knowledge, the current report is the first study to investigate the regulation of OATP4C1 in kidney in a pathological model *in vivo*.

It is interesting that MDR1 and OATP1A4 participate in transport of both bile acids and digoxin[33]. Also, there is marked similarity in the method of excretion for bile acids and digoxin in obstructive cholestasis. OATP4C1 may also participate in the excretion of bile acids by the kidney through increased uptake at the basolateral membrane, although the data is conflicting. To date, two studies assessed the transport of bile acids in Madin-Darby canine kidney cells transfected with a plasmid containing OATP4C1, one showed no transport of taurocholate[17], while the other showed transport of both chenodeoxycholate and glycocholate[34]. Our study showed upregulation of OATP4C1 in cholestasis which would increase uptake of bile acids by proximal tubule cells with subsequent excretion at the apical membrane by MDR1.

Bile acids activate the nuclear hormone receptors farnesoid-X-receptor and pregnane-X-receptor (PXR) and in cholestasis there were increased activations of these receptors[35,36]. MDR1 and OATP1A4 are both PXR-responsive and their expression increased in cholestasis. OATP4C1 expression is induced through transitional factor Aryl hydrocarbon receptor (AhR) through binding of the xenobiotic responsive element[37]. Previous studies have shown that AhR is activated in cholestasis[38] through the action of PXR[39]. We propose that the increased expression of OATP4C1 in cholestasis is best explained by this mechanism.

This is an exploratory research to study how the body responds to increased digoxin during cholestasis. Further studies are needed to confirm the implications by measuring digoxin tissue distributions and digoxin concentrations in urine and along the intestinal tract from the duodenum to the ileum. We believe that the findings from the current study will serve as a base for future study of digoxin clearance mediated by renal-expressed OATP4C1 during cholestasis.

## CONCLUSION

In conclusion, under physiological conditions, the main route of elimination of digoxin is renal excretion which is closely correlated with glomerular filtration rate. Biliary excretion is the major non-renal route. Enterohepatic cycle has minor importance[6]. Our finding demonstrated that under pathological condition, cholestasis in the current study, cell membrane digoxin transporters are regulated which is in favor of an increase in digoxin excretion in renal tubules and a decrease in its absorption from the tubules of intestine. These changes compensate the reduced digoxin clearance due to cholestasis. This finding could have clinical application by modifying transporters' activities through pharmaceutical approaches for improving digoxin clearance during cholestasis.

## ARTICLE HIGHLIGHTS

### *Research background*

The heart and liver are inextricably linked by virtue of blood flow and metabolism of medications.

Drugs with biliary elimination, such as digoxin, decrease clearance with cholestasis.

### *Research motivation*

We performed this study to better understand the effect of extrahepatic cholestasis on regulations of membrane transporters involving digoxin and its implication for digoxin clearance.

### *Research objectives*

The efflux transporter, multidrug resistance 1 (MDR1), and influx transporters, organic anion transporting polypeptides (OATP)1A4 and OATP4C1 in kidney, intestine and liver were examined.

### *Research methods*

Twelve adult Sprague Dawley rats were included in this study; baseline hepatic and renal laboratory values and digoxin pharmacokinetic (PK) studies were established before evenly dividing them into two groups to undergo bile duct ligation (BDL) or a sham procedure. After 7 d repeat digoxin PK studies were completed and tissue samples were taken to determine the expressions of MDR1, OATP1A4 and OATP4C1 by quantitative western blot and real-time polymerase chain reaction.

### *Research results*

Digoxin clearance was decreased and liver function, but not renal function, was impaired in BDL rats. BDL resulted in significant up-regulation of MDR1 expression in the liver and kidney and its down-regulation in the small intestine. OATP1A4 was up-regulated in the liver but down-regulated in intestine after BDL. OATP4C1 expression was markedly increased in the kidney following BDL.

### *Research conclusions*

The results suggest that cell membrane transporters of digoxin are regulated during cholestasis. These regulations are favorable for increasing digoxin excretion in kidney and decreasing its absorption from intestine in order to compensate the reduced digoxin clearance due to cholestasis.

### *Research perspectives*

The current study was designed as an exploratory research for providing clues for future study in this field. Previous studies on the transporters in kidney and intestine were done only by *in vitro* experiments. To the best of our knowledge, the current report is the first study to investigate the regulation of the digoxin transporters in kidney and intestine in animal model of cholestasis. Our results does demonstrate that the cell membrane transporters were regulated which is in favor of digoxin excretion during cholestasis. To confirm our finding, more detailed PK studies need to be done, for example, tissue distributions of digoxin and digoxin concentrations in urine and in intestine. Knock-out (KO) animal lacking the transporters, especially tissue-specific KO, will be a powerful tool in further study.

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## FOOTNOTES

**Author contributions:** Giroux P, Nowicki MJ, Tan C and Liu H participated in conception and research design; Giroux P, Kyle PB, and Liu H conducted experiments; Giroux P, Nowicki MJ, Tan C, Edwards JD, and Liu H performed data analysis and interpretation; Giroux P, Kyle PB, Nowicki MJ, Tan C, Edwards JD, and Liu H wrote or contributed to the reversion of the manuscript; and all authors have read and approved the final manuscript.

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**ORCID number:** Parker Giroux 0000-0002-5644-7720; Patrick B Kyle 0000-0003-0154-7078; Chalet Tan 0000-0001-7489-7699; Joseph D Edwards 0000-0003-4979-2937; Michael J Nowicki 0000-0001-9395-3027; Hua Liu 0000-0002-7936-5330.

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ORIGINAL ARTICLE

**Retrospective Study**

# Increasing thirty-day readmissions of Crohn's disease and ulcerative colitis in the United States: A national dilemma

Dushyant Singh Dahiya, Abhilash Perisetti, Asim Kichloo, Amandeep Singh, Hemant Goyal, Laura Rotundo, Madhu Vennikandam, Hafeez Shaka, Gurdeep Singh, Jagmeet Singh, Sailaja Pisipati, Mohammad Al-Haddad, Madhusudhan R Sanaka, Sumant Inamdar

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**Dushyant Singh Dahiya, Asim Kichloo,** Department of Internal Medicine, Central Michigan University College of Medicine, Saginaw, MI 48601, United States

**Abhilash Perisetti,** Division of Gastroenterology, Parkview Cancer Institute, Fort Wayne, IN 46845, United States

**Amandeep Singh, Madhusudhan R Sanaka,** Division of Gastroenterology and Hepatology, Cleveland Clinic Foundation, Cleveland, OH 44195, United States

**Hemant Goyal,** Department of Internal Medicine, The Wright Center for Graduate Medical Education, Scranton, PA 18505, United States

**Hemant Goyal,** Department of Internal Medicine, Mercer University School of Medicine, Macon, GA 31207, United States

**Laura Rotundo,** Section of Digestive Diseases, Yale New Haven Hospital, New Haven, CT 06510, United States

**Madhu Vennikandam,** Department of Gastroenterology and Hepatology, Sparrow Hospital/Michigan State University College of Human Medicine, Lansing, MI 48912, United States

**Hafeez Shaka,** Department of Internal Medicine, John H. Stroger, Jr. Hospital of Cook County, Chicago, IL 60612, United States

**Gurdeep Singh,** Department of Internal Medicine, Our Lady of Lourdes Memorial Hospital, Binghamton, NY 13905, United States

**Jagmeet Singh,** Department of Internal Medicine, Guthrie Robert Packer Hospital, Sayre, PA 18840, United States

**Sailaja Pisipati,** Division of Gastroenterology and Hepatology, Mayo Clinic, Scottsdale, AZ 85259, United States

**Mohammad Al-Haddad,** Division of Gastroenterology and Hepatology, Indiana University School of Medicine, Indianapolis, IN 46202, United States

**Sumant Inamdar,** Division of Gastroenterology and Hepatology, University of Arkansas for Medical Sciences, Little Rock, AR 72205, United States

**Corresponding author:** Dushyant Singh Dahiya, MD, Doctor, Department of Internal Medicine, Central Michigan University College of Medicine, 1015 S Washington Ave, Saginaw, MI 48601, United States.
dush.dahiya@gmail.com

## Abstract

***BACKGROUND***

The prevalence of Crohn's disease (CD) and ulcerative colitis (UC) is on the rise worldwide. This rising prevalence is concerning as patients with CD and UC may frequently relapse leading to recurrent hospitalizations and increased healthcare utilization.

***AIM***

To identify trends and adverse outcomes for 30 d readmissions for CD and UC.

***METHODS***

This was a retrospective, interrupted trends study involving all adult (≥ 18 years) 30 d readmissions of CD and UC from the National Readmission Database (NRD) between 2008 and 2018. Patients < 18 years, elective, and traumatic hospitalizations were excluded from this study. We identified hospitalization characteristics and readmission rates for each calendar year. Trends of inpatient mortality, mean length of hospital stay (LOS) and mean total hospital cost (THC) were calculated using a multivariate logistic trend analysis adjusting for age, gender, insurance status, comorbidity burden and hospital factors. Furthermore, trends between CD and UC readmissions were compared using regression of the interaction coefficient after adjusting for age and gender to determine relative trends between the two populations. Stata® Version 16 software (StataCorp, TX, United States) was used for statistical analysis and $P$ value ≤ 0.05 were considered statistically significant.

***RESULTS***

Total number of 30 d readmissions increased from 6202 in 2010 to 7672 in 2018 for CD and from 3272 in 2010 to 4234 in 2018 for UC. We noted increasing trends for 30-day all-cause readmission rate of CD from 14.9% in 2010 to 17.6% in 2018 ($P$-trend < 0.001), CD specific readmission rate from 7.1% in 2010 to 8.2% in 2018 ($P$-trend < 0.001), 30-day all-cause readmission rate of UC from 14.1% in 2010 to 15.7% in 2018 ($P$-trend = 0.003), and UC specific readmission rate from 5.2% in 2010 to 5.6% in 2018 ($P$-trend = 0.029). There was no change in the risk adjusted trends of inpatient mortality and mean LOS for CD and UC readmissions. However, we found an increasing trend of mean THC for UC readmissions. After comparison, there was no statistical difference in the trends for 30 d all-cause readmission rate, inpatient mortality, and mean LOS between CD and UC readmissions.

***CONCLUSION***

There was an increase in total number of 30 d readmissions for CD and UC with a trend towards increasing 30 d all-cause readmission rates.

**Key Words:** Inflammatory bowel disease; Crohn's disease; Ulcerative colitis; Readmissions; Trends



**Core Tip:** This retrospective interrupted trend study analyzed 30 d readmissions of Crohn's disease (CD) and ulcerative colitis (UC) in the United States from 2010–2018. There was a rising trend for 30 d all-cause readmission rate of CD and UC, and CD- and UC-specific readmission rate throughout the study period. However, we noted no change in the risk adjusted trends of inpatient mortality and mean length of hospital stay (LOS) for 30 d readmissions of CD and UC. Furthermore, there was no statistical difference in the trends for 30 d all-cause readmission rate, inpatient mortality, and mean LOS between CD and UC readmissions.

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## INTRODUCTION

Inflammatory bowel disease (IBD) is characterized by chronic inflammation of the gastrointestinal tract with a propensity of remission and relapse over time[1]. It consists of Crohn's disease (CD) and ulcerative colitis (UC)[2]. The exact pathogenesis of IBD is relatively unknown, but researchers believe that factors such as immune response dysregulation, gut microbiota dysbiosis, environmental changes and genetic variants play a key role[3]. In 2017, there were 6.8 million patients with IBD worldwide with studies reporting continuously rising incidence and prevalence, particularly in North America[4]. The rising rates of IBD are concerning as it is associated with a poor quality of life and places significant social and economic burden on individuals and the United States healthcare system[5,6].

Despite outpatient management by gastroenterologists, patients with IBD are at increased risk of readmission due to relapse, complications of the disease or for additional interventions after index hospitalization. This further exacerbates the impact of the disease on individuals and the healthcare system. Additionally, studies have demonstrated that about 9%–50% of IBD readmissions are preventable and may be directly linked to the quality of hospital care and inadequate post-discharge care[7]. Hence, hospital systems have developed scoring systems to identify individuals at the highest risk of readmission and implemented strategies to reduce readmissions and improve the overall quality of care[8].

In current literature, a majority of the studies investigating readmissions of IBD have been single-center experiences or primarily focused on surgical patients[9,10]. There continues to be relative paucity of data on early (30 d) readmissions of CD and UC in the United States. Hence, this national, retrospective, interrupted trends study was designed to identify the hospitalization characteristics and estimate readmission rates of CD and UC in the United States between 2010–2018. We also identified the trends of inpatient mortality to determine improvements in therapeutic management of the disease. Furthermore, we calculated the burden of the disease on the United States healthcare system in terms of healthcare utilization and hospitalization costs.

## MATERIALS AND METHODS

### *Design and data source*

This was a retrospective interrupted trends study involving all adult readmissions of IBD (UC and CD) in the United States between 2010–2018. Data for analysis was extracted from the Nationwide Readmissions Database (NRD) which is a part of the Agency for Healthcare Research and Quality (AHRQ) Healthcare Cost and Utilization Project (HCUP) State Inpatient Databases (SID)[11]. It allows for weighted analysis to obtain 100% of the United States hospitalizations within a given calendar year [11]. The data for NRD is collected using the International Classification of Diseases, Ninth/Tenth Revision, Clinical Modification (ICD-9/10-CM/PCS) codes.

### *Study population*

The study involved all adult (≥ 18 years) 30 d readmissions of CD and UC from the NRD for the years 2010, 2012, 2014, 2016 and 2018. We used all available ICD-9-CM/PCS codes for CD (555X) and UC (556X) along with the equivalent ICD-10-CM/PCS codes K50X and K51X for CD and UC, respectively. The precedence for the utilization of these codes has been established in prior published studies[12]. Individuals < 18 years of age, elective and traumatic hospitalizations were excluded from the analysis. Using unique hospitalization identifiers, index hospitalizations of CD and UC were identified and one subsequent hospitalization within 30 d was tagged as a readmission.

### *Statistical analysis and outcome measures*

The data was analyzed using Stata® Version 16 software (StataCorp, TX, United States). All analyses were conducted using weighted samples for national estimates. *P* value ≤ 0.05 was set as the threshold for statistical significance. We highlighted hospitalization trends and obtained the 30 d all-cause readmission rate, disease specific readmission rate and readmission proportion for specific calendar years. The comorbidity burden was assessed using Sundararajan's adaptation of the modified Deyo's

Charlson comorbidity index[13]. Trends of inpatient mortality, mean length of stay (LOS) and mean hospital cost (THC) for CD and UC readmissions were calculated using a multivariate logistic trend analysis adjusting for age, gender, insurance status, comorbidity burden and hospital factors. The total hospital cost was obtained using the HCUP Cost-to-Charge Ratio files and adjusted for inflation using the Medical Expenditure Panel Survey index for hospital care, with 2018 as the reference point[14,15]. Additionally, trends between CD and UC readmissions were compared using regression of the interaction coefficient after adjusting for age and gender to determine relative trends between the two populations. Furthermore, we report no missing data in this study.

### Ethical considerations

The NRD database lacks patient and hospital-specific identifiers. Hence, this study was exempt from Institutional Review Board (IRB) approval for analysis as per guidelines put forth by our institutional IRB for research on database studies.

### Data availability statement

The NRD is a large publicly available, multi-ethic, all-payer inpatient care database in the United States, containing data on more than 18 million hospital stays/year. The database can be accessed at: https://www.hcup-us.ahrq.gov/nrdoverview.jsp.

## RESULTS

### CD: Hospitalization characteristics and outcomes for 30 d readmissions

The total number of 30 d readmissions of CD increased from 6202 in 2010 to 7672 in 2018 (Figure 1). The mean age increased from 41.8 ± 0.9 in 2010 to 43.9 ± 0.7 years in 2018. A female predominance was noted throughout the study period (Table 1); however, a statistically significant trend for gender was absent. Additionally, 30 d readmissions of CD were noted to have an increasing comorbidity burden with time (Table 1). Furthermore, metropolitan teaching hospitals had the majority of the readmissions with a statistically significant trend towards increasing readmissions from 52.1% in 2010 to 77% in 2018 (Table 1).

There was a statistically significant trend towards increasing 30 d all-cause readmission rate of CD from 14.9% in 2010 to 17.6% in 2018 ($P$-trend < 0.001) (Figure 2). The CD specific readmission rate also had a statistically significant increasing trend with an increase from 7.1% in 2010 to 8.2% in 2018 ($P$-trend < 0.001). However, we did not observe a significant change in the risk adjusted trends of inpatient mortality, mean LOS, and mean THC for these readmissions.

### UC: Hospitalization characteristics and outcomes for 30 d readmissions

Similar to CD, the total number of 30 d readmissions of UC increased from 3272 in 2010 to 4234 in 2018 (Figure 1). The mean age for these readmissions increased from 49.8 ± 1.6 in 2010 to 51.2 ± 0.8 years in 2018. A female predominance without a statistical trend for gender and increasing comorbidity burden with time was also noted. Furthermore, metropolitan teaching hospitals had an increasing trend of readmissions from 53.6% in 2010 to 76.3% in 2018 (Table 2), similar to that for CD.

A rising trend was noted for 30 d all cause readmission rate of UC from 14.1% in 2010 to 15.7% in 2018 ($P$-trend = 0.003) (Figure 2) and for UC specific readmission rate from 5.2% in 2010 to 5.6% in 2018 ( $P$-trend = 0.029). Additionally, the mean THC increased from $13783 in 2010 to $15929 in 2018 ($P$-trend = 0.009) with a rising trend unlike CD. However, similar to CD, a significant change in the risk adjusted trends was absent for of inpatient mortality and mean LOS (Table 3).

### Comparison of trends for 30 d readmissions of CD and UC

Although CD had higher number of 30 d readmissions every year, we did not observe a statistically significant difference in the in the trends for 30 d all-cause readmission rate (interaction $P$-trend = 0.087), inpatient mortality (interaction $P$-trend = 0.231), and mean LOS (interaction $P$-trend = 0.388). However, there was a statistically significant trend towards increasing mean THC for 30 d readmissions of UC relative to 30 d readmissions of CD (interaction $P$-trend < 0.001).

## DISCUSSION

It is essential to identify early (30 d) readmissions of IBD as they may be associated with quality of inpatient care, increased risk of adverse outcomes and place significant burden on the United States healthcare system in terms of healthcare costs and resource utilization. Additionally, as providers become aware of the magnitude of these readmissions and the patient demographics most effected, efforts could be directed at index admissions to further optimize medical therapy before discharge, promote patient education and encourage a greater degree of involvement in their care, and increase

**Table 1 Biodemographic characteristics and hospitalization trends for 30 d readmissions of Crohn's disease**

| Variable | Year | | | | |
|---|---|---|---|---|---|
| | 2010 | 2012 | 2014 | 2016 | 2018 |
| **Number of readmissions** | 6202 | 6580 | 6475 | 8278 | 7672 |
| **Age (mean ± SE, yr)** | 41.8 ± 0.9 | 41.6 ± 1.1 | 41.2 ± 0.8 | 42.5 ± 0.7 | 43.9 ± 0.7 |
| **Gender (%)** | | | | | |
| Males | 45.5 | 44.0 | 45.7 | 46.7 | 46.5 |
| Females | 54.5 | 56.0 | 54.3 | 53.3 | 53.5 |
| **Charlson comorbidity index score (%)** | | | | | |
| 0 | 69.7 | 72.0 | 69.9 | 64.9 | 61.3 |
| 1 | 19.2 | 15.5 | 17.3 | 19.5 | 20.0 |
| 2 | 5.9 | 6.1 | 6.7 | 7.5 | 9.0 |
| ≥ 3 | 5.2 | 6.4 | 6.1 | 8.1 | 9.7 |
| **Insurance type (%)** | | | | | |
| Medicare | 20.5 | 29.1 | 29.3 | 28.9 | 30.6 |
| Medicaid | 21.5 | 24.9 | 26.4 | 25.5 | 24.7 |
| Private | 41.2 | 37.1 | 37.0 | 40.8 | 39.0 |
| Uninsured | 8.8 | 8.9 | 7.3 | 4.8 | 5.7 |
| **Household income quartile (%)** | | | | | |
| 1st | 27.8 | 29.2 | 27.9 | 29.0 | 28.6 |
| 2nd | 23.4 | 25.6 | 28.5 | 26.8 | 30.0 |
| 3rd | 24.9 | 25.1 | 22.5 | 24.5 | 23.7 |
| 4th | 23.9 | 20.1 | 21.1 | 19.7 | 17.7 |
| **Hospital characteristics** | | | | | |
| **Hospital bed size (%)** | | | | | |
| Small | 9.9 | 9.9 | 14.2 | 13.3 | 15.0 |
| Medium | 22.4 | 22.4 | 27.3 | 26.9 | 26.3 |
| Large | 67.7 | 67.7 | 58.5 | 59.8 | 58.7 |
| **Teaching status (%)** | | | | | |
| Metropolitan non-teaching | 39.2 | 34.4 | 25.2 | 21.8 | 17.3 |
| Metropolitan teaching | 52.1 | 56.8 | 68.4 | 72.3 | 77.0 |
| Non-metropolitan | 8.7 | 8.8 | 6.4 | 5.9 | 5.7 |
| **Hospital volume quintiles (%)** | | | | | |
| Q1 | 1.8 | 1.9 | 1.5 | 1.7 | 1.3 |
| Q2 | 4.3 | 5.4 | 5.1 | 4.2 | 4.5 |
| Q3 | 10.3 | 10.0 | 10.2 | 8.4 | 10.4 |
| Q4 | 19.4 | 18.1 | 18.1 | 18.6 | 19.1 |
| Q5 | 64.2 | 64.6 | 65.1 | 67.1 | 64.7 |

outpatient follow-up, thereby decreasing early readmissions. A single center retrospective study from 2007–2010 revealed that about 5% patients with IBD were readmitted within 1 wk of hospital discharge, 14% within 1 mo, 23% within 3 mo and about 39% within the year[16]. Another study in the United States reported similar findings with a readmission rate of 18% within 1 mo of hospital discharge[17]. In 2013, an NRD-based study estimated 3037 (7%) readmissions of IBD at 30 d[7].

**Table 2 Biodemographic characteristics and hospitalization trends for 30 d readmissions of ulcerative colitis**

| Variable | Year | | | | |
|---|---|---|---|---|---|
| | 2010 | 2012 | 2014 | 2016 | 2018 |
| **Number of readmissions** | 3272 | 3399 | 3426 | 4449 | 4234 |
| **Age (mean ± SE, yr)** | 49.8 ± 1.6 | 49.6 ± 1.5 | 48.4 ± 1.1 | 49.9 ± 1.0 | 51.2 ± 0.8 |
| **Gender (%)** | | | | | |
| Males | 48.1 | 45.6 | 47.5 | 46.7 | 49.4 |
| Females | 51.9 | 54.4 | 52.5 | 53.3 | 50.6 |
| **Charlson comorbidity index score (%)** | | | | | |
| 0 | 57.8 | 59.6 | 60.6 | 55.6 | 50.9 |
| 1 | 20.3 | 20.0 | 18.6 | 19.4 | 20.7 |
| 2 | 9.4 | 9.0 | 8.5 | 10.6 | 10.3 |
| ≥ 3 | 12.5 | 11.4 | 12.3 | 14.4 | 18.1 |
| **Insurance type (%)** | | | | | |
| Medicare | 36.3 | 36.6 | 32.4 | 35.1 | 34.8 |
| Medicaid | 17.8 | 17.0 | 22.3 | 17.5 | 19.5 |
| Private | 39.4 | 37.0 | 40.1 | 42.2 | 40.4 |
| Uninsured | 6.5 | 9.4 | 5.2 | 5.2 | 5.3 |
| **Household income quartile (%)** | | | | | |
| 1st | 25.5 | 29.2 | 26.5 | 27.2 | 25.0 |
| 2nd | 22.5 | 23.1 | 25.9 | 27.5 | 26.7 |
| 3rd | 26.4 | 24.6 | 22.9 | 25.0 | 26.1 |
| 4th | 25.6 | 23.1 | 24.7 | 20.3 | 22.2 |
| **Hospital characteristics** | | | | | |
| **Hospital bed size (%)** | | | | | |
| Small | 10.2 | 9.8 | 13.2 | 13.5 | 16.8 |
| Medium | 19.8 | 22.4 | 26.8 | 25.7 | 24.3 |
| Large | 70.0 | 67.8 | 60.0 | 60.8 | 58.9 |
| **Teaching status (%)** | | | | | |
| Metropolitan non-teaching | 37.3 | 38.2 | 26.1 | 24.5 | 19.3 |
| Metropolitan teaching | 53.6 | 53.5 | 67.7 | 70.3 | 76.3 |
| Non-metropolitan | 9.1 | 8.3 | 6.2 | 5.2 | 4.4 |
| **Hospital volume quintiles (%)** | | | | | |
| Q1 | 2.4 | 2.4 | 2.5 | 2.1 | 2.0 |
| Q2 | 6.0 | 7.4 | 5.9 | 5.8 | 5.5 |
| Q3 | 11.8 | 10.5 | 11.7 | 10.3 | 12.3 |
| Q4 | 20.2 | 20.1 | 19.0 | 20.4 | 21.4 |
| Q5 | 59.6 | 59.6 | 60.9 | 61.4 | 58.8 |

In our study, the total number of 30 d readmissions of CD increased from 6202 in 2010 to 7672 in 2018 and for UC from 3,272 in 2010 to 4,234 in 2018, both with a female predominance (Tables 1 and 2). This coincides with rising prevalence of CD and UC in the general population[18]. We also noted an increasing trend for 30 d all-cause readmission rates and disease specific readmission rates for 30 d readmissions of CD and UC (Table 3). These findings may, in part, be due to a rising prevalence of IBD in the general population which increased significantly from 0.9% (2 million adults) in 1999 to 1.3% (3 million adults) in 2015, an increase in the flare-ups of IBD which may account for about 50% of the

**Table 3 Readmission rates, inpatient mortality, and healthcare burden for 30 d readmissions of Crohn's disease and ulcerative colitis**

| Outcomes | Year | | | | | *P* trend |
|---|---|---|---|---|---|---|
| | 2010 | 2012 | 2014 | 2016 | 2018 | |
| **Crohn's disease** | | | | | | |
| All-cause readmission rate (%) | 14.9 | 15.5 | 15.2 | 18.9 | 17.6 | < 0.001 |
| Crohn's disease specific readmission rate (%) | 7.1 | 6.9 | 7.0 | 8.9 | 8.2 | < 0.001 |
| Crohn's disease readmission proportion (%) | 54.9 | 51.8 | 53.0 | 55.8 | 54.6 | 0.002 |
| Inpatient mortality (%) | 0.9 | 1.4 | 0.7 | 0.7 | 1.0 | 0.059 |
| Mean length of stay (d) | 5.9 | 5.9 | 5.3 | 6.0 | 6.2 | 0.927 |
| Mean total hospital cost (USD) | 12327 | 13068 | 10988 | 13421 | 14260 | 0.210 |
| **Ulcerative colitis** | | | | | | |
| All-cause readmission rate (%) | 14.1 | 14.2 | 13.5 | 16.6 | 15.7 | 0.003 |
| Ulcerative colitis specific readmission rate (%) | 5.2 | 5.3 | 5.2 | 6.1 | 5.6 | 0.029 |
| Ulcerative colitis readmission proportion (%) | 42.6 | 42.4 | 43.4 | 43.0 | 41.0 | 0.566 |
| Inpatient mortality (%) | 2.5 | 1.8 | 2.2 | 2.0 | 2.3 | 0.912 |
| Mean length of stay (d) | 6.8 | 6.8 | 6.3 | 6.8 | 6.9 | 0.452 |
| Mean total hospital cost (USD) | 13783 | 13568 | 13790 | 15358 | 15929 | 0.009 |

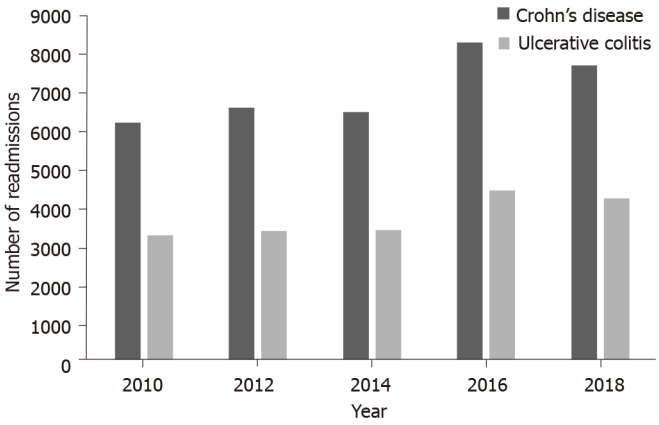


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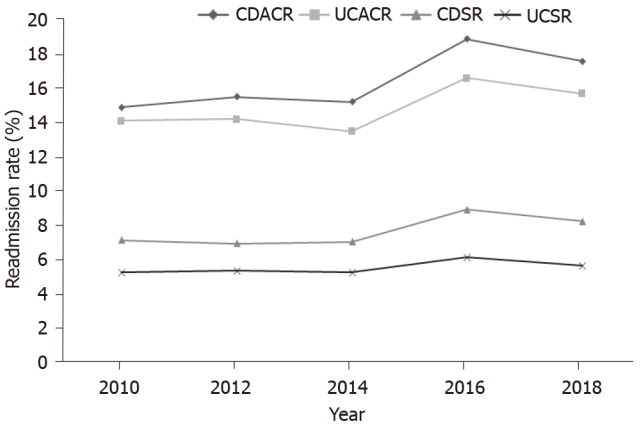
**Figure 1 Total number of 30 d readmissions of Crohn's disease and ulcerative colitis.**

readmissions or due to non-IBD related causes such as infections secondary to the widespread use of biological agents or immunosuppressants[16,18,19]. We performed a trend comparison between 30 d all-cause readmission rate of CD and UC. It was not statistically significant and signified that all-cause readmissions for both CD and UC were increasing proportionately in the United States.

The mean age for 30 d readmissions increased for both CD and UC without a statistically significant trend. The difference in the mean age between the two groups is approximately 7 years. These finding align with current literature which reports that patients with CD tend to be younger and the mean age at the time of diagnosis of CD is usually 5–10 years earlier than that of UC[20]. From a gender standpoint, there is a lower risk of CD until puberty for females when compared to males, after which there is a reversal of this risk[21]. For UC, males and females have a similar incidence until the age of 45 after which males exhibit higher risk of incident UC than females[21]. However, for readmissions of CD and UC, a slight female predominance has been noted in literature[22]. Similarly in our study, a slight female predominance was noted for CD and UC readmissions. Furthermore, we did not find a statistically significant readmission trend for gender over time which implied that the readmission rates for both genders have remained relatively stable. Moreover, we noted an increase in the overall comorbidity burden for 30 d readmissions of CD and UC. This was expected as readmissions for individuals with multiple concurrent co-morbidities have been increasing.




**DOI**: 10.4291/wjgp.v13.i3.85 **Copyright** ©The Author(s) 2022.

**Figure 2 Trends of 30 d readmission following Crohn's disease and ulcerative colitis hospitalizations.** CDACR: Crohn's disease all-cause readmission; UCACR: Ulcerative colitis all-cause readmission; CDSR: Crohn's disease specific readmission; UCSR: Ulcerative colitis specific readmission.

From a hospital perspective, large bed-sized hospitals had the highest proportions of 30 d readmissions of CD and UC. This may be due to the fact that larger hospitals have a higher capacity of in-patient admissions. Additionally, metropolitan teaching hospitals consistently had the highest readmission rates with an increasing trend. This may be because these hospitals are usually tertiary care referral center accepting complex patients from large geographical areas and hence, are well equipped with the necessary resources and specialists to manage these readmissions and their complications. Moreover, an urban location, consisting of a greater population density which may be attributed to a demographic shift of non-urban/rural population to urban locations between 2010 and 2018, is more likely to yield higher readmissions[23].

Furthermore, IBD readmissions have been associated with significant inpatient mortality and healthcare burden. As per literature, frailty and length of intensive care unit stay is independently associated with higher rates of inpatient mortality for IBD readmissions[16,24]. From 2010–2014, a study reported that the inpatient mortality for 30 d readmissions of CD was 2.85% per year, the LOS was 6 d, and cost of hospitalization was $11402[25]. In 2017, for 30 d readmissions of UC, literature reported an inpatient mortality of 1.99% along with longer LOS and higher hospitalization costs compared to index admission[26]. In our study, despite an increasing co-morbidity burden (CCI) for the study period, inpatient mortality, and mean LOS for 30 d readmissions of CD and UC did not have a significant change in the risk adjusted trend (Table 3) over time. These stable mortality and LOS trends may reflect optimal guideline driven therapeutic management for the study period. However, the mean THC for 30 d readmission of UC increased from $13783 in 2010 to $15929 ($P$-trend = 0.009) with an increasing trend, while no trend in THC was identified for CD readmissions. Furthermore, a trend comparison of mean THC between CD and UC yielded a statistically significant trend towards increasing mean THC for 30 d readmissions of UC relative to 30 d readmissions of CD. The exact reason for these THC findings is unclear but may be attributed to an increased complexity and complications of UC readmission requiring immediate higher level of care, additional endoscopic interventions, and a multi-disciplinary team approach for management.

Directing our focus to individual calendar years, we noted a decrease in the total number of readmissions for both CD and UC from 2016 to 2018 (Tables 1 and 2). Similarly, the 30 d all-cause readmissions rate and disease specific readmission rate also decreased from 2016 to 2018 (Table 3). These findings may be due to an overall decrease in the readmissions for one particular calendar year and do not reflect an overall trend. In fact, as discussed earlier, when trended from 2010 to 2018, we noted an increasing trend for all-cause readmissions rate and disease specific readmission rate, and with respect to 2010, there was an overall increase in the total number of 30 d readmissions of CD and UC. Hence, future larger studies are needed to assess rate of readmissions from 2018 to evaluate the trends further.

### *Strength and limitations*

The key strengths of this study include the study population, unique study design, and methodology which allowed for a comprehensive analysis. As the data was collected from one of the largest databases containing information on readmissions from hospitals across the United States, the results are applicable to hospitals throughout the United States. Additionally, we studied a 9-year time frame which helped us establish meaningful trends. However, important limitations exist with this study. The NRD does not contain data on the severity of the disease and therefore, we were unable to further stratify the readmissions based on the severity of CD or UC. The NRD also lacks data on the total duration of the illness and the exact duration after discharge to readmissions, limiting our ability to

assess index admissions more prone to earlier readmissions. Furthermore, it does not contain information on the pharmacological treatment, hospital course and management of IBD readmissions. Hence, we could not comment on the treatment aspects of these readmissions. Moreover, this study is amenable to all biases associated with retrospective studies. Finally, the NRD is an administrative database and therefore, susceptible to coding errors. Despite these limitations, this study helps us better understand the hospitalizations characteristics and trends of 30 d readmissions for CD and UC which is critical for management of these patients.

## CONCLUSION

In conclusion, the total number of 30 d readmission for CD and UC increased. UC readmissions were older than CD readmissions. We noted an increasing trend for 30 d all-cause readmission rate for CD and UC. However, there was no statistical change in the risk adjusted trends of inpatient mortality and mean LOS for these readmissions. The mean total healthcare cost for 30 d readmissions of UC had a rising trend while no trend was observed for CD readmissions. Future prospective studies are needed to further study these findings.

## ARTICLE HIGHLIGHTS

### *Research background*

The prevalence of inflammatory bowel disease (IBD) continues to be on the rise around the globe. Despite outpatient management, these patients are at increased risk of relapse leading to hospitalizations and subsequent readmissions.

### *Research motivation*

Through this study, we attempted to outline the magnitude, characteristics and outcomes of early (30 d) readmissions of IBD in the United States.

### *Research objectives*

This national, retrospective, interrupted trends study aimed to identify hospitalization characteristics, readmission rates, adverse outcomes, and healthcare burden for 30 d readmissions of Crohn's disease (CD) and ulcerative colitis (UC) in the United States between 2010-2018.

### *Research methods*

This was a retrospective, interrupted trends which analyzed data from the National Readmission Database (NRD) on all adult 30 d readmissions of CD and UC in the United States between 2010-2018. Patients < 18 years of age, elective and traumatic hospitalizations were excluded from the analysis. Hospitalization characteristics, readmission rates, adverse outcomes and the healthcare burden was identified. *P*-values ≤ 0.05 were considered statistically significant.

### *Research results*

Total number of 30 d readmissions increased from 6202 in 2010 to 7672 in 2018 for CD and from 3272 in 2010 to 4234 in 2018 for UC. There was an increase in the 30 d all-cause readmission rate of CD and UC for the study period. We did not observe a change in the risk adjusted trends of inpatient mortality and mean length of hospital stay (LOS) for CD and UC readmissions. However, there was a rising trend of mean THC for UC readmissions. After comparison, there was no statistical difference in the trends for 30 d all-cause readmission rate, inpatient mortality, and mean LOS between CD and UC readmissions.

### *Research conclusions*

From 2010 to 2018, there was an increase in the total number of 30 d readmissions with a trend towards increasing 30 d all-cause readmission rates for CD and UC. However, there was no change in the risk adjusted trends of inpatient mortality.

### *Research perspectives*

This study helps clinicians better understand the magnitude and characteristics of 30 d readmissions of CD and UC in the United States. Through this study, we also aim to encourage and promote future research on readmissions of IBD.

## FOOTNOTES

**Author contributions:** Dahiya DS, Kichloo A and Sumant Inamdar S contributed to the conception and design; Dahiya DS, Kichloo A, Al-Haddad M contributed to the administrative support; Kichloo A and Shaka H contributed to the provision, collection, and assembly of data; Dahiya DS, Perisetti A, Singh A, Al-Haddad M, Sanaka MR and Sumant Inamdar S revised the key components of manuscript; and All authors reviewed the literature, drafted the manuscript, finally approved the manuscript, and agreement to be accountable for all aspects of the work.

**Institutional review board statement:** As the National Readmission Database does not contain patient-specific and hospital-specific identifiers, this study was exempt from the Institutional Review Boards (IRB) as per guidelines put forth by the IRB for research on database studies.

**Informed consent statement:** Patients were not required to give informed consent to the study because the analysis used anonymous clinical data that was obtained after analysis of a national database.

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**Country/Territory of origin:** United States

**ORCID number:** Dushyant Singh Dahiya 0000-0002-8544-9039; Abhilash Perisetti 0000-0003-4074-6395; Asim Kichloo 0000-0003-4788-8572; Amandeep Singh 0000-0001-8581-1408; Hemant Goyal 0000-0002-9433-9042; Laura Rotundo 0000-0002-4094-3682; Hafeez Shaka 0000-0002-9456-4581; Gurdeep Singh 0000-0001-6044-7419; Jagmeet Singh 0000-0001-7179-1020; Sailaja Pisipati 0000-0000-0000-0001; Mohammad Al-Haddad 0000-0003-1641-9976; Madhusudhan R Sanaka 0000-0003-2506-8602; Sumant Inamdar 0000-0002-1002-2823.

**Corresponding Author's Membership in Professional Societies:** American College of Gastroenterology, No. 59498; American Gastroenterological Association, No. 1550524.

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ORIGINAL ARTICLE

Observational Study

# Utility of FibroScan-based scoring systems to narrow the risk group of nonalcoholic fatty liver disease with comorbidities

Kouichi Miura, Hiroshi Maeda, Naoki Morimoto, Shunji Watanabe, Mamiko Tsukui, Yoshinari Takaoka, Hiroaki Nomoto, Rie Goka, Kazuhiko Kotani, Hironori Yamamoto

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**Kouichi Miura, Hiroshi Maeda, Naoki Morimoto, Shunji Watanabe, Mamiko Tsukui, Yoshinari Takaoka, Hiroaki Nomoto, Rie Goka, Hironori Yamamoto,** Department of Medicine, Division of Gastroenterology, Jichi Medical University School of Medicine, 3311-1 Yakushiji, Shimotsuke 329-0498, Tochigi, Japan

**Kazuhiko Kotani,** Division of Community and Family Medicine, Center for Community Medicine, Jichi Medical University, 3311-1 Yakushiji, Shimotsuke 329-0498, Japan

**Corresponding author:** Kouichi Miura, Doctor, Associate Professor, Department of Medicine, Division of Gastroenterology, Jichi Medical University School of Medicine, 3311-1 Yakushiji, Shimotsuke 329-0498, Tochigi, Japan. miura385@jichi.ac.jp

## Abstract

### *BACKGROUND*

Vibration-controlled transient elastography (VCTE) is proposed as a second step of examination to assess liver fibrosis in patients with nonalcoholic fatty liver disease (NAFLD) after triaging by the fibrosis-4 (FIB-4) index. Recently, VCTE-based scoring systems, including FibroScan-AST (FAST), Agile 3+, and Agile 4, emerged to determine the status of NAFLD. However, the significance of these scoring systems remains unknown in narrowing the high-risk group of NAFLD patients with comorbidities, including hepatocellular carcinoma (HCC) and esophagogastric varices (EGV).

### *AIM*

To clarify the significance of VCTE-based scoring systems to narrow the high-risk group of NAFLD patients with comorbidities.

### *METHODS*

We performed a cross-sectional study to investigate the usefulness of VCTE-based scoring systems and other fibrosis markers to narrow the high-risk group of patients with NAFLD. FIB-4 index was used for the first triage. Risk groups of FAST, Agile 3+, and Agile 4 were stratified according to the published data. Among the 191 patients with NAFLD, there were 26 (14%) and 25 patients (13%) with HCC and EGV, respectively.

### *RESULTS*

When 1.3 was used as a cutoff value, the FIB-4 index narrowed the risk group to

120 patients, in which all patients with HCC and/or EGV were included. High risk group of Agile 3+ could subsequently narrow the risk group. The prevalence of HCC and EGV at this step were 33% (26/80) and 31% (25/80), respectively. In further narrowing of EGV, Agile 4 aggregated the patients with EGV into 43 patients, of whom 23 (53%) had EGV. FAST failed to narrow the risk group of patients with comorbidities. When 2.6 was used as a cutoff value of the FIB-4 index, three patients with HCC and two patients with EGV were missed at the first triage.

*CONCLUSION*

Agile 3+ and Agile 4 are useful to narrow the NAFLD patient group, in which patients may have HCC and/or EGV.

**Key Words:** Nonalcoholic fatty liver disease; Vibration controlled transient elastography; Non-invasive test; Hepatocellular carcinoma; Varix



**Core Tip:** It is necessary to narrow the high-risk group of nonalcoholic fatty liver disease (NAFLD) patients with comorbidities, including hepatocellular carcinoma (HCC) and esophagogastric varices (EGV). Although the fibrosis-4 index is an excellent formula to narrow the high-risk group, there remain many patients to be ruled out. Vibration controlled transient elastography (VCTE) is proposed as a second step examination. FibroScan-AST, Agile 3+, and Agile 4 emerged as VCTE-based scoring systems to determine the status of patients with NAFLD. Here, we demonstrated that Agile 3+ and Agile 4 are good tools to narrow the high-risk group of patients with HCC and/or EGV.

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## INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is the most common chronic liver disease worldwide. A subset of patients with NAFLD can progress to liver cirrhosis, in which patients may have comorbidities, including hepatocellular carcinoma (HCC) and esophagogastric varices (EGV). Current studies have demonstrated that liver fibrosis is a prognostic factor of patients with NAFLD because comorbidities of NAFLD are noted in patients with liver fibrosis[1,2]. Thus, the assessment of liver fibrosis is essential to identifying patients with comorbidities.

Although liver biopsy remains the gold standard to assess liver fibrosis, it is costly and has a risk of complications, including bleeding. In addition, it is difficult to perform liver biopsy in all patients with NAFLD because the global prevalence of patients with NAFLD is approximately 25%[3]. Thus, the demand for noninvasive tests (NITs) to assess liver fibrosis is expanding. Currently, there are several markers and formulae to assess liver fibrosis using clinical parameters without liver biopsy[4]. In addition, imaging studies, including elastography and magnetic resonance imaging (MRI), are used as NITs for the assessment of liver fibrosis. Each method has both advantages and disadvantages. Among NITs, the fibrosis-4 (FIB-4) index is a widely used formula because this formula uses only 4 components, including age, aspartate aminotransferase (AST), alanine aminotransferase (ALT), and platelet count[5], which are easily available not only for hepatologists but also for general physicians. The merits of using the FIB-4 index are high accuracy and low cost[6]. In addition, many validation studies have been performed in chronic liver diseases, including NAFLD. Furthermore, the FIB-4 index is useful for identifying NAFLD patients with extrahepatic comorbidities, including cardiovascular diseases[7]. However, elderly patients tend to show a high score. In addition, there are many patients who show an intermediate risk for liver fibrosis. As a result, the FIB-4 index is used in the first step to narrow the high-risk group of patients who may have comorbidities of NAFLD.

FibroScan, a vibration-controlled transient elastography (VCTE), is proposed as the second step of NIT that can identify such patients[8]. Liver stiffness measurement (LSM) ≥ 11.9 KPa by FibroScan is highly suspected of liver fibrosis over F4[9]. Although FibroScan shows high sensitivity and specificity in the diagnosis of liver fibrosis, some patients have unexpectedly high LSM, probably due to the presence of obesity and the examiners' skill. Thus, a combination of LSM and laboratory data may reflect a more accurate status of patients with NAFLD. To this end, FibroScan-based scoring systems,

including FibroScan-AST (FAST)[10], Agile 3+[11] and Agile 4[12], have been developed. These scoring systems use data obtained from FibroScan and some clinical parameters, including age, sex, AST, ALT, platelet count, and diabetes status. Among these scoring systems, FAST was designed to identify NAFLD patients with liver fibrosis F ≥ 2. Agile 3+ and Agile 4 were designed to identify NAFLD patients with liver fibrosis at F3-F4 and F4, respectively. Although these FibroScan-based scoring systems are correlated with liver fibrosis, little data are available on the significance of identifying NAFLD patients with comorbidities. Thus, the aim of the present cross-sectional study was to investigate the utility of these FibroScan-based scoring systems to narrow the high-risk group of NAFLD patients with comorbidities after triaging by the FIB-4 index.

## MATERIALS AND METHODS

### Patients

We investigated 191 patients with NAFLD who visited our hospital between April 2019 and March 2022. The diagnosis of NAFLD was made as follows: Steatosis was determined by an ultrasonographic examination conducted by well-experienced gastroenterologists. Steatosis pointing out past examinations was included. Men who used alcohol > 30 g/d and women who used > 20 g/d were excluded. Patients with HBV infection (positive for HBs antigen), HCV infection (positive for HCV antibody) and other liver diseases, including autoimmune hepatitis and primary biliary cholangitis, were also excluded. In addition, we used data obtained from FibroScan as well as blood tests, including the FIB-4 index and *Wisteria floribunda* agglutinin-positive Mac2-binding protein glycosylation isomer (M2BPGi). Diagnosis of diabetes was defined as a fasting blood glucose ≥ 126 mg/dL, hemoglobin A1c ≥ 6.5% and/or antidiabetic drug use. All patients in the present study had FibroScan examination as well as blood tests. This study was approved by the Institutional Review Board of Jichi Medical University (20-175). The study was performed according to the ethical guidelines of the Declaration of Helsinki.

### FibroScan-based scoring systems

Transient elastography was performed with FibroScan (Echosens, Paris, France), using an M probe. The FIB-4 index, FAST score, Agile 3+, and Agile 4 were calculated according to published formulae using age, controlled attenuation parameter (CAP), LSM, AST, ALT, platelet count, and presence of diabetes (Supplementary Figure 1). The impact of these parameters on the scoring systems were shown in Supplementary Table 1. Blood data obtained on the same day of FibroScan examination or within 1 mo from the examination were used (Supplementary Figure 2). CAP and LSM were the mean data of 10 consecutive examinations.

Risk assessments for each formula and factor are shown in Supplementary Table 2. In addition, Baveno VI criteria[13], expanded Baveno VI criteria[14], and New NFLD-cirrhosis criteria[15] were also assessed in narrowing the risk group of patients with EGV.

### Diagnosis of HCC and EGV

The diagnosis of HCC was made by hepatologists and radiologists using contrast-enhanced computed tomography and/or contrast-enhanced MRI and/or contrast-enhanced ultrasonography. Histologically proven HCC were also added. Form 1 ≤ were defined as having EGV in patients who underwent esophagogastroduodenal endoscopy (EGD)[16,17]. Patients with histories of HCC and/or endoscopic variceal treatment were included as shown in Supplementary Figure 2. If patients did not have EGD examination within 1 year, we interviewed a history of gastrointestinal bleeding from gastrointestinal varices. If patients reported no history of variceal bleeding, the patient was defined as having no EGV.

### Statistical analysis

Statistical analyses were performed using Stata 17 (STATA Corporation, College Station, United States). Sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) were calculated. For patient background evaluation, analyses were performed by the chi-square test or Fisher's test as appropriate. In addition, the Mann-Whitney U test was used in a comparison of two groups. In a comparison of three groups, one-way analysis of variance was used. All $P$ values < 0.05 were considered statistically significant.

## RESULTS

### The characteristics of NAFLD patients with HCC and/or EGV

Table 1 shows the characteristics of patients. The median age was 62 years old, 81 (42.4%) were male, and 75 (39.3%) had diabetes. There were 26 patients with HCC and 25 patients with EGV. Among these patients with HCC and/or EGV, 17 had HCC alone, 16 had EGV alone, and 9 had both HCC and EGV.

**Table 1 Characteristics of patients with nonalcoholic fatty liver disease**

| | |
|---|---|
| Patients (*n*) | 191 |
| Age (years old) | 62 (20-90) |
| Men (%) | 81 (42.4) |
| diabetes (%) | 75 (39.3) |
| HCC | 17 |
| EGV | 16 |
| Both HCC and EGV | 9 |
| AST (U/L) | 36 (13-208) |
| ALT (U/L) | 40 (10-214) |
| Platelet count ($\times 10^9$/L) | 207 (45-445) |

AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; EGV: Esophagogastric varices; HCC: Hepatocellular carcinoma.

Then, we investigated the scores and values of each formula and marker in patients with HCC and/or EGV (Figure 1). In FIB-4 and FAST, the maximum and the minimum of scores were similar among patients with HCC and/or EGV. In Agile 3+, patients with HCC and/or EGV aggregated into a zone of high score. In Agile 4, LSM, and M2BPGi, the score and values tended to show a stepwise increase from HCC, EGV, and both HCC and EVG.

### *The high to intermediate-risk group of FIB-4 index includes all patients with HCC and/or EGV*

In a stratification of the FIB-4 index, there were 71, 51, and 69 patients in the low-, intermediate-, and high-risk groups, respectively. No patients with HCC and/or EGV were noted in the low-risk group of the FIB-4 index, while three patients with HCC and two patients with EGV were in the intermediate stage. The remining patients with HCC and/or EGV were in the high-risk group (Tables 2 and 3). Thus, the high to intermediate-risk group of FIB-4 index is suitable for the first triage.

### *The high-risk group of Agile 3+ includes all patients with HCC and/or HGV*

Then, we investigated the prevalence of patients with HCC and/or EGV (Tables 2 and 3). When the patients were divided into two groups, including low-risk and high to intermediate-risk, there were no patients with HCC and/or EGV in the low-risk group of Agile 3+ (Table 2). In addition, Agile 3+ was the only examination that included all patients with HCC and/or EGV in the high-risk group (Table 3). As a result, Agile 3+ showed extremely high sensitivity and NPV. In contrast, there were patients with HCC in the low-risk group of FAST, Agile 4, LSM, and M2BPGi and patients with EGV in the low-risk group of FAST, Agile 4, and M2BPGi (Table 2), suggesting that FAST, Agile 4, LSM, and M2BPGi are unsuitable for screening of patients with HCC and/or EGV. Thus, Agile 3+ is a good tool to narrow the high-risk group of patients with HCC and/or EGV.

### *Agile 4 is a potential tool to narrow the patients with EGV*

Although the Agile 3+ could narrow the patients with EGV, we further attempted to narrow the patients with EGV. Patients with EGV tended to have a more advanced stage of fibrosis based on Agile 4, LSM, and M2BPGi (Figure 1). Although there were no patients with EGV in the low-risk group of LSM, the PPV was 21% (Table 2). In contrast, the high-risk groups of Agile 4 and M2BPGi missed one patient with EGV, their PPVs were higher than that of LSM. In addition, the PPV of the high-risk group of Agile 4 was 56%, the highest among tests (Table 3). Despite the high-risk group of Agile 4 missed two patients with EGV, Agile 4 is a potential tool to narrow the risk group of patients with EGV.

### *Baveno VI and its derivatives did not work in our patient group*

Baveno VI criteria, expanded Baveno VI criteria, and new NAFLD-cirrhosis criteria, using LSM and platelet count, are simple tools to rule out patients with varices needing treatment. There were 13 (52%), 17 (68%), and 19 patients (76%) with EGV who were defined as "rule out "of the Baveno VI criteria, expanded Baveno VI criteria, and new NAFLD-cirrhosis criteria, respectively (Table 4). Thus, it was difficult to narrow the patients with EGV using a combination of LSM and platelet count.

### *Agile 3+ and Agile 4 are good tools to narrow the patients with HCC and/or EGV*

We applied our patient group to determine whether VCTE-based scoring systems and other fibrosis markers can narrow the risk group of patients with HCC and/or EGV after triaging by the FIB-4 index (Figure 2A). There were 26 patients with HCC (14%) and 25 patients with EGV (13%) among 191

**Table 2 Sensitivity, specificity, positive predictive value, and negative predictive value of each score and marker (L *vs* I-H)**

| | FIB-4 | | FAST | | Agile 3+ | | Agile 4 | | LSM | | M2BPGi | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Risk | L | I-H | L | I-H | L | I-H | L | I-H | L | I-H | L | I-H |
| *n* | 71 | 120 | 87 | 104 | 96 | 95 | 131 | 60 | 73 | 118 | 102 | 89 |
| HCC | 0 | 26 | 10 | 16 | 0 | 26 | 7 | 19 | 4 | 22 | 5 | 21 |
| *P* value | < 0.01 | | 0.44 | | < 0.01 | | < 0.01 | | < 0.01 | | < 0.01 | |
| Sensitivity | | 1 | | 0.62 | | 1 | | 0.73 | | 0.85 | | 0.81 |
| Specificity | | 0.43 | | 0.53 | | 0.58 | | 0.79 | | 0.44 | | 0.62 |
| PPV | | 0.22 | | 0.17 | | 0.27 | | 0.36 | | 0.19 | | 0.25 |
| NPV | | 1 | | 0.90 | | 1 | | 0.95 | | 0.95 | | 0.95 |
| EGV | 0 | 25 | 6 | 19 | 0 | 25 | 1 | 24 | 0 | 25 | 1 | 24 |
| *P* value | < 0.01 | | 0.02 | | < 0.01 | | < 0.01 | | < 0.01 | | < 0.01 | |
| Sensitivity | | 1 | | 0.76 | | 1 | | 0.96 | | 1 | | 0.96 |
| Specificity | | 0.43 | | 0.52 | | 0.58 | | 0.79 | | 0.44 | | 0.61 |
| PPV | | 0.21 | | 0.19 | | 0.26 | | 0.41 | | 0.21 | | 0.27 |
| NPV | | 1 | | 0.94 | | 1 | | 0.99 | | 1 | | 0.99 |

L: Low-risk; I: Intermediate-risk; H: High-risk; HCC: Hepatocellular carcinoma; EGV: Esophagogastric varix; HCC: Hepatocellular carcinoma; EGV: Esophagogastric varices; PPV: Positive predictive value, NPV: Negative predictive value; M2BPGi: Mac2-binding protein glycosylation isomer; FAST: FibroScan-AST; FIB-4: Fibrosis-4; LSM: Liver stiffness measurement.

**Table 3 Sensitivity, specificity, positive predictive value, and negative predictive value of each score and marker (L-I *vs* H)**

| | FIB-4 | | FAST | | Agile 3+ | | Agile 4 | | LSM | | M2BPGi | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Risk | L-I | H | L-I | H | L-I | H | L-I | H | L-I | H | L-I | H |
| *n* | 122 | 69 | 146 | 45 | 111 | 80 | 148 | 43 | 136 | 55 | 148 | 43 |
| HCC | 3 | 23 | 18 | 8 | 0 | 26 | 12 | 14 | 10 | 16 | 11 | 15 |
| *P* value | < 0.01 | | 0.35 | | < 0.01 | | < 0.01 | | < 0.01 | | < 0.01 | |
| Sensitivity | | 0.89 | | 0.31 | | 1 | | 0.54 | | 0.62 | | 0.58 |
| Specificity | | 0.74 | | 0.89 | | 0.67 | | 0.90 | | 0.82 | | 0.90 |
| PPV | | 0.35 | | 0.30 | | 0.33 | | 0.45 | | 0.36 | | 0.47 |
| NPV | | 0.98 | | 0.89 | | 1 | | 0.93 | | 0.93 | | 0.93 |
| EGV | 2 | 23 | 14 | 11 | 0 | 25 | 2 | 23 | 3 | 22 | 4 | 21 |
| *P* value | < 0.01 | | 0.01 | | < 0.01 | | < 0.01 | | < 0.01 | | < 0.01 | |
| Sensitivity | | 0.92 | | 0.44 | | 1 | | 0.92 | | 0.88 | | 0.84 |
| Specificity | | 0.74 | | 0.88 | | 0.67 | | 0.89 | | 0.82 | | 0.89 |
| PPV | | 0.34 | | 0.36 | | 0.31 | | 0.56 | | 0.42 | | 0.54 |
| NPV | | 0.98 | | 0.91 | | 1 | | 0.99 | | 0.98 | | 0.97 |

EGV: Esophagogastric varices; HCC: Hepatocellular carcinoma; PPV: Positive predictive value; NPV: Negative predictive value; M2BPGi: Mac2-binding protein glycosylation isomer; FAST: FibroScan-AST; FIB-4: Fibrosis-4; LSM: Liver stiffness measurement.

patients. At the first triage using the FIB-4 index at 1.3 (high to intermediate-risk group), we could narrow the risk group to 120 patients, in whom all patients with HCC and/or EGV were included. In the first step, the prevalence of HCC and EGV was 22% (26/120) and 21% (25/120), respectively. Then, we narrowed the patients using Agile 3+ at the second step, in which all patients with HCC and/or

**Table 4 The prevalence of esophagogastric varices in Baveno VI criteria and its derivatives**

| | Baveno VI | | Exp. Baveno VI | | New NASH C.C | |
|---|---|---|---|---|---|---|
| | LSM | platelet | LSM | platelet | LSM | platelet |
| | < 20 | 150 < | < 25 | 110 < | < 30 | 110 < |
| EGV/rule in (*n*) | 12/26 | | 8/13 | | 6/9 | |
| EGV/rule out (*n*) | 13/165 | | 17/178 | | 19/182 | |

Exp. Baveno VI: Expanded Baveno VI; New NASH C.C: New NASH cirrhosis criteria; LSM: Liver stiffness measurement (KPa); Platelet count ($\times 10^9$/L); EVG: Esophagogastric varix.

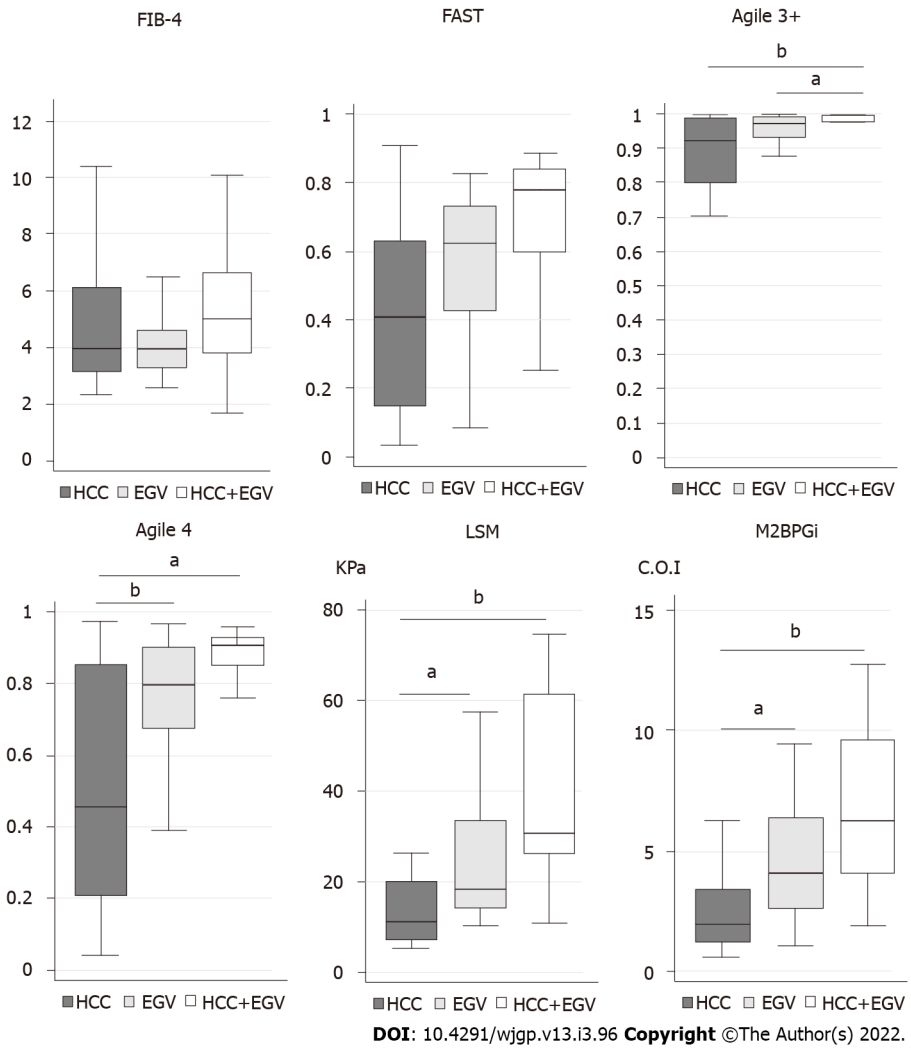


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**Figure 1 Scores (Fibrosis-4, FibroScan-AST, Agile 3+, Agile 4) and values (Liver stiffness measurement, Mac2-binding protein glycosylation isomer) of patients with hepatocellular carcinoma (*n* = 17), esophagogastric varices (*n* = 16), and both hepatocellular carcinoma and esophagogastric varices (*n* = 9).** $^aP < 0.05$, $^bP < 0.01$. HCC: Hepatocellular carcinoma; EGV: Esophagogastric varices; LSM: Liver stiffness measurement; FAST: FibroScan-AST; M2BPGi: Mac2-binding protein glycosylation isomer.

EGV were included. When the high to intermediate-risk group of Agile 3+ was used, the prevalence of HCC was 27% (26/95) and 26% (25/95), respectively. When the high-risk group of Agile 3+ was used, the prevalence of HCC was 33% (26/80) and 31% (25/80), respectively. Because the low-risk group of Agile 4, LSM, and M2BPGi included patents with HCC, further narrowing was difficult without missing patients with HCC.

Then, we attempted to narrow the patients with EGV. The high to intermediate and high-risk of Agile 3+ groups subsequently narrowed the patients with EGV. Although the high to intermediate-risk group of LSM successfully narrowed the risk group without missing patients with EGV, the prevalence was a

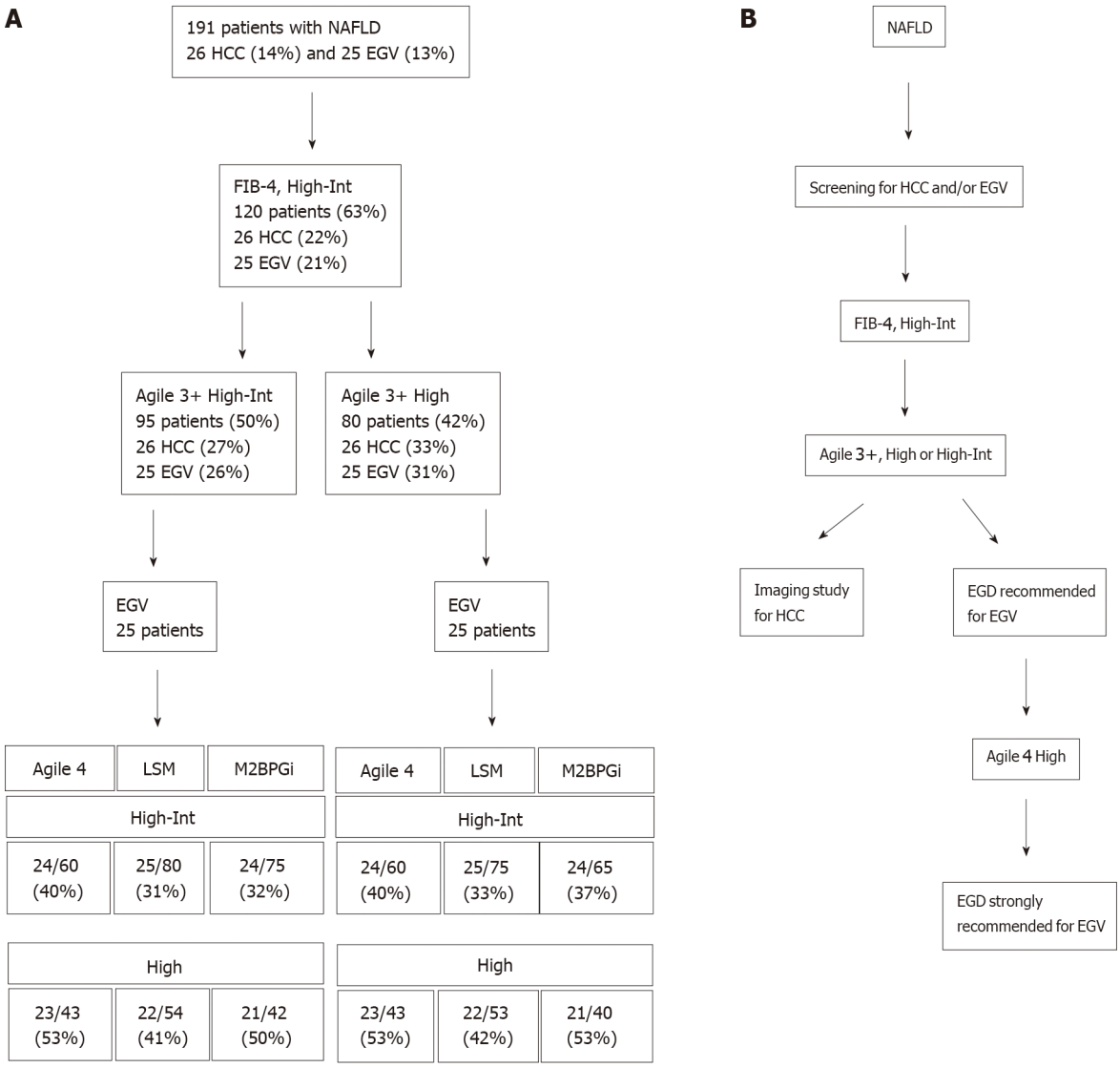


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**Figure 2 Flow chart.** A: A flowchart in sorting nonalcoholic fatty liver disease (NAFLD) patients using the fibrosis-4 index, Agiles, and other fibrosis markers; B: A proposal algorithm to narrow the high-risk group of NAFLD patients with hepatocellular carcinoma and/or esophagogastric varices. HCC: Hepatocellular carcinoma; EGV: Esophagogastric varices; LSM: Liver stiffness measurement; M2BPGi: Mac2-binding protein glycosylation isomer; NAFLD: Nonalcoholic fatty liver disease.

small increase, up to 33% (25/75). In contrast, high-risk group of Agile 4 could concentrated the patients with EGV. Although the high-risk group of Agile 4 missed two patients (8%), the prevalence of patients with EGV increased to 53% (23/43). Thus, Agile 4 is a good tool to further narrow the risk group of patients with EGV.

Based on our results, sorting patients using the FIB-4 index, Agile 3+, and Agile 4 is a potential screening method to narrow the high-risk group of NAFLD patients with comorbidities (Figure 2B).

## DISCUSSION

The requirement for NITs to narrow the risk group of patients with comorbidities is expanding because a quarter of people in the world have NAFLD, a risk factor for HCC and/or EGV. The FIB-4 index, which is simple and inexpensive, was used in the first triage to narrow the high-risk group of NAFLD patients with comorbidities. However, there remain many patients even after triage. In the present study, we demonstrated that Agile 3+ and Agile 4, VCTE-based scoring systems, were good tools for further narrowing the high-risk group of patients with HCC and/or EGV at the second and third steps, respectively.

Agile 3+, developed by Yonoussi's group, was suitable to narrow the risk group of patients with HCC and/or EGV in the present study. Agile 3+ has been designed to optimize PPV and reduce cases of intermediate stage (Gray zone) among patients with advanced liver fibrosis[11]. Our data demonstrated

that Agile 3+ had high sensitivity and high NPV for HCC and EGV. Although the number of patients in the high-risk group of Agile 3+ was larger than that of other scoring systems and fibrosis markers, Agile 3+ did not miss the patients with HCC and/or EGV, which is contrast to other tools, including FAST, Agile 4, LSM, and M2BPGi. Indeed, all patients with HCC and/or EGV were included in the high-risk group of Agile 3+, suggesting that Agile 3+ is useful for screening patients with HCC and/or EGV. Because the background liver of NAFLD patients with HCC is often characterized by less fibrosis[18], fibrosis markers sometimes fail to identify patients with HCC. Some patients with HCC were included in the low-risk group of Agile 4, LSM, and M2BPGi. The Agile 3+ scoring system includes age, AST, ALT, platelet count, LSM, sex, and diabetes. Because old age and diabetic individuals are prone to HCC [19], it is reasonable to include these variables in the scoring system to find HCC.

Agile 4, also developed by Yonoussi's group, was suitable to narrow the high-risk group of patients with EGV. Agile 4 was designed to identify patients with NASH cirrhosis. Agile 4 showed high specificity and high PPV for EGV. There were 23 (92%) and 24 patients (96%) with EGV in the high- and high to intermediate-risk groups, respectively. We also applied our patient group to the Baveno VI criteria, expanded Baveno VI criteria, and New NAFLD-cirrhosis criteria, which are combinations of LSM and platelet count. However, more than half of the patients were included in the rule-out group. In the Asian cohort, the Baveno VI criteria performed better than the expanded Baveno VI criteria[20], suggesting that Asian people may have EGV at lower LSM and higher platelet counts than people in the USA and Europe. Although it remains unknown why the Baveno VI criteria and its derivatives did not work in the present study, further studies are required. As a result, Agile 4 can be used at the third step to identify patients with EGV.

FAST failed to narrow the high-risk group of patients with HCC and/or EGV. FAST showed low sensitivity to identify such patients. In addition, there were 10 (38%) with HCC and 6 patients (23%) with EGV in the low-risk (rule out) group, respectively. FAST, designed for identifying patients with NAFLD activity score ≥ 4 and fibrosis stage (F ≥ 2), is calculated using LSM, CAP, and AST. However, the FAST score did not include risk factors for HCC, including age, sex, and diabetes. The association between the grade of CAP, fat content in the liver, and HCC remains unknown. Izumi *et al*[21] reported that CAP was significantly lower in the HCC group than in the non-HCC group in patients with NAFLD. Indeed, our data revealed that CAP tended to be low in patients with HCC (data not shown). Thus, FAST is unlikely suitable for the screening of patients with HCC and/or EGV. However, patients with high FAST scores should be followed up because these patients have a risk of progressive NASH in the future.

There are a couple of limitations in the present study. Our study is a single-center study, and the number of patients examined was small. Thus, the bias of NAFLD population is noted. In a previous study, the proportions of patients in the low- and high-risk FIB-4 index groups were 58.3% and 10.2%, respectively, among patients with biopsy-proven NAFLD[22]. The proportions in the present study showed small size of the low-risk group (37.2%) but large size of the high-risk group (36.1%). In addition, a total of 42 patients (22.0%) had HCC and/or EGV among patients with NAFLD. Because our hospital is a referral center, patients with comorbidities were aggregated into our hospital. In addition, the present study counted patients with histories of HCC and/or EGV, suggesting that scores of FIB-4 and Agile 3+ may be higher than those when comorbidities first developed. Thus, prospective study will clarify the significance of Agiles for finding patients with HCC and/or EGV. At least, the stream from FIB-4 index to Agiles worked in narrowing the high-risk patients with HCC and/or EGV in the present study.

## CONCLUSION

In conclusion, Agile 3+ and Agile 4 can narrow the high-risk group of patients who may have HCC and/or EGV after triaging by the FIB-4 index. Because Agile 3+ and Agile 4 share common parameters, including LSM and clinical data, they have a potential use in screening for such patients.

## ARTICLE HIGHLIGHTS

### *Research background*

It is necessary to narrow the high-risk group of nonalcoholic fatty liver disease (NAFLD) patients with comorbidities, including hepatocellular carcinoma (HCC) and esophagogastric varices (EGV).

### *Research motivation*

Although the fibrosis-4 index is an excellent formula to narrow the high-risk group, there remain many patients to be ruled out.

### *Research objectives*
This study aimed to assess the utility of VCTE-based scoring systems to narrow the risk group of nonalcoholic fatty liver disease with comorbidities.

### *Research methods*
We performed a cross-sectional study to investigate the usefulness of VCTE-based scoring systems and other fibrosis markers to narrow the high-risk group of patients with NAFLD.

### *Research results*
The high-risk group of Agile 3+ could narrow the patients with HCC and/or EGV without missing one patient. The high-risk group of Agile 4 showed a high PPV for patients with EGV.

### *Research conclusions*
The brand new VCTE-based scoring systems, Agile 3+ and Agile 4, are useful to narrow the NAFLD patient group, in which patients may have HCC and/or EGV.

### *Research perspectives*
Agile 3+ and Agile 4 will be used for screening of NAFLD patients with HCC and/or EGV.


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## FOOTNOTES

**Author contributions:** Miura K designed the study and performed acquisition, analysis, interpretation of data, and drafted the initial manuscript; Maeda H participated in acquisition and analysis of data; Morimoto N participated in acquisition of data; Watanabe S participated in acquisition of data; Tsukui M participated in acquisition of data; Takaoka Y participated in acquisition of data; Nomoto H participated in acquisition of data; Goka R participated in acquisition of data; Kotani K, a specialist of biostatistics, reviewed the statistical analysis and revised the draft carefully; Yamamoto H revised the draft carefully.

**Institutional review board statement:** The present study was reviewed and approved by the Institutional Review Board of Jichi Medical University (20-175).

**Informed consent statement:** A written informed consent was waived because of the retrospective nature of this study. Instead, opt-out consent documents were shown on the website of Jichi Medical University for patients who did not wish to participate in the study.

**Conflict-of-interest statement:** There are no conflict of interest to report.

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**Country/Territory of origin:** Japan

**ORCID number:** Kouichi Miura 0000-0001-8036-6544; Hiroshi Maeda 0000-0003-4558-3935; Naoki Morimoto 0000-0001-9600-7407; Shunji Watanabe 0000-0002-5449-6553; Mamiko Tsukui 0000-0002-2407-0842; Yoshinari Takaoka 0000-0003-3753-6063; Hiroaki Nomoto 0000-0002-3776-2418; Rie Goka 0000-0003-4359-9220; Kazuhiko Kotani 0000-0001-8119-633X; Hironori Yamamoto 0000-0002-3601-1153.

**Corresponding Author's Membership in Professional Societies:** The Japanese Society of Gastroenterology, No. 030879.

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CASE REPORT

# Gastric cancer with concurrent pancreatic schwannoma: A case report

Mateus Barradas Ribeiro, Emerson Shigueaki Abe, André Kondo, Adriana Vaz Safatle-Ribeiro, Marina Alessandra Pereira, Bruno Zilberstein, Ulysses Ribeiro Jr

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**Mateus Barradas Ribeiro, Emerson Shigueaki Abe, André Kondo, Adriana Vaz Safatle-Ribeiro, Marina Alessandra Pereira, Bruno Zilberstein, Ulysses Ribeiro Jr,** Department of Gastroenterology, Instituto do Cancer, Hospital das Clínicas da Faculdade de Medicina da Universidade de São Paulo, Sao Paulo 01249000, Brazil

**Corresponding author:** Ulysses Ribeiro Jr, PhD, Chief Doctor, Surgeon, Department of Gastroenterology, Instituto do Cancer, Hospital das Clínicas da Faculdade de Medicina da Universidade de São Paulo, Av Dr Arnaldo 251, Sao Paulo 01249000, Brazil.
ulysses.ribeiro@hc.fm.usp.br

## Abstract

*BACKGROUND*

The differential diagnosis of abdominal masses is somewhat troublesome, especially when there is a malignancy to be evaluated. We report herein a unique case of gastric adenocarcinoma concurrent with a pancreatic schwannoma. Correct assessment of intraoperative findings is essential for adequate tumor staging and to decide the proper management of a concurrent pancreatic lesion.

*CASE SUMMARY*

Computed tomography scan performed for gastric cancer staging revealed a solid and cystic pancreatic mass that had no signs of local invasiveness. Surgical resection of the pancreas was decided preoperatively since a radical approach of the gastric tumor could be performed. There were no signs of distant metastases, and the large pancreatic mass was in contact with the posterior gastric wall. Histopathological study revealed a pancreatic schwannoma, which is an uncommon neoplasm that arises from Schwann cells around peripheral nerves.

*CONCLUSION*

Therefore, pancreatic masses deserve special attention regarding the differential diagnosis in patients with gastric cancer. The presence of a large pancreatic mass should not preclude the potentially curative intent of the gastric cancer treatment.

**Key Words:** Stomach neoplasms; Gastric adenocarcinoma; Schwannoma; Pancreas; Case report

**Core Tip:** We display here the first case of synchronous gastric cancer and pancreatic schwannoma, highlighting the relevance of the differential diagnosis in approaching pancreatic masses in the context of staging gastric neoplasm. Correct intraoperative staging was essential in treatment decision-making.

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## INTRODUCTION

Accurate staging is essential in gastric cancer treatment decision-making, and any lymph nodes or masses observed in staging assessment should be investigated[1]. Schwannomas, also referred to as neurilemmomas, are rare neoplasms that arise from Schwann cells around peripheral nerves, usually epineurium of either autonomic sympathetic or parasympathetic fibers[2,3]. Pancreatic locations are unusual, with about 70 cases reported in the last 40 years, and most of them are benign. However, malignancy can be found in up to 15% of cases, especially in lesions greater than 6 cm[3-5]. Schwannomas are usually well-encapsulated firm masses, and two-thirds may undergo degenerative changes, which can be cystic formation, calcification, and hemorrhage, among others[2,6]. Due to these alterations, they can mimic cystic pancreatic lesions or metastasis of a different primary site tumor in radiologic investigation, including gastric cancer.

## CASE PRESENTATION

### *Chief complaints*

A 73-year-old woman presented with epigastric pain and weight loss.

### *History of present illness*

She had a history of non-insulin-dependent diabetes mellitus, arterial hypertension, and elevated cholesterol level.

### *History of past illness*

She did not report a history of other previous illnesses.

### *Personal and family history*

She was unaware of a family history of cancer.

### *Physical examination*

Abdominal examination did not detect any marked change.

### *Laboratory examinations*

All laboratory data were normal, including hemoglobin of 12.2 g/dL. Serum amylase was 50 U/mL, serum CEA was 1.3 ng/mL, and CA19-9 was 12.7 U/mL.

### *Imaging examinations*

Upper gastrointestinal endoscopy revealed an ulcerated and infiltrative (Borrmann III) lesion measuring 4 cm in the lesser curvature extending to the posterior wall of the antrum and body region. Biopsy revealed a moderately differentiated adenocarcinoma. Preoperative evaluation using computed tomography (CT) scan showed a well-defined 8 cm × 5 cm solid and cystic tumor in the body and tail of the pancreas in close contact to the posterior wall of the gastric body. No sign of infiltration in the surrounding tissue was detected. No liver mass, peripancreatic lymph node swelling, or free peritoneal fluid was detected (Figure 1).

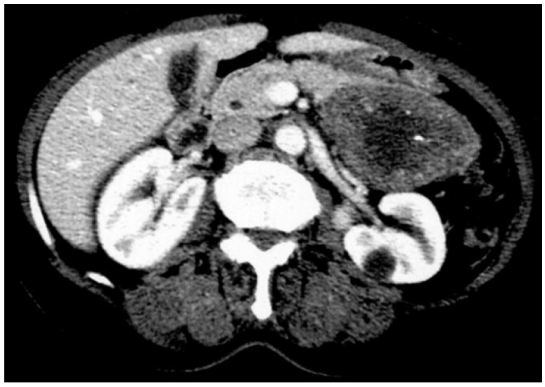

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**Figure 1 Computed tomography scan showing solid and cystic tumor in the body and tail of the pancreas (pancreatic schwannoma).**

## MULTIDISCIPLINARY EXPERT CONSULTATION

Laparotomy disclosed a localized gastric tumor in the body and a distinct solid, well-encapsulated tumor at the body of the pancreas without signs of inflammation or neoplastic infiltration. However, the lesion was in close contact to the posterior gastric wall (Figures 2 and 3). Due to the locoregional infiltration of the gastric tumor, absence of distant metastases, and proximity to a large pancreatic lesion, a total gastrectomy with D2 lymph node dissection plus distal pancreatectomy and splenectomy was performed. The final gastric cancer stage was pT2N0, with 0/73 lymph nodes examined (Figure 4). The cut surface of the excised 8 cm pancreatic tumor was pale yellow with hemorrhage foci. On microscopic examination, the lesion showed spindle cells with Antoni A and B patterns and was strongly positive for S100 protein (Figure 5).

## FINAL DIAGNOSIS

Gastric adenocarcinoma and concurrent pancreatic schwannoma.

## TREATMENT

Total gastrectomy with D2 lymph node dissection, plus distal pancreatectomy and splenectomy.

## OUTCOME AND FOLLOW-UP

The patient recovered without any complication, and she was discharged after 12 d. After 44 mo of follow-up, the patient has no evidence of recurrence.

## DISCUSSION

In this case report, the patient presented unspecific symptoms including epigastric pain and weight loss. Therefore, it was not possible to define if these symptoms were related to the gastric cancer or if it was a symptomatic case of pancreatic schwannoma. Pancreatic schwannoma appear to be indolent, corroborating its benign nature, and around one-third of the pancreatic schwannomas are asymptomatic. Abdominal pain is the most displayed symptom, ranging from 30% to 57% of patients. Other symptoms are reported less frequently, such as back pain, jaundice, anorexia, vomiting, weight loss, anemia, abdominal mass, and gastrointestinal bleeding[7,8].

CT scan performed for gastric cancer staging showed a solid and cystic pancreatic mass, and it was necessary to make differential diagnosis with a primary pancreatic neoplasm or metastases from the gastric tumor. CT scan may be beneficial in pancreatic schwannoma initial evaluation, and most of them revealed low density or cystic masses, as presented in this case[9,10]. Moreover, magnetic resonance imaging appears to be more helpful in characterizing schwannomas by their typical encapsulation, hypointensity on T1-weighted images, and hyperintensity on T2-weighted images[11,12]. These characteristics are typical radiological features of Antoni A areas, suggesting that these should be classified as

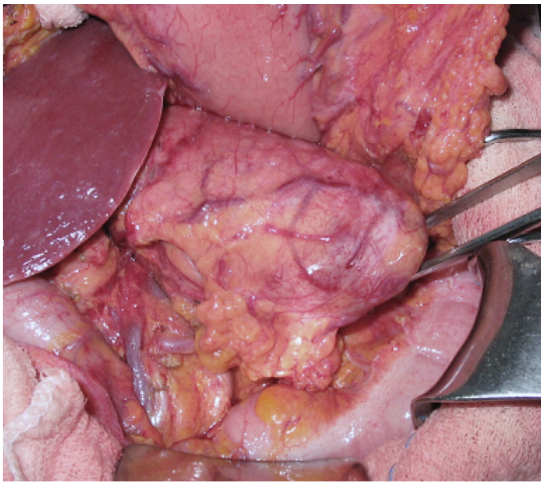

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**Figure 2 Laparotomy view of pancreatic body mass.**

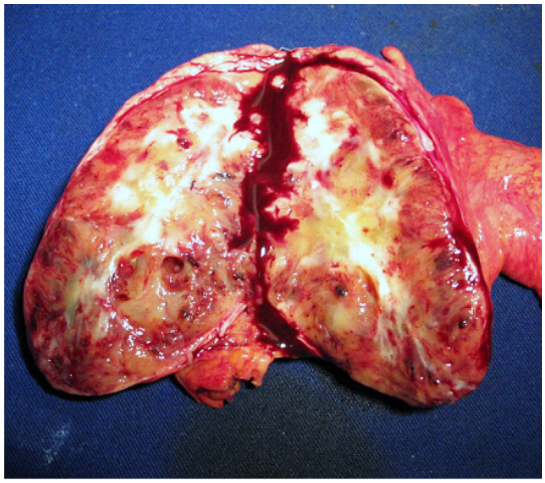

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**Figure 3 Macroscopic examination showed a well-encapsulated, pale yellow solid pancreatic tumor with areas of hemorrhage.**

solid hypervascularized tumors of the pancreas. Meanwhile, type Antoni B tumor areas are characterized by a significant cystic component, in which differential diagnosis must be made from a large amount of pancreatic cystic neoplasms[9,12]. Fluorodeoxyglucose-positron emission tomography-CT usually demonstrates a hypermetabolic appearance[8,9]. Complementary magnetic resonance imaging and fluorodeoxyglucose-positron emission tomography-CT were not performed in this patient but would be helpful in better characterizing morphological tumoral features.

Endoscopic ultrasound-guided fine needle aspiration may be useful, but this method remains controversial due to high false-negative rate. In two reviews, only 44% and 50% of patients were correctly diagnosed with pancreatic schwannoma[4,8].

Intraoperative analysis is also a helpful tool in diagnosis, especially to ensure negative margins and correct resection of pancreatic neoplasms, as demonstrated in this case. One review showed that 47% of pancreatic schwannomas were correctly diagnosed, and 33% were reported as benign[8], showing that the intraoperative assessment of these tumors may aid the decision making in these cases.

Surgical treatment includes Whipple procedure (pancreaticoduodenectomy) or distal pancreatectomy with or without splenectomy, either because a definite diagnosis was not made pre- or intraoperatively or due to large tumor size[13,14] (Table 1). Enucleation should be considered a surgical option when preoperative histopathology confirms the diagnosis. However, a tumor size larger than 6.0 cm, vascular encasement, or visceral invasion should elicit suspicion of malignant transformation, and a more radical approach should be chosen[4].

Gastrectomy with D2 lymph node dissection is a gold standard treatment considering the gastric neoplasm; however due to the pancreatic tumor size and the proximity to the posterior gastric wall harboring the tumor, it was decided to perform a partial pancreatectomy with splenectomy in addition

**Table 1 Summary of literature review on pancreatic schwannoma surgical management**

| Ref. | Type of study | Number of patients | Case presented in the article | | Literature review presented in the article | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | Moment of diagnosis | Surgery performed | Size (cm) | Mean size/range (cm) | Type of surgery performed | | | Malignancy, % |
| | | | | | | | Types of pancreatectomy or pancreatoduodenectomy, % | Enucleation | Surgical resection otherwise non-specified, % | |
| Paranjape *et al* [3], 2004 | Case report and review | 40 | Postoperative | Enucleation | 3.5 | 8.79 | 27 (67.5) | 4 (10.0) | 5 (12.5) | 5 (12.5) |
| Ma *et al*[4], 2017 | Case report and review | 68 | Postoperative | Whipple pancreaticoduodenectomy | 6 × 5 | 6.1 ± 5.7 (1-33) | 40 (59.0) | 8 (12.0) | 14 (21.0) | 8 (12.0) |
| Su *et al*[5], 2016 | Case report and review | 65 | Intraoperative frozen pathology | Central pancreatectomy | 1.6 × 1.1 × 1.1 | 5.83 ± 4.59 (1-20) | 40 (61.5) | 9 (13.8) | 13 (20.0) | 5 (7.7) |
| Gupta *et al*[6], 2009 | Case report and review | 37 | Postoperative | Whipple pancreaticoduodenectomy | 7.9 × 8.3 | - | 19 (51.3) | 6 (16.2) | 9 (24.3) | - |
| Moriya *et al* [7], 2012 | Case report and review | 47 | Intraoperative frozen pathology | Enucleation | 4 × 4 × 3 | 6.2 ± 5.1 (1-20) | 25 (53.0) | 7 (15.0) | 12 (26.0) | 5 (11.0) |
| Zhang *et al*[8], 2019 | Case report and review | 75 | Postoperative | Central pancreatectomy | 2.8 and 4.0 | 5.5 ± 5.0 (1.0-30.0) | 45 (60.0) | 11 (15.0) | 14 (19.0) | 4 (5.0) |
| Watanabe *et al* [9], 2018 | Case report | 1 | Postoperative | Subtotal stomach-preserving pancreaticoduodenectomy | 5.4 × 5.4 | - | - | - | - | - |
| Wang *et al* [11], 2019 | Case report | 1 | Postoperative | Distal pancreatectomy with splenectomy | 2.0 × 2.0 × 1.8 | - | - | - | - | - |
| Shi *et al*[14], 2021 | Case series and systematic review | 6 | Postoperative | Pancreaticoduodenectomy 5 (83%) and distal pancreatectomy 1 (17%) | 3.7 (range 2.0-6.4) | 4.3 ± 2.2 (1.4-10) | - | - | - | - |
| Kimura *et al* [15], 2021 | Case report | 1 | Postoperative | Distal pancreatectomy with splenectomy | 1.1 × 0.8 | - | - | - | - | - |

to the gastric resection.

Microscopically, schwannomas are divided in two main subareas: Antoni A areas, displaying an organized hypercellular component, characterized by closely packed spindle cells with occasional nuclear palisading; and Antoni B areas, featuring a hypocellular component with loose myxoid stroma, often with degenerative changes[4,7]. Immunohistochemistry is crucial to the differential diagnosis since immunostaining is strongly positive for S-100 protein, vimentin, and CD56 and negative for cytokeratin AE1/AE3, desmin, smooth muscle myosin, CD34, and CD117[4,7,15]. In this case, diagnosis was confirmed by the presence of these typical findings in pathology: Antoni A and B areas as well as immunohistochemistry with strong S-100 (+) staining.

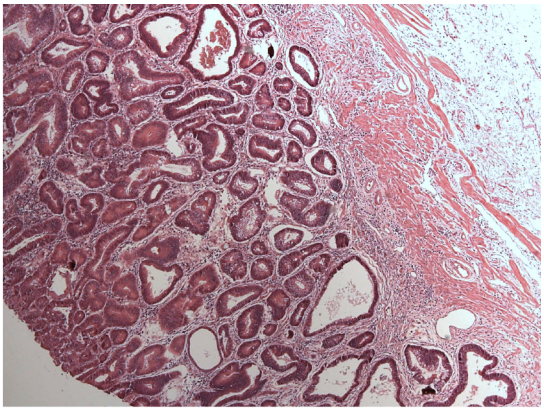

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**Figure 4 Representative area of moderately differentiated gastric adenocarcinoma.** Hematoxylin and eosin; Magnification × 50.

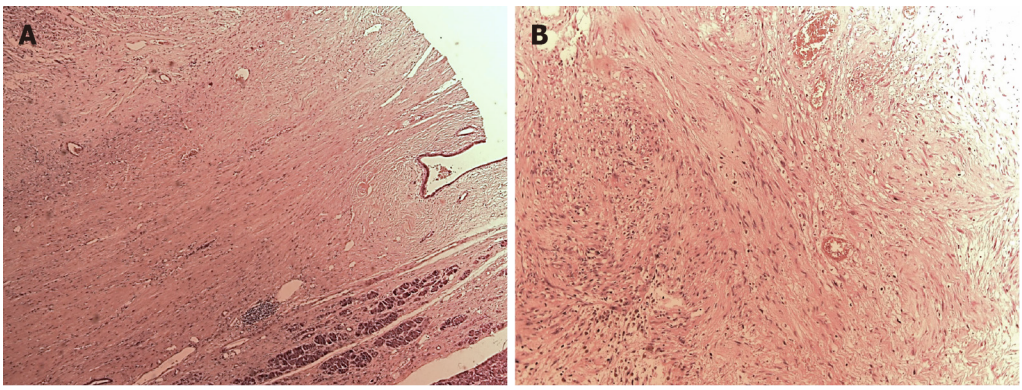


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**Figure 5 Microscopic examination.** A and B: Representative areas of pancreatic schwannoma; Hematoxylin and eosin; Magnification × 20).

Pancreatic schwannomas usually have good prognosis, showing no rates of recurrence over a mean follow-up of 19 mo[4,8].

## CONCLUSION

Therefore, we present the first case of synchronous gastric cancer and pancreatic schwannoma reported in the literature. Intraoperative staging examination was decisive in the adequate management of this patient. The presence of a large pancreatic mass should not preclude the potentially curative intent of the gastric cancer treatment.

## FOOTNOTES

**Author contributions:** Ribeiro MB contributed to the study design and drafting of the manuscript; Abe ES, Kondo A, and Safatle-Ribeiro AV contributed to data retrieval and manuscript review; Pereira MA and Zilberstein B contributed to manuscript review; Ribeiro Jr U conceived the study and contributed to critical analysis and manuscript review.

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**ORCID number:** Mateus Barradas Ribeiro 0000-0001-8702-0079; Emerson Shigueaki Abe 0000-0001-6054-5705; André Kondo 0000-0003-4842-3363; Adriana Vaz Safatle-Ribeiro 0000-0001-7686-8859; Marina Alessandra Pereira 0000-0002-6865-0988; Bruno Zilberstein 0000-0002-1809-8558; Ulysses Ribeiro Jr 0000-0003-1711-7347.

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ORIGINAL ARTICLE

Case Control Study

# Common polymorphisms of protein tyrosine phosphate non-receptor type 2 gene are not associated with risk of Crohn's disease in Indian

Kaushik Chatterjee, Amit Kumar Dutta, Ashish Goel, Rekha Aaron, Vijayalekshmi Balakrishnan, Ajith Thomas, Anoop John, Rajeeb Jaleel, Deepu David, Reuben Thomas Kurien, SD Chowdhury, Ebby George Simon, AJ Joseph, Prasanna Premkumar, Anna B Pulimood

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**Kaushik Chatterjee, Amit Kumar Dutta, Ashish Goel, Rekha Aaron, Vijayalekshmi Balakrishnan, Ajith Thomas, Anoop John, Rajeeb Jaleel, SD Chowdhury, Anna B Pulimood,** Department of Gastrointestinal Sciences, Christian Medical College and Hospital, Vellore 632004, Tamil Nadu, India

**Deepu David, Reuben Thomas Kurien, Ebby George Simon, AJ Joseph,** Department of Gastroenterology, Christian Medical College, Vellore 632004, Tamil Nadu, India

**Prasanna Premkumar,** Departments of Biostatistics, Christian Medical College, Vellore 632004, Tamil Nadu, India

**Corresponding author:** Amit Kumar Dutta, MD, Professor, Department of Gastrointestinal Sciences, Christian Medical College and Hospital, Ground Floor, Williams Building, Vellore 632004, Tamil Nadu, India. akdutta1995@yahoo.co.in

## Abstract

### *BACKGROUND*

Multiple genetic risk factors for Crohn's disease (CD) have been identified. However, these observations are not consistent across different populations. The protein tyrosine phosphate non-receptor type 2 (*PTPN2*) gene plays a role in various aspects of host defense including epithelial barrier function, autophagy, and innate and adaptive immune response. Two common polymorphisms in the *PTPN2* gene (rs2542151 and rs7234029) have been associated with risk of CD in Western countries.

### *AIM*

To evaluate the association of *PTPN2* gene polymorphisms with risk of CD in Indian population.

### *METHODS*

We conducted a prospective case-control study. Patients with CD were recruited, and their clinical and investigation details were noted. Controls were patients without organic gastrointestinal disease or other comorbid illnesses. Two common polymorphisms in the *PTPN2* gene (rs2542151 and rs7234029) were assessed. DNA was extracted from peripheral blood samples of cases and controls

and target DNA was amplified using specific sets of primers. The amplified fragments were digested with restriction enzymes and the presence of polymorphism was detected by restriction fragment length polymorphism. The frequency of alleles was determined. The frequencies of genotypes and alleles were compared between cases and controls to look for significant differences.

***RESULTS***

A total of 108 patients with CD (mean age 37.5 ± 12.7 years, females 42.6%) and 100 controls (mean age 39.9 ± 13.5 years, females 37%) were recruited. For the single nucleotide polymorphism (SNP) rs7234029, the overall frequency of G variant genotype (AG or GG) was noted to be significantly lower in the cases compared to controls (35.2% *vs* 50%, $P = 0.05$). For the SNP rs2542151, the overall frequency of G variant genotype (GT or GG) was noted to be similar in cases compared to controls (43.6% *vs* 47%, $P = 0.73$). There were no significant differences in minor allele (G) frequency for both polymorphisms between the cases and controls. Both the SNPs had no significant association with age of onset of illness, gender, disease location, disease behaviour, perianal disease, or extraintestinal manifestations of CD.

***CONCLUSION***

Unlike observation form the West, polymorphisms in the *PTPN2* gene (rs7234029 and rs2542151) are not associated with an increased risk of developing CD in Indian patients.

**Key Words:** *PTPN2* gene; Crohn's disease; Genetic polymorphism; Case-control study; Asia; Risk factor



**Core Tip:** Several genetic risk factors have been associated with Crohn's disease and they have provided valuable insights into the pathogenesis of the disease. However, some of the genetic changes are not observed uniformly across all populations and hence it is essential to determine their occurrence in different populations. In this prospective case-control study, we investigated the association of two common polymorphisms in the protein tyrosine phosphate non-receptor type 2 (*PTPN2*) gene (rs7234029 and rs2542151) with risk of Crohn's Disease in an Asian country. Our results showed that contrary to observation form the West, polymorphisms in the *PTPN2* gene were not associated with an increased risk of developing Crohn's disease.

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## INTRODUCTION

Crohn's disease (CD) is a chronic inflammatory disorder of the gastrointestinal tract with the peak age of onset in the third and fourth decades of life[1]. The frequency of CD is increasing in several Asian countries including India[1,2]. Environmental factors, gut microbiota, and host genetic factors are considered to be the key players in the pathogenesis of CD resulting in dysregulated immune response. Research on genetic factors has made significant contribution in understanding the pathogenesis of CD [3,4]. These include defective innate immunity and intracellular bacterial killing (*CARD15/NOD2, IL23R*, and *LRRK2* genes), defective autophagy (*ATG16L1* and *IRGM* genes), and dysregulated adaptive immune responses, namely, the interleukin-23 (IL-23) and T helper 17 (Th17) cell pathway (*IL23R, IL12B, STAT3, JAK2*, and *TYK2* genes)[4]. Some of the genetic alterations identified in CD are not observed uniformly across different populations[5]. For example, the mutation in the *NOD2/CARD15* gene present in the Western population with CD was not detected in Indian patients with CD[6]. Hence, it is important to investigate the presence of known genetic defects in different populations to understand their contribution to the pathogenesis of disease in that group.

Protein tyrosine phosphate non-receptor type 2 (PTPN2), also known as T-cell protein tyrosine phosphatase, is a cytosolic tyrosine phosphatase and is almost ubiquitously expressed in embryonic and adult tissues[7,8]. It has an N-terminal phosphatase domain and a nuclear localization sequence. It can

dephosphorylate targets in both the cytosol and nucleus. PTPN2 has two variants arising out of alternate splicing[8]. The larger 48 kD form has a C-terminal hydrophobic domain that masks the nuclear localisation sequence and it remains attached to the endoplasmic reticulum[9]. The small 45 kD form does not have any hydrophobic sequence and can help the protein translocate to the nucleus. PTPN2 has been shown to affect various aspects of host defense including epithelial barrier function, autophagy, and innate and adaptive immune response[7,10-13]. Several studies have shown an association between polymorphism in the *PTPN2* gene and CD. In a meta-analysis, two single nucleotide polymorphisms (SNPs) in the *PTPN2* gene, rs7234029 (odds ratio [OR] = 1.36, 95% confidence interval [CI]: 1.16-1.59) and rs2542151 (OR = 1.22, 95%CI: 1.15-1.3), have been shown to increase the risk of CD. We aimed to study the association of these two SNPs in the *PTPN2* gene (rs7234029 and rs2542151) with risk of CD in an Asian country (India) which has a total estimated burden of inflammatory bowel disease of about 1.4 million persons (highest in Asia)[2].

## MATERIALS AND METHODS

We conducted a prospective case-control study to determine the association of polymorphisms in the *PTPN2* gene with risk of CD. Adult patients (age > 18 years) with a diagnosis of CD were the cases. The diagnosis of CD was based on a combination of clinical, endoscopic, histological and radiological features as suggested by the Asia Pacific consensus criteria[14]. Adult subjects (age > 18 years) with dyspeptic symptoms and unrelated to cases were screened for inclusion as controls. Those with normal upper gastrointestinal endoscopy, normal haemoglobin, normal blood sugar, normal liver and renal function tests, and absence of significant comorbid illnesses were recruited as controls. The age and gender distribution of controls were kept similar to those of cases. Cases and controls were recruited after obtaining written informed consent. Patients were recruited from 2016 to 2018. The study was approved by the local institute review board and ethics committee.

As there were no previous studies from India on the SNPs in *PTPN2*, we could not make assumptions for sample size calculation and planned to recruit about 100 cases and controls each. The clinical, demographic, and investigation details of the cases were recorded in a predesigned form. These included age at the diagnosis of CD, extent of disease, disease behaviour, presence of extra-intestinal manifestations (EIM), and previous surgery for CD. The demographic details of controls were also recorded.

Blood samples were collected from the cases and controls and were stored at -80 °C till analysis. Genomic DNA was extracted from leukocytes using phenol-chloroform method and target DNA fragments were amplified by polymerase chain reaction (PCR) using specific primers (rs2542151: forward, 5′-TGCTGTGCTGCGTGAGTT-3′ and reverse, 5′-CACCATTGAGCGAAGTCC-3′; rs7234029, forward, 5′-GGCAGTGCTGAAACGAGA-3′ and reverse, 5′-TCCCACCACCTACCTACGG-3′). The steps of PCR included initial denaturation at 94 °C for 5 min, 35 cycles of denaturation at 94 °C for 35 s, annealing at 58 °C for 30 s, and extension at 72 °C for 35 s, and final extension at 72 °C for 7 min. The PCR products were electrophoresed on a 2% agarose gel with TBE buffer for 45 min and visualized under a gel imaging system (Bio-Rad Gel Doc-2000, United States). The PCR products (5 μL) were digested by the appropriate restriction enzyme (Bsp1286I for rs2542151 and Hpy188I for rs7234029) for about 10 h, followed by electrophoresis on a 2.5% agarose gel. The digested product was gel documented and results were analysed and reported.

### *Statistical analysis*

Continuous variables are summarised as the mean ± standard deviation or median with range and categorical variables as percentages. Categorical variables were compared using the chi-square test and continuous variables using independent *t*-test. A *P* value ≤ 0.05 was considered significant. Presence of Hardy-Weinberg equilibrium was assessed for both the SNPs in cases and controls. Data analyses were done using statistical software package SPSS v13.

## RESULTS

We recruited 108 cases of CD and 100 control subjects during the study period. Table 1 shows the baseline characteristics of the patients with CD in this study. Their mean age was 37.5 ± 12.7 years and 42.6% were females. One patient with ileocolonic disease had coexisting upper gastrointestinal involvement while upper gastrointestinal disease alone was noted in one case. EIM were present in 17.6% cases, among which arthropathy was most frequent (17 cases) followed by uveitis (3 cases) and primary sclerosing cholangitis (1 case). Two patients had more than one EIM. About one-third of patients had previous surgery for CD, which included bowel resection in 30 patients. History of appendectomy was noted in three patients. Family history of IBD was present in two cases. The mean age of the 100 control subjects was 39.9 ± 13.5 years and 37% were females. The age and gender distri-

**Table 1 Baseline characteristics of patients with Crohn's disease**

| Characteristic | Frequency (*n* = 108) |
|---|---|
| Age (yr) | 37.5 + 12.7 |
| Sex (females) | 46 (42.6%) |
| Age at diagnosis | |
| < 17 yr | 7 (6.5%) |
| 17-40 yr | 75 (69.4%) |
| > 40 yr | 26 (24.1%) |
| Disease behaviour | |
| Inflammatory | 43 (39.8%) |
| Stricturing | 48 (44.4%) |
| Penetrating | 13 (12.1%) |
| Stricturing and penetrating | 4 (3.7%) |
| Disease location | |
| Ileal | 49 (45.4%) |
| Colonic | 16 (14.8%) |
| Ileo-colonic | 42 (38.9%) |
| Upper gastrointestinal | 1 (0.9%) |
| Perianal disease (Yes) | 15 (13.9%) |
| Surgery for Crohn's disease (Yes) | 37 (34.3%) |
| Extra-intestinal manifestations (Yes) | 19 (17.6%) |
| Smoking (Yes) | 12 (11.1%) |

bution were not significantly different from those of cases (age, $P = 0.19$; sex, $P = 0.41$).

The frequencies of the two SNPs in the *PTPN2* gene evaluated in this study among cases and controls are shown in Table 2. Figures 1 and 2 illustrate the examples of digestion pattern noted for polymorphisms in both the SNPs. Both the SNPs were in Hardy-Weinberg equilibrium in cases (rs7234029, $P = 0.21$; rs2542151, $P = 0.65$) as well as controls (rs7234029, $P = 0.47$; rs2542151, $P = 0.42$). Results for the *PTPN2* SNP rs7234029 were obtained for all cases and 98 controls. In the remaining two controls, the results of laboratory test were not clear, which hence were not included in the analysis. For the SNP rs7234029, the wild type (AA) was noted in 64.8% of the cases and in 50% of the controls. Homozygous variant (GG) was noted in 7.4% and heterozygous variant (AG) in 27.8% of the cases. The overall frequency of G variant (AG or GG) was noted to be significantly lower in the cases compared to controls (35.2% *vs* 50%, $P = 0.05$). In addition to the genotype, we also compared the minor allele frequency between cases and controls (Table 3). The frequency of the minor allele (G) was 20.9 % in cases and 27.6% in controls. Although the minor allele was more common in controls, the difference was not significant statistically ($P = 0.17$).

Results for the *PTPN2* SNP rs2542151 were obtained for 101 cases and all control subjects (Table 2). The results of laboratory tests were not clear in the remaining seven cases with CD, which hence were excluded from the analysis. For the SNP rs2542151, the wild type (TT) was detected in 56.4% of the cases and in 53% of the controls. Homozygous variant (GG) was seen in 4% and heterozygous variant (GT) in 39.6% of the cases. The overall frequency of G variant (GT or GG) was noted to be similar in cases compared to controls (43.6% *vs* 47%, $P = 0.73$). On comparing the alleles, the frequency of minor allele (G) was 23.8 % in cases and 25.5% in controls, which was not significantly different ($P = 0.77$, Table 3).

We evaluated the association of the two SNPs in the *PTPN2* gene with patient and disease characteristics (Table 4). The *PTPN2* SNP rs7234029 GG or GA genotype was more common in patients with perianal disease and less common in patients with disease onset after 40 years although the difference did not reach statistical significance. There was no association of this polymorphism with gender, disease behaviour, disease location, EIM, or requirement of surgery (Table 4). The *PTPN2* SNP rs2542151 GT or GG genotype appeared to have a negative association with history of surgical intervention for CD. Among patients who underwent surgery for CD in past, 28.6% had the variant genotype (GT or GG) while 50% of patients without prior surgery had this variant and the difference was close to being statistically significant ($P = 0.07$). Other features like gender, age of onset of illness, disease behaviour, disease location, and perianal disease were not associated with the *PTPN2* SNP

**Table 2 Frequency of the single nucleotide polymorphisms in protein tyrosine phosphate non-receptor type 2 gene in cases and controls**

| | ***PTPN2* SNP rs7234029** | | |
|---|---|---|---|
| | **Homozygote (GG)** | **Heterozygote (AG)** | **Wild type (AA)** |
| Cases ($n$ = 108) | 8 (7.4%) | 30 (27.8%) | 70 (64.8%) |
| Controls ($n$ = 98) | 5 (5.1%) | 44 (44.9%) | 49 (50%) |
| $P$ value[1] | 0.05 | | |
| | *PTPN2* SNP rs2542151 | | |
| | Homozygote (GG) | Heterozygote (GT) | Wild type (TT) |
| Cases ($n$ = 101) | 4 (4%) | 40 (39.6%) | 57 (56.4%) |
| Controls ($n$ = 100) | 4 (4%) | 43 (43%) | 53 (53%) |
| $P$ value[1] | 0.73 | | |

[1]Wild *vs* variant (homozygote or heterozygote). SNP: Single nucleotide polymorphism; PTPN2: Protein tyrosine phosphate non-receptor type 2.

**Table 3 Minor allele frequency of the single nucleotide polymorphisms in protein tyrosine phosphate non-receptor type 2 gene in cases and controls**

| | **Minor allele** | **Cases** | **Controls** | ***P* value** |
|---|---|---|---|---|
| rs7234029 | G | 20.9% | 27.6% | 0.17 |
| rs2542151 | G | 23.8% | 25.5% | 0.77 |

SNP: Single nucleotide polymorphism; PTPN2: Protein tyrosine phosphate non-receptor type 2.

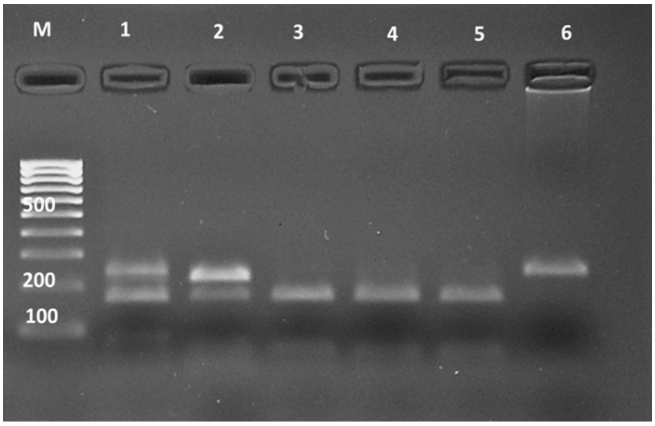


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**Figure 1 Restriction digestion pattern for the single nucleotide polymorphism rs7234029.** M lane shows 100 bp ladder. Lanes 3, 4, and 5 show wild type digestion pattern (AA), lanes 1 and 2 show heterozygous digestion pattern (AG), and lane 6 shows homozygous digestion pattern of the variant (GG).

rs2542151 (Table 4).

## DISCUSSION

The two common SNPs in the *PTPN2* gene (rs2542151, rs7234029) were not associated with an increased risk of CD among Indian patients based on the observation made in the current study. In fact, the *PTPN2* SNP rs7234029 was more frequent in control subjects compared to cases and hence had a negative association with CD. This highlights the heterogeneity of genetic risk factors for CD in different populations.

**Table 4 Association of the single nucleotide polymorphisms in protein tyrosine phosphate non-receptor type 2 gene with patient and disease characteristics**

| Characteristic | | rs7234029 | | | rs2542151 | | |
|---|---|---|---|---|---|---|---|
| | | Variant (n=38, GG or GA) | Wild type (n=70, AA) | *P* value | Variant (n=44, GG or GT) | Wild type (n=57, TT) | *P* value |
| Sex | Female | 37% | 63% | 0.74 | 38.1% | 61.9% | 0.46 |
| | Male | 33.9% | 66.1% | | 47.5% | 52.5% | |
| Age at onset of illness (yr) | Up to 40 yr | 39% | 61% | 0.21 | 43.4% | 56.6% | 0.96 |
| | > 40 | 23.1% | 76.9% | | 44% | 56% | |
| Disease behaviour | NSNP | 29.6% | 70.4% | 0.42 | 45% | 55% | 0.98 |
| | SP | 39.1% | 60.9% | | 42.6% | 57.4% | |
| Disease location | Ileal | 34.7% | 65.3% | 0.98 | 42.9% | 57.1% | 0.28 |
| | Colonic | 35.3% | 64.7% | | 23.5% | 76.5% | |
| | Ileocolonic | 36.6% | 63.4% | | 43.9% | 56.1% | |
| Perianal disease | Present | 46.7% | 53.3% | 0.32 | 46.7% | 53.3% | 0.79 |
| | Absent | 33.3% | 66.7% | | 43% | 57% | |
| Surgery for Crohn's disease | Yes | 32.4% | 67.6% | 0.67 | 29.4% | 70.6% | 0.07 |
| | No | 36.6% | 63.4% | | 50.1% | 49.9% | |
| Smoking | Yes | 25% | 75% | 0.43 | 41.7% | 58.3% | 0.89 |
| | No | 36.5% | 63.5% | | 43.8% | 56.2% | |
| EIM | Yes | 40% | 60% | 0.62 | 29.4% | 70.6% | 0.31 |
| | No | 34.1% | 65.9% | | 46.4% | 53.6% | |

SNP: Single nucleotide polymorphism; PTPN2: Protein tyrosine phosphate non-receptor type 2; NSNP: Non-stricturing and non-penetrating; SP: Stricturing and/or penetrating; EIM: Extraintestinal manifestations.

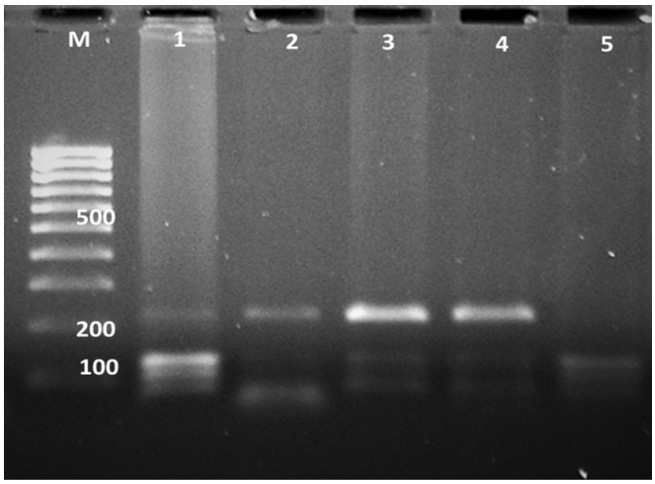


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**Figure 2 Restriction digestion pattern for the single nucleotide polymorphism rs2542151.** M lane shows 100 bp ladder. Lanes 1 and 5 show wild type digestion pattern (TT), lanes 3 and 4 show heterozygous digestion pattern (GT), and lane 2 shows homozygous digestion pattern of the variant (GG).

More than 240 genetic susceptibility loci have been identified for IBD[15]. Many of them are also associated with risk of other diseases. *PTPN2* was first reported as a susceptibility gene for CD in the genome wide association studies (GWAS) by the Wellcome Trust Case Control Consortium[16]. The *PTPN2* SNP rs2542151 was significantly associated with CD (trend $P = 4.56 \times 10^{-8}$, genotypic $P = 2.03 \times 10^{-7}$). Another polymorphism, the *PTPN2* SNP rs7234029 (located in intronic region), was also

subsequently found to be associated with CD. Polymorphisms in the *PTPN2* gene have also been associated with rheumatoid arthritis, celiac disease, type II diabetes mellitus, *etc*.[7,17-19]. The *PTPN2* gene affects several components of immune response and mice deficient in this gene develop severe systemic inflammatory illness[20]. They also show dysbiosis of intestinal microbiota[21, 22]. Patients with loss of function variant of *PTPN2* demonstrate increased markers of Th1 and Th17 cell activation and reduced Treg cell activity[12,21]. PTPN2 is an important negative regulator of STAT1 and 3 activities and restricts TNF-alpha related release of inflammatory mediator[23]. It also affects epithelial barrier function[11,24]. Interestingly, administration of Tofacitinib (an inhibitor of Janus kinase), a drug approved for treatment of ulcerative colitis, was shown to correct the epithelial barrier defect induced by functional defect in the *PTPN2* gene[25]. Observations in PTPN2 knockout mouse showed overexpression of cation-selective pore-forming molecule claudin-2, which allows para-cellular passage of molecules like sodium[24]. *In vitro* studies have also shown increased transcellular passage of macromolecules in PTPN2 deficient cells. PTPN2 in intestinal epithelial cells inhibits the expression of several autophagy-associated molecules, including beclin-1, ATG5, ATG7, ATG12, and ATG16L[26]. siRNA induced knockdown of the *PTPN2* gene has also demonstrated the role of this gene in regulating autophagy[27].

After the initial report, several studies have subsequently assessed the frequency of polymorphisms in the *PTPN2* gene in patients with CD[28]. A meta-analysis of data from these studies has shown an increased risk of CD associated with G variant in the *PTPN2* SNP rs2542151[29]. Thirteen studies in this meta-analysis studied this variant in CD. Subjects with genotype GG or GT had an OR of 1.22 (95%CI: 1.15-1.3, $P < 0.001$) of having CD compared to genotype TT. Subjects with G allele had an OR of 1.22 (95%CI: 1.15-1.28, $P < 0.001$) of having CD compared to T allele. However, among studies from Asia, which included two studies from China and one from Japan, there was no significant association with CD at the genotype ($P = 0.06$) or allele level ($P = 0.18$)[29]. This is consistent with the observation made in our study where we did not find any significant association of CD with the *PTPN2* SNP rs2542151. We found no significant difference either at the genotype ($P = 0.73$) or allele level ($P = 0.77$).

The above meta-analysis by Zhang *et al* also showed an increased risk of CD associated with G variant in the *PTPN2* SNP rs7234029[29]. However, only two studies (one each from Japan and Germany) assessed this polymorphism. Genotype GG or AG was associated with an OR of 1.36 (95%CI: 1.16-1.59, $P < 0.001$) of developing CD compared to AA genotype. A significant association was also noted at the allelic level and subjects with G allele had an OR of 1.33 (95%CI: 1.15-1.52, $P < 0.001$) of developing CD compared to A allele[29]. This finding could not be replicated in our study. There was no significant difference in the frequency of G allele between cases and controls ($P = 0.17$). Interestingly, the variant genotype (GG or GT) was more common in controls compared to cases ($P = 0.05$). The differences in genetic susceptibility loci between populations are not unexpected and have been noted for several other genes as well[5]. The phenotype of CD shows some variation between Western and Asian countries and the genetic differences may contribute to this in addition to environmental factors.

In addition to susceptibility to disease, polymorphisms in *PTPN2* have also been linked with disease phenotype and response to therapy. In a study from New Zealand with 315 cases with CD and 481 controls, the *PTPN2* SNP rs2542151 was associated with penetrating disease behaviour, need for bowel resection, late age at first diagnosis, and smoking[30]. However, our observations suggest a negative association of the *PTPN2* SNP rs2542151 GT or GG genotype with history of surgical intervention for CD. Other characteristics of the disease were not affected by this variant in our patients. Van der Heid *et al* observed that the *PTPN2* SNP rs2542151 increases the risk of CD only in smokers[31]. Another study from Germany revealed an association of the *PTPN2* SNP rs7234029 with stricturing disease[9]. We found the *PTPN2* SNP rs7234029 GG or GA genotype to be more frequent in patients with perianal disease and in those with onset of CD before the age of 40 years, although the difference was not significant statistically. A recent study by Hoffman *et al*[32] showed reduced response to anti IL-12/23 therapy in CD patients with G allele of the *PTPN2* SNP rs7234029 compared to A allele (67.6% *vs* 89.9%, $P = 0.005$). As multiple factors may affect the disease phenotype and behavior, association with SNPs needs to be interpreted with caution. This may also explain the variability of effects in different studies and causality assessment would require a GWAS study with adjustment for other factors.

Our study evaluated two well-known mutations in the *PTPN2* gene but there may be mutations in other parts of the gene which would require sequencing of the entire gene. This is one of the limitations of this study. However, our aim was to evaluate the previously known mutations and hence this did not affect our study objective.

## CONCLUSION

In conclusion, we did not find a positive association of variants in the *PTPN2* SNP rs2542151 and rs7234029 with risk of CD in Indian patients. As PTPN2 has several effects on immune function, whole gene sequencing studies may provide an insight on whether variations at other sites in this gene are associated with risk of CD in this population.

## ARTICLE HIGHLIGHTS

### *Research background*

The frequency of Crohn's disease (CD) has been increasing in several Asian countries. Although its exact pathogenesis is still being elucidated, host genetic, gut microbiota, and environmental factors are key players involved. Research on genetic factors have provided valuable insight into the pathogenesis of the disease. However, some of the genetic abnormalities identified are not consistently seen across different populations and observations from one region cannot be extrapolated to other regions.

### *Research motivation*

Protein tyrosine phosphate non-receptor type 2 (PTPN2) plays an important role in autophagy, innate and adaptive immune response, and maintaining epithelial barrier function. Single nucleotide polymorphisms (SNP) in the *PTPN2* gene have been associated with an increased risk of CD. However, this needs to be confirmed in different populations.

### *Research objectives*

Two SNPs in the *PTPN2* gene (rs7234029 and rs2542151) have been associated with risk of CD. The objective of the current study was to assess the association of these two polymorphisms with CD in a large Asian country.

### *Research methods*

A prospective case-control study was conducted where cases were patients with CD. Two SNPs in the *PTPN2* gene (rs2542151 and rs7234029) were assessed using restriction fragment length polymorphism. The frequencies of polymorphisms between cases and controls were compared.

### *Research results*

The study included 108 patients with CD and 100 controls. The two SNPs in the *PTPN2* gene were not associated with an increased risk of CD. In addition, no association was observed between the two SNPs and disease characteristics.

### *Research conclusions*

The current study did not show an increased risk of CD with polymorphisms in the *PTPN2* gene contrary to observations in Western population.

### *Research perspectives*

This study reemphasizes on the heterogeneity of genetic risk factors for CD across different population and the need to evaluate them in different populations.

## FOOTNOTES

**Author contributions:** Chatterjee K recruited the patients, collected the samples, and critically revised the manuscript; Dutta AK designed the study, analyzed the data, and wrote the manuscript; Balakrishnan V and Aaron R carried out laboratory analysis and critically revised the manuscript ; Samuel P helped with study design, data analysis, and critical revision; Goel A, Thomas A, John A, Jaleel R, David D, Kurien RT, Chowdhury SD, Simon EG, Joseph AJ, and Pulimood AB helped with data collection and critical revision of the manuscript; all authors have read and approved the final manuscript.

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**ORCID number:** Kaushik Chatterjee 0000-0002-1983-8546; Amit Kumar Dutta 0000-0002-5111-7861; Ashish Goel 0000-0003-1659-2103; Rekha Aaron 0000-0002-6376-8071; Vijayalekshmi Balakrishnan 0000-0001-9398-4958; Ajith Thomas 0000-0002-9498-6792; Anoop John 0000-0001-5335-6658; Rajeeb Jaleel 0000-0002-6059-6139; Deepu David 0000-0001-9462-9202; Reuben Thomas Kurien 0000-0003-4456-816X; SD Chowdhury 0000-0001-6484-0218; Ebby George Simon 0000-0002-6328-2434; AJ Joseph 0000-0003-0099-5370; Prasanna Premkumar 0000-0002-7157-4138; Anna B Pulimood 0000-0003-0186-8584.

**Corresponding Author's Membership in Professional Societies:** Indian Society of Gastroenterology, LM 001733.

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LETTER TO THE EDITOR

# Hepatomusculoskeletal disorders: Coining a new term might improve the management of the musculoskeletal manifestations of chronic liver disease

Christos Tsagkaris, Stavros P Papadakos, Dimitrios V Moysidis, Andreas S Papazoglou, Alexandra Koutsogianni, Marios Papadakis

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**Christos Tsagkaris,** Public Health and Policy Working Group, Stg European Student Think Tank, Amsterdam, Netherlands

**Stavros P Papadakos, Alexandra Koutsogianni,** Laiko General Hospital of Athens, National and Kapodistrian University of Athens, Athens 18233, Greece

**Dimitrios V Moysidis,** Hippokration University Hospital, Aristotle University of Thessaloniki, Thessaloniki 54642, Greece

**Andreas S Papazoglou,** Athens Naval Hospital, Athens 18233, Greece

**Marios Papadakis,** Department of Surgery II, University Hospital Witten-Herdecke, University of Witten-Herdecke, Wuppertal 42283, Germany

**Corresponding author:** Christos Tsagkaris, MD, Academic Fellow, Public Health and Policy Working Group, Stg European Student Think Tank, Postjeskade 29, 1058 DE, Amsterdam, Netherlands. chriss20x@gmail.com

## Abstract

Chronic liver disease can affect many body systems including the musculoskeletal system. The pathogenetic crosstalk between the liver and organs such as the brain and the kidneys has already been described with compound terms merging the organs affected by the pathology, such as the hepatorenal syndrome. Nevertheless, the musculoskeletal manifestations of chronic liver disease have not been coined with such a term to date. Because of this shortage, documenting the musculoskeletal implications of chronic liver disease in both research and clinical practice is challenging. To fill this gap, the authors propose the term hepatomusculoskeletal disorders, a compound term of Greek origin that encompasses all the body structures involved in the aforementioned pathologic crosstalk.

**Key Words:** Chronic liver disease; Hepatomusculoskeletal disorders; Musculoskeletal system; Hepatology; Pathophysiology; Osteodystrophy

**Core Tip:** The authors recommend coining the umbrella term "hepatomusculoskeletal disorders" in response to the need to expand knowledge about chronic liver disorders and capitalize it in the form of practice guidelines.

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## TO THE EDITOR

Chronic liver disease (CLD) is the 11th leading cause of mortality globally accounting for up to 2% of disability-adjusted life years worldwide[1]. It encompasses ailments of infectious (viral hepatitis) and non-infectious (alcohol abuse, non-alcoholic steatohepatitis, cancer) origin leading to progressive structural and functional depletion of hepatic physiology in the form of liver cirrhosis. CLD is associated with multisystem complications involving the kidneys, the heart, the nervous system and the musculoskeletal system[2]. Research in the field has recently sought hematological and electrocardiographic CLD biomarkers addressing CLD's extrahepatic manifestations as a potential standpoint for the management of the disease and for the identification of novel therapeutic targets[3-5]. Nevertheless, research regarding the musculoskeletal implications of CLD remains limited. Action is needed to expand the existing knowledge and its clinical applications.

The impact of CLD on the musculoskeletal system has been better understood during the last years [6]. The musculoskeletal manifestations can be classified into two categories according to their etiology: (1) On the causative disease which insults the liver; and (2) On the type and the degree of liver disease. In more detail, Hepatitis C is frequently associated with rheumatologic phenomena. Polyarthralgia either in the context of mixed cryoglobulinemia triad of purpura, fatigue and arthralgia or alone as hepatitis C virus (HCV)-induced arthritis is documented frequently[7,8]. Overt arthritis and fibromyalgia are less frequently diagnosed in parallel with HCV infection. Polyarthritis and polyarthralgia are commonly presented as manifestations of hepatitis B virus, hepatitis A virus and hepatitis E virus infections[7] while erosive arthritis is encountered in anti-cyclic citrullinated peptide positive type I autoimmune hepatitis[9]. In regards to the alcoholic liver disease, ethanol exerts direct cytotoxic effects into the muscular system causing alcoholic myopathy while affects bone metabolism causing matrix decomposition and suppression of bone synthesis[10]. Nonalcoholic fatty liver disease is frequently associated with low bone mineral density[11] while in diseases characterized by defective metabolism of metals (*e.g.*, copper in Wilson's disease and iron in haemochromatosis), arthritis, chondrocalcinosis and muscle stiffness and pain are regularly noticed[7,12]. On the other side, the severity of liver disease impacts the musculoskeletal health. Alterations in endogenous steroid metabolism and the use of proton pump inhibitors and diuretics results in fluctuations of mineral metabolism which result in hepatic osteodystrophy[13]. The defective immune responses due to poor complement system and opsonization sufficiency, portosystemic shunt and bacterial intestinal overgrowth render the patients prone to infections like septic arthritis, osteomyelitis, cellulitis and necrotizing fasciitis[14]. Finally, sarcopenia [15], non-traumatic osteonecrosis[16] and a higher rate of periprosthetic complications[17] are manifestations from the musculoskeletal system that compromise severely the quality of a patient's life.

On these grounds, healthcare professionals specializing in the management of musculoskeletal conditions (rheumatologists, orthopedic surgeons, physiatrists, physiotherapists, *etc*) can substantially contribute to CLD management. Prevention-wise, patients with CLD history can benefit from regular screening for osteopenia and osteoporosis and from falls' prevention training[18]. Similarly, physiotherapy to maintain muscle mass, improve patients' functionality and prevent sarcopenia-associated injury and disability can also be provided[19]. Treatment-wise, orthopedists and rheumatologists need to be aware of septic arthritis in CLD patients presenting with joint pain, and for spondylodiscitis and vertebral tuberculosis - in regions where the disease is endemic-in CLD patients presenting with low back pain[20-22]. Performing orthopedic surgery should also entail special considerations in CLD patients. Given their 3.5-fold higher risk for periprosthetic infections, cellulitis and necrotizing fasciitis, conservative management of fractures or osteoarthritis can be prioritized. In case of surgery, the patients and their formal and informal caregivers need to be instructed about the risk of infection and the need to carefully inspect surgical wounds and areas of plaster casting and seek medical attention when appropriate[23].

To contribute towards this end, musculoskeletal healthcare professionals need updated practice guidelines and relevant training. Developing concrete guidelines in turn requires systematic research in the field, with large scale observational studies and clinical trials confirming the existing knowledge and

optimizing the recommended interventions. Currently, it appears that research in the field is heterogeneous, with the majority of studies being observational and having been conducted independently in inconsistent time intervals.

A search for relevant publications on Medline, Scopus and other databases reveals a plethora of terms used to describe CLD musculoskeletal implications. The wording is often alternating (musculoskeletal disorders in patients with CLD, hepatic osteodystrophy) and rather descriptive words addressing particular alterations associated with CLD (sarcopenia, osteosarcopenia, skeletal muscle mass) rather than the phenomenon as a whole[2,24-26]. A term grouping all of the aforementioned together has not been included in the Medical Subject Headings thesaurus and in the International Disease Classification (ICD10) system to date. To the best of the authors' knowledge, no relevant term can be found in hospital records and documentation systems as well. Therefore, the lack of a consistent nomenclature poses significant obstacles to the appraisal of the existing knowledge, let alone its expansion.

The authors recommend coining the umbrella term "hepatomusculoskeletal disorders" in response to the need to expand relevant knowledge and capitalize it in the form of practice guidelines. The term is a compound word of Greek origin. It emphasizes the implications of liver conditions (hepato-) on muscles (musculo-), bones and connective tissue (skeletal). The composition of the term is similar to other relevant clinical terms such as the hepatorenal or the cardiorenal syndrome. In both these examples, the organs whose pathologies affect each other (liver, heart and kidneys respectively) are merged in a single term. Coining the new term in a similar linguistic format to other terms that are established in clinical practice makes it easily comprehensible to physicians and researchers. Therefore, the proposed term can benefit future research, clinical practice and medical education. Certainly, to address the musculoskeletal implications of CLD sufficiently, several steps involving clinicians, researchers, health bodies, healthcare administrators and stakeholders are required. Nonetheless, the new term can hopefully serve as common ground underlining the need to take relevant action.

## FOOTNOTES

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**Country/Territory of origin:** Switzerland

**ORCID number:** Christos Tsagkaris 0000-0002-4250-574X; Stavros P Papadakos 0000-0003-1583-1125; Dimitrios V Moysidis 0000-0001-9083-0267; Andreas S Papazoglou 0000-0003-4981-8121; Alexandra Koutsogianni 0000-0002-4215-3078; Marios Papadakis 0000-0002-9020-874X.

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## REVIEW



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*REVIEW*

# Electrical neuromodulation therapy for inflammatory bowel disease

Farah Yasmin, Abdul Moiz Sahito, Syeda Lamiya Mir, Govinda Khatri, Somina Shaikh, Ambresha Gul, Syed Adeel Hassan, Thoyaja Koritala, Salim Surani

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**Farah Yasmin, Abdul Moiz Sahito, Syeda Lamiya Mir, Govinda Khatri, Somina Shaikh,** Department of Medicine, Dow University of Health Sciences, Karachi 74200, Pakistan

**Ambresha Gul,** Department of Medicine, People's University of Medical and Health Sciences, Nawabshah 67480, Pakistan

**Syed Adeel Hassan,** Department of Medicine, University of Louisville, Louiseville, KY 40292, United States

**Thoyaja Koritala,** Department of Medicine, Mayo Clinic, Rochester, NY 55902, United States

**Salim Surani,** Department of Medicine, Texas A&M University, College Station, TX 77843, United States

**Salim Surani,** Department of Anesthesiology, Mayo Clinic, Rochester, MN 55902, United States

**Corresponding author:** Salim Surani, FACP, FCCP, MD, MSc, Doctor, Professor, Department of Medicine, Texas A&M University, 00 Bizzell St, College Station, TX 77843, United States. srsurani@hotmail.com

## Abstract

Inflammatory bowel disease (IBD) is an inflammatory disease of the gastrointestinal (GI) tract. It has financial and quality of life impact on patients. Although there has been a significant advancement in treatments, a considerable number of patients do not respond to it or have severe side effects. Therapeutic approaches such as electrical neuromodulation are being investigated to provide alternate options. Although bioelectric neuromodulation technology has evolved significantly in the last decade, sacral nerve stimulation (SNS) for fecal incontinence remains the only neuromodulation protocol commonly utilized use for GI disease. For IBD treatment, several electrical neuromodulation techniques have been studied, such as vagus NS, SNS, and tibial NS. Several animal and clinical experiments were conducted to study the effectiveness, with encouraging results. The precise underlying mechanisms of action for electrical neuromodulation are unclear, but this modality appears to be promising. Randomized control trials are required to investigate the efficacy of intrinsic processes. In this review, we will discuss the electrical modulation therapy for the IBD and the data pertaining to it.

**Key Words:** Inflammatory bowel disease; Sacral nerve stimulation; Vagus nerve stimulation; Tibial nerve stimulation; Electrical neuromodulation; Crohn's disease;

Ulcerative colitis; Neuromodulation



**Core Tip:** Inflammatory bowel disease (IBD) is an inflammatory disease of the gastrointestinal tract with no known available treatment. Electrical neuromodulation is the use of electric stimulation of nerves or brain regions as a therapeutic technique. Electrical neuromodulation therapy has been studied as a possible treatment regimen for IBD. There are several forms of neuromodulation that use various types of nerves, such as sacral nerve stimulation, vagal NS (VNS), and tibial NS. As indicated by many clinical investigations, VNS as a potential therapy for IBD has a lot of promise. More research is needed to assess the possibility of VNS as a viable cure for IBD.

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## INTRODUCTION

Inflammatory bowel disease (IBD) comprises ulcerative colitis (UC) and Crohn's disease (CD). In these conditions, neutrophils and macrophages produce cytokines, proteolytic enzymes, and free radicals, leading to inflammation and ulceration of the intestinal lining. Both UC and CD share similar manifestations, including abdominal pain, diarrhea, weight loss, and hematochezia. Malnutrition, anemia, fatigue, fever, mouth ulcers, joint pain, and skin lesions, including erythema nodosum or pyoderma gangrenosum, are the common findings[1].

The exact etiology of IBD is unknown, but the altered immune system is suggested as a possible explanation. Risk factors include race, family history, ethnicity, cigarette smoking, and non-steroidal drugs. Colon cancer, skin infection, eye and joint infection, pharmaceutical side effects, and blood clots are all common complications of CD and UC[2].

Diagnostic procedures for IBD include blood work (for anemia and infection), endoscopic procedures (colonoscopy, flexible sigmoidoscopy, upper endoscopy, capsule endoscopy, and balloon aided enteroscopy), and imaging treatments (X-ray, computerized tomography scan, magnetic resonance imaging)[2].

The common medical treatment consists of antibiotics, corticosteroids, immune regulators, aminosalicylates, Janus kinase inhibitor (JAK), anti-tumor necrosis factor-alpha (Anti-TNF-α), anti-integrin, and anti-interleukin (IL) 12/IL23. Adverse reactions include itching, erythema, and delayed allergic reactions can be seen in patients due to these medication use[3]. Therefore, more effective, and safer therapeutic choices are needed. Nerves or brain structures electrical stimulation is being studied as an intervention in a growing number of conditions, including Parkinson's disease, arthritis, and depressive disorders. The idea that bioelectrical neuromodulation can be used to treat gastrointestinal (GI) disorders has piqued the interest of the medical community[4].

## ELECTROMODULATION THERAPY FOR IBDS

The usage of electric stimulation of nerves or brain centers as a therapeutic tool is being tested in a wide variety of conditions as Parkinson's disease and schizophrenia. This approach is called neuromodulation or bioelectric neuromodulation, or electroceuticals[5]. GI tract is connected to the central nervous system *via* vagus and sacral nerve, providing disease-modifying bioelectric neuromodulation therapy opportunities[4]. Electrical neuromodulation (ENM) has been used effectively to treat variety of gastrointestinal disorders including GERD, dyspepsia, gastroparesis, fecal incontinence and constipation as shown in Figure 1. Neuromodulation may be central, as in thalamic stimulation or transmagnetic stimulation; spinally, as in spinal cord stimulation for ache and movement in spinal cord damage; vagal as regional, as in auricular stimulation for seizures; sacral, as in stimulation for genitourinary (GU)/GI dysfunction; and peripherally, as in electrified stimulation for GU/GI dysfunction peripherally, as in electroacupuncture; and enteric, as in gastric/GI electrical stimulation (GES)[6]. Sacral nerve stimulation (SNS) is the most effective neuromodulation protocol for GI disease that is currently in use[7]. Because of the dysregulation of brain-gut interactions in IBD, ENM can be

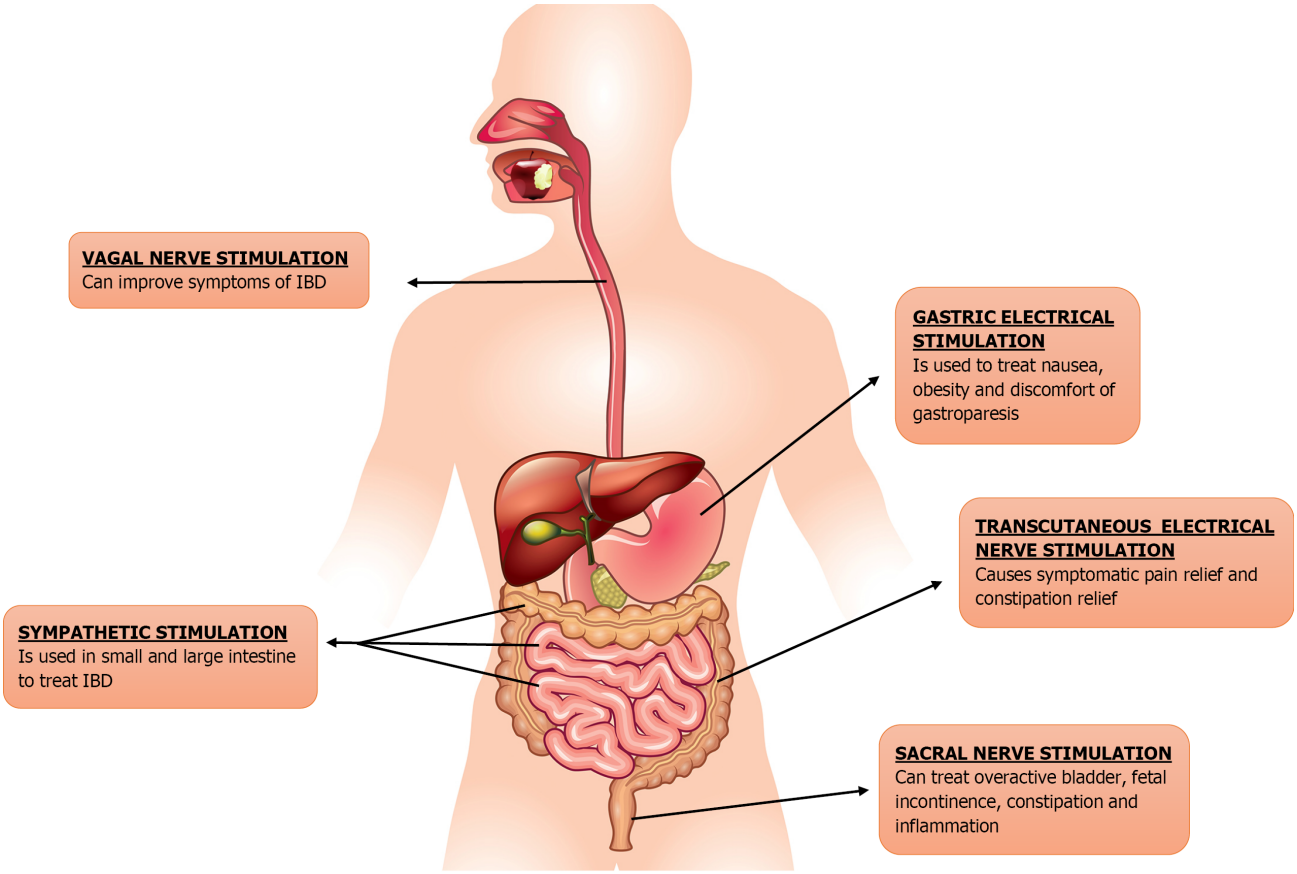


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**Figure 1 Sites of bioelectric neuromodulation to improve gastro-intestinal symptoms.** In animal research and experimental clinical settings, neuromodulation has been used to treat a ramification of gastrointestinal (GI) illnesses at numerous sites on neurons innervating the gastrointestinal tract. Some of the neuromodulation techniques such as transcutaneous electrical nerve stimulation, sympathetic stimulation, vagal nerve stimulation, and gastric electrical stimulation are mentioned in the figure above that relieve the symptoms related to inflammatory bowel disease. IBD: Inflammatory bowel disease.

considered as a treatment option[8]. Numerous electrical neuromodulation techniques for treating IBD, *i.e.*, we will be discussing vagus NS (VNS), SNS, and tibial NS (TNS), in this review.

## INTERPLAY BETWEEN BRAIN-GUT AXIS/EXTRINSIC GI INNERVATION

The GI tract (GIT) has intrinsic (enteric nervous system) as well as extrinsic innervation (gut-brain axis). The gut-brain axis is bidirectional in nature, mediated through hormonal, neural, metabolic, and immunological responses. It carries different sensations such as GIT pressure changes, ischemia, poisons, bacterial infection, gastric acidity, and inflammation of the brain through afferent fibers, as demonstrated in Figure 2[9]. These fibers then carry information to the brain, which sends efferent signals to the gut and associated organs, causing toxic substances to be removed, decreasing acid production, increasing satiety, and nausea, to name a few. Recently, the gut microbiota is also included in the gut-brain axis[10], which links intestinal microbiota and the brain[11].

Accurate extrinsic innervation is crucial for the proper functioning of the gut as well as for the balanced emotional and psychological responses through dual connections between brain and gut[12]. Various researches have listed the effects of the brain on the gut or vice versa, signaling, *e.g.*, how depression and impaired brain functioning can increase an individual's vulnerability to IBD. Whereas other experimentations have shown the prevalence of psychic and anxiety-related disorders in IBD patients, these researches show a close interplay between the gut and the brain[8].

The complex pathway connects the central nervous system (CNS), sympathetic ganglia, enteric nervous system, and gastrointestinal effector tissues. The nucleus tractus solitarius receives the communications *via* the vagal afferents, while the thoracolumbar spinal cord receives the input *via* the spinal afferents. Cervical afferents also link the esophagus to the cervical spinal cord. Intestinal-fugal neurons that amplify from the intestine to the CNS are involved in certain afferent routes. To accurately understand the specifics of the extrinsic innervation in the form of the gut-brain axis, the various pathways through which the dual interaction between the gut and the brain takes place are described in Figure 2.

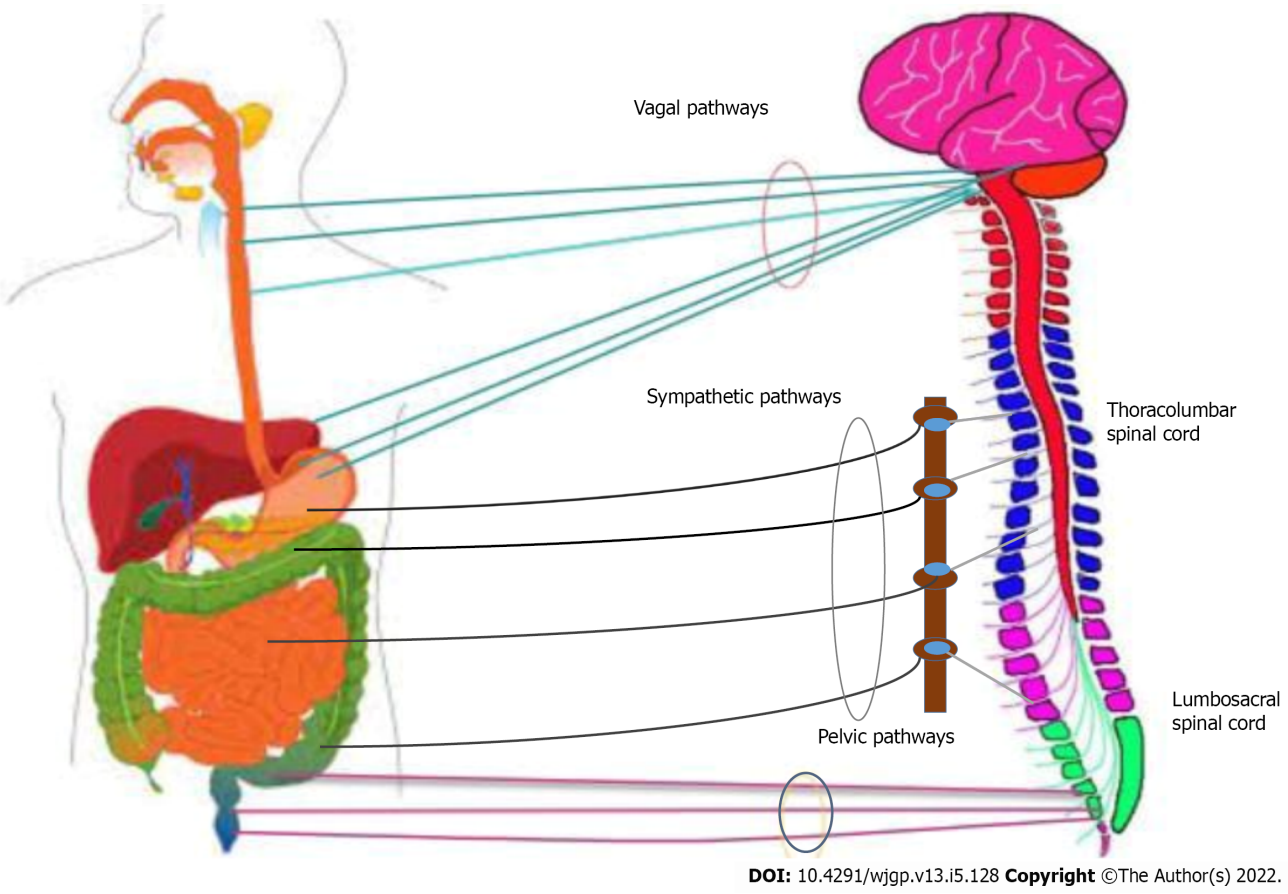


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**Figure 2 Gastrointestinal tract's extrinsic innervation.**

## NEURAL CONTROL OF GUT INFLAMMATION

### *Influence of vagal pathway on gut inflammation*

Preganglionic neurons of cranial nerve corticofugal fibers protrude from the medulla' dorsal motor nucleus of the vagus nerve and innervate the muscular and tissue layer layers of the gut, each within the lamina propria and also the muscularis externa of the viscus wall[13]. Food, antigens, potential pathogens, and symbiotic intestinal microbiota are always present in the gastrointestinal system, and some of them may present as risk factors for intestinal inflammation[14]. TNF-α, a cytokine, is released by activated macrophages, nerve fiber cells, and different tissue layer cells in response to the infective toxin and other harmful stimuli to cause inflammation[15,16]. Counter-regulatory mechanisms consist of capable immune cells and anti-inflammatory cytokines that inhibit inflammatory mediators' transfer into the circulation. As an anti-inflammatory mechanism, there is a fine relationship between neurological and immune system processes. The dorsal vagus complicated (DVC), which has the sensory nuclei of the solitary tract (NTS), the area postema (AP), and also the dorsal motor core of the cranial nerve (DMN), responds to higher current levels of TNF-α by increasing motor levels activity within the vagus nerve[17]. Two studies have shown that electrical cranial nerve stimulation will suppress inflammation in models of inflammation[18,19]. Furthermore, due to the lack of control on immunological mediating cells, the sub-diaphragmatic vagotomy increases inflammation in the gut. The brain can monitor immunological states and detect peripheral inflammation through two mechanisms.

### *Neural pathway*

Stimulation of the vagus nerve is triggered directly or indirectly by cytokines discharged by nerve fiber cells, macrophages, and different vagus-associated immune cells and indirectly by chemoreceptors[20]. Visceral afferent vagus fibers within the neural structure nodosum principally end in the DVC of the medulla oblongata. DVC includes NTS, the dorsal motor nucleus of the vagus (DMV), and also the post-mammary region (AP)[21]. DMN is a critical region for the formation of preganglionic vagus efferent fibers. The majority of sensory vagal input is received by the NTS[22]. The paraventricular nucleus (PVN) of hypothalamus, receives signals from the NTS. PVN causes the production and release of corticotropin-releasing hormones (CRH), which is an important chemical on the hypothalamus-pituitary-adrenocortical (HPA) axis (described below)[23].

### *Humoral pathway*

Circulating cytokines in the humoral route interact directly with areas of the brain involved in anti-inflammatory response. Circulatory IL-1 and TNF can move across the blood-brain barrier through a saturated transport mechanism to get into the CSF and interstitial space of the brain and spinal cord, where they can directly stimulate the brain to produce an anti-inflammatory reaction[24]. Circumventricular organs that lack regular blood-brain barrier protection uses cytokine-to-brain transmission. Postrema is the most well-known circumventricular organ[23].

Followings are a few pathways that are included in the neural control of gut inflammation.

### *HPA axis pathway*

The HPA axis is composed of three major components (the hypothalamus, the anterior and posterior pituitary gland, and the adrenal cortex). Steinlein[25] demonstrated the role of vagal afferents in the neuro-immune axis in the control of the HPA axis. According to L E Goehler and co-workers, peripheral administration of lipopolysaccharides (LPS), a pro-inflammatory cytokine that stimulates vagal afferents *via* IL-1 receptors, induces the production of IL-1, a pro-inflammatory cytokine[26]. The vagal nerve is susceptible to peripheral pro-inflammatory cytokines generated by macrophages and other immune cells, such as IL-1, IL-6, and TNF-α[27]. Vagal afferent receptors (IL-1 beta) convey information to the parvo-cellular zone of the paraventricular nucleus of the hypothalamus (PVH) around corticotrophin-releasing-factor (CRF)-containing neurons. These CRF neurons subsequently drive the hypophysis to release the adreno-corticotrophin hormone, which stimulates the adrenal glands to release glucocorticoids, reducing peripheral inflammation[27]. Glucocorticoids affect the inflammatory response by suppressing immune cell release of pro-inflammatory cytokines, as well as inhibiting vasodilation and vascular permeability caused by inflammation. The brain can influence the activity of functional intestinal effector cells such as immune cells, smooth muscle cells, epithelial cells, interstitial cells of Cajal, enteric neurons, and enterochromaffin cells through neuronal and hormonal communication lines [24]. These cells, on the other hand, are influenced by the gut microbiome. The internal organ microbiota encompasses a vital influence on the intestinal axis, not solely through native interaction with intestinal cells conjointly with the enteric systema nervosum, but also through direct effects on the system and metabolic processes[28]. Emerging evidence supports the function of gut bacteria in anxiety and depressive-like behavior[29].

### *Cholinergic anti-inflammatory pathway (ach axis)*

Acetylcholine is a crucial neurochemical and neuromodulator within the brain, mediates neuronal transmission in sympathetic and parasympathetic neurons, and acts as a primary neurotransmitter in parasympathetic/pneumogastric neural structure corticoefferent neurons[23]. This neurotransmitter acts through two varieties of receptors: muscarinic (metabotropic) and nicotinic (ionotropic)[30,31]. The seven component of the nicotinic acetylcholine receptor is displayed on phagocytes[32]. TNF production from human macrophages used by endotoxins is considerably reduced by acetylcholine through a post-transcriptional mechanism and is concentration-dependent. The authors demonstrated the connection of a bungarotox-insensitive vasoconstrictor receptor in suppressing cytokine production in vitro by neurotransmitter mistreatment specific muscarinic and nicotinic agonists and antagonists[23]. Apart from TNF, acetylcholine suppresses alternative endotoxin-inducible pro-inflammatory proteins reminiscent of IL-1, IL-6, and IL-18 through a post-transcriptional mechanism. However, acetylcholine has no effect on the discharge of the anti-inflammatory cytokine IL-10 from endotoxin-stimulated macrophages[32]. Nicotinic acetylcholine receptors are a ligand-gated pentameric ion channel family. The HPA axis (afferent vagal Fibers) activates the cholinergic anti-inflammatory pathway. Proinflammatory cytokines discharged throughout the immunologic response will activate vagal receptive signals, resulting in direct or indirect activation (via the core of the neurons of the solitary tract NTS) of the vagal efferents in the DMN. As a result, the sensory vagal afferents and motor vagus efferents produce an inflammatory reflex that constantly monitors and modifies the inflammatory condition in the periphery[33]. Since the tetravalent guanyl hydrazone CNI1493 induces the activation of the vagus and, by activating the cholinergic anti-inflammatory signal pathway, confers anti-inflammatory effects in each native and general model of inflammation, it's going to be attainable to activate the cholinergic anti-inflammatory pathway (with centrally active substances)[34].

### *Vagal sympathetic pathway*

The celiac, superior mesenteric, and inferior mesenteric ganglia contain the cell bodies of the bulk of postganglionic sympathetic neurons that innervate the gastrointestinal tract[35]. In gut noradrenaline (NA) is the primary neurotransmitter released from sympathetic postganglionic nerve terminals; however, ATP and neuropeptide Y (NPY) can also engage in sympathetic neurotransmission within the GI tract[36,37]. The vagal afferent Fibers terminate in the NTS, which ultimately activates the central autonomic network (CAN). The sympathetic outlet is operated by 5 CAN brain regions (the paraventricular nucleus of the neural structure HPV, the noradrenergic cluster A5, the area of the caudal raphe, the rostral ventrolateral medulla, and ventromedial medulla)[38]. By increasing sympathetic outflow, the vagal nerve can generate a non-direct anti-inflammatory reaction. Abe *et al*[39] explained

the role of the C1 adrenergic cluster. They concluded that these neurons are concerned with protecting the result of stress in reperfusion injuries because of nephritic anemia *via* a sympathetic pathway. They conjointly mentioned how activation of vagal afferents in mice twenty-four hours before injury considerably reduced acute excretory organ inflammation and plasma levels of TNF-α[30]. Tyrosine hydroxylase is found in the lamina propria, the submucosa, the ganglia of the nerve plexus, and lymph follicles (Peyer' plaques)[40]. Adrenergic receptors of diverse types are expressed by macrophages. In vitro, beta receptors mediate the anti-inflammatory effects of agonists on macrophages derived from the intestine[41]. Although sympathetic nerves decrease gut inflammation, persistent nerve stimulation should be avoided as it can promote stasis and aggravate bacterial growth in Crohn's illness[4].

### *Vagal splenic pathway*

Through an association between the VN and the splenic nerve, the Vago-splenic pathway works collectively[42]. In general inflammatory conditions, the spleen is a crucial supply of inflammatory cytokines, and excision considerably reduces circulating TNFα levels in mouse endotoxemia[43]. Tracey *et al* identified the vagal splenic route, finding that VNS caused the celiac ganglion to produce acetylcholine (Ach), which subsequently adhered to the c7nAChR of the splenic neve to release norepinephrine (NE) in the spleen[24]. Following that, it binds to beta two adrenergic receptors of splenic lymphocytes, which produces acetylcholine, which will act on the α c7nAChR of splenic macrophages limiting release of TNF, resulting in an anti-inflammatory impact[44]. According to Martelli *et al*[45], there is also a non-nervous relationship between the vagus and splenic sympathetic nerves. In another article, Martelli *et al*[46] noted how the sympathetic nerve, not the vagal nerve, is the efferent mediator of the cholinergic anti-inflammatory pathway (splenic nerve).

## CAPSAICIN-SENSITIVE AFFERENTS AND INFLAMMATION REGULATION

Electrical and physiological stimulation of receptive neurons, particularly afferent nerves of the digestive tract, generates the release of transmitters at their peripheral ends, most often tachykinin and the amide (CGRP) linked to the calcitonin gene[47]. CGRP serves a number of purposes *via* serving as a modulator, transmitter, and hormone. CGRP-containing nerve Fibers are numerous surrounding blood vessels, particularly arterioles, suggesting that they may have a physiologic role in regulating blood flow to the gastric mucosa[48]. Capsaicin-sensitive afferent Fibers conduct protective anti-inflammatory activities in the gastrointestinal tract by releasing peptides from their peripheral ends[49-52]. Sensory inputs innervating the stomach generate CGRP, which reduces mucosal damage and improves mesenteric and mucosal blood flow in stomachic ulcer models in rats and mice[50,51]. Once administered at the time of injury, capsaicin promotes the discharge of neuropeptides and reduces the extent of ethanolin-induced gastric injury in rats[49,52]. This impact is operated by the discharge of CGRP from receptive nerve endings before their degeneration, which happens hours or days after the capsaicin injection. Numerous studies have shown that hCGRP (837), a fraction of human CGRP lacking the cyclic loop at the amino terminus of native CGRP, inhibits the action of exogenous CGRP[53,54].

## VNS FOR INTESTINAL BOWEL DISEASE

VNS is a unique therapeutic method for chronic TNF-mediated inflammatory illnesses in the framework of bioelectronic medicine, with the objective of employing tiny stimulators to provide electrical nerve signals for therapeutic, rather than pharmaceutical, purposes[55-57]. VNS is already used to treat depression and epilepsy which is resistant to drugs[58]. There is currently no recognized curative medicine for IBD. Current medicines reduce disease activity, and when therapy is stopped, the condition recurs. TNF is one of the most significant cytokines in IBD, and anti-TNF medicines have transformed the therapy of the disease[59]. New compounds are available that target pro-inflammatory cytokines such as IL-12, IL-23, anti-integrin, and anti-JAK therapies[60,61]. In the case of treatment failure or an IBD consequence (perforation, abscess, stenosis), surgery is an option, although the disease reappears after the procedure. While anti-TNF medications are effective in IBD, there is a 20%-30% initial non-response rate, and the yearly chance of anti-TNF reactivity is 13% per patient year for infliximab and 20% per patient year for adalimumab[62-64]. This lack of secondary response is attributable to I the formation of autoantibodies, particularly for infliximab but also, to a lesser extent, for adalimumab, or (ii) secondary failure due to insufficient dose[65,66]. As a result of the risk of adverse effects and the requirement for ongoing therapy for these disorders, patients are increasingly hesitant to begin and maintain these treatments once they are in remission. The non-compliance rate is 30%-50% [67,68]. Therefore, targeted therapy for pro-inflammatory cytokines such as TNF-alpha and others using CAP could be extremely helpful with fewer side effects, no compliance issues, and cheaper than biologics (*i.e.*, anti TNF-alpha). In this case, targeting the VN's anti-inflammatory characteristics might be of interest. VNS, particularly as a non-drug therapy, has the potential to be employed as an

alternative to conventional biological therapies. A number of animal and clinical research have been undertaken in recent years to investigate the efficacy of VNS in the treatment of IBD (Supplementary Table 1).

### *Animal evidence*

Vagotomy has been found in several studies to enhance the disease activity index (DAI), gross and pro-inflammatory cytokine levels in mice[69-71]. To replicate UC, Chen and colleagues employed dextran sodium sulphate (DSS) colitis in mice. They observed that VNS eased cerebral cortical microinfarcts induced by a two-photon laser and reduced DSS colitis. This neuroprotection was linked to decreased blood-brain barrier permeability and inflammatory processes[72].

### *Human evidence*

Indirect data suggests that a vagal anti-inflammatory action plays a role in IBD. Vagal activity has been demonstrated to be inversely associated with inflammatory markers in healthy and cardiac patients as evaluated by HRV spectral analysis[73]. VNS might be an attractive method for the treatment of IBD based on pre-clinical results in rats with colitis and two recent clinical pilot trials targeting two distinct categories of patients with active CD, either ignorant of anti-TNF on inclusion or resistant to biologics [74].

## LABORATORY AND CLINICAL STUDIES

### *Animal studies*

Miceli and Jacobson[75] published the first data on the anti-inflammatory effects of VN in digestive inflammation. Colitis in rats with 2,4,6 trinitrobenzenesulfonic acid (TNBS) improved with early treatment of anticholinesterase medications such as neostigmine, which does not cross the blood-brain barrier, or physostigmine. This impact was more pronounced with physostigmine, indicating a dominating central mechanism. In mice, vagotomy aggravated experimental colitis, indicating that NV serves a protective function[76]. It was demonstrated that in the non-vagotomized watchful rat, 3 h per day for five consecutive days, low-frequency VNS (5 Hz) led in an improvement in TNBS colitis in rats [31] VNS inhibited weight loss and inflammatory indicators.

An improvement in a multivariate measure of colitis was also observed as an anti-inflammatory impact (which includes body mass, temperature, and motor function, macroscopic area of the lesions, histological and biological parameters such as myeloperoxidase activity, cytokines, and mRNAs related to cytokines)[77]. Sun *et al*[32] showed that chronic VNS increased the clinical activity index, the histological scores, the biological inflammation due to myeloperoxidase activity, the iNOS, TNF, and IL-6 Levels among rats with colitis, and the inflammatory response induced by LPS in cells of the human epithelial colorectal adenocarcinoma (Caco2) by ACh *in vitro*. In 2000, Kevin Tracey's team first described CAP[78,79]. They found that there is an inflammatory reflex in which proinflammatory cytokines stimulate vagus afferents, which activate vagus efferents, causing the production of these cytokines by tissue macrophages, mainly TNF, but also other pro-inflammatory cytokines such as IL-6. IL-1b, but not IL-10, an anti-inflammatory cytokine VN has anti-inflammatory effects because it inhibits pro-inflammatory cytokines.

### *Human studies*

Decreased vagus activity was observed to be related to systemic inflammatory markers in both IC and CD patients[80,81]. VNS improved several inflammatory markers in rats' small intestines, including fecal quality, inflammatory processes, and leukocyte infiltration. Furthermore, considerable cardiac and respiratory changes happened with supra-threshold cervical VNS, while abdominal VNS caused alterations. Due to the lack of side effects and effectiveness in reducing inflammation, abdominal VNS appears to be a viable alternative to cervical VNS. This evidence supports the application of this novel peripheral nerve network for abdominal VNS as a potential therapy for IBD like CD[82]. A pilot study on VNS was carried out for the first time in patients with moderate to severe celiac disease as an alternative to drug anti-TNF therapy or in untreated patients in a translational approach from the laboratory to the bedside[56]. A VNS device and electrode were implanted in nine patients. At the time of implantation, two patients had failed immunosuppressive drugs (azathioprine), while the other seven received no treatment[56]. ENV was carried out on a continuous basis over a period of one year. In April 2012, the first patient was implanted, and then the last in March 2016.

Due to increasing condition, two patients were removed from the trial after three months of neurostimulation: The first had ileocecal resection but elected to continue neurostimulation until the end of the study due to an early good response and rejection of pharmaceutical therapy. The second patient took infliximab and azathioprine and continued to use an active VNS. Six patients were in remission owing to neurostimulation alone after one year of follow-up, while the seventh was in relapse. In April 2012, the first patient to get the implant was in remission from azathioprine in ileal CD with a history of ileocecal resection[56]. In conclusion, five out of seven patients who received the one-year VNS attained

clinical improvement (CDAI 150), and all gained the CDAI70 response (CDAI decreased 70 points from baseline). Similarly, the Endoscopic CD Severity Score (CDEIS) decreased from 60% to 100% in five patients. Other than complaints caused by the output current/intensity of the device, no adverse events were observed[56]. In patients with UC decrease activity has been linked with autonomic function[83].

### Devices and methods

Currently, the generally used VNS therapeutic equipment is invasive and implantable. The VNS Therapy System consists of an implanted pulse generator, a bipolar VNS electrode, a small handheld device, programming software, a programming stick, and hand magnets. VNS is traditionally used to treat epilepsy and depression, as well as in the two pilot studies in patients with CD.

It is invasive, generally performed by a neurosurgeon who is experienced in the surgery, and lasts 1 h with minimal side effects. Noninvasive (n) VNS may be beneficial in certain patients who are afraid to have surgery in a vasculo-nervous location, such as the vein or the external carotid artery, which are close to the VN. Furthermore, if the device is removed, the electrode wrapped around the VN is normally kept in place, although some writers have removed it without causing significant nerve and artery damage[84]. Anesthesia is necessary for the operation, which requires two small incisions. The bipolar lead is looped around the left cervical VN and the pulse generator is positioned in the top-left chest. Physicians program the stimulator with a small handheld device, programming software, and a programming stick. After implantation, patients are given a wearable magnet to manipulate the stimulation on their own. The left vagus, which is more intimately linked to cardiovascular activities, is considered more suitable than the right cervical vagus. In the treatment of epilepsy, right-sided VNS has been observed in numerous patients[85-87]. Right-sided VNS appears to be as effective as, if not more successful than, left VNS[88]. Gadgets stimulating the VN on the cervical degree or on the auricular degree were produced (Figure 3). Certainly, the cymbal concha of the external ear is innervated by means of a sensory auricular branch of the VN that sends projection inside the NTS in cats and human beings[89-91]. These noninvasive devices have not been associated with any significant major side effects. In comparison to invasive VNS, n-VNS has the disadvantage of low compliance, which is a major concern in the treatment of IBD. Indeed, 30%-40% of IBD patients fail to take their medicine[92]. One can wonder if the same problem arises with these noninvasive devices. Furthermore, in the case of the Gamma core device, the repeatability of the placement of the discs in contact with the VN is unknown. Finally, ta-VNS was less efficient than VNS in decreasing the LPS-induced serum cytokine (TNF, IL-1, and IL-6) response in a septic shock animal[93].

### Mechanism of VNS

An unexpected receptor mechanism underpins the anti-inflammatory effect of the Vagus nerve. In comparison to many "classical" physiological activities, which might be managed with the aid of metabotropic mAChRs, the anti-inflammatory effects of the Vagus nerve are mediated *via* ionotropic nicotinic acetylcholine receptors (nAChRs)[94]. The frequency of stimulation for VN activation is critical to the function of various therapies[95-100]. A couple of studies have indicated that mAChRs, especially the M1 mAChR, play a role in this regulation in endotoxemia, inflammatory bowel disorder (colitis), hemorrhagic shock, and other illnesses[101,102-104].

Increased cholinergic transmission in the brain with centrally acting acetylcholinesterase inhibitors, particularly galantamine, leads to inhibition of unusual inflammatory responses generated by vagus nerve impulses in mice models of endotoxemia, colitis, and lupus[105-107]. The most recent work, which used targeted optogenetic stimulation and sophisticated pharmacological methods, discovered that forebrain signal transduction and M1 mAChR play a unique role in the modulation of peripheral inflammatory responses in endotoxemia mice *via* vagus nerve transduction[108]. VNS blocks splenic TNF, which has been identified as a primary contributor to systemic TNF. It is critical to understand how the vagus nerve regulates cytokines in the spleen. The vagus nerve innervates the celiac ganglia and the superior mesenteric ganglion, which have been shown to provide neurons to the splenic nerve [101].

## SNS

The sacral nerves are divided into five pairs. Each contains an afferent and efferent component, allowing for effective interaction between the lower GIT and the nervous system. The activity of the lower GIT (descending colon, rectum), sexual organs, and urinary bladder is modulated by the parasympathetic component of sacral nerves. The principal somatic nerve of the sacral plexus is the pudendal nerve (S2-S4). It is both sensory and motor. The external anal sphincter, which is under our conscious control, receives sensory and motor innervation from it. It also gives sensation to the external genitalia, the skin around the anal area, the anal canal, the perineum, and motor innervation to the external urethral sphincter.

SNS, also known as "sacral neuromodulation", is a relatively new and promising treatment option. SNS uses an implanted device that stimulates the S3 nerve root and offers a wide range of applications

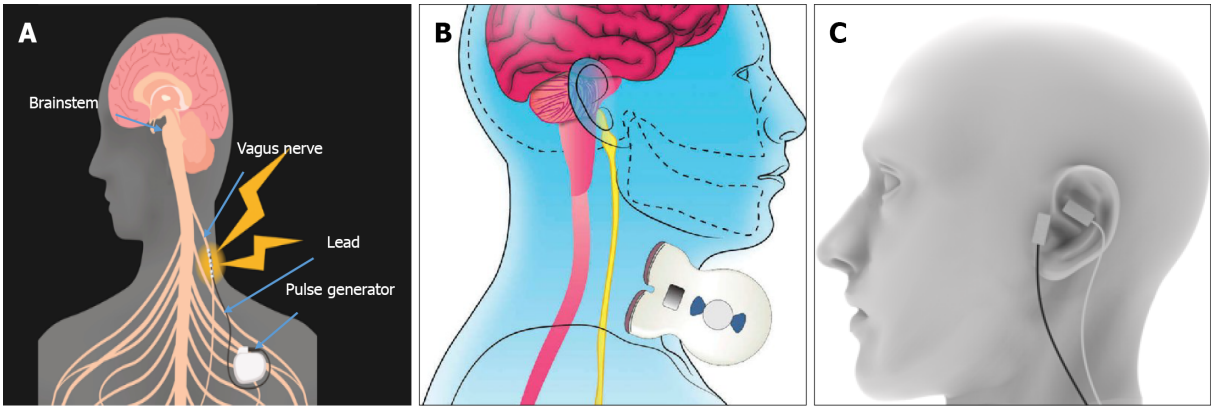


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**Figure 3 Vagus nerve stimulation.** A: Direct vagus nerve stimulation; B and C: Noninvasive vagus nerve stimulation; transcutaneous cervical vagus nerve stimulation (VNS) (B); transcutaneous auricular VNS (C).

in conditions such as urgency urinary incontinence, pelvic pain, detrusor stimulation with transurethral approach, FI, *etc*. Following are some applications of SNS in relation to IBD[3].

## SACRAL NEUROMODULATION FOR INFLAMMATION AND INTESTINAL BARRIER IN IBD

Experimental and clinical evidence from several studies signifies the potential of SNS as a treatment option for IBD Patients who have received SNS had less severe mucosal lesions than those who have not received SNS. SNS also improves the recovery of enema caused by trinitrobenzene sulfonic acid (TNBS enema). With elevated TNF1 and trypsin levels, SNS also increases the number of mucosal neutrophils. SNS also stopped TNBS-induced inflammatory factors, including IL-4 and IL-1, from rising. All of these variables indicate that sacral neuromodulation is beneficial in restoring the intestinal barrier following mucosa injury[109]. In IBD, SNS has a significant anti-inflammatory impact. SNS enhanced the spinal afferent-vagal efferent pathway and improved autonomic function by increased vagal efferent activity. SNS also causes anti-inflammatory effects due to the SNS-mediated release of Ach[110]. In a study using the TNBS rat model, sacral neuromodulation lowered the level of pro-inflammatory cytokines and improved colonic inflammation[111].

## SACRAL NEUROMODULATION IN FECAL INCONTINENCY

The inability to regulate bowel movements, which can range from modest rectum leaks to total bowel control, is known as FI. Viability of sacral neuromodulations as a treatment option for FI is tremendous. Many studies have demonstrated that FI responds positively to SNS. SNS has proven to be a reliable method for dealing with FI in children[112]. Clinical trials have also shown that SNS can help with FI [113]. Another extensive approach conveys the benefits of SNS in patients with neuropathic FI[114].

## OTHER METHODS OF NEUROMODULATION

Other than SNS and VNS, other neuromodulation methods to treat IBD are TNS and Spinal Cord Stimulation (SCS). The sensory, motor and autonomic fibers in the tibial nerve make it a mixed nerve. It is caused by the L4-S3 nerves, which feed the colorectum, bladder, and pelvic floor. TNS uses electrical impulses to treat bladder and pelvic floor issues. TNS is classified into two types: Percutaneous TNS (PTNS) and transcutaneous TNS (TCTNS) (TTNS). The former makes use of a needle electrode, whilst the latter makes use of a sticky electrode[3]. PTNS is a minimally invasive method that has been demonstrated to be beneficial in treating overactive bladder, FI, and pelvic discomfort. Having few side effects is highly convenient, but it is limited by the necessity that patients visit the clinic weekly to obtain the series of treatments[115].

The actual mechanism of TNS is uncertain however it appears to involve excitation of afferent pathways to the sacral spinal cord as well as regulation of efferent nerves[116]. Retrospective research looked at 183 individuals with refractory overactive bladder (OAB) who had 30-min PTNS sessions for 12 wk during nine years. There was a significant improvement in micturition frequency, nocturia, and urge incontinence episodes in the PTNS group, with the impact obvious by week 10 of therapy. With a

wide range of PTNS times, 61.5 percent of subjects self-proclaimed > 50% improvement in signs and symptoms, raising the subjective accomplishment percentages[117]. For a 12-wk treatment period, a recent randomized research of forty women with nocturia of weekly TTNS periods compared pelvic floor muscle training and behavioral therapy. Both medicines improved sleep quality by reducing the number of times people awoke to pee (45 percent reduced by 1 in both groups)[118]. A spinal cord stimulator (SCS) is surgically implanted under the skin and delivers a weak electrical current to the spinal cord. Current from a pulse generator is carried to the spinal cords' nerve fibers by thin wires. When the SCS is activated, it stimulates the nerves in the area where a person is feeling pain. The pain signal is altered and masked by electrical impulses, prohibiting it from going to the brain[119].

For more than a half-century, spinal cord stimulation (SCS) has been used to treat chronic pain. Several studies have demonstrated that SCS can help with stomach discomfort[120]. Randomized trial has shown that SCS can lessen diarrhea and pain pain in persons with irritable bowel syndrome[121]. Although it has been quite successful, some people might experience device-related challenges such as pain at the implantation site or subsequent infections. But it doesn't cause any serious complications like paralysis or hemorrhage in the epidural space[122,123].

## CONCLUSION

The digestive system's broad and approachable interaction with the CNS, the predominance of IBD, and the lack of effective treatment options make it an appealing target for bioelectrical neuromodulation therapy for digestive system innervation. A wide range of gastrointestinal problems has been treated with various degrees of success. This approach has been tried with different degrees of effectiveness in a range of gastrointestinal diseases. SNS for faecal incontinence has become a popular bio-electric therapy for gastrointestinal disorders. The development of bioelectrical digestive system neuromodulation medicines requires investigation. The advancement of our understanding of the multiple roles of the mixed nerve components, such as vagus nerves and sympathetic routes to the intestines, should allow us to take IBS treatment to a new level.

## FOOTNOTES

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**Country/Territory of origin:** United States

**ORCID number:** Farah Yasmin https://orcid.org/0000-0002-5264-6140; Abdul Moiz Sahito 0000-0001-7748-3440; Syeda Lamiya Mir 0000-0001-9327-4390; Govinda Khatri 0000-0002-7233-753X; Somina Shaikh 0000-0002-9943-9907; Ambresha Gul 0000-0002-0618-1397; Syed Adeel Hassan 0000-0002-8484-0319; Thoyaja Koritala 0000-0002-1020-9882; Salim Surani 0000-0001-7105-4266.

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*REVIEW*

# Epilepsy and the gut: Perpetrator or victim?

Mohammed Al-Beltagi, Nermin Kamal Saeed

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**Mohammed Al-Beltagi,** Department of Pediatrics, Faculty of Medicine, Tanta University, Tanta 31527, Algharbia, Egypt

**Mohammed Al-Beltagi,** Department of Pediatrics, University Medical Center, King Abdulla Medica City, Arabian Gulf University, Manama 26671, Bahrain

**Mohammed Al-Beltagi,** Department of Pediatrics, University Medical Center, King Abdulla Medical City, Dr. Sulaiman Al Habib Medical Group, Manama 26671, Bahrain

**Nermin Kamal Saeed,** Medical Microbiology Section, Department of Pathology, Salmaniya Medical Complex, Ministry of Health, Kingdom of Bahrain, Manama 26612, Bahrain

**Nermin Kamal Saeed,** Department of Microbiology, Irish Royal College of Surgeon, Busaiteen 15503, Muharraq, Bahrain

**Corresponding author:** Mohammed Al-Beltagi, MBChB, MD, MSc, PhD, Chairman, Professor, Department of Pediatrics, Faculty of Medicine, Tanta University, Al Bahr Streat, Tanta 31527, Algharbia, Egypt. mbelrem@hotmail.com

## Abstract

The brain and the gut are linked together with a complex, bi-path link known as the gut-brain axis through the central and enteric nervous systems. So, the brain directly affects and controls the gut through various neurocrine and endocrine processes, and the gut impacts the brain *via* different mechanisms. Epilepsy is a central nervous system (CNS) disorder with abnormal brain activity, causing repeated seizures due to a transient excessive or synchronous alteration in the brain's electrical activity. Due to the strong relationship between the enteric and the CNS, gastrointestinal dysfunction may increase the risk of epilepsy. Meanwhile, about 2.5% of patients with epilepsy were misdiagnosed as having gastrointestinal disorders, especially in children below the age of one year. Gut dysbiosis also has a significant role in epileptogenesis. Epilepsy, in turn, affects the gastrointestinal tract in different forms, such as abdominal aura, epilepsy with abdominal pain, and the adverse effects of medications on the gut and the gut microbiota. Epilepsy with abdominal pain, a type of temporal lobe epilepsy, is an uncommon cause of abdominal pain. Epilepsy also can present with postictal states with gastrointestinal manifestations such as postictal hypersalivation, hyperphagia, or compulsive water drinking. At the same time, antiseizure medications have many gastrointestinal side effects. On the other hand, some antiseizure medications may improve some gastrointestinal diseases. Many gut manipulations were used successfully to manage epilepsy. Prebiotics, probiotics, synbiotics, postbiotics, a ketogenic diet, fecal microbiota transplantation, and

vagus nerve stimulation were used successfully to treat some patients with epilepsy. Other manipulations, such as omental transposition, still need more studies. This narrative review will discuss the different ways the gut and epilepsy affect each other.

**Key Words:** Epilepsy; Epilepsy with abdominal pain; Gut; Gastrointestinal diseases; Gut-brain-microbiota axis; Abdominal aura; Ketogenic diet; Abdominal migraine



**Core Tip:** The brain and the gut have an intense but complex interaction through a strong relationship between the enteric and the central nervous systems. Epilepsy and the gut may affect each other in diverse ways. About 2.5% of patients with epilepsy are misdiagnosed as gastrointestinal disorders, especially at an early age. Gut dysbiosis also has a significant role in epileptogenesis. Epilepsy affects the gastrointestinal tract in different forms, such as abdominal aura, epilepsy with abdominal pain, and the adverse effects of antiseizure medications on the gut and the gut microbiota. Simultaneously, many gut manipulations successfully managed some cases of epilepsy.

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## INTRODUCTION

The human body organs and systems interact with each other in harmony. However, the interaction between the brain and the gut is overly complex, forming a two-way link known as the gut-brain axis through the central and the enteric nervous system. The enteric nervous system is the most crucial autonomic nervous system component. It has common structural and functional similarities with the brain, consequently named the second brain, forming 90%-95% of total body serotonin[1]. It is uniquely prepared with intrinsic microcircuits to orchestrate the gastrointestinal functions independent of the central nervous system (CNS) control[2]. The brain directly affects the stomach and intestines and controls the gut through various neurocrine and endocrine processes[3].

On the other hand, the gut impacts the brain *via* different mechanisms, including neuropeptide and neurotransmitter release such as leptin and serotonin, vagus nerve activation, immune signaling through controlling the release of secretory IgA, affecting the integrity of mucous membrane barrier through Zonulin protein, and local production of short-chain fatty acids such as butyrate by gut microbiota[4]. The gut-brain axis explains the effects of the emotional and cognitive centers of the brain and its control over peripheral intestinal functions. It also describes how a chronic painful abdominal condition such as irritable bowel syndrome (IBS) can affect the cognitive and psychological function of the body[5]. Many neurological disorders, including hereditary, metabolic, infectious, vascular, inflammatory, and metabolic diseases, may affect the brain and gastrointestinal tract. Consequently, the clinical neurological or gastrointestinal findings may assist in confirming the diagnosis or reducing the differential diagnosis[6]. This review sheds some light on the relationship between epilepsy, a common neurological disorder, and its effects on the abdomen and vice versa.

## EPILEPSY AND SEIZURE DISORDERS IN GASTROINTESTINAL DISORDERS

Epilepsy is a CNS disorder with abnormal brain epileptic activity, causing repeated seizures or periods of sudden abnormal motor or sensory behavior and sometimes impaired or even loss of consciousness due to a transient excessive or synchronous alteration in the brain's electrical activity. Any part of the brain can be affected by epileptic activity, especially the mesial part of the temporal lobes[7]. Epilepsy is a common neurological condition, affecting about 5%-10% of the population at a particular time of their life and about 0.5%-1.0% of children. It can affect any age or sex and all races[8].

According to the etiology, there are four main types of epilepsy, idiopathic, symptomatic, provoked, and cryptogenic, resulting from genetic, structural/metabolic, immunological, infectious, or unknown causes. Idiopathic epilepsy is pure epilepsy resulting from a single gene disorder or complex inheritance. Symptomatic epilepsy has predominately genetic or developmental causation such as childhood epilepsy syndromes, progressive myoclonic epilepsies, neurocutaneous syndromes, other single-gene

neurologic disorders, chromosomal disorders, developmental cerebral structure anomalies, perinatal and infantile causes, cerebral trauma, tumor, or infection, cerebrovascular disorders, cerebral immunologic disorders, or degenerative brain diseases. Provoked epilepsy could arise from provocation factors like fever or menses or reflex epilepsy such as photosensitive or reading epilepsies. Cryptogenic epilepsies are "unknown" and more common in adults than in the pediatric age[9,10]. Due to the strong relationship between the enteric nervous system and the CNS is always single and never be multiple, gastrointestinal dysfunction can be seen in neurological disorders, and neurological dysfunction can be seen in gastrointestinal disorders[11]. About 2.5% of patients with epilepsy were misdiagnosed with gastrointestinal disorders, especially in children below the age of one year[12].

### Gastroesophageal reflux and gastroesophageal reflux disease

Gastroesophageal reflux disease (GERD) is a common childhood disorder. It can simulate epileptic seizures and may be misdiagnosed as epilepsy. Sandifer Syndrome is a distinct clinical entity presented with GER, irritability, and abnormal head and body movements with spasmodic contractions of the neck. It may appear as paroxysms with abnormal neurobehavior like crying, irritability, torticollis, head/eye version, and extensor spasm of the neck with dystonic posturing. These paroxysms may simulate epilepsy and can be misdiagnosed with specific types of epilepsy, particularly infantile spasms [13].

On the other hand, epilepsy can be missed as GERD. Sweetman *et al*[14] reported a gelastic seizure due to hypothalamic hamartomas misdiagnosed as GERD[14]. Eating epilepsy is a type of feeding-related reflex focal epilepsy. It may be misdiagnosed as GERD, especially in very young infants[15]. Eating epilepsy should be considered if the history, clinical examination, and investigations for GER and apparent life-threatening events are absent[16].

Meanwhile, GERD is a common comorbidity in children with neurological problems such as cerebral palsy, frequently complicated with epilepsy. Early-onset neurological disease, abnormal electroencephalogram (EEG), and the presence of mitochondrial disorder are significant risk factors for severe GERD [17]. The presence of GERD in such patients may jeopardize their management and mimic refractory seizures[18]. Asymptomatic gastroesophageal reflux can induce laryngospasm during sleep. This nocturnal laryngospasm causes non-rapid eye movement parasomnias, which clinically simulate sleep-related hypermotor epilepsy. Video-EEG can differentiate between the two conditions[19]. The nocturnal choking sensation is a scary condition that may complicate insular epilepsy, nocturnal laryngospasm, and gastroesophageal reflux[20]. Acid reflux can induce obstructive laryngospasm and subsequent respiratory arrest, a probable mechanism of sudden unexpected death in epileptic patients. Proper GERD management and antiseizure medication significantly improve the prognosis[21].

### Peptic ulcer

Peptic ulcers are up to eight times more prevalent in patients with epilepsy than in the general population[22]. At the same time, epilepsy can be misdiagnosed as a peptic ulcer, as reported by Magon [23]. At the same time, a perforated peptic ulcer may provoke or complicate a generalized tonic-clonic seizure. Consequently, we should carefully consider the vital signs during seizure episodes. Omeprazole is a proton pump inhibitor effectively used to treat peptic ulcers. It has effective anticonvulsant activity through carbonic anhydrase inhibition but with rapid tolerance[24].

### Celiac disease

Celiac disease is a well-known systemic autoimmune disease characterized by gluten-induced autoimmune intestinal villous atrophy, malabsorption, and various systemic and gastrointestinal symptoms. The older the patient with celiac disease is, the more the prevalence of systemic symptoms not related to the gastrointestinal tract, including neurological symptoms[25]. About 10% of patients with celiac disease develop neurological complications, including seizures. At the same time, about 0.78% to 9.10% of patients with epilepsy develop celiac disease[26,27]. The exact mechanism of neurological manifestations is poorly understood, probably related to immune mechanisms. This hypothesis is advocated by the presence of anti-Purkinje cells and anti-ganglioside antibodies in patients with celiac disease who developed neurological manifestations[28]. Another possible hypothesis is neurological damage due to deficiencies of the neurotrophic and neuroprotective vitamins (*e.g.*, vitamin D, vitamin E, thiamine, and vitamin B12) resulting from the malabsorption associated with celiac disease[29]. The prevalence of drug-resistant epilepsy is more common in children who have celiac disease as a comorbidity. Most patients with celiac disease and epilepsy have been cured with adherence to a gluten-free diet. Adherence to a gluten-free diet and adequate antiseizure medications can also reduce the seizure frequency and severity in patients with celiac disease and drug-resistant epilepsy[30].

### Gut dysbiosis

Gut dysbiosis strongly relates to autoimmune diseases, which are closely linked with epilepsy, suggesting an association between epilepsy and gut dysbiosis[3]. Huang *et al*[31] showed that mild gastroenteritis precedes the development of benign infantile convulsions. This temporal relation links

the infection-induced gut dysbiosis with epileptogenesis[31]. Şafak *et al*[32] found a significant increase in Fusobacteria prevalence in patients with epilepsy (10.6%) but not in the healthy control. This considerable shift and drift in the intestinal microbiota and the subsequent gut dysbiosis may be present in certain epilepsy types[32]. Meanwhile, the gut microbiome differs in patients with drug-resistant epilepsy (*e.g.*, *Cronobacter, Bacteroides, Bifidobacterium,* and *Erysipelatoclostridium*) from patients with drug-sensitive epilepsy with an abnormally increased richness of rare flora. On the other hand, patients with drug-sensitive epilepsy have a gut microbiome composition like the healthy controls, enforcing the evidence of the effects of gut dysbiosis in the development of epilepsy and drug-resistant epilepsy[33, 34].

### IBS

IBS is a constellation of symptoms occurring together, such as repeated abdominal pain and changes in bowel habits, such as diarrhea, constipation, or both. It affects about 7%-21% of the population[35]. IBS is associated with increasing the incidence of epilepsy, particularly temporal lobe epilepsy. A large population-based cohort study by Chen *et al*[36] showed that IBS increased the epilepsy risk with a cumulative incidence of epilepsy of 2.54/1000 person-years *vs* 1.86/1000 person-years in the cohort without IBS with an adjusted hazard ratio of 1.30[36]. Studies also showed that the incidence of IBS increases five times in patients with epilepsy than in controls[37]. There is also an increased incidence of functional gastrointestinal disorders, including IBS, in children with epilepsy than in matching controls [38]. Epilepsy with abdominal pain could also be misdiagnosed as IBS[39]. The cumulative data from these studies showed the bidirectional link between IBS and epilepsy. The exact cause of this increase in epilepsy risk is not known. It is probably related to the shared pathophysiological mechanisms and risk factors such as disturbed brain-gut axis, microbiota imbalance of the gastrointestinal tract, increased incidence of dietary allergies, neuroimmune interactions, and mucosal inflammatory mediator deregulation in the gastrointestinal tract[40-42]. Patients with epilepsy with IBS as a comorbidity have an increased rate of depressive and anxiety disorders[43]. If IBS is present in patients with drug-resistant epilepsy, most of the seizures occur during the period of altered bowel movements[44].

### Inflammatory bowel diseases

Inflammatory bowel diseases (IBD) are chronic autoimmune and immune-mediated inflammatory disorders affecting the digestive system with gastrointestinal and systemic manifestations, including the central and peripheral nervous systems. IBDs include ulcerative colitis, Crohn's disease, and unclassified IBD[45]. Neurological complications occur in 0.25% to 47.50% of patients with IBDs. Seizures of all types, including status epilepticus, can be observed during the clinical course of IBDs, especially in severe cases[46]. Many underlying mechanisms explain the occurrence of seizures in IBDs. These mechanisms include autoimmune-mediated neuroinflammation, gut dysbiosis with brain-gut-microbiota axis dysfunction, the associated nutritional deficiencies, especially thiamine and vitamin B12, increased incidence of infections, arterial and venous thromboembolism, and possible side effects of medications especially sulfasalazine, metronidazole, steroids, tumor necrosis factor-α inhibitors, and anti-integrin antibodies[47]. Seizures in patients with IBDs indicate the need to rule out a cranial thromboembolic event[48].

### Gastrointestinal disorders in children with autism

Gastrointestinal disorders occur in 46%-84% of children with autism. The most common gastrointestinal problems observed in children with autism are motility disorders such as chronic constipation or diarrhea, nausea, vomiting, gastroesophageal reflux or disease, chronic flatulence, abdominal discomfort, ulcers, inflammatory bowel disease, colitis, food allergies or intolerance, and failure to thrive. The severity of autism strongly correlates positively with gastrointestinal symptoms[49]. Meanwhile, abnormal EEG is present in 60% of children with autism (compared to 6%-7% of typically developed children), while epilepsy is present in 10% to 30% of children with autism. Children with autism have a high rate of celiac disease and gut dysbiosis, which increases the incidence of epilepsy [50].

### Situation-related seizures (Convulsions associated with gastrointestinal infections CwG)

Gastrointestinal infections were first reported to cause epileptiform activity development by Japanese researcher Morooka in 1982 and were called "situation-related seizures"[51]. It occurred in a previously healthy child who developed nonfebrile convulsions following mild gastroenteritis and mild dehydration for 1–5 d without apparent acid intoxication or electrolyte imbalance. It usually occurs during the winter, mainly by the rotavirus, which can reach the brain and cause encephalitis, cerebropathy, or convulsions[52]. The convulsions may present as single or multiple attacks of generalized tonic-clonic or focal seizure with characteristic normal interictal EEG, normal electrolytes, serum glucose, and cerebrospinal fluid. Stool analysis may test positive for rotavirus, norovirus, adenovirus, sapovirus, and coxsackievirus. It occurs in young children with an immature nervous system, like febrile convulsions[53]. Unfortunately, the prevalence of this type of convulsion is on the rise and has not been affected by the introduction of the rotavirus vaccination[54]. The etiology and pathophysiology are not yet thoroughly explained. However, it could be related to direct microbial invasion of the CNS

due to the indirect effects of specific mediators triggered by gastrointestinal infections[55]. This type of seizure has a favorable prognosis with infrequent relapse and typically normal development without the need for long-term antiseizure therapy[56].

## EFFECTS OF EPILEPSY ON THE GUT

As the brain has a bidirectional relationship with the gut, neurological disorders may impact the gastrointestinal tract. Examples of this impact include the occurrence of sialorrhea, anorexia, dysphagia, gastroparesis, and motility disorders such as diarrhea, intestinal pseudo-obstruction, constipation, and fecal incontinence[57]. Hence, epilepsy, in turn, affects the gastrointestinal tract in different forms, such as abdominal aura, epilepsy with abdominal pain, and the adverse effects of medications on the gut and the gut microbiota.

### *Abdominal aura*

An 'aura' is subjective warning feelings, experiences, movements, or events (*e.g.*, specific memory, music, song, or swirling colors) some people with epilepsy may experience, usually before or at the onset of a tonic-clonic seizure. Auras occur in about 70% of patients with generalized epilepsy[58]. Auras arise due to the activation of a functional cortex by aberrant, unilateral, focal, and short neuronal discharge[59]. It is a form of an aware focal seizure that develops into another type of seizure. It usually occurs at the seizure onset before impairment or loss of consciousness and is usually memorized afterward. We should differentiate auras from the premonitory or prodromal sensations, which occur at least 30 min before the seizures[60]. There are different forms of auras depending on the epileptogenic zone. Auras could be visual, auditory, olfactory, gustatory, somatosensory, psychic, autonomic, or even sexual. Hence, auras are accurate anatomical markers of the epileptogenic zone[61]. However, auras could be multiple, as reported in 6% of patients with epilepsy. Multiple auras are associated with multifocal epilepsy or activation of a neural network that involves more than one functional region. The presence of aura has an essential role in diagnosing, localization, and classification of epilepsy. Epileptic aura could assist in differentiating partial from generalized seizures[62].

Gustatory aura or gustatory hallucination epilepsy are a type of simple partial seizures. They are characterized by taste sensations, including sweet, bitter, acidic, salty, or metallic tastes, as the first clinical manifestation of the seizure. It is one of the parietal, temporal, or temporoparietal seizure manifestations and often evolves into complex partial seizures[59]. It occurs in the form of a sudden taste sensation of short duration, primarily seconds, that usually follow or is accompanied by the olfactory hallucination that resembles the perceived taste in the absence of an actual stimulus of the sensation. Both gustatory and olfactory auras are often linked together and are difficult to differentiate [63]. Gustatory auras arise from the mesial temporal region, particularly the left side, and are a manifestation of mesial temporal sclerosis or tumors[64].

An epigastric aura (visceral aura) is a somatosensory (*e.g.*, pain) aura that typically demonstrates an increasing epigastric sensation. It may appear as visceral sensations (*e.g.*, abdominal discomfort), visceromotor symptoms (*e.g.*, vomiting, borborygmi, or tachycardia), or vegetative symptoms (*e.g.*, blushing or sweating). Epigastric aura occurs due to abnormal neuronal activation and discharges in the sensory cortex representing the abdominal viscera[65]. This type of aura is frequently seen in migraine or epilepsy. Epigastric auras are the most prevalent aura in medial temporal lobe epilepsy. It also may have an insular origin[66]. The presence, type, and severity of epigastric aura and other forms of autonomic manifestations depend on the seizure onset location and timing, propagation pathway, lateralization, and the persistence of interictal autonomic dysfunction. The presence of a severe autonomic aura can expect the occurrence of sudden death[67].

### *Abdominal skin temperature in focal epilepsy*

Thermographic studies showed that the abdominal wall has colder spots and areas in patients with focal-onset epilepsy than in controls. It could be related to the visceral-somatic and somatic-visceral neurological interactions[68]. We can use infrared thermography mapping and thermochromic/thermo-sensitive silicone to locate the irritative epileptogenic areas in patients with focal epilepsy. Their accuracy and safety are like electrocorticography. This thermographic localization of the epileptogenic activity can be used to locate the irritative zones in neurosurgery, particularly epilepsy surgery[69].

### *Epilepsy with abdominal pain (abdominal epilepsy)*

Abdominal pain is one of the most frequent complaints, especially in pediatric age. It may result from a wide range of causes, both intra- and extra-abdominal. Systemic causes of abdominal pain may include hereditary, infectious, inflammatory, metabolic diseases, and neurologic disorders[70]. Many neurologic diseases can cause abdominal pain. For example, abdominal migraine, epilepsy, peripheral neuropathy, or even cerebral tumors can present with abdominal pain[71,72]. Occasionally the cause of the abdominal pain is ill-defined, making the diagnosis of abdominal pain without evident abdominal abnormality a puzzle for most physicians.

Epilepsy with abdominal pain is an uncommon condition of abdominal pain. It is a type of temporal lobe epilepsy that usually presents with abdominal auras and is characterized by recurrent episodic paroxysms of abdominal and periumbilical pain with various abdominal symptoms (*e.g.*, nausea and vomiting) accompanied or followed by disturbed brain functions. Epilepsy with abdominal pain usually occurs in childhood, but it is also reported in adults[73]. The characteristic postictal manifestations (such as lethargy, drowsiness, headache, blindness, paraesthesia, or even convulsions) help to differentiate it from the abdominal migraine[74].

The exact mechanism of epilepsy with abdominal pain is not fully understood but could be related to abnormal neuronal activation of the temporal lobe involving the amygdala. Amygdala then serves as a signal conductor to the gut through direct projections to the dorsal motor part of the vagus nerve nucleus. The vagus nerve then transmits the electrical activity to the target organs causing different gastrointestinal symptoms, especially abdominal pain (Figure 1)[75]. It is usually idiopathic; however, it may manifest temporal lobe lesions such as prematurity, febrile seizures, neuronal migration defects, cortical malformations, arterio-venous malformations, neuroendocrine dysfunction, mesial temporal lobe sclerosis, gliotic damage resulting from encephalitis, or brain tumors such as dysembryoplastic neuroepithelial tumors, benign tumors, cerebral astrocytoma, or gliomas[76,77].

Epilepsy with abdominal pain has a characteristic tetrad[78]: (1) Paroxysmal gastrointestinal and autonomic complaints (abdominal pain, vomiting, nausea, flushing, palpitation, and stuttering) of unapparent cause; (2) CNS disturbance symptoms (*e.g.*, alteration of mental status, headache, dizziness, and convulsions); (3) Abnormal EEG findings characteristic of epileptic activity; and (4) Improvement of the symptoms with antiseizure medications.

The diagnosis of epilepsy with abdominal pain is essentially clinical. To properly diagnose epilepsy with abdominal pain, we should rule out organic causes in the gastrointestinal tract and the nervous system. Other causes of recurrent abdominal pain should also be ruled out, such as porphyria, familial Mediterranean fever, abdominal migraine, and cyclic vomiting[79]. Describing the abdominal attacks by emphasizing the presence or absence of aura and postictal events may help reach the diagnosis. Complete physical, abdominal, and neurological examinations should be performed in suspected patients. Serum prolactin could increase within 20 min of the attack in epilepsy with abdominal pain. The sample should be taken within two hours. Presumably, the prolactin release is due to the propagation of epileptic activity from the temporal lobe spreading to the hypothalamic-pituitary axis. High serum prolactin could help to differentiate epilepsy with abdominal pain from psychogenic or functional causes of abdominal pain[80]. The presence of abnormal epileptogenic activity by EEG accompanying the pain paroxysm or between the attack confirms the diagnosis. Computed tomography or magnetic resonance imaging of the brain may be needed to rule out neurologic diseases or tumors. Other laboratory tests to rule out the gastrointestinal causes of abdominal pain are tailored according to the clinical finding. Abdominal ultrasound could also help[77].

Epilepsy and migraine are frequent comorbid conditions and shared genetic susceptibility[81]. Abdominal migraine has many shared features with epilepsy with abdominal pain: Auras, abdominal pain, nausea, vomiting, and headache. So, when a patient with epilepsy with abdominal pain presents with a headache, it will be challenging to differentiate it from abdominal migraine (Table 1). The duration of the symptoms could help in diagnosis, as headache is usually prolonged in abdominal migraine rather than in abdominal epilepsy. Postictal manifestations, abnormal EEG, and high postictal serum prolactin could help confirm epilepsy with abdominal pain[79]. Treatment of epilepsy with abdominal pain with antiseizure medications is usually successful, with very few relapse rates. There are no current recommendations on the type of antiseizure medications, but many studies recommend using oxcarbazepine[82].

### *Postictal abdominal manifestations*

Postictal states are transient brain conditions following seizures (most common complex partial and tonic-clonic seizures), manifested as neurological deficits (confusion, weakness, memory impairment, and headache) with/without psychiatric manifestations of variable severity and duration, frequently associated with EEG slowing or suppression, and persist for minutes to days[83]. The duration of these symptoms usually corresponds to the intensity and duration of the ictal period. The mechanism of postictal states is related to robust cortical inhibitory mechanisms that try to inhibit and terminate the seizures, producing changes in membrane receptors and alteration of neurotransmitter release together with cerebrovascular changes, contributing to the development of these postictal events. Postictal event type depends on the type of epilepsy, the location of the epileptogenic activity, and the severity of the seizure[84,85]. Sometimes it is challenging to differentiate between ictal and postictal events, especially in nonconvulsive seizures[86]. The EEG and magnetic resonance imaging brain changes usually relate to the postictal manifestations with characteristic slowing and temporary signal increases[87].

Postictal hypersalivation is rare but occurs entirely in seizures of mesial origin in temporal lobe epilepsy, mainly from the left side[88]. Hypersalivation reflects a purposeful response to hypersecretion following regaining consciousness after a complex partial seizure. It is prevalent in patients with temporal lobe epilepsy, especially mesial temporal lobe epilepsy[89]. This postictal event is more common in females than males supporting the sex differences in epilepsy[90]. Postictal hyperphagia and compulsive water drinking were reported in a few case reports in patients with secondary epilepsy due

**Table 1 Differences between epilepsy with abdominal pain and abdominal migraine**

| Parameter | Epilepsy with abdominal pain | Abdominal migraine |
|---|---|---|
| Age | Mainly pediatric age (4-9 yr), scarce in adults | It starts in childhood (3-10 yr with a peak at 7), though it may occur in adults |
| Sex | More in males during childhood, more in females in adulthood | More in females |
| Prevalence | Very rare | More common affect 2% to 4% of children |
| Etiology | Focal partial temporal lobe epilepsy due to idiopathic or secondary causes | Food allergy, Mitochondrial DNA mutation (cytopathy), Corticotropin-releasing factors abnormalities, Endogenous prostaglandin release |
| Family history | | Strong family history of migraine |
| Duration of episodes | Usually 10-30 min, 4–5 times/month | Usually, more than an hour (3-4 h), at least twice/6 mo |
| Aura | May present | May present |
| Headache if present | Short duration | Long duration |
| Consciousness | May be altered | Not affected |
| Postictal tiredness or confusion | May present | absent |
| EEG | Abnormal epileptogenic electrical activity of focal temporal epilepsy | Usually, normal |
| Postictal serum Prolactin | Usually, high | Usually normal, it may be high, especially in females |
| Prevention | Prevention and treatment of the cause in secondary cases and sleep hygiene in idiopathic cases | Good sleep hygiene, hydration, stress reduction, and avoiding dietary triggers |
| Prophylaxis therapy | Antiseizure medications | Amyltryptine, propranolol, cryoheptadine, pizotifen |

EEG: Electroencephalogram.

to temporal lobe lesions. It showed a dramatic response to carbamazepine[91]. It was also reported in secondary epilepsy due to frontal lobe lesions[92]. Remick *et al*[93] described three patients who experienced postictal hyperphagia[93].

### *Effects of antiseizure medications on the gastrointestinal tract*

Antiseizure medications generally have a narrow therapeutic window with many adverse effects, especially on the gastrointestinal tract. According to the reporting method, the prevalence of the antiseizure side effects ranges between 10%-90% of the patients[94]. Over the last one and half centuries, the adverse effects of antiseizure medications remain the primary cause of treatment failure. About 10%-30% of the patients with epilepsy did not tolerate these side effects and stopped the drugs, especially with polytherapy[95]. Gastrointestinal side effects were observed in many antiseizure medications. Table 2 summarizes the common gastrointestinal side effects of the commonly used antiseizure medications.

On the other side, some antiseizure medications can improve some gastrointestinal manifestations. For example, gabapentin can improve functional dyspepsia, which is resistant to other conventional therapies[96]. Gabapentin also decreases rectal mechanosensitivity and enhances rectal compliance in patients suffering from diarrhea-predominant IBS[97]. Another interesting finding by Liu *et al*[98] is the ability of valproate to prevent peritoneal adhesion following abdominal injury through chymase inhibition[98]. Valproate also decreased intestinal inflammation in inflammatory bowel disease[99].

Meanwhile, Patel and Patel[100] showed that sodium valproate could experimentally inhibit the proliferation of carcinogenic cells in colon cancer associated with diabetes mellitus[100]. As valproate is a GABA agonist, it can modulate gastrointestinal motility and the anal sphincter. Valproate can normalize the activity of the human lower esophageal sphincter and reduces the number of reflux episodes in health and GERD[101]. Phenobarbital is effective and safe for preventing prenatal and treating postnatal hyperbilirubinemia through its effects on the hepatic enzymatic elimination of bilirubin[102,103].

## ABDOMINAL MANIPULATIONS TO MANAGE EPILEPSY

As the gut-brain axis has a bidirectional effect on both gut and brain, modulation of the gut microbiota

**Table 2 Common gastrointestinal side effects of antiseizure medications[122-129]**

| Antiseizure medications | Common gastrointestinal side effects |
|---|---|
| Carbamazepine | Dry mouth, mouth sores, glossitis, loss of appetite, dysphagia, nausea, vomiting, hurt burn, gastritis, stomach/abdominal pain, constipation, diarrhea, abnormal liver functions, cholestatic and/or hepatocellular jaundice, hepatitis; hepatic failure (very rare), and pancreatitis (rare), eosinophilic colitis |
| Ethosuximide | Anorexia, nausea, vomiting, gastric pain, diarrhea, gastric and intestinal atony with decreased peristaltic activity |
| Phenobarbital | Diarrhea, sore throat, swelling of the tongue/throat, nausea, vomiting, constipation, dysphagia, and heartburn. As it is a cytochrome P450 hepatic enzyme inducer, it can cause abnormal hepatic function, hepatitis, liver damage, cholestasis, toxic hepatitis, and jaundice |
| Phenytoin | Changes in taste sensation, gingival overgrowth, sore throat, mouth ulcers, diarrhea, nausea, vomiting, constipation, dysphagia, heartburn, idiosyncratic hepatotoxicity (< 1% of the patients), reduced gastrointestinal absorption of calcium, reduced hepatic synthesis of 25-hydroxycholecalciferol, cause a relative vitamin K deficiency |
| Valproate | Diarrhea, nausea, vomiting, constipation, dysphagia, gastritis with heartburn, several distinctive forms of acute and chronic liver injury, and vitamin D deficiency |
| Gabapentin | Vomiting, constipation, gastritis, pancreatitis |
| Topiramate | Taste perversion, anorexia, nausea, abdominal pain, indigestion, diarrhea, constipation |
| Lamotrigine | Dry mouth, nausea, vomiting, gastritis, diarrhea, or constipation |

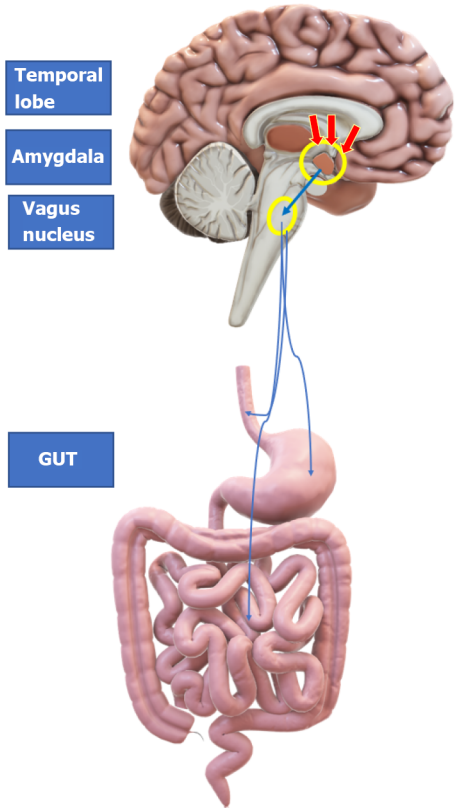


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**Figure 1 Mechanism of epilepsy with abdominal pain.**

could positively impact managing diverse types of epilepsy. The gut microbiota may influence brain functions in several ways, including the CNS, the hypothalamic-pituitary-adrenal axis, immune and inflammation modulation, and neuromodulators. Therefore, gut microbiota modulation could exert a beneficial role in epilepsy management. Prebiotics, probiotics, synbiotics, postbiotics, a ketogenic diet, and fecal microbiota transplantation are probable methods to treat epilepsy *via* modulation of the microbiota-gut-brain axis[104]. Probiotics are living organisms able to provide the host with health benefits when supplied in an appropriate dose. At the same time, prebiotics is selective nutritious substrates for specific types of host microorganisms to confer health benefits to the host. Synbiotics are a mixture of both pre- and probiotics. Postbiotics are the metabolic end products of the probiotic

organisms that can confer health benefits to the host[105].

Gómez-Eguílaz *et al*[106] found a reduction in seizure frequency by 50% in about 28.9% of patients with drug-resistant epilepsy when supplied with a probiotic mixture as adjuvant therapy for four months. This effect persisted for another 4 mo after probiotic discontinuation in 78.9% of those who showed improvement[106]. The gut microbiota can modulate brain activity by the peripheral production of GABA, metabolizing serotonin precursors, and modulating brain-derived neurotrophic factors that correlate with epilepsy severity. The bacterial production of short-chain fatty acids, which have anti-inflammatory effects, is another factor explaining the probiotic effects in treating epilepsy. Gut microbiota also modulates the endocannabinoid system with its inflammatory suppressor effects on seizure events[107]. At the same time, some gut microbiota strains can metabolize anticonvulsants affecting their antiseizure effect. For example, the gut microbiota can metabolize the antiseizure zonisamide into pharmacologically inactive 2-sulfamoyl-acetyl-phenol[108]. Fecal microbiota transplantation is a promising approach to reconstructing the gut microbiota. It is successfully used to treat various diseases, including neurological disorders. He *et al*[109] successfully treated a girl with long-term Crohn's disease and epilepsy for 17 years with fecal microbiota transplantation, which could prevent seizure relapse during 20 mo of follow-up[109]. However, we need more time to have a valuable experience with the efficacy of fecal microbiota transplantation in treating epilepsy.

The ketogenic diet is an old modality used to treat drug-resistant epilepsy and metabolic diseases since 1920. Though the precise mode of action is not well known, its activity could be related to modifying the gut microbiota composition and function. The gut microbiota modification causes alteration of beta-hydroxybutyrate levels and elevates the hippocampal GABA compared to the glutamate content[110]. In addition, the ketogenic diet modification of the gut microbiota reduces the alpha diversity and increases proposed beneficial bacteria like *Akkermansia muciniphila* and *Parabacteroides spp*. This microbiota modulation changes the colonic luminal metabolome, with a decrease in gamma-glutamyl amino acids and an increase in the brain GABA/glutamate content by reducing the blood gamma-glutamyl amino acids[111]. A ketogenic diet also alters neuronal metabolism by reducing cerebrospinal fluid glucose levels, increasing ketone bodies, and reducing cortical hyperexcitability with reduced seizure frequency[112]. Ketone bodies such as acetoacetate exerted a broad-spectrum anticonvulsant effect through modulation of neurotransmitter release and modification of ATP-sensitive potassium channels[113]. Additionally, ketone bodies have a direct inhibitory influence on the vesicular glutamate transport[114].

Vagus nerve stimulation was approved by the Food and Drug Administration in 1997 as adjuvant treatment in patients with multidrug resistant epilepsy who are not fit for epilepsy surgery. The vagus nerve is a vital brain-gut axis component and plays an essential role in inflammation modulation, intestinal homeostasis maintenance, food intake, satiety regulation, and energy homeostasis[115]. Vagus nerve stimulation leads to electrical energy discharge into a wide brain area, disturbing the unusual brain activity that produces seizures[116]. At the same time, vagal stimulation has anti-inflammatory properties affecting the gastrointestinal tract through hypothalamic-pituitary-adrenal axis activation and vasovagal reflex-induced cortisol release, which has an anti-tumor necrosis factor effect[117]. Consequently, vagus nerve stimulation can be used to treat multidrug resistant epilepsy and at the same time can treat gut inflammatory disorders such as IBD, which at the same time is a risk factor to increase the incidence of epilepsy[118].

Omentum is a large double peritoneal flat sheet of fatty tissue that hangs from the greater and the lesser gastric curvature to float on the intraperitoneal organs, including large and small intestines. It has many functions: Fat storage, immune regulation, neovascularization, tissue regeneration, and healing. Omental transposition or graft was used in various surgeries, including abdominal, cardiac, thoracic, orthopedic, plastic, vascular, urogenital, gynecological, and neurosurgeries[119]. Omental transposition on the brain surface enhances neoangiogenesis by generating plentiful new vessel connections between the omentum and the brain, which induces healing of neural injury by increasing the cerebral blood flow and the available oxygen to the neural tissues, releasing omental neurotransmitters, such as acetylcholine, dopamine, and noradrenaline. It also releases neurotrophic factors such as gangliosides and nerve growth factors that help to restore neurologic functions[120]. Rafael *et al*[121] used omental transplantation to treat two patients with uncontrolled temporal lobe epilepsy. They transplanted the omental tissues directly upon the epileptic focus on the left temporal lobe and the anterior perforated space. One patient showed complete recovery, while the other showed about 85% improvement in seizure frequency and severity[121]. However, there are few reported cases, and there is a need for long-term follow-up to have a better experience with omental transplantation to treat epilepsy.

## CONCLUSION

There is a strong interaction between the gut and the brain. This interaction forms the typical gut-brain axis. Consequently, gastrointestinal dysfunction can be seen in neurological disorders, and neurological dysfunction can be seen in gastrointestinal disorders. There is an increase in epilepsy incidence in various gastrointestinal diseases. On the other hand, epilepsy, in turn, affects the gastrointestinal tract in

different forms, such as abdominal aura, epilepsy with abdominal pain, and the adverse effects of antiseizure medications on the gut and the gut microbiota. Various gut manipulations could help manage epilepsy, such as gut microbiota modification, fecal microbiota transplantation, ketogenic diet, vagus nerve stimulation, and omentum transplant. Understanding the strong relationship between epilepsy and the gut could help alleviate epileptic and gastrointestinal disorders.

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**Country/Territory of origin:** Bahrain

**ORCID number:** Mohammed Al-Beltagi 0000-0002-7761-9536; Nermin Kamal Saeed 0000-0001-7875-8207.

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*MINIREVIEWS*

# Influence of the COVID-19 pandemic in the gastrointestinal oncology setting: An overview

Breno Bittencourt de Brito, Hanna Santos Marques, Filipe Antônio França da Silva, Maria Luísa Cordeiro Santos, Glauber Rocha Lima Araújo, Lara de Araujo Valente, Fabrício Freire de Melo

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**Breno Bittencourt de Brito, Filipe Antônio França da Silva, Maria Luísa Cordeiro Santos, Glauber Rocha Lima Araújo,** Instituto Multidisciplinar em Saúde, Universidade Federal da Bahia, Vitória da Conquista 45029-094, Bahia, Brazil

**Hanna Santos Marques, Lara de Araujo Valente,** Campus Vitória da Conquista, Universidade Estadual do Sudoeste da Bahia, Vitória da Conquista 45055-380, Bahia, Brazil

**Fabrício Freire de Melo,** Instituto Multidisciplinar em Saúde, Universidade Federal da Bahia, Vitória da Conquista 45029-094, Brazil

**Corresponding author:** Fabrício Freire de Melo, MSc, PhD, Postdoc, Professor, Instituto Multidisciplinar em Saúde, Universidade Federal da Bahia, Instituto Multidisciplinar em Saúde, Universidade Federal da Bahia, Rua Hormindo Barros, 58, Quadra 17, Lote 58, Vitória da Conquista 45029-094, Bahia, Brazil. freiremeloufba@gmail.com

## Abstract

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection has been impacting healthcare in various ways worldwide and cancer patients are greatly affected by the coronavirus disease 2019 (COVID-19) pandemic. The reorganization of the health facilities in order to supply the high demand resulting from the aforementioned infection as well as the social isolation measures led to impairments for the diagnosis and follow-up of patients with gastrointestinal cancers, which has had an impact on the prognosis of the oncologic patients. In that context, health authorities and organizations have elaborated new guidelines with specific recommendations for the management of individuals with gastrointestinal neoplasms during the pandemic. Of note, oncologic populations seem to be more susceptible to unfavorable outcomes when exposed to SARS-CoV-2 infection and some interactions involving virus, tumor, host immune system and anticancer therapies are probably related to the poorer prognosis observed in those COVID-19 patients. Moreover, vaccination stands out as the main prevention method against severe SARS-CoV-2 infection and some particularities have been observed regarding the seroconversion of vaccinated oncologic patients including those with gastrointestinal malignancies. In this minireview, we gather updated information regarding the influence of the pandemic in the diagnosis of gastrointestinal neoplasms, new recommendations for the management of gastrointestinal cancer patients, the occurrence of SARS-CoV-2 infection in those individuals and the scenario of the vaccination against

the virus in that population.

**Key Words:** Gastrointestinal cancer; COVID-19; Treatment; Diagnosis; Vaccination; Pandemic



**Core Tip:** The coronavirus disease 2019 pandemic has impacted the care of patients with serious chronic conditions such as cancer. In this minireview, we gather updated information regarding the influence of the pandemic in the diagnosis of gastrointestinal neoplasms, new recommendations for the management of gastrointestinal cancer patients, the occurrence of severe acute respiratory syndrome coronavirus 2 infection in those individuals and the scenario of the vaccination against the virus in that population.

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## INTRODUCTION

The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) outbreak emerged in 2019 which soon spread worldwide becoming a pandemic[1]. Coronavirus disease 2019 (COVID-19) infection is one of the greatest threats to global public health and by March 2022 the World Health Organization had already identified 464809377 confirmed cases and 6062536 deaths[2]. The course of the disease ranges from asymptomatic to fatal infection and its clinical presentation is mainly characterized by respiratory symptoms such as cough and dyspnea but it can also affect other systems leading to cardiac, gastrointestinal, renal, neurological, cutaneous and hematological disorders[3]. Severe COVID-19 primarily affects patients with comorbidities including individuals with cancer who are often immunocompromised[4]. Gastrointestinal neoplasms including colorectal, gastric, liver, esophageal and pancreatic cancers are relatively frequent and some of them are among the malignancies that kill the most in the world, such as gastric and colorectal cancers[5]. In the context of the new coronavirus pandemic, tumors that affect the gastrointestinal tract are the most common malignancies among patients infected with COVID-19 in various investigations[6]. Disturbingly, SARS-Cov-2 infection in oncologic patients is linked to higher rates of intensive care unit (ICU) admission, greater need for mechanical ventilation and increased propensity to death[7-9].

The clinical practice of oncologists and the routine of cancer patients were significantly affected by the effects of the COVID-19 pandemic[10]. The measures adopted to prevent the spread of the disease and the overload of health services around the world impacted the diagnosis of some malignancies, especially those that require invasive procedures such as colorectal and gastric cancers[11]. In addition, cancer health care including oncologic surgeries, visits to the health system, outpatient consultations and anti-cancer therapies were negatively affected by experiencing delays or interruptions during treatment[12]. Finally, vaccination is the main available strategy to prevent the SARS-CoV-2 infection. However, studies have highlighted particularities involving the effectiveness of the available immunizers in the oncologic population[13-15].

This minireview focuses on addressing the key challenges faced by oncologists and patients with gastrointestinal malignancies in face of the changes that follow the aforementioned pandemic. The aim is to highlight the main aspects discussed in the current scientific evidence regarding diagnosis, treatment, vaccination and infection prevention among patients with gastrointestinal cancer in that context.

## METHODS

In order to review the repercussions of SARS-CoV-2 infection in patients with gastrointestinal cancer, a search was performed for relevant articles published in English in the National Library of Medicine (PubMed) database until March 5, 2022. In this sense, two researchers acted independently using the following descriptors: COVID-19; SARS-CoV-2 in combination with Gastrointestinal cancer; Gastric cancer; Esophageal cancer; Colorectal Cancer; Treatment; Cancer diagnosis; Vaccination. The selection of studies was made by screening the titles and abstracts of articles. We included studies that evaluated

outpatients and inpatients with confirmed SARS-CoV-2 infection who had cancer, outpatients and inpatients with confirmed SARS-CoV-2 infection who had gastrointestinal cancer, prospective, retrospective, cross-sectional studies, systematic reviews and narratives.

## IMPACTS OF THE COVID-19 PANDEMIC OVER GASTROINTESTINAL CANCER DIAGNOSIS

Healthcare systems around the world have been broadly impacted by the COVID-19 pandemic. Many health facilities had to be reorganized in order to uphold the high demand for medical assistance imposed by the aforementioned disease[16]. Financial, structural and personal resources have been redirected to supply the unexpected consequences that follow such an unprecedented health problem [17]. Other issues also impaired the access of populations to healthcare providers including the burden of the pandemic over the economy as well as the difficulties and fears faced by populations to reach healthcare centers in the presence of lockdowns and other measures for contagion containment[18]. In addition, the interruption of nonurgent medical procedures, including diagnostic tests, in order to avoid viral dissemination, was another trouble in that setting[19]. Unfortunately, these changes undoubtedly prejudiced the proper assistance and early diagnosis of serious chronic conditions such as gastrointestinal malignancies.

A population-based study performed by Maringe *et al*[20] in England aimed at estimating the influence of the pandemic over cancer deaths due to delays in diagnosis in that country, gathering 24975 individuals with colorectal cancer and 6744 persons with esophageal malignancy. They estimated an increase of about 15.3%-16.6% in the number of colorectal cancer-related deaths and an enhancement of 5.8%-6.0% in esophageal cancer-associated deaths within the first 5 years after diagnosis. Another study carried out with the Chilean population estimated the impact of the COVID-19 outbreak on the diagnosis and survival of breast, cervix, colorectal, prostate and stomach cancers. The results predicted a larger percentage of individuals diagnosed with cancer at advanced stages between 2020 and 2022 which leads to a lower 5-year net survival. They previsied 3542 extra deaths from 2022 to 2030 (95% UI 2236–4816) associated with these cancers, led by colorectal cancer, which accounts for 1389 excess deaths (95% UI 364–2567), whereas stomach cancer will probably be the cause of 6.0% of those additional deaths[21].

In addition, an investigation performed in an academic health center in New York (United States) compared the number of diagnostic and resection specimens for the detection of gastrointestinal malignancies during the years 2018, 2019 and 2020. They included 949 patients, gathering 1028 pathology samples, and observed a reduction of 57% in the number of samples in 2020 compared to the preceding year ($P < 0.01$). Moreover, a drop in the number of colorectal cancer specimens from older patients was found when pre- and post-COVID-19 periods were compared ($P < 0.01$)[22]. Alarmingly, a retrospective Japanese study evaluated 5167 patients (4218 before the pandemic and 949 diagnosed with gastrointestinal cancer during the pandemic) and observed that during the pandemic period there was a significant decrease in diagnoses of stage 0 colorectal cancers ($P = 0.008$, stage I ($P = 0.003$) and stage II (0.01) and an increase in diagnoses in stage III malignancies ($P < 0.001$)[11]. These data evidence the repercussions of the pandemic on the diagnosis of gastrointestinal cancers as well as the impact of the delay for diagnosis on the prognosis of oncologic patients. Interestingly, a study with 298 patients carried out in an Italian hospital observed a lower number of elective colorectal cancer screening colonoscopies, but a higher detection of colorectal cancer cases during the pandemic[23]. They found five cases (8%) of the malignancy among individuals ($n = 60$) evaluated from March 9 to May 4, 2020 (lockdown group), and only 3 cases (1%) among the patients ($n = 238$) who underwent the diagnostic assessment in the same period of 2019 (control group, $P < 0.01$). Moreover, the prevalence of patients with more high-risk factors for the disease, such as a familiar positive history and significant symptoms (*e.g.*, rectal bleeding), was higher in the lockdown group. These results suggest that the presence of meaningful risk factors for colorectal cancer probably made patients prioritize the diagnosis of the disease despite the risk of acquiring SARS-CoV-2 infection.

## TREATMENT OF GASTROINTESTINAL CANCER PATIENTS DURING THE PANDEMIC

Since the World Health Organization declared the SARS-CoV-2 outbreak a pandemic, the impacts of the infectious disease on cancer treatment have become a major concern around the world. Patient protection and continuity of treatment became challenging factors within that context in which social isolation and reduced displacement were the main measures to be taken.

In Europe, one of the first continents that became the epicenter of transmission, health authorities and governments decided to postpone consultations for patients with gastric cancer or carry them out remotely, treatment plans were reformulated and many clinical trials on gastrointestinal malignancies had their development impaired. In Italy and the United Kingdom (UK), for example, some health units

were designated for the exclusive care of patients with COVID-19 and others to assist individuals without the infection, and even so it is estimated that more than 200000 weekly exams were unable to be performed in the UK[24].

In a Japanese cross-sectional study carried out with 61 patients undergoing treatment for gastrointestinal cancer, it was observed that the pandemic caused a reduction in the number of exits and more caution regarding the prevention of infections ($P < 0.001$) as well as an increase in the occurrence of anxiety and insomnia in those patients during treatment ($P < 0.01$). Of note, most patients do not wish to change their treatment plans as recommended by guidelines developed during the pandemics[25] and this may be due to the fear and insecurity in face of the chance of having a worse prognosis because of a decrease in the frequency of care measures. Another American study that compared 25666 patients being treated for gastrointestinal cancer in 2020 and 23530 patients followed up in 2019, observed that there were statistically significant decreases in the number of radiotherapies and surgeries in patients with gastrointestinal neoplasms[26]. Sozutek *et al*[27] recently observed a reduction of about 70% in the volume of cases of colorectal cancer at an academic center during the pandemic. This study also showed that there was a lower proportion of cancer resections ($P = 0.01$), with a decrease of about 15% in the number of colorectal cancer surgical therapies ($P = 0.04$)[22]. These results indicate that the pandemic, indeed, has had negative impacts on the treatment of patients with various gastrointestinal malignancies.

The international survey in question focused on the preoperative screening of asymptomatic patients aiming to elucidate the current global situation of surgical practice under the COVID-19 pandemic. A total of 936 centers in 71 countries completed the survey; the survey respondents were a total of 1173 surgeons who represented the centers' surgical departments. Results show that the majority of them (73.8 per cent) performed preoperative COVID-19 testing exclusively based on symptoms or suspicious radiologic findings, but only 22.8 per cent of the overall centers performed routine screening by chest-computer tomography (CT) scan. To test every surgical patient for COVID-19 was a guideline recommended in barely 17 per cent of the centers. Results also show that 27.5 percent of the centers reported asymptomatic COVID-19 patients who tested positive postoperatively; most centers (81.9 per cent), only then, changed testing policies and preventive measures in surgical practice[28].

The surgeon's personal feelings were also investigated in the survey; in total, 1124 surgeons replied to the questions. When asked about the personal fear of getting sick or infecting others, the respondents overall reported a relatively high score of 37 ± 13, 1 point meaning "never" and 5 points meaning "always". Just over 50 per cent of the surgeon's said to be satisfied with the hospital's preventive measures, agreeing that their centers were taking enough preventive measures to avoid in-hospital transmission. The survey clarified the current surgeons' fear of getting infected was particularly associated with shortage of gloves, gown, hand sanitizer and medical masks. That, in addition to experiencing in-hospital infection, which was reported in 31.5% of the overall centers and the majority of these centers failed to trace it. Social support for the surgeons' fear and secure working environment with enough personal protective equipment (PPE) supply have shown to be unwarranted[29].

Despite all the risks involved in performing surgical procedures during the pandemic, a 60-d observational study of 177 patients with gastrointestinal cancer observed that there was no SARS-CoV-2 infection in any staff member or patient who underwent tumor resection during the study period. They concluded that even in a hospital that takes care of patients with COVID-19, if there are adequate prevention measures for both the patients and the medical staff, the procedure can be performed safely, thus optimizing the treatment of these patients[27]. It is important to point out that, unfortunately, this was not the reality of most underdeveloped countries which had little availability of adequate infrastructure and resources for the implementation of proper preventive methods to avoid SARS-CoV-2 contagion and had to postpone many surgical procedures due to the high chance of infection in a hospital environment[30].

While a guideline for clinicians published by the World Health Organization states that patients who have confirmed COVID-19 infection should be assessed for holding anticancer therapy until they are deemed medically clear, it is unquestionable that surgery and adjuvant therapies cannot always be postponed; emergency surgery is still recommended in certain diagnoses[31]. Studies show that patients who underwent chemotherapy or surgery in the past month before diagnosis with COVID-19 had a higher risk of severe clinical events than those not receiving chemotherapy or surgery. Therefore, the necessity of any interventional procedure must be balanced against the increased risk during a pandemic and should be evaluated on a case-by-case basis[9,32]. The potential benefit of chemotherapy remains unchanged during a pandemic, but the risk of harm would be increased to a degree that cannot be quantified. Undoubtedly, cancer patients need to be made aware that myelosuppressive treatment could carry greater risk during a pandemic so they may well make an informed choice[31,33]. Moreover, it is clear that an intentional postponement of adjuvant chemotherapy or elective surgery for stable cancer should be considered for patients with acute SARS-CoV-2 or other infections[32].

However, delays for surgery or curative adjuvant chemotherapy can only be considered within acceptable periods for each disease. While some cases can be postponed indefinitely, the majority of them are associated with progressive diseases that will continue to advance at variable disease-specific rates. For instance, while some asymptomatic breast cancer tumors can be followed up until the pandemic is more controlled or over, chemotherapies against stage III colorectal cancers can only be

safely delayed up to 8 wk post -surgery, but more than 12 wk of delay is not recommended, being associated with worse outcomes[34,35].

To spare this group of patients the possibly irreparable consequences of delayed treatment in this uncertain pandemic setting, it is imperative that each hospital should review its own facilities and provide these patients with treatment when possible. During the COVID-19 pandemic, one of the points to be considered when making the decision for surgery in cancer patients is the current condition of the hospital. Operating rooms are high-risk areas for contact contamination through airway or possible splash; to avoid the risk is it a demand that they should be very well-designed to deal with this type of high contamination risk situation; a minimum number of people should enter and leave patient rooms for all types of work and procedures. The widespread use of hand washing, antiseptic procedures and PPE should be ensured by the hospital and usage rules should be strictly followed. In cases of required emergency surgery for a patient with both cancer and ongoing SARS-CoV-2 infection, it has to previously be defined in detail the operational, perioperative and postoperative management including prevention and control measures for the medical staff, operating rooms and surgical tools as well as the protection of the wards, healthcare personnel and other patients. Hospital resources should be evaluated with a multidisciplinary approach and a personalized treatment protocol should be developed for each patient[36-38].

### *Considerations for gastric and esophageal cancer*

Upper gastrointestinal tract (esophageal and gastric) malignancies rank among the ten most common malignancies worldwide while gastric cancer still remains one of the leading causes of cancer-associated deaths. The incidence of upper GI malignancies varies widely and regions with high COVID-19 incidence such as, China, Japan, Central, and South America, also represent areas with the highest occurrence of esophageal and non-cardiac gastric cancer[39].

With regard to the treatment of these malignancies, the Society of Surgical Oncology affirms that most upper gastrointestinal tract cancer surgeries are not elective. If there are inadequate resources to manage potential complications then surgery may need to be delayed or, if necessary, referred to centers with resources to perform the procedure. Discussion of cases remains critical to assert priorities, resources, and personalized treatment plans based on the hospital, patient and tumor specificities. However, a few organ-specific approaches are determined: cT1a lesions amenable to endoscopic resection may preferentially undergo endoscopic management where resources are available; cT1b cancers should be resected; cT2 or higher and node-positive tumors should be treated with neoadjuvant systemic therapy. Given the concerns regarding laparoscopic surgery in COVID-19 patients, since the SARS-CoV-2 may be present in the smoke caused by the cautery devices, consideration may be given to proceeding straight to neoadjuvant treatment in COVID-19 positive patients.

Patients completing neoadjuvant chemotherapy may stay on chemotherapy if responding to and tolerating treatment. If patients are not responding to systemic treatment, resection and/or referral may be considered. Patients with gastric outlet obstruction or hemorrhage should be treated with endoscopic measures to allow for enteral nutrition or control of bleeding; proceed to surgery if these measures fail. In less biologically aggressive cancers, such as gastrointestinal stromal tumors - unless symptomatic or bleeding - surgery may be considered for short-term deferral[40].

### *Considerations for colorectal cancer*

Guidelines have been published by several associations based on the experience gained from colorectal cancer patients in China and Italy, during the pandemic, reciting recommendations to protect both patients undergoing cancer treatments and healthcare professionals. These guidelines all converge to a general direction: It is critical to postpone elective surgery as much as possible but to perform emergency surgery provided that general measures are taken. The Society of American Gastrointestinal and Endoscopic Surgeons published similar guidelines recommending that surgical intervention should be performed in cancer patients who are likely to progress or who require emergency intervention. The situation is not all that simple with regard to colorectal cancers. It is accepted that surgery should be performed in life-threatening conditions such as cancer patients with perforating, obstructing, actively bleeding tumors or septic patients, but other conditions might require further consideration such as looking into the status of the patient, the stage of the tumor, the risk of the surgical procedure and the condition of the respective hospital[41]. Asymptomatic stage I-II patients can have their elective colon cancer surgery deferred for 30 d and have a new decision made at the end of this period; they will not be affected unfavorably by the deferral up to approximately 6 wk. However, the need for a further deferral at the end of the 60-d-period warrants radiological staging for decision making in those patients. In asymptomatic stage III colon cancer patients, deferral longer than 30 d should involve discussing a plan of neoadjuvant chemotherapy. In asymptomatic stage IV colon cancer patients, guidelines recommend initiating chemotherapy and planning surgery depending on the radiological response after three courses of chemotherapy[42]. Figure 1 summarizes the recommended approach to colon cancer in the context of COVID-19.

Rectal surgery can wait no longer than 60 d between the diagnosis and the treatment or the rate of survival will be considerably lower. In a stage I asymptomatic rectal cancer, a 30-d deferral might not affect the oncological outcomes. At the end of the 30-d delay, depending on the patient's symptoms,

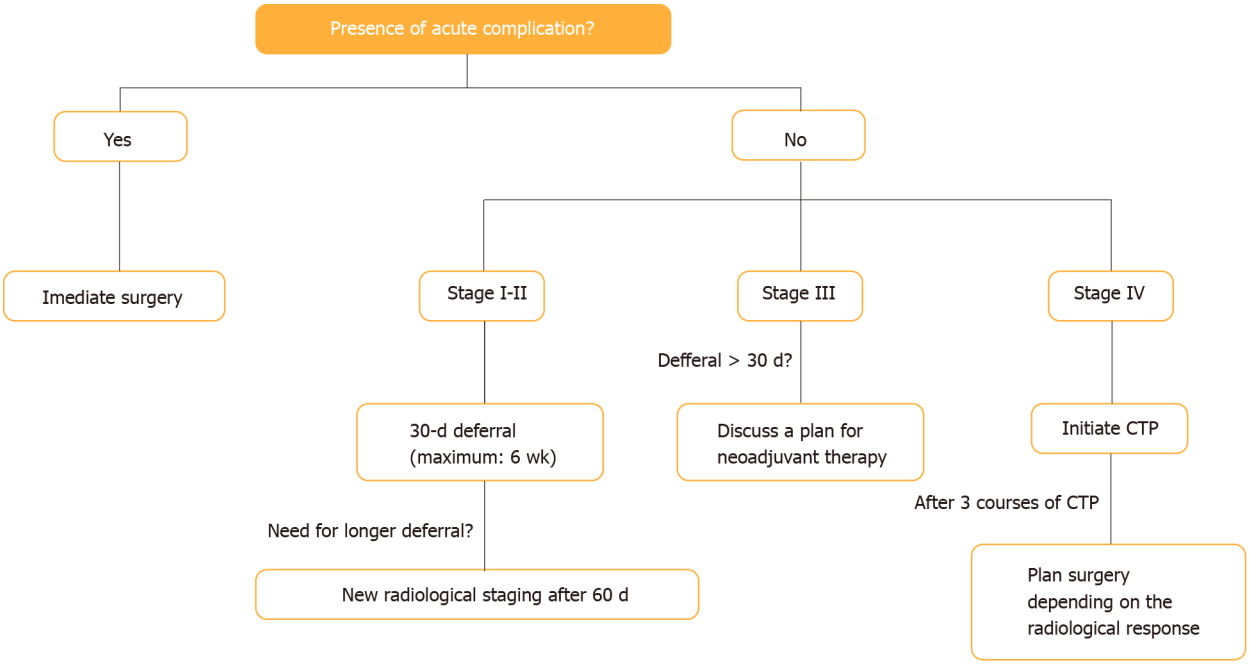


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**Figure 1 Summarizes the recommended approach to colon cancer in the context of coronavirus disease 2019.** CTP: Chemotherapy.

treatment can be deferred for another month but radiological staging is necessary to make any new decisions. In stage II-III rectal cancers, radiotherapy should be administered; its response should be evaluated in the 8th week after radiotherapy. If there is a regression with radiotherapy, surgery could wait for a period of up to 12 or even 16 wk while the patient is closely monitored. If, however, results show no regression at the 8th week with radiotherapy, the decision for surgery can be made depending on the infrastructure of the hospital[43].

Symptomatic rectal cancer patients, usually between the stages II-IV, should make the decision for the treatment depending on the severity of symptoms and findings and their effect on the quality of life. Radiology staging is necessary; patients who are symptomatic but can wait, should, preferably, defer the surgery as described for asymptomatic stage I, II and III. As for patients who have been diagnosed with malignant polyps, it is appropriate to postpone prophylactic surgeries. Whichever the decision regards the patient's treatment, it is necessary to choose protocols that will minimize the patient's hospitalization for both surgery, radiotherapy and chemotherapy procedures. It is peremptory that all the staff should be careful and follow the protocols during the preoperative and postoperative period to prevent infection for themselves and all other patients hospitalized[36].

An issue that should not be forgotten is the fact that because of the aforementioned higher risk of viral transmission in laparoscopic surgeries, open surgeries are the most suitable for COVID-19 patients. If the surgery has to be performed laparoscopically, fixed pressure insufflators, a closed-circuit smoke absorption system, a negative pressure operating room and a carbon dioxide filter should be used to discharge the smoke to reduce the aerosol effects of insufflation. On the other hand, laparoscopic surgery is associated with earlier recovery and discharge and might benefit individuals who are not currently infected with the virus. In summary, minimally invasive surgery, ideally, should not be used in cases known to be infected with SARS-CoV-2 and should only be used after all necessary precautions have been taken[40].

## SARS-COV-2 INFECTION AMONG PATIENTS WITH GASTROINTESTINAL CANCER

The current scientific evidence indicates that individuals with cancer might be more susceptible to a severe course of infection with SARS-CoV-2[44]. The greater likelihood of severe development is probably explained by the immunosuppression that often accompanies malignancies and oncological therapies[45]. However, data on the repercussions of SARS-Cov-2 infection in cancer patients are still being developed with the possibility of inconsistencies regarding the conclusions on the subject[46]. In addition, most studies address various types of neoplasms with a focus on lung and blood cancer, with limited information on gastrointestinal malignancies. In a case-control analysis with 73.4 million cancer patients, including colorectal cancer, the authors concluded that cancer carriers are at increased risk of SARS-CoV-2 infection and that the occurrence of the infection is associated with higher rates of hospitalization and mortality in that population. It confirms the occurrence of worse outcomes among infected

oncologic patients and, interestingly, these findings were especially substantial among African Americans[4].

Furthermore, two meta-analyses had similar conclusions regarding COVID-19 infection in cancer patients. The first included 38 studies and 7094 patients with COVID-19, with a pooled cancer prevalence of 2.3%, and demonstrated that cancer significantly contributed to the occurrence of severe course and death in SARS-CoV-2 infections. The second covered a total of 110 studies with a combined prevalence of cancer as a comorbidity of 2.6% in hospitalized patients with COVID-19 and indicated that the risk of mortality is about five times higher among oncologic patients when compared to non-elderly SARS-CoV-2-infected individuals without comorbidities[44]. One of the first cohorts on the subject evaluated characteristics and clinical outcomes of 105 individuals with gastrointestinal cancer and COVID-19 and 536 non-oncologic SARS-CoV-2-positive patients. Their findings revealed that patients with COVID-19 and gastrointestinal cancer had worse outcomes regarding mortality, ICU admissions, the prevalence of at least one severe or critical symptom and the need for invasive mechanical ventilation when compared to the non-oncologic patients[47]. In addition, a retrospective study with 52 oncologic COVID-19 patients found that some complications such as liver injury (36.5%), acute respiratory distress syndrome (17.3%), sepsis (15.4%), myocardial injury (15.4%), renal failure (7.7%) and multiple organ dysfunction syndrome (5.8%) are common in cancer patients infected with SARS-Cov-2 and, therefore, these individuals may be more prone to more severe outcomes[45].

A study looked at COVID-19-related clinical symptoms, survival rate and risk of infection among cancer patients, including colon cancer and gastric cancer, and the results suggested that thrombocytopenia, anemia and diarrhea are symptoms that increase independently the risk of death in oncologic patients with COVID-19[48]. Another study portrayed gastrointestinal manifestations in 36 cancer patients, of whom 8 had gastrointestinal cancer. Their results concluded that the most prevalent gastrointestinal symptoms in the hospitalized patients were anorexia (52%), diarrhea (39%) and vomiting (35%) and that elevations in hepatic transaminases were associated with a higher occurrence of gastrointestinal symptoms[49].

From an immunological point of view, viral infections and neoplasms are associated with high levels of proteins that activate the T cell-mediated response leading to inflammation which may play important roles in cancer progression[50]. In this sense, some signaling pathways can be affected by both COVID-19 infection and cancer, influencing the expression of type-I IFN and androgen receptor as well as the activation of immune checkpoint signaling pathways, and alterations at these points of the immune response have the potential to lead to the development of a cytokine storm that is closely associated with acute respiratory distress syndrome, organ failure and death in severe COVID-19[51]. Furthermore, ACE2 receptors are highly consumed in SARS-CoV-2 infection due to their ability to assist the virus in cell entry[52]. For this reason, there is a decrease in the availability of those receptors and, as a consequence, important functions played by these receptors may be compromised[52]. In this context, low ACE2 activity has the potential to contribute to severe inflammation and is related to some types of gastrointestinal malignancies such as gallbladder cancer and pancreatic ductal adenocarcinoma[53,54]. Another well-established issue in cancer patients is the immunosuppression caused by the depletion of leukocytes and the use of glucocorticoids in addition to other oncological therapies that compromise the ability of the immune system to respond to viral infections such as SARS-CoV-2 infection, leading to a course of more serious illness[55].

Despite what has been discussed so far, some studies present results that contrast with the conclusions that associate cancer with worse COVID-19 infection outcomes. A prospective cohort that included 9842 patients found that the incidence and severity of clinical presentation of COVID-19 infection in cancer patients are not significantly different from those observed in the general population [46]. In agreement with the aforementioned results, in an observational study gathering 78 cancer patients positive for SARS-Cov-2, only one developed the severe form of the disease and only three developed symptoms[56].

## VACCINATION AGAINST COVID-19 IN GASTROINTESTINAL CANCER PATIENTS

During the new coronavirus pandemic, as soon as vaccination schemes were implemented, certain priority groups were identified, taking into account the epidemiological data obtained so far. In this sense, cancer patients were considered as a priority group, mainly, the worst prognosis of the disease among these individuals including a higher mortality rate. In this context, institutions such as the Asian Oncology Society, the European Society for Medical Oncology and the National Comprehensive Cancer Network recommended that cancer patients be a priority thus including individuals undergoing treatment or about to undergo treatment and those who underwent treatment for at least 6 mo[51,57].

However, despite the priority for vaccination, little is known about the immune response of these individuals after the application of the immunizer. It is necessary to take into account that cancer patients, including those with gastrointestinal involvement, have conditions linked to the disease and to the treatments adopted that can compromise the effective response to the vaccine. In this context, chemotherapy, by causing bone marrow suppression can cause thrombocytopenia and neutropenia. In

addition, radiotherapy, because it is capable of damaging the DNA of cells, including lymphocytes, is also capable of causing lymphopenia. Associated with this, therapies that use corticosteroids and other immunosuppressive elements can further compromise the full functioning of the immune system of individuals undergoing cancer therapy and directly influences the immune response to vaccination. In addition, the initial clinical trials did not include individuals with cancer and the literature addressing the relationship between the vaccine and cancer patients is scarce[58].

Thus, given the need to better understand the immune response to the vaccine in cancer patients, some studies were carried out bringing results with the ability to directly influence the care provided to this group. However, studies focusing exclusively on patients with gastrointestinal involvement seem to have not yet been performed.

Among the parameters adopted by the studies to analyze the immune response to vaccines, anti-Spike (anti-S) IgG antibodies were the most used. Thus, the Coronavirus Disease 2019 Antiviral Response in a Pan-tumor Immune Monitoring (CAPTURE) trial, which included 585 participants, including 87 with gastrointestinal cancer (19%), evaluated individuals immunized with the BNT162b2 (Pfizer–BioNTech) or AZD1222 vaccines (Oxford–AstraZeneca) and found 85% seroconversion after the application of two doses of the immunizer in the general group of patients with solid cancer. In addition, they reported that older age is related to a lower titer of neutralizing antibodies[59].

In this context, studies evaluating seroconversion after the application of the CORONAVAC vaccine were also carried out. In this sense, Yasin *et al*[60] defined an IgG level ≥ 50 AU/mL as seropositive in a study that included 776 cancer patients, including 174 (22.4%) with gastrointestinal involvement, and 715 non-cancer volunteers. The seropositivity rate and antibody level were significantly lower in individuals with cancer when compared to the control group ($P < 0.001$). In this context, the seropositivity rate was 85.2%, with a mean antibody titer of 363.9 AU/mL in the patient group and 97.5%, with a mean antibody titer of 656.5 AU/mL in the control group. In addition, as the CAPTURE study pointed out, age was a factor associated with a lower rate of seropositivity ($P < 0.001$). The study also pointed to ongoing chemotherapy in the group of cancer patients ($P = 0.038$) as a factor capable of negatively influencing seropositivity rates, the opposite was pointed out by the Vaccination Against COVID in Cancer (VOICE) and CAPTURE trials[61]. Table 1 summarizes the seroconversion rates of the immunizers among oncologic patients.

Another important point is linked to the increase in antibody titers that were observed after the application of the second dose. Thus, it is noted that only one dose of the immunizer provides immunity much lower than that which can be obtained with the application of two doses[62]. In this context, Becerril-Gaitan *et al*[57] reported that cancer patients with an incomplete vaccination schedule, when compared to individuals in the control group without cancer, had a 55% reduced probability of reaching anti-S IgG titers above the stipulated threshold (RR 0.45; CI95% 0.35-0.58). For those with a complete vaccination schedule, the reduced probability was 31% (RR 0.69; 95%CI 0.56-0.84).

Given the above, although individuals with cancer reach acceptable seroconversion rates, despite being reduced compared to the "healthy" population, studies indicate that the application of booster doses is indicated for individuals with compromised immunity[63,64]. Thus, in August 2021, the Food and Drug Administration (FDA) authorized the application of the booster dose to immunosuppressed individuals[13]. In this context, Ligumsky *et al*[14] when analyzing the response of 72 cancer patients and 144 "healthy" individuals (control group) to the booster dose of the BNT162b2 vaccine (Pfizer–BioNTech), they initially observed that before the application of the third dose, 20 cancer patients (28%) and two in the control group (1%) were seronegative. However, after the application of the booster dose, only three cancer patients and none of the control group remained seronegative. In addition, when comparing the absolute concentration of anti-SARS-CoV-2 S IgG antibodies, they observed that there was a significant increase in levels in both groups ($P < 0.0001$). In this context, studies also point out that the application of the booster dose can guarantee a better response to variants of concern such as Delta and Omicron[15].

Therefore, it is evident that cancer patients have a less pronounced immune response to vaccination, even with the application of the third dose, when compared to "healthy" individuals in the control group, although satisfactory in most individuals. In addition, it is noted that the application of the booster dose is capable of guaranteeing greater seroconversion in this group and therefore should be encouraged. Finally, more studies are needed to better understand the immune response of cancer patients to currently available vaccines, given that these individuals are subject to variables related to cancer and to the different treatments that can be applied which influence immunity in different ways.

### *Adverse effects associated with vaccination against COVID-19*

Another factor that should be taken into account are the adverse events that may occur as a result of vaccination. In this sense, studies were carried out to analyze the acceptance of cancer patients to immunization. In one of these studies, which involved the participation of 364 cancer patients, when asked if they would take the vaccine as soon as it became available, 41.8% answered "yes", 37.6% answered they were "not sure", and 20.6% answered who would not get the vaccine. Among the factors that encourage cancer patients to be vaccinated are the fear of getting sick, trust in the recommendations of health professionals and the desire to contribute to herd immunity. As for those who expressed doubt or refusal of the vaccine, fear and concern about possible adverse effects were present in 24.5% of the

**Table 1 Seroconversion of immunizers in oncologic patients**

| Immunizer | Ref. | Cancer patients, *n* | % GIC | Seroconversion, % |
|---|---|---|---|---|
| BNT162b2 (Pfizer–BioNTech), OR, AZD1222 (Oxford–AstraZeneca) | Fendler *et al*[59] | 585 | 19 | 85 |
| CORONAVAC | Yasin *et al*[60] | 776 | 22.4 | 85.2 |

GIC: Gastrointestinal cancer.

participants[65].

In this sense, studies suggest that, as in the general population, cancer patients tend to have mild to moderate effects. Thus, a study that included 291 participants immunized with BNT162b2 reported adverse events following immunization in 14.78% of subjects. These include local reactions, pyrexia, fatigue, headache and chills. Furthermore, the risk of developing these events was higher in women ($P$ = 0.001) and young patients ($P$ = 0.009). Another study, which evaluated the BNT162b2 vaccine in 326 participants diagnosed with cancer, reported similar results, without any serious reaction[66,67].

However, despite the majority of events being mild or moderate, the possibility of serious complications exists. Thus, there are case reports that associate certain events with vaccination. In this context, Chong *et al*[68] reported severe thrombocytopenia 3 d after the application of the first dose of the Moderna vaccine and Brage *et al*[69] reported fulminant myocarditis after receiving the third dose of the Moderna vaccine. In this context, it is evident that serious adverse events can occur, but most patients have mild or moderate events. However, more studies are needed to better clarify the effects presented and understand the possible interactions between the different types of anti-cancer treatment and the epidemiological factors of each individual with the development of mild, moderate or severe reactions.

Nevertheless, it is still the role of health professionals to inform their patients about the risks and benefits of vaccination helping them to make effective decisions.

## CONCLUSION

The COVID-19 pandemic has been negatively impacting the diagnosis, treatment and prognosis of gastrointestinal cancer. Although most studies indicate that having cancer, in general, implies a greater risk of severe COVID-19, it is an ongoing pandemic with still limited studies and only a few investigations are specific for neoplasms from the gastrointestinal tract. The immunosuppression caused by cancer and its related therapies probably make the patient more vulnerable to infections; however, the measures adopted to avoid the contagion in this population can also impair anticancer therapies. Therefore, it is essential that research on the subject continues to evolve towards a better understanding of how the pandemic caused by the new coronavirus interferes with the context of gastrointestinal cancer in order to improve the approach to cancer patients and solve remaining challenges in that context.

## FOOTNOTES

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**Country/Territory of origin:** Brazil

**ORCID number:** Breno Bittencourt de Brito 0000-0002-1831-7909; Hanna Santos Marques 0000-0001-5741-1570; Filipe Antônio França da Silva 0000-0002-0550-1109; Maria Luísa Cordeiro Santos 0000-0001-7078-9789; Glauber Rocha Lima Araújo 0000-0002-5195-3632; Fabrício Freire de Melo 0000-0002-5680-2753.

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*MINIREVIEWS*

# COVID-19 in patients with gastrointestinal stromal tumors: Recommendations for management and vaccination

Violeta Snegarova, Dimitrina Miteva, Milena Gulinac, Monika Peshevska-Sekulovska, Hristiana Batselova, Tsvetelina Velikova

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**Violeta Snegarova,** Clinic of Internal Diseases, Naval Hospital – Varna, Military Medical Academy, Medical Faculty, Medical University, Varna 9000, Bulgaria

**Dimitrina Miteva,** Faculty of Biology, Department of Genetics, Sofia University "St. Kliment Ohridski", Sofia 1164, Bulgaria

**Milena Gulinac,** Department of General and Clinical Pathology, Medical Faculty, Medical University of Plovdiv, Plovdiv 4000, Bulgaria

**Monika Peshevska-Sekulovska,** Department of Gastroenterology, University Hospital Lozenetz, Sofia 1407, Bulgaria

**Monika Peshevska-Sekulovska, Tsvetelina Velikova,** Medical Faculty, Sofia University St. Kliment Ohridski, Sofia 1407, Others, Bulgaria

**Hristiana Batselova,** Department of Epidemiology and Disaster Medicine, Medical University, Plovdiv, University Hospital "St George", Plovdiv 6000, Bulgaria

**Tsvetelina Velikova,** Department of Clinical Immunology, University Hospital Lozenetz, Sofia 1407, Bulgaria

**Corresponding author:** Tsvetelina Velikova, MD, PhD, Assistant Professor, Chief Doctor, Department of Clinical Immunology, University Hospital Lozenetz, Kozyak 1 Street, Sofia 1407, Bulgaria. tsvelikova@medfac.mu-sofia.bg

## Abstract

The coronavirus disease 2019 (COVID-19) pandemic profoundly affected the management and treatment of patients with malignancies. Based on the progress reported in the literature, we reviewed the recommendations for treatment and vaccination in patients with gastrointestinal stromal tumor (GIST) during COVID-19. We focus on whether there is a risk and what could be the possible effects of vaccinating patients with GIST/cancer. Since the situation is quickly changing, and the health services have been severely disrupted, the diagnosis, treatment and recommendations for vaccination of these patients against COVID-19 are still not updated. The approval of vaccines in the pandemic gave hope that we would soon be able to return to a more normal life. However, the oncology community needs to adapt and provide the most effective treatment and care models for patients with rare cancer, such as GIST. Collecting data on the impact of

vaccination in patients with GIST/cancer also will be beneficial in expanding knowledge about the future planning of treatment strategies and optimizing care in the event of a subsequent pandemic.

**Key Words:** Gastrointestinal stromal tumor; GIST; Cancer; COVID-19 vaccination; efficacy; Treatment strategy; Side effects



**Core Tip:** Even under normal operating conditions, appropriate monitoring and treating patients with gastrointestinal stromal tumors (GISTs) require complex decision-making. Given the growing number of deaths worldwide and the failure of many countries to control the pandemic, vaccination against COVID-19 in these patients must be accelerated. The data show no significant difference in the efficacy of vaccines for the GIST population compared to that of other cancers. Vaccination between cycles of therapy and after waiting periods for patients with stem cell transplantation and immunoglobulin therapy can be used to reduce the risks while protecting patients from risk groups.

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## INTRODUCTION

The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) pandemic in China at the end of 2019 and coronavirus disease 2019 (COVID-19) are considered risk factors for severe outcomes in cancer patients[1]. Statistics indicate that by March 13, 2022, there have been > 6 million deaths caused by COVID-19 worldwide, and the number of confirmed cases recorded is > 455 million[2].

In line with this, according to a number of reports, diseases such as diabetes, hypertension, cardiovascular diseases, respiratory diseases, and cancer are associated with an increased risk of fatality in patients diagnosed with COVID-19[3]. In addition, an international study involving 1035 patients with COVID-19 who have concomitant cancer showed that these patients had a higher risk of hospitalization and need for intensive care and mechanical ventilation, regardless of the type of malignancy and antitumor therapy[4].

Patients with malignant diseases represent a heterogeneous group. Therefore, it remains to be determined which factors related to tumor type and treatment increase the risk of infection with COVID-19 and adverse outcomes[5]. According to a study that aimed to identify the risk factors of severe COVID-19 infection in patients with malignancy, the administration of antitumor treatment (chemotherapy, radiotherapy, targeted therapy or immunotherapy) within 14 d of diagnosis significantly increases the risk[6].

To assist health care facilities and minimize the negative effects of the pandemic associated with COVID-19 in patients with malignancies, the European Society for Medical Oncology (ESMO), the American Society of Clinical Oncology, the National Comprehensive Cancer Network (NCCN) and other organizations have developed recommendations for patient categorization based on the Ontario Health Cancer Care criteria[7].

Gastrointestinal tumors are a relatively new tumor group that has emerged in recent decades from other mesenchymal tumors in this field, mainly neurinomas and leiomyomas, thanks to the achievements of modern medicine in molecular biology and pharmacotherapy. Therefore, the justification for a separate tumor form merits an in-depth multidisciplinary study. Furthermore, it represents a model for successfully applying targeted therapy in treating solid tumors[8].

Gastrointestinal stromal tumors (GISTs) are rare neoplasms of the gastrointestinal tract associated with high rates of malignant transformation. They represent 1%–2% of all gastrointestinal neoplasms [9]. The mean age at diagnosis is 58 years, with most patients being between the ages of 40 and 80 years [10].

Although the risk of SARS-CoV-2 infection is not increased in GIST patients, they may experience other consequences during the COVID-19 pandemic, such as delay in treatment, delayed surgery or long waiting period for elective surgery, a heavy burden on medical resources, and the need for emergency surgery[11]. Additionally, neoadjuvant imatinib is routinely used to shrink locally advanced GISTs and if there is a danger of positive margins, unresectable, or borderline resectable tumors[12]. Imatinib may be a beneficial alternative to minimize the possibility of tumors developing in

intermediate or high-risk cancers bearing imatinib-sensitive mutations that would otherwise be excised during a time of limited access to surgical therapy[12]. Even if imatinib is generally well tolerated, patients may develop adverse effects such as myelosuppression (grade 3 in up to one-fifth of all patients), which might be concerning if the patient becomes infected with SARS-CoV-2[13]. Finally, initial watchful waiting would not rule out the possibility of starting imatinib if the tumor progressed.

The term GIST was introduced by Mazur and Clarck in 1983 for a group of nonepithelial mesenchymal tumors of the gastrointestinal tract (most often leiomyomas, leiomyosarcomas and neurinomas), which differ from the eponymous tumors in other areas of the body in their immunohistochemical characteristics[14]. It is now commonly accepted that GISTs derive from so-called pacemaker cells in the intestinal tract – the interstitial cells of Cajal or similar stem cells[15]. Cajal cells are intermediates of gastrointestinal autonomic nervous system cells and smooth muscle cells and regulate the motility and autonomic nerve conduction and function activity. They are positive for Kit and Kit-ligand (stem cell marker), localized around the myenteric plexus and in the stratum muscularis propria along the entire gastrointestinal tract. Cajal cells can either be or include a subclass of multipotent, stem-like cells that can differentiate into smooth muscle cells if the Kit signaling pathway is disrupted[16]. In most cases, GISTs are specifically Kit (CD117) positive or caused by mutations in *Kit* or *PDGFRA* genes, and are the primary mesenchymal tumors of the gastrointestinal tract with characteristic histological features[17].

In the 1990s, GIST were found to express CD34 antigen, which has been identified as a distinguishing feature of neurinomas and leiomyomas. However, in a new study phase, GISTs were found to have standard immunohistochemical and ultrastructural features with Cajal interstitial cells or related stem cells, as stated above. For this reason, studying Kit (CD117) expression in tumor cells is the best immunostaining method for identifying GIST[14,18-20].

GISTs have malignant and insufficiently predictable biology and behavior, even with benign histological features. Morphologically, GISTs vary from spindle cell tumors to epithelioid and pleomorphic tumors. GISTs have approximately the same distribution in both sexes. Most are localized in the stomach (50%–60%) and the small intestine (30%). Esophageal, colorectal and rectal GISTs are rare (3%)[21].

The diagnosis of GIST is based on pathomorphological evidence by histological examination of biopsy material, and when taking a biopsy, the recommendations of NCCN. The NCCN organized a multidisciplinary panel composed of experts in surgery, pathology, medical oncology and molecular diagnostics to discuss the optimal approach for the care of patients with GIST at all stages of the disease [22,23].

## SEARCH STRATEGY

We performed a modified form of a narrative review where a search through scientific databases combined solid evidence from studies on vaccine effectiveness and safety in patients with gastrointestinal tumors and GISTs. The first literature search was carried out in Medline (PubMed) and Scopus bibliographic databases. Both MeSH and relevant free-text terms were used, as follows: (COVID-19 OR SARS-CoV-2) AND (GIST OR gastrointestinal stromal tumor) AND (vaccine* OR mRNA). Our search was confined to articles published up to April 2022. Finally, references of retrieved publications were further hand-searched for supplements.

### *Official recommendations for COVID-19 vaccination in patients with GIST*

Up to date, no specific and official recommendations are included in the ESMO–EURACAN–GENTURIS Clinical Practice Guidelines for diagnosis, treatment and follow-up for GIST (2022)[24]. However, ESMO statements on vaccination against COVID-19 in people with cancer conclude that all the approved COVID-19 vaccines could be administered to patients with cancer taking into account their effectiveness and safety, according to the official international recommendations[25]. Furthermore, ESMO has confirmed that the mass vaccination program is a crucial strategy for protecting against severe infection. This also stands for vulnerable patients, such as cancer patients, who take advantage of the most preferable benefit–risk ratio[25]. Since some patients with cancer, especially those with active malignancies, may experience a greater risk of severe SARS-CoV-2 infection, ESMO recommends COVID-19 vaccination. Despite reduced effectiveness for specific subgroups of cancer patients, the protection is still meaningful, and vaccination is strongly advised. Patients with hematological malignancies, particularly those undergoing cytotoxic chemotherapy, anti-CD20, CAR-T cell, or stem-cell-transplant-based treatments, are also among these populations.

### *Effectiveness and safety of COVID-19 vaccines in patients with GIST*

Prior to the COVID-19 pandemic, there was little evidence of the humoral and cellular immune responses to antiviral vaccination in cancer patients. Additionally, this primarily addressed the influenza vaccination[26,27]. Despite a general exclusion of cancer patients from the major clinical studies of COVID-19 and COVID-19 vaccination, subsequent results repeatedly proved the effectiveness

and safety of SARS-CoV-2 immunization in these patients. Overall, after complete COVID-19 immunization, persons with cancer have clinically significant seroconversion rates[28-32]. Although the efficiency of mRNA and adenoviral vector vaccines appears almost identical[30], there is a lack of comparative effectiveness data, particularly in cancer patients. Notably, when only one dose of an mRNA vaccine is delivered, the incidence of seroconversion is much reduced, emphasizing the necessity of vaccination completion and, eventually, booster for cancer patients[33,34].

However, there are not enough data from studies for COVID-19 vaccination in patients with GIST. There have been a few studies[30,35-39] that mainly recruited patients with gastrointestinal tumors, some with GIST, as summarized in Table 1. Thakkar *et al*[30] and Suenaga *et al*[35] demonstrated that even on chemotherapy, patients with gastrointestinal tumors tolerated COVID-19 vaccines well. Additionally, the effectiveness was assessed as adequate for SARS-CoV-2 infection protection. This observation was also valid for immunocompromised patients due to cancer treatment[36-38]. Given the scientific and logistical challenges in identifying cancer patients with weak or decreasing immunity, the global strategy of a booster dosage vaccination should be investigated for cancer patients. However, until better quality information on booster dosage benefits becomes available, international recommendations considering the risk of poor COVID-19 outcomes in cancer patients, vaccine availability/access, immunization progress, and the pandemic burden should be followed.

### *Are there any risks for vaccination of patients with GIST/cancer*

The most significant driver for public health protection is the availability and equal access to COVID-19 immunization, with conformity to international criteria to be encouraged and supported. Therefore, vaccination plans have been established worldwide to prioritize vaccine delivery in various groups, including cancer patients. On the other hand, cancer patients do not constitute a homogenous group. And GISTs are among the rare cancer types.

In general, cancer patients can be divided into three groups: patients with active disease undergoing treatment, patients with chronic illness following specific therapy, and patients in the survival phase. Vaccination is essential to protect all of these patient groups[25]. If we translate this knowledge to the patients with GIST, we can assume that COVID-19 vaccination is strongly advised for them.

However, despite increasing compliance rates and existing evidence/data, 10%–20% of patients remain skeptical about the COVID-19 vaccine. These patients are at a higher risk of developing severe COVID-19 illness. In addition, they are a more likely source of SARS-CoV-2 transmission to other, more sensitive cancer patients[25]. It is critical to reinforce trust, education, and easy, transparent communication with those patients and their relatives based on the accumulated knowledge and better understanding of their concerns and hesitancy. In addition, communication of available data on vaccine safety and efficacy to people with cancer should also include assuring them that COVID-19 vaccines will not interfere with their cancer treatment[40]. Furthermore, there is no indication that COVID-19 immunizations substantially influence anticancer medication's efficacy or safety profile, such as cytotoxic chemotherapy, immune checkpoint inhibitors, or targeted therapies. Thus, COVID-19 vaccination is strongly advised[25]. More data on the preference for a specific type of vaccine and potential unusual interactions of SARS-CoV-2 vaccines with antineoplastic therapy should be collected by in-trial, post-trial, and registry monitoring.

Suppose an anticancer medication is urgently required for disease control. It is advised that suitable medication be implemented first, followed by COVID-19 immunization, as soon as the patient is clinically stable and significant symptoms are under control. To minimize misattribution of any short-term reactions/side effects, providers may consider administering anticancer medication and COVID-19 vaccinations on different days[41].

Therefore, since we do not have studies on the effectiveness and safety of COVID-19 vaccination for patients with GISTs, we have to rely on the official recommendations for patients with cancer generally. The data for rare diseases usually accumulate slowly. To protect patients from a "double jeopardy", informed consent and collaborative decision-making should be the rule when discussing the advantages and risks of COVID-19 immunization and SARS-CoV-2 infection.

## CONCLUSION

Before the COVID-19 pandemic, most vaccination research with cancer patients was conducted for vaccines against hepatitis B, influenza and other infections. However, as the immune response is reduced in those patients, the risk of severe COVID-19 should be noted. Therefore, patients have to receive complete vaccination and booster doses to acquire higher levels of protection. This is also valid for patients with GIST. COVID-19 vaccination could be administered to patients who are even on therapy if some vaccine components are not contraindicated. The data show no significant difference in the efficacy of vaccines for the GIST/cancer population compared to other cancers. Oncologists have extensive experience in vaccinating cancer patients who are being treated, so they can effectively help save their patients' lives.

**Table 1 Studies of COVID-19 vaccination in patients with gastrointestinal tumors**

| Ref. | Type of study | Type of COVID-19 vaccine | Participants | Efficacy/effectiveness | Adverse effects |
|---|---|---|---|---|---|
| Suenega *et al* [35], 2022 | Retrospective observational study | mPNA (BNT162b2 or mRNA-1273) | Gastrointestinal cancer patients, *n* = 52 | BNT162b2 (approximately 95%), mRNA-1273 (approximately 94%) | 82.2% had adverse events: Injection site pain (approximately 67%), fatigue (approximately 12%), fever (approximately 6%), headache (approximately 4%), gastrointestinal problems (approximately 4%), redness (approximately 2%), insomnia (approximately 2%); no vaccine-related deaths |
| Fendler *et al* [36], 2022 | Retrospective observational study | BNT162b2; mRNA-1273 | 115 | mRNA vaccines (against omicron approximately 75%) (against delta approximately 79%); against omicron increased from 47.8% to 88.9% following a third vaccine dose | Injection site pain (approximately 63%), local swelling (9%), muscle pain (34%), fatigue (34%), headache (16%), fever (10%), chills (10%) and gastrointestinal events (10%); no vaccine-related deaths |
| Thakkar *et al* [30], 2021 | Retrospective study | BNT162b2, mRNA-1273, Ad26.COV2.S | 27 (14%) from 200 are with GIST | BNT162b2 (95%), mRNA-1273 (94%), Ad26.COV2.S (85%) | Sore arm (20%–37%), fatigue (5%–16%), muscle ache (5%–17%), fatigue (1%–5%), rash (1%–3%), redness (approximately 2%), other (1%–5%); no vaccine-related deaths |
| Embi *et al* [37], 2021 | Observational study | BNT162b2; mRNA-1273 | 20 101 immunocompromised patients | BNT162b2 (71%), mRNA-1273 (81%) | Sore arm (20%–47%), fever (10%), fatigue (1%–5%), other (1%–5%); no vaccine-related deaths |
| Karacin *et al* [38], 2021 | Prospective observational study | CoronaVac vaccine | 47 | Sero-response rate 63.8% | Pain at the injection site (4.2%), fever (2.1%), fatigue (4.2%–10.5%), headache (2.1%), and myalgia (2.1%), There were no serious side effects or toxic deaths |
| Ariamanesh *et al*[39], 2022 | Prospective study | BBIBP-CorV | 364 (32 patients with gastrointestinal tumors) | Sero-response rate 86.9% | Injection site pain, fever, fatigue, headache |

## FOOTNOTES

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**Country/Territory of origin:** Bulgaria

**ORCID number:** Violeta Snegarova 0000-0003-0754-6439; Dimitrina Miteva 0000-0002-5931-2426; Milena Gulinac 0000-0001-7970-9378; Monika Peshevska-Sekulovska 0000-0002-8468-0132; Hristiana Batselova 0000-0002-6201-848X; Tsvetelina Velikova 0000-0002-0593-1272.

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Editorial Board Member of *World Journal of Gastrointestinal Pathophysiology*, Narongrit Thongon, PhD, Associate Professor, Division of Physiology, Department of Biomedical Sciences, Faculty of Allied Health Sciences, Burapha University, Muang 20131, Chonburi, Thailand. narongritt@buu.ac.th

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The primary aim of the *World Journal of Gastrointestinal Pathophysiology* (*WJGP*, *World J Gastrointest Pathophysiol*) is to provide scholars and readers from various fields of gastrointestinal pathophysiology with a platform to publish high-quality basic and clinical research articles and communicate their research findings online.

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ORIGINAL ARTICLE

Prospective Study

# Knowledge regarding celiac disease among healthcare professionals, patients and their caregivers in Turkey

Yasin Sahin, Eylem Sevinc, Nevzat Aykut Bayrak, Fatma Ilknur Varol, Ulas Emre Akbulut, Ayşegül Bükülmez

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**Yasin Sahin,** Department of Pediatric Gastroenterology, Dr. Ersin Arslan Training and Research Hospital, Gaziantep, Turkey

**Yasin Sahin,** Gaziantep Islam Science and Technology University, Faculty of Medicine, Gaziantep 27560, Gaziantep, Turkey

**Eylem Sevinc,** Department of Pediatric Gastroenterology, Karabuk University, Faculty of Medicine, Karabuk 78100, Karabuk, Turkey

**Nevzat Aykut Bayrak,** Department of Pediatric Gastroenterology, Zeynep Kamil Women and Children's Training and Research Hospital, University of Health Sciences, Istanbul 34668, Istanbul, Turkey

**Fatma Ilknur Varol,** Department of Pediatric Gastroenterology, Inonu University, Faculty of Medicine, Malatya 244280, Malatya, Turkey

**Ulas Emre Akbulut,** Department of Pediatric Gastroenterology, University of Health Sciences, Antalya Training and Research Hospital, Antalya 07100, Antalya, Turkey

**Ayşegül Bükülmez,** Department of Pediatric Gastroenterology, Afyonkarahisar Health Sciences University, Afyonkarahisar 03200, Afyonkarahisar, Turkey

**Corresponding author:** Yasin Sahin, MD, Academic Editor, Associate Professor, Department of Pediatric Gastroenterology, Dr. Ersin Arslan Training and Research Hospital; Gaziantep Islam Science and Technology University, Faculty of Medicine, Gaziantep 27560, Gaziantep, Turkey. ysahin977@gmail.com

## Abstract

### *BACKGROUND*

Celiac disease (CD) is one of the most prevalent chronic disorders. The clinical manifestations of CD are diverse and may present with gastrointestinal findings, extra-intestinal findings or no symptoms. Although there has been a marked increase in the prevalence of CD in the past 30 years, up to 95% of patients with CD remain undiagnosed. As most cases have atypical signs or no symptoms, the diagnosis of CD is either missed or delayed. In addition, one of the most important reasons for the delay in diagnosis may be the poor knowledge of healthcare professionals (HCPs) regarding CD.

### *AIM*

To evaluate the knowledge of HCPs, patients and their caregivers (parents) regarding CD.

***METHODS***

The current study was carried out between June 2021 and February 2022 prospectively, as part of the Focus IN CD project. Patients with CD and their caregivers participated in the study from 6 different cities in Turkey. General practitioners, pediatricians, pediatricians with other subspecialities and pediatric gastroenterologists from different cities participated in the study.

***RESULTS***

The questionnaire was completed by 348 HCPs, 34 patients with CD, and 102 mothers and 34 fathers of patients with CD. Most of the participants were general practitioners (37.07%). There were 89 (25.57%) pediatricians and 72 (20.69%) pediatric gastroenterologists in the study. The highest score in all categories was achieved by pediatric gastroenterologists. There were significant differences between the four groups of HCPs in terms of the subsections of overall mean score, epidemiology and clinical presentation, treatment and follow-up. No significant difference was found between the groups (patients with CD, mothers of patients with CD and fathers of patients with CD) in terms of the questionnaire subsections.

***CONCLUSION***

The level of knowledge on CD among HCPs, patients and their caregivers was unsatisfactory. We consider that it is necessary to increase awareness and to develop e-learning activities on CD among HCPs, patients and their caregivers. Consequently, they may benefit from e-learning programs similar to the one created as part of the EU-funded project Focus IN CD (https://www.celiacfacts.eu/focusincd-en).

**Key Words:** Celiac disease; Healthcare professionals; Knowledge; Patients



**Core Tip:** In this study, we aimed to evaluate the knowledge of healthcare professionals (HCPs), patients and their caregivers (parents) regarding celiac disease (CD). We found that the level of knowledge on CD among HCPs, patients and their caregivers was unsatisfactory. We consider that it is necessary to increase awareness and to develop e-learning activities on CD among HCPs, patients and their caregivers. Patients, their caregivers, and HCPs may benefit from e-learning programs similar to the one created as part of the EU-funded project Focus IN CD (https://www.celiacfacts.eu/focusincd-en).

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## INTRODUCTION

One type of systemic autoimmune illness is celiac disease (CD), which is characterized by a combination of various degrees of small bowel damage and clinical manifestations triggered by gluten ingestion in people who are genetically vulnerable[1,2]. It is one of the most common chronic disorders. The prevalence of CD is estimated to be approximately 1% in the general population worldwide[1,2].

The clinical manifestations of CD are diverse and may present with gastrointestinal findings, extra-intestinal findings or no symptoms[1-3]. Constipation, recurrent abdominal pain, bloating, and chronic diarrhea are the primary gastrointestinal symptoms. Short stature, iron deficiency anemia, and poor growth, decreased bone mineral density, dermatitis herpetiformis, delayed puberty, alopecia, neurological symptoms, headache, joint manifestations, fatigue, stomatitis, infertility, and unexplained abnormal liver enzymes are common extra-intestinal symptoms[1]. The definitive diagnosis of CD is carried out by evaluating clinical findings, positivity of CD specific serological tests, and characteristic histological findings in the small intestinal mucosa[1].

In the past 30 years, there has been a noticeable rise in the prevalence of CD, which may be attributed to a combination of factors including greater medical education and awareness of CD as well as the utilization of very sensitive and specific diagnostic tests[4,5]. Due to increased awareness, up to 95% of patients with CD remain undiagnosed[6,7]. It has been reported that the delay in diagnosis is between 4

and 10 years[8-10]. Undiagnosed cases are very high even in developed countries. As most cases have atypical signs or no symptoms, the diagnosis of CD is either missed or delayed[11,12]. Other factors that may contribute to delayed or missed diagnosis include the scarcity of serological diagnostic tests in developing countries and a scarcity of experienced specialists in this field[13].

Early diagnosis is crucial in order to prevent long-term complications of CD such as malnutrition, osteoporosis, infertility, small bowel cancer, and lymphoma[14].

One of the most important reasons for the delay in diagnosis may be the poor knowledge of healthcare professionals (HCPs) regarding CD. In addition, insufficient information on CD may affect adherence to a gluten-free diet. As CD affects many systems such as neurological, hematological and reproductive systems, it is very important to adhere to a strict gluten-free diet to prevent long-term complications[2,11]. There are limited studies investigating the knowledge regarding CD among HCPs, patients and their caregivers. To our knowledge, there are no studies on this issue in Turkey. The aim of the present study was to evaluate the knowledge of HCPs, patients and their caregivers (parents) regarding CD.

## MATERIALS AND METHODS

The current study was carried out between June 2021 and February 2022 prospectively, as part of the Focus IN CD project. The local Ethics Committee approved the study (Sanko University, Gaziantep, Turkey, June 2, 2021/06).

### *Participants and study design*

Patients with CD and their caregivers participated in the study from 6 different cities in Turkey. General practitioners, pediatricians, pediatricians with other subspecialities and pediatric gastroenterologists from different cities participated in the study.

Patients with CD who were followed up and treated in pediatric gastroenterology outpatient clinics were selected. Face to face communication with patients was conducted. Those who voluntarily agreed to participate were included in the study. Communication with HCPs was established by face to face communication and by phone, and then a link was sent *via* WhatsApp to those who voluntarily participated in the study. Also, HCPs and patients, who did not answer all the questions, were excluded from the study.

We analyzed the differences in the knowledge on CD among HCPs and differences in the knowledge between patients with CD and their caregivers.

HCPs, patients with CD and their caregivers were asked to answer and complete web-based questions on CD (for HCPs https://tr.surveymonkey.com/r/Q2_Focus_in_CD_TUR) (for patients with CD and their caregivers https://tr.surveymonkey.com/r/Q3_CD_in_Focus_TUR).

The questionnaire for HCPs included 21 questions in total, which were divided into 3 subgroups: Epidemiology and clinical presentation (7 questions), diagnostic methodology (7 questions), and treatment with follow-up (7 questions). Fourteen questions were included in the questionnaire for patients and parents, and they were categorized into two subgroups: Epidemiology, clinical presentation, and diagnostic methods (7 questions) and treatment with follow-up (7 questions). All 14 questions were similar to the questions for HCPs. Nine of those questions were exactly the same. The remaining 5 questions required fewer answers from patients and their relatives.

### *Statistical analysis*

Version 22.0 of the Statistical Package for Social Sciences program was used for the statistical analysis (SPSS Inc; Chicago, IL, United States). Descriptive statistics were used for frequency, percentage, and mean ± standard deviation (SD). To ascertain if the data distribution adhered to a normal distribution, the Kolmogorov-Smirnov test was utilized. For nominal data, the independent samples *t*-test was performed. To compare ranges of numerical variables, the Mann-Whitney U test was employed. For the comparison of categorical variables, the chi-square test was used. One-way analysis of variance (ANOVA) for independent groups was used to compare the groups. When there was a significant difference between the groups, Post Hoc Multiple Comparison Tests were performed to determine which groups showed a statistically significant difference.

## RESULTS

### *Analysis of healthcare professionals' knowledge*

The questionnaire was completed by 348 HCPs. Most of the participants were general practitioners (37.07%). There were 89 (25.57%) pediatricians and 72 (20.69%) pediatric gastroenterologists in the study (Table 1). Forty-six HCPs who did not answer all the questions, were excluded from the study.

**Table 1 The distribution of health care professionals according to specialty**

| Specialty | Number (%), *n* = 348 |
|---|---|
| General practitioners | 129 (37.07) |
| Pediatricians | 89 (25.57) |
| Pediatricians with other subspecialities | 58 (16.67) |
| Pediatric gastroenterologists | 72 (20.69) |

The highest score in all categories was achieved by pediatric gastroenterologists. There were significant differences between the four groups of HCPs in terms of the subsections of overall mean score, epidemiology and clinical presentation, treatment and follow-up ($P < 0.001$). There was a significant difference between the four groups of HCPs in terms of the subsections of diagnostic procedure ($P = 0.023$). After performing Post Hoc Multiple Comparison Tests, a difference was detected between pediatric gastroenterologists and the other groups. No one answered all the questions correctly. When analyzing the questionnaire subsections, we detected a lower mean score in the subsection on diagnostic procedure in the pediatricians with different subspecialities in comparison to the other HCPs (Table 2).

Healthcare professionals mostly received information on CD from books (68.32%), the internet (67.6%), at seminars, lectures, and congresses (66.0%) and medical journals (56.7%).

### *Analysis of patients and caregivers' knowledge*

The questionnaire was completed by 34 patients with CD, 102 mothers and 34 fathers of patients with CD. Thirty-two caregivers, who did not answer all the questions, were excluded from the study.

No significant difference was found between the groups (patients with CD, mothers of patients with CD and fathers of patients with CD) in terms of all the questionnaire subsections ($P > 0.05$) (Table 3). None of the patients with CD or their caregivers answered all the questions correctly. The highest mean score in all subsections was achieved by the fathers of patients with CD. Of the 168 patients with CD and their caregivers (parents), 19 (11.3%) of them were members of the Local Celiac Society.

There was no significant difference between the groups (patients with CD, mothers of patients with CD and fathers of patients with CD) in terms of duration of diagnosis ($P > 0.05$). In addition, no significant difference was found between the groups (patients with CD, mothers of patients with CD and fathers of patients with CD) in terms of educational level ($P > 0.05$).

## DISCUSSION

Celiac disease is one of the most common systemic diseases. The clinical manifestations of CD are very diverse[1,3]. Delayed diagnosis can result in many complications such as growth retardation, osteopenia, delayed puberty, infertility, and malignancy[2,14,15]. Despite the development of sensitive and specific tests in recent years, the majority of patients with CD is still not diagnosed[1,2,10].

One of the most important reasons for delays in diagnosis may be poor knowledge of HCPs regarding CD[16,17]. The delay in diagnosis has been reported to be up to 10 years[8-10]. According to reports, the number of undiagnosed cases is estimated to be very high. Due to the lack of clinically obvious symptoms in most CD patients, the diagnosis is often missed or delayed[11,12]. Therefore, awareness in HCPs regarding CD is very important in order to diagnose more patients.

In the present study, family physicians and pediatricians had lower scores in the survey than pediatric gastroenterologists, and there was a statistically significant difference between them. It is very important to increase the knowledge of family physicians and pediatricians on CD, as they represent the first HCP for potential patients with CD[16,17]. Consistent with the present findings, Riznik *et al*[17] and Zipser *et al*[18] also strongly suggested that the level of knowledge in family physicians regarding CD symptoms and related diseases should be increased. Both our study and the results of these two studies have revealed that increasing the level of knowledge and awareness of CD in family physicians and pediatricians in order to refer patients thought to have CD to pediatric gastroenterologists may reduce the delay in CD diagnosis.

Assiri *et al*[16] reported that the level of knowledge in young doctors is better. As CD is not a rare disease, more detailed information on CD is now known about the disease in medical faculties. On the other hand, Barzegar *et al*[19] found that the level of knowledge regarding diagnosis and treatment by doctors who have been practicing medicine for more than 10 years was higher than that in young doctors. In contrast to these studies, no difference was detected in the present study concerning this issue.

**Table 2 Results achieved by healthcare professionals according to the different questionnaire subsections on celiac disease**

| | General practitioners | Pediatricians | Pediatricians with other subspecialities | Pediatric gastroenterologists | *P* value |
|---|---|---|---|---|---|
| Overall mean score | 54.18 ± 21.11 | 55.20 ± 20.90 | 50.29 ± 22.26 | 66.37 ± 15.32 | < 0.001 |
| Epidemiology and clinical presentation | 66.87 ± 17.98 | 67.17 ± 17.79 | 62.01 ± 18.98 | 74.79 ± 17.12 | < 0.001 |
| Diagnostic procedure | 40.38 ± 24.15 | 45.24 ± 24.78 | 40.29 ± 25.36 | 51.64 ± 22.94 | 0.023 |
| Treatment and follow-up | 55.29 ± 32.47 | 53.18 ± 31.76 | 48.56 ± 33.08 | 72.68 ± 18.82 | < 0.001 |

**Table 3 Results of celiac patients and parents according to the questionnaire**

| | Mothers of patients with CD | Fathers of patients with CD | Patients with CD | *P* value |
|---|---|---|---|---|
| | *n* = 102 | *n* = 34 | *n* = 34 | |
| Overall mean score | 45.78 ± 18.10 | 48.63 ± 19.31 | 38.28 ± 19.22 | 0.055 |
| Epidemiology, clinical presentation and diagnosis | 47.65 ± 15.03 | 51.95 ± 17.12[a] | 41.00 ± 17.40[a] | 0.018 |
| Treatment and follow-up | 43.90 ± 28.02 | 45.31 ± 27.24 | 35.56 ± 27.72 | 0.260 |

[a]There was a significant difference between two groups. CD: Celiac disease

In the current study, excluding the pediatric gastroenterologists, approximately half of the questions were answered correctly. Interestingly, even pediatric gastroenterologists answered about half of the questions correctly on the diagnostic procedure. These results were unsatisfactory but in line with previous studies[16,17,19-22].

As expected, pediatric gastroenterologists scored highest of all the groups in the study, their awareness of CD was high, but an average of 50% correct answers were given in the section on diagnostic procedure. As we found that pediatric gastroenterologists have insufficient knowledge of the 2020 ESPGHAN guideline for diagnosing CD in the survey, we considered that the current ESPGHAN guideline is not followed entirely by pediatric gastroenterologists. Poor knowledge among HCPs leads to increased numbers of undiagnosed cases[19,20,23-25].

In the present study, we determined that the knowledge and awareness levels of the patients and their caregivers on CD were both low and unsatisfactory.

The fathers had a mean score greater than 50% in the subsection on epidemiology, clinical presentation and diagnosis, the mean scores of patients with CD, and parents of patients with CD were below 50% in all other subgroups. We found that the level of knowledge in the subsection on epidemiology, clinical presentation and diagnosis in patients with CD, mothers of patients with CD and fathers of patients with CD was higher than that in the subsection of treatment and follow-up. There are not only compatible studies but also incompatible studies with the present study[17,26-28]. In contrast to our study, higher scores were found in the subsection on treatment and follow-up[17,26]. The authors concluded that families are in charge of their children's nutrition and are more cautious around them[17,26]. It has been shown that 46%-52% of the parents were members of the Celiac Society; therefore, the authors thought that the scores were low[27,28]. Consistent with previous studies, only 11.3% of the parents were members of the Regional Celiac Support Association. Membership of associations is very important in terms of informing and raising awareness of the disease. We suggest that patients and their caregivers should be directed to membership of these associations. Also, we should increase the level of knowledge by organizing conferences on CD at regular intervals.

The mean score of the patients with CD was lower than those of parents in the current study. The results of our study also support the view that education is an important factor in increasing knowledge and awareness regarding CD in patients. It was also shown that knowledge of epidemiology, diagnosis and treatment increases significantly after a training program[29,30].

**Limitations:** There are several limitations in the current study. First, as the current study was web-based, we excluded 46 HCPs and 32 celiac patient caregivers who did not complete the entire questionnaire. Second, we were unable to make regional comparisons between HCPs and caregivers, as the majority of HCPs and celiac patient caregivers did not specify the region in which they lived. Third, a small number of patients and their caregivers participated in the study.

## CONCLUSION

Despite these limitations, the level of knowledge on CD among HCPs, patients and their caregivers was unsatisfactory. We consider that it is necessary to increase awareness and to develop e-learning activities on CD among HCPs, patients and their caregivers. They may benefit from e-learning programs similar to the one created as part of the EU-funded project Focus IN CD (https://www.celiacfacts.eu/focusincd-en). A higher level of knowledge will substantially reduce the number of undiagnosed patients, allow for earlier diagnosis, and enhance overall quality of life. Patients with CD and their caregivers should be guided and encouraged to become members of regional Celiac Support Associations. E-learning activities should be organized through these associations. It is very important for the patients to be more informed regarding the disease in terms of compliance with the gluten-free diet. The better the compliance with the diet, the fewer complications will arise.

## ARTICLE HIGHLIGHTS

### *Research background*

Celiac disease (CD) is a systemic autoimmune disorder characterized by a combination of various degrees of small bowel damage and diverse clinical manifestations triggered by gluten ingestion in people who are genetically vulnerable. It is one of the most prevalent chronic disorders. The clinical manifestations of CD are diverse and may present with gastrointestinal findings, extra-intestinal findings or no symptoms. Up to 95% of patients with CD remain undiagnosed. As most cases have atypical signs or no symptoms, the diagnosis of CD is either missed or delayed. In addition, one of the most important reasons for the delay in diagnosis may be the poor knowledge of healthcare professionals (HCPs) on CD.

### *Research motivation*

There are limited studies investigating the knowledge on CD among HCPs, patients and their caregivers. To our knowledge, there are no studies on this issue in Turkey. Thus, we aimed to evaluate the knowledge on CD among HCPs, patients and their caregivers.

### *Research objectives*

To evaluate the knowledge on CD among HCPs, patients and their caregivers.

### *Research methods*

The current study was carried out between June 2021 and February 2022 prospectively, as part of the Focus IN CD project. Patients with CD and their caregivers participated in the study from 6 different cities in Turkey. In addition, general practitioners, pediatricians, pediatricians with other subspecialities and pediatric gastroenterologists from different cities participated in the study.

### *Research results*

The questionnaire was completed by 348 HCPs, 34 patients with CD, 102 mothers and 34 fathers of patients with CD. Most of the participants were general practitioners (37.07%). There were 89 (25.57%) pediatricians and 72 (20.69%) pediatric gastroenterologists in the study. The highest score in all categories was achieved by pediatric gastroenterologists. There were significant differences between the four groups of HCPs in terms of the subsections on overall mean score, epidemiology and clinical presentation, treatment and follow-up. There was no significant difference between the groups (patients with CD, mothers of patients with CD and fathers of patients with CD) in terms of the questionnaire subsections.

### *Research conclusions*

The level of knowledge on CD among HCPs, patients and their caregivers was unsatisfactory. We consider that it is necessary to increase awareness and to develop e-learning activities on CD among HCPs, patients and their caregivers. They may benefit from e-learning programs similar to the one created as part of the EU-funded project Focus IN CD (https://www.celiacfacts.eu/focusincd-en). A higher level of knowledge will substantially reduce the number of undiagnosed patients, allow for earlier diagnosis, and improve the quality of life.

### *Research perspectives*

According to the current study, we believe that patients, their caregivers, and HCPs may benefit from e-learning programs similar to the one created as part of the EU-funded project Focus IN CD (https://www.celiacfacts.eu/focusincd-en).

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## FOOTNOTES

**Author contributions:** Sahin Y designed the study, analyzed the data, interpreted the data, conceived the study, was involved in the statistical analysis, and wrote the manuscript; Sevinc E, Bayrak NA, Varol FI, Akbulut UA, and Bukulmez A collected the data, and analyzed the data; All authors have read and approved the final manuscript.

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**Informed consent statement:** Informed consent was obtained from all participants.

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**Country/Territory of origin:** Turkey

**ORCID number:** Yasin Sahin 0000-0002-7394-4884; Fatma Ilknur Varol 0000-0001-5212-218X; Ayşegül Bükülmez 0000-0002-6013-5172.

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