GAME CHANGERS IN INFLAMMATORY BOWEL DISEASES

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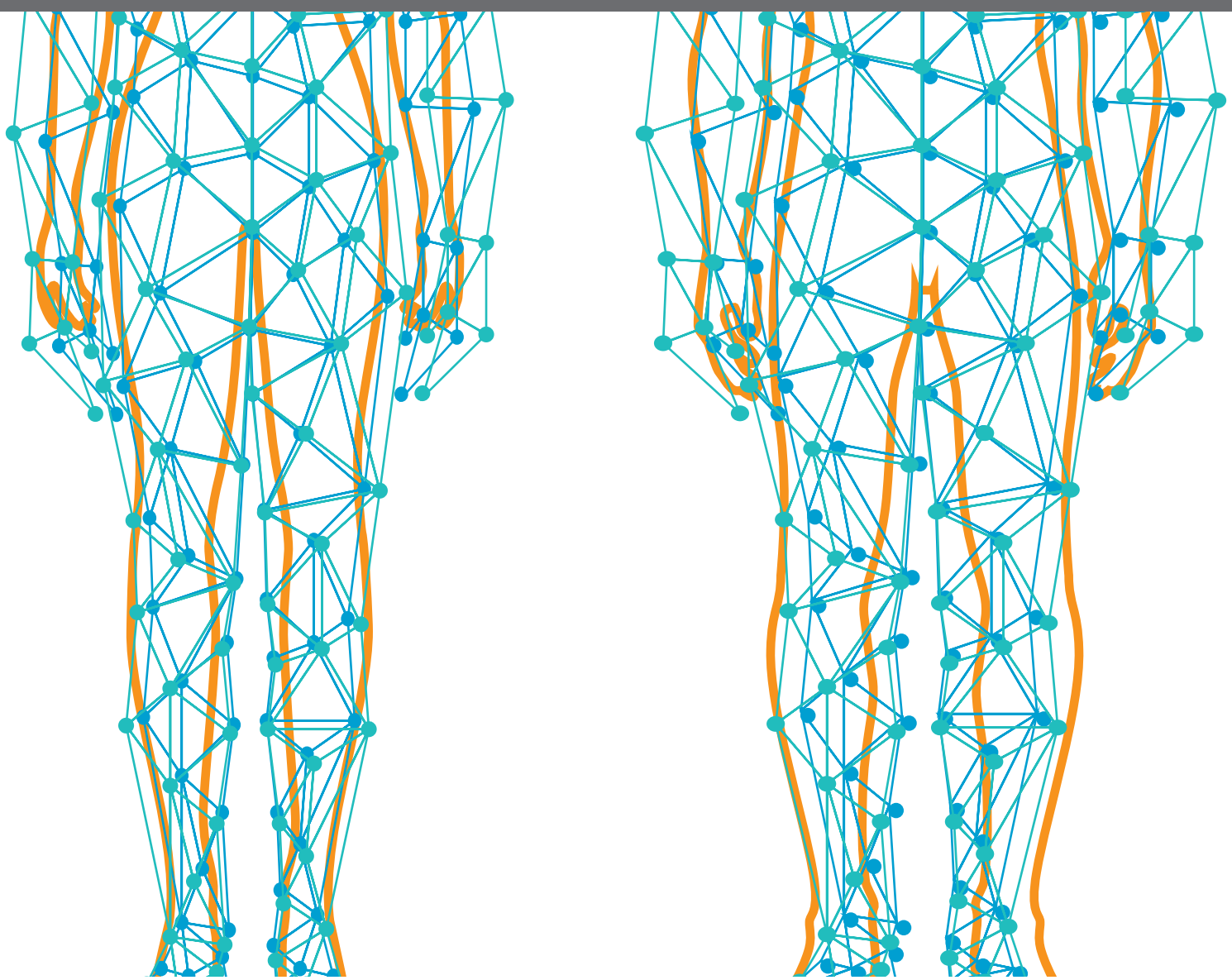
PUBLISHED IN: Frontiers in Medicine



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ISSN 1664-8714
ISBN 978-2-88974-019-2
DOI 10.3389/978-2-88974-019-2

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GAME CHANGERS IN INFLAMMATORY BOWEL DISEASES

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Citation: Bálint, A., Kopylov, U., Rutka, M., Lovasz, B. D., Kolar, M., eds. (2021). Game Changers in Inflammatory Bowel Diseases. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-88974-019-2

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frontiers in Medicine

EDITORIAL
published: 18 November 2021
doi: 10.3389/fmed.2021.795597

Editorial: Game Changers in Inflammatory Bowel Diseases

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Keywords: inflammatory bowel disease, Crohn's disease, ulcerative colitis, biological therapies, biomarkers, microbiome, new advanced technologies

Editorial on the Research Topic

Game Changers in Inflammatory Bowel Diseases

BACKGROUND

The accurate etiology of inflammatory bowel disease (IBD)—[Crohn's disease (CD) and ulcerative colitis (UC)] is still unknown. According to the accepted theory, the disease seems to be the consequence of an abnormal immune response induced by luminal antigen exposure. In the absence of clear etiology, there is no targeted treatment that could be cure IBD.

Moreover, the heterogeneity and complexity of UC and CD greatly complicate their treatment. The disease dramatically decreases the patients' quality of life and accounts for substantial costs to the healthcare system and society. Currently, the therapeutic goal includes not only clinical and endoscopic remission but also mucosal healing, i.e., deep remission, therefore basic and clinical research are both needed.

Considering these facts, the significance of the scientific research and innovations of the last decades, which have enriched the therapeutic tools of the gastroenterologist, are evident. Numerous studies have been published on new management lines, innovative drugs, serum, and fecal markers. Are the introduction of new molecules as anti-integrin antibodies, anti-interleukin (IL)-23 antibodies, Janus Kinase (JAK) inhibitors, or mesenchymal stem cells game changers? Or is the application of right predictors and excellent monitoring the key? How could we improve patient management? What new opportunities do we have?

ETIOPATHOLOGY OF IBD: FOCUS ON GUT MICROBIOME

Thanks to the next-generation technologies (including 16S rRNA, 18S rRNA, internal transcribed spacer sequencing, shotgun metagenomic sequencing, metatranscriptomic sequencing, and viromic sequencing), the connection of gut microbiome, and a wide range of diseases was confirmed. The more associations we explore, the more the function encoded in the intestinal microbiome appears to be significant. Microbiome analysis software tools are constantly evolving. While amplicon-based sequencing (16S rRNA, 18S rRNA) methods usually target a single gene, shotgun metagenomic sequencing adds a detailed layer to the taxonomic characterization of the microbial community by providing information on the gene composition and the functional capacity of the gut microbiome. Moreover, metatranscriptomic sequencing allows researchers to identify the activity of the microbe, in parallel activity of gene expression. Although it is a very promising tool, it must be taken into account that sometimes multiple metagenomic analysis methods may produce inconsistent results even if the same databases are used, therefore standardization of data processing and analysis is warranted (1).

OPEN ACCESS

Edited and reviewed by:
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University of Zaragoza, Spain

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Specialty section:
This article was submitted to Gastroenterology, a section of the journal Frontiers in Medicine

Received: *15 October 2021*
Accepted: *28 October 2021*
Published: *18 November 2021*

Citation:
Bálint A and Lovász BD (2021) Editorial: Game Changers in Inflammatory Bowel Diseases. Front. Med. 8:795597. doi: 10.3389/fmed.2021.795597

However, several IBD research results have been achieved recently by using these technologies. Individuals with genetic susceptibility when exposed to external triggers are at increased risk at developing IBD. As a summary by Cai et al. shows, the accumulation of potentially pathogenic microorganisms in the gut can be responsible for intestinal inflammation. Moreover, these microorganisms appear to be shared with those of periodontitis and may play a role in the comorbidity of periodontitis and IBD. Changes in the gut microbiome can be responsible for not only the development of IBD, but even for the recurrence after surgical resection. Alteration in gut microbiota can be associated with relapse of CD. These changes are even more important in high-risk patients who need surgical treatment. However, the efficacy of microbial-based therapies in preventing postoperative recurrence of CD is still limited. In the systematic review by Zhuang et al., the mucosa-associated microbiota in surgical biopsy of CD patients is significantly distinct from that in normal mucosa from healthy subjects. Recolonization with recurrence-associated bacteria of gut microbiome after intestinal resection might be associated with post-operative recurrence in CD patients.

Besides dysbacteriosis, long-standing, chronic infections might be another interesting issue in the etiopathology of IBD and even more in the development of complications. Patients with long-standing IBD and cytomegalovirus (CMV) infections are at increased risk of gastrointestinal neoplasms. The connection between them is supported by a case report of a family with an aggressive ATP4A-mutated gastric neuroendocrine tumor (gNET) who had a concurrent inflammatory disease. The progression of ileitis-associated neoplasia was compatible with IBD and CD. Results described by Calvete et al. are important since they contribute to describing the genetic landscape of these clinical associations: inflammation in the intestine was observed as a primary disease and not derived from the gastric neoplasm. Therefore, the findings of this report are directing the attention of gastroenterologists to monitor patients with inflammatory diseases that may trigger other severe pathologies.

DIAGNOSTIC TOOLS: WHAT'S NEW IN THE FIELD OF ENDOSCOPY?

Endoscopy plays a defining role in the diagnosis and management of IBD, additionally, it is fundamental to determine one of our therapeutic goal, the deep remission. We need more and more precise methods to identify inflammation and other pathologies on gut mucosa as prevention becomes the focus of patient management. Recently, most of advances of endoscopy aims to get as close as possible to microscopic findings. New diagnostic methods have been developed, such as dye-less chromoendoscopy (narrow band imaging, Fuji intelligent color enhancement, i-scan, blue light imaging, linked color imaging, etc.), endocytoscopy–endomicroscopy, and molecular imaging, which is based on the topical or intravenous administration of specific label substance and the subsequent visualization of targeted structures through simultaneous confocal imaging. The new pan-enteric video capsules with latest technologies provide more effective inflammation detection. The benefits of artificial intelligence system are reduction of operator-subjectivity and inter-observer variability (2). In the near future, artificial intelligence can be a useful tool in the real-time assistance of doctors' diagnostic decisions during endoscopy in everyday practice. Deep learning algorithms have a high accuracy in the detection of CD ulcers with video capsule endoscopy. This could be a practical application for example in the differential diagnosis of CD and non-steroidal anti-inflammatory drugs (NSAID) ulcers. Non-steroidal anti-inflammatory drugs-induced ulcers are one of the most important differential diagnosis for small bowel ulcers in patients evaluated for suspected CD. A deep learning network was trained by Klang et al., using Crohn's ulcer images from capsule endoscopy and evaluated its performance for NSAID ulcers.

THERAPEUTIC ADVANCES: NEW MOLECULES, OLD MOLECULES…NEW COMBINATIONS

The first drug that reduced IBD mortality by orders of magnitude was the corticosteroid. Thiopurines have been another cornerstone in the treatment of IBD. There is a growing interest in optimizing thiopurine therapy, especially in the era of biological therapies. Gargallo-Puyuelo et al. are reviewing the pros and cons of the efficacy and safety issues of conventional thiopurine therapy and the benefits from personalized therapy with thiopurines.

The introduction of biological agents was a real breakthrough in the management of severe IBD. Nevertheless, treatment non-response, allergy or infusion reactions, and other adverse events limit the use of these drugs. There are several studies that may represent new therapeutic options, like anti-integrin antibodies (vedolizumab, etrolizumab, AJM300, ontamalimab), blockade of interleukin-23 (ustekinumab, briakinumab, brazikumab, risankinumab, mirikizumab, guselkumab), JAK inhibitors (upadacitinib, filgotinib), modulation of sphingosine-1-phosphate receptors (fingolimod, ozanimod, etrasimod, amiselimod), phosphodiesterase inhibitors (apremilast), oligonucleotide-based drugs (morgensen, alicaforsen, cobitolimod), stem cell therapy, and fecal microbiota transplantation (3). Promising research is under process related to the efficacy and safety of V565-oral anti-TNF therapy (4).

The choice of treatment depends on the phenotype, activity, and complications of the IBD. To note, several factors play a role in the effectiveness of therapy, even the doctors themselves, as well as their preferences. Abdullah et al. evaluated the adherence of gastroenterologists at an IBD center, to anti-TNF (anti-tumor necrosis factor) combination therapy. Tight disease control, the timing of biological therapy, and monitoring of serum antibody levels can be affected by different groups of gastroenterologists according to the age and interest in IBD.

PROGNOSIS: PREDICTORS OF TREATMENT RESPONSE AND IBD OUTCOME

Appropriate diagnosis, treatment decision, and monitoring can have a significant impact on beneficial IBD outcomes. What can we do? The sole predictor or biomarker, which is sensitive and specific enough to be used alone in the prognosis and the outcome of IBD does not exist, but we have some useful, low-cost, and reliable tools like C-reactive protein (CRP), antibodies (pANCA: perinuclear anti-neutrophil cytoplasmic antibodies, ASCA: Anti-*Saccharomyces cerevisiae* antibodies), and fecal calprotectin. Although there have been some promising results from studies, some biomarkers suitability is questionable and requires further investigation: LL-37 (serum cathelicidin), TFF3 (trefoil factor 3), cytokines (IL-6, TNF, etc.), fibrinogen, anti-outer membrane protein, antibodies to flagellin, anti-12 antibody, anti-carbohydrate antibody, fecal lactoferrin, fecal neopterin, fecal polymorphonuclear neutrophil elastase, fecal S100A12 (5). A lot of research are being carried out about the role of circulating non-coding microRNA besides metabolomics and proteomics studies. Some serum immunoglobulins were associated with specific disease phenotypes of CD. Yang et al. revealed that neuron-specific enolase and CRP could be used as non-invasive tests in detecting the location and severity of disease in patients with CD in daily routine practice. The mucosal healing index [including carcinoembryonic antigen-related cell adhesion molecule, vascular cell adhesion molecule, CRP, serum amyloid A, angiopoietin-1, angiopoietin-2, matrix metalloproteinase (MMP)-1, MMP-2, MMP-3, MMP-9, extracellular MMP inducer, transforming growth factor-α, and IL-7] was created using multiple logistic regression models to predict mucosal healing in patients with CD. The same in UC patients who received anti-TNF therapy was established with CRP, CHI3L1 (anti-chitinase-3-like protein 1), LL-37, and neutrophil count (6). Therapeutic drug monitoring has been suggested to be applied in case of monitoring for dose escalation, de-escalation, or switch treatment (7).

In the treatment of pediatric patients with CD, risk-stratification is mandatory during diagnosis in order to identify high-risk patients who need early intensive therapy to prevent poor outcomes. The consensus guidelines of ESPGHAN/ECCO describe predictors of poor outcome (POPO) as means for risk stratification. De Laffolie et al. investigated therapy stratifying potential comparing POPO-positive and -negative CD patients from CEDATA-GPGE®, a German-Austrian Registry for Pediatric Inflammatory Bowel Disease to describe "POPO" criteria test statistical properties. They found that POPO with complicated courses of disease were common, hence an early intensified management for pediatric CD patients with POPO-positivity should be considered.

Unfortunately, varying degrees of disability are common in IBD. This is due to different underlining mechanisms, but certain prognostic factors could help to identify the group of patients at significantly higher risk. Bian et al. reported that disability is mostly correlated with disease activity as well as BMI.

CONCLUSIONS

A great number of landmark studies have been published on new therapeutic strategies, disease monitoring, and complication management in recent years. It also means that we must constantly educate and train ourselves to provide up-to-date and optimal care to our patients. Due to the multitude of new as well as already established treatments, we have the opportunity to apply patient-tailored therapy that have a great significance in achieving better disease outcome.

AUTHOR CONTRIBUTIONS

Both authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

FUNDING

This work was supported by the research grants of the National Research, Development and Innovation Office (Grant Nos. 125377, 129266, and 134863), by the New National Excellence Program of the Ministry of Human Capacities (UNKP-20-5-SZTE-161) and Janos Bolyai Research Grant (BO/00598/19/5), and the Géza Hetényi Research Grant by the Faculty of Medicine, University of Szeged.

ACKNOWLEDGMENTS

The authors would like thank to Uri Kopylov, Martin Kolar, and Mariann Rutka for their editorial work in helping to create the special issue Game Changers in Inflammatory Bowel Diseases.

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frontiers in Medicine

SYSTEMATIC REVIEW
published: 28 January 2021
doi: 10.3389/fmed.2020.615858

Gut Microbiota Profiles and Microbial-Based Therapies in Post-operative Crohn's Disease: A Systematic Review

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Background and Aims: Gut microbiota recolonization after intestinal resection had been reported to be associated with post-operative recurrence in Crohn's disease (CD). However, the results of different studies are inconsistent and even contradictory. In addition, knowledge on the efficacy of microbial-based therapies in preventing post-operative recurrence of CD is limited. Therefore, the aim of this review was to investigate gut microbiota profiles in patients with CD before and after surgery and evaluate microbial-based therapies in preventing post-operative recurrence.

Methods: Electronic databases were searched from inception to 31 June 2020 using predefined terms. Studies that investigated gut microbiota pre- and post-intestinal resection, and microbial-based therapies in preventing post-operative recurrence, were eligible. Study quality was assessed using either the Newcastle–Ottawa scale or Jadad scoring system.

Results: Twelve studies investigating gut microbiota of CD patients suffering from operation, and other 12 studies evaluating the efficacy of antibiotics and probiotics, were included in our review. The mucosa-associated microbiota in surgical biopsy of CD patients is significantly distinct from that in normal mucosa from healthy subjects. Gut microbiota recolonization following surgery might be associated with post-operative recurrence in CD patients. Furthermore, CD patients with post-operative recurrence presented a gain in pro-inflammatory pathogenic bacteria and a loss in short-chain fatty acid-producing bacteria before and after surgery. However, no consistent bacteria or metabolites were found to predict the post-operative recurrence of CD. Additionally, microbial-based therapies are deficient and present restricted widespread clinical utility due to several deficiencies.

Conclusion: Recurrence-associated bacteria observed pre- and post- operation might be promising in preventing the post-operative recurrence of CD. Furthermore, potential microbe biomarkers for predicting subsequent disease recurrence should be validated with larger sample sizes using more rigorous and standardized methodologies.

Keywords: crohn's disease, mucosa-associated microbiota, feces-associated microbiota, post-operative recurrence, microbial-based therapies

OPEN ACCESS

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Specialty section:
This article was submitted to Gastroenterology, a section of the journal Frontiers in Medicine

Received: *10 October 2020*
Accepted: *14 December 2020*
Published: *28 January 2021*

Citation:
Zhuang X, Tian Z, Li N, Mao R, Li X, Zhao M, Xiong S, Zeng Z, Feng R and Chen M (2021) Gut Microbiota Profiles and Microbial-Based Therapies in Post-operative Crohn's Disease: A Systematic Review. Front. Med. 7:615858. doi: 10.3389/fmed.2020.615858

INTRODUCTION

Crohn's disease (CD) is a chronic relapsing inflammatory bowel disease (IBD) with multifactorial pathogenesis and is characterized by recurrent transmural inflammation (1). Eventually, the recurrent inflammation can lead to intestinal stricture and fistulae (often perianal) complications or the creation of abscesses (2). Surgical resection is required in ∼70–80% of CD patients, owing to the penetrative nature of the disease, the development of structural changes, and the failure of medical therapy (3–5). However, operative management is not curative, and up to 75% of CD patients will experience post-operative disease recurrence (clinical, endoscopic, or surgical recurrence) over time (6–8). As a consequence, ∼30% of patients require a second surgical resection within 5 years (9, 10). Given the significant recurrence risk after surgical resection in CD, elucidating the specific factors that predispose patients to post-operative CD recurrence is a high priority. Multiple clinical risk factors, including active smoking, perforating disorders, prior resection, myenteric plexitis, younger age of disease onset, short disease duration, CD behavior, histologic involvement of resection margins, remnant disease post-operation, and length of the resected segment, have been associated with post-operative CD recurrence; however, these factors are far from being adequate in predicting disease recurrence (11–13).

Gut microbiota alterations have been identified as key contributors to the pathogenesis of CD; the crucial link between gut microbiota dysbiosis and post-operative disease recurrence has been documented by numerous studies (14–16). However, the post-operative role of microbial communities in patients with CD remains unknown, largely owing to heterogeneous studies with highly diverse results. In order to facilitate the use of gut microbiota in improving the diagnosis and treatment of post-operative CD, it is imperative to elucidate the bacteria that are associated with disease recurrence or its absence and evaluate whether these microbial factors could predict the post-operative CD recurrence.

Although there is compelling evidence pointing to a critical role of gut microflora in the post-operative CD recurrence, microbial-based therapies for preventing CD recurrence following surgery remain limited (17). Antibiotics and probiotic supplements aimed at altering gut microbiota composition have both been studied in terms of their ability to prevent post-operative disease recurrence; however, there is currently no evidence-based consensus on the topic (18–20). Antibiotics may be the most cost-effective strategy to prevent post-operative disease recurrence in patients who can tolerate the treatment, but the long-term effects beyond antibiotic cessation are questionable (10). In addition, the prolonged administration of these antibiotics is not feasible, owing to a high rate of side effects, significant toxicity, and bacterial resistance (18). Accumulating evidence has implicated that manipulating gut microflora with probiotics is an appealing alternative in reducing the postsurgical CD relapse rate by counterbalancing harmful bacteria (21). However, there are currently design limitations in probiotic trials for the prevention of post-operative CD recurrence; these limitations include small sample sizes, short observation periods, or the co-administration of other drugs.

The aim of this review was to summarize the results of studies investigating gut microbiota alterations in CD individuals suffering from operative management and to evaluate whether specific gut microbiota variations are associated with the post-operative recurrence (PR) of the disease. Furthermore, we revisited previous randomized controlled trials and high-quality uncontrolled studies in an effort to better elucidate the role of microbial-based therapies in preventing the PR of CD.

Abbreviations: CD, Crohn's disease; IBD, Inflammatory bowel disease; PR, Post-operative recurrence; SCFA, Short chain fatty acid.

METHODS

Search Protocol

The protocol for this systematic review was registered on the International Prospective Register of Systematic Reviews (PROSPERO) with the ID number CRD42020200956, and the Preferred Reporting Items for Systematic Review and Meta-Analyses (PRISMA) checklist was used as a guideline. A comprehensive search was performed on public databases, including PubMed, Web of Science, Embase, Scopus, and the Cochrane Library (last search: 31 June, 2020), with no date or language restrictions. The MeSH term and free-text word combinations that we used were the following: "Crohn's disease," "CD," "post-operative," "surgery," "resection," "recurrence," "microbiota," "microbiome," "microflora," "bacterial flora," "antibiotic," "probiotic." Boolean operators (AND, OR, NOT) were used to widen and narrow the search results.

Eligibility Criteria and Study Selection

The articles were selected on the basis of certain criteria: observational studies that focused on gut microbiota profiles associated with the post-operative disease course in CD patients or clinical trials that evaluated the effect of microbial-based therapies (antibiotics and probiotics) on the prevention of the PR of CD. The microbial communities in these studies were assessed from either fecal or mucosal samples. Two independent investigators screened titles and abstracts from the databases according to the eligibility criteria. Subsequently, the included articles were subjected to whole-paper reading, and the accompanying references were checked to identify additional potentially eligible articles. Any discrepancies between the investigators were resolved through discussion until consensus was reached, and a third reviewer was involved if necessary.

Data Extraction and Quality Assessment

For studies investigating gut microbiota profiles in the PR of CD, we extracted demographic, clinical, and bacterial richness and diversity and taxonomic bacterial composition (phylum, class, order, family, and genus), as well as information on the methodology applied to the microbiota analysis. For each of the studies that evaluated the effect of microbial-based therapies on preventing the PR of CD, we extracted data on the first author of the study, the year of publication, the population examined,

intervention details, treatment duration, recurrence definition, follow-ups, and the outcomes.

The quality of the included studies was evaluated using the Newcastle–Ottawa Scale (NOS) for cohort studies (22). The NOS contains three criteria: selection (representativeness of the exposed cohort, selection of the non-exposed cohort, ascertainment of exposure, demonstration that the outcome of interest was not present at start of study), comparability (comparability of cohorts on the basis of the design or analysis), and exposure (assessment of outcome, whether follow-up was long enough for outcomes to occur, adequacy of follow-up of cohorts). A quality score ranging from 0 to 9 was obtained by the use of a rating algorithm previously described: 0–3 (poor), 4–6 (moderate), and 7–9 (high).

The Jadad quality scoring system, which is based on randomization, blinding, and dropouts (withdrawals), was used to assess the quality of the randomized controlled trials (23). The quality scale ranges from 0 to 5 points, with a score of $\leq$2 indicating a low-quality report and a score of $\geq$3 indicating a high-quality report.

RESULTS

Study Selection and Quality Assessment

The initial database search yielded 3,712 records. After the automatic removal of duplicate, 3,346 unique abstracts were screened and 54 records were selected for full-text review. Finally, 24 original articles were included in this systematic review (**Figure 1**). These articles included 12 studies reporting gut microbiota profiles in post-operative, and five and seven studies evaluating the efficacy of antibiotics and probiotics, respectively, in preventing the PR of CD (24–47). The quality scores of the included studies were assessed and are reported in **Supplementary Table 1**.

Characteristics of Studies Investigating Gut Microbiota in Post-operative CD

The demographic and clinical characteristics of the patients from the included studies investigating gut microbiota are detailed in **Table 1**. The studies were performed in different geographical regions (including Sweden, Belgium, France, Canada, Australia, France, and the USA) from 2002 to 2020, and almost all of their subjects were adults. In patients with CD, either ileal or ileocecal resections were performed to remove diseased areas from the ileum and right colon with ileocolonic anastomosis. Approximately, 3–18 months after intestinal surgery, a post-operative colonoscopy was performed to assess the endoscopic recurrence based on the Rutgeerts score (48); and the PR was assigned a Rutgeerts score of $\geq$i2. In addition, the endoscopic recurrence rates were reported as ranging between 33.3 and 73.7% in the included studies.

Handling and Analysis of Samples

Mucosal and/or fecal samples were collected at the time of surgery or at different time points in the follow-up period. The various differences in the handling and analysis of samples that were observed among the individual studies are shown in **Supplementary Table 2**. In the majority of the studies, the samples were preserved at either −20 or −80°C. In approximately half of the studies, DNA extraction was used for microbiota analysis, using reliable kits from different manufacturers, while other studies did not provide information on the methods they used. Most of the analyzed studies employed sequencing techniques based on the hypervariable regions of the 16S ribosomal ribonucleic acid (rRNA) gene for the gut microbiota analysis. However, two early studies analyzed specific bacterial strains using fluorescence *in situ* hybridization (FISH) and culture-based methods. In addition, taxonomic classification was assigned via multiple databases, with Greengenes and Silva being the most commonly used ones.

Mucosa-Associated Microbiota Profiles in Surgical Biopsy Samples of CD Patients

In order to characterize the mucosa-associated microbiota in CD patients, we compared the surgical biopsies of CD patients and the normal samples from healthy subjects in **Table 2**. With regard to the microbiota community diversity, the alpha-diversity of mucosa-associated microbiota from surgical specimens decreased significantly in CD patients. In addition, beta-diversity analysis revealed that the mucosa-associated microbiota in the surgical biopsies of CD patients deviated significantly from those of healthy controls.

A comparison of the relative bacterial abundance at different taxonomic levels revealed that the mucosal microbiome composition of CD patients and control cases differed in a manner of ways. At the phylum level, the relative abundance of Bacteroidetes and Firmicutes in surgical biopsies of CD patients at the time of resection decreased; on the contrary, the relative abundance of Proteobacteria and Fusobacteria increased. At the family level, surgical specimens from CD patients contained an increase in *Clostridiaceae*, *Enterobacteriaceae*, and *Enterococcaceae* populations and a decrease in *Bacteroidaceae* and *Lachnospiraceae* populations. Moreover, in CD patients who suffered from mucosal microbiota dysbiosis, which also showed mucosal microbiota dysbiosis at the deeper genus level, 21 bacterial genera were found in different abundances. More specifically, the *Actinomyces*, *Enterococcus*, *Fusobacterium*, *Lactobacillus*, *Streptococcus*, and *Veillonella* populations increased, while the *Bacteroides*, *Blautia*, *Clostridium*, *Coprococcus*, *Dorea*, *Faecalibacterium*, *Lachnobacterium*, *Lachnospira*, *Odoribacter*, *Paraprevotella*, *Phascolarctobacterium*, *Prevotella*, and *Ruminococcus* populations decreased. However, the changes in the *Bifidobacterium* and *Eubacterium* numbers reported from surgical biopsies are divergent and even contradictory in the included studies. A more comprehensive list of specific mucosa-associated microbiota alterations of CD patients at the phylum, class, order, family, and genus levels is presented in **Supplementary Table 3**.

Dynamic Alterations of Gut Microbiota During the Postsurgical Disease Course

Intestinal resection may play a crucial role in the gut microbiota recolonization process. Several studies have investigated

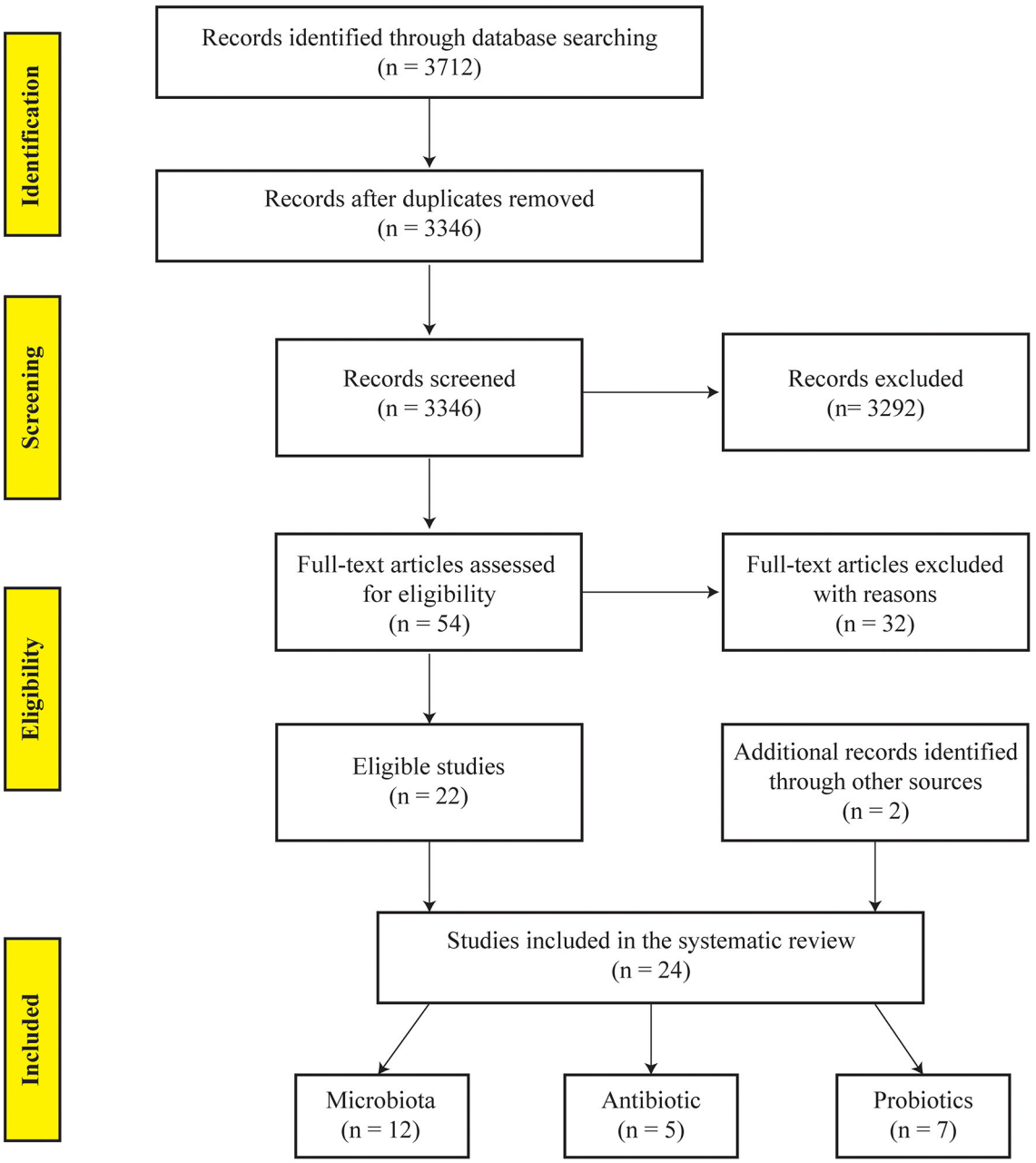

FIGURE 1 | PRISMA flow diagram summarizing the studies identified during the selection process.

temporal alterations of the mucosal microbiota community structure in CD patients before and after surgery (23–34). The taxonomic differences in the mucosa-associated microbiota of CD patients following surgery compared to baseline are presented in **Table 3** and **Supplementary Table 4**. There were no significant alpha-diversity differences between the surgical samples and those obtained from the post-operative follow-up. In addition, it was observed that the beta-diversity of the gut microbiota of post-operative CD patents differed from that at the time of resection in three studies, whereas one study reported no significant differences.

At the phylum level, CD patients had reduced Actinobacteria and elevated Fusobacteria levels during the postsurgical disease course, while the Bacteroidetes levels were inconclusive in three studies. Following resection, the mucosa of CD patients was enriched with members of the *Lachnospiraceae* family; on the contrary, *Bifidobacteriaceae*, *Clostridiaceae*, *Pseudomonadaceae*, *Sphingomonadaceae*, *Staphylococcaceae*, and *Streptococcaceae* levels in these patients decreased. No clear overall conclusion could be drawn from the included studies in terms of the bacterial genera populations. Post-operative CD patients had higher relative abundances of the *Anaerostipes*, *Bacteroides*, *Blautia*,

TABLE 1 | Characteristics of studies investigating gut microbiota profiles in patients with CD.

Studies	Country	Year	Sample size	Gender (male %)	Age	Resection	Ileocolonoscopy time	Recurrence evaluation	Endoscopic recurrence	Medicine before resection	Medicine after resection	Follow-up time	RR (%)
Hamilton et al.	Australia	2020	CD, *n* = 130	44	M: 36	Ileocecal resection	6 and 18 m	Endoscopic recurrence	Rutgeerts score	–	Metronidazole (23), Thiopurine (72), Adalimumab (35)	6, 12, 18 m	6 m: 34.78 18 m: 42.86
Strömbeck et al.	Sweden	2020	CD, *n* = 21	66.7	R: 17–63	Ileocecal resection	52 (41–58) w	Endoscopic recurrence	Rutgeerts score	No medication (4), 5-ASA (7), Corticosteroids (13), Thiopurines (10), Anti-TNF (1)	No medication (8), 5-ASA (8), Corticosteroids (2), Thiopurines (6)	3–10 w; 1 y	1 y: 38.1
			Ctrl, *n* = 7	42.9	R: 20–36								
Machiels et al.	Belgium	2020	CD, *n* = 120	47.5	–	Ileocecal resection	6 m	Endoscopic recurrence	Rutgeerts score	Corticosteroids (25), Anti-TNF (22), Immunosuppressants (24), Antibiotics (21)	Thiopurines (6), Anti-TNF (10), Vedolizumab (1)	1, 3, 6 m	6 m: 43
			Ctrl, *n* = 39	–	–								
Sokol et al.	France	2019	CD, *n* = 201	49	M: 34.6	Ileal resection	6–12 m	Endoscopic recurrence	Rutgeerts score	Steroids (65), Immunosupressant (63), Anti-TNF (95), Antibiotics (68)	Immunosupressant (48), Anti-TNF (68), Antibiotics (13)	6–12 m	6–12 m: 50
Keshteli et al.	Canada	2018	CD, *n* = 38	34.2	R: 18.6–66	Ileocolonic resection	6–12 m	Endoscopic recurrence	Rutgeerts score	–	No medication (12), 5-ASA (7), AZA/6-MP (13), MTX (5), Corticosteroids (2), Adalimumab (4), Infliximab (6)	6–12 m	6–12 m: 73.7
Laffin et al.	Canada	2018	CD, *n* = 45	37.8	M: 43.2	Ileocolonic resection	6 m	Endoscopic recurrence	Rutgeerts score	Steroids (15), Biologic therapy (22), 5-ASA (6), AZA (17), MTX (5)	Steroid (2), Biologic therapy (25), 5-ASA (5), AZA (16), MTX (3), Antibiotics (18)	6 m	6 m: 33.3
Wright et al.	Australia	2017	CD, *n* = 34	41	R: 23–43	Ileal and ileocecal resection	6 and/or 18 m	Endoscopic recurrence	Rutgeerts score	–	Metronidazole (6), Thiopurine (22), Adalimumab (6)	6 and/or 18 m	6 m: 37.0 18 m: 58.3
			Ctrl, *n* = 12	33	R: 46–83								
Mondot et al.	France	2015	CD, *n* = 20	–	–	Ileocolonic resection	6 m	Endoscopic recurrence	Rutgeerts score	Antibiotics (0)	*Lactobacillus johnsonii* LA1	6 m	6 m: 50
Cruz et al.	Australia	2014	CD, *n* = 12	58.3	R: 19–50	Ileocecal resection	6 m	Endoscopic recurrence	Rutgeerts score	Antibiotics (0), Probiotics (0)	No medication (2), Thiopurine (7), Adalimumab (3)	6 m	6 m: 50
			Ctrl, *n* = 10	50	R: 26–66								
Dey et al.	USA	2013	CD, *n* = 6	50	M: 34.6	Ileocolic resection	5–10 m	Endoscopic recurrence	Rutgeerts score	5-ASA (1), Anti-TNF (5), Antibiotics (4), Anti-4-integrin (1), Probiotics (5)	No medication (1), Adalimumab (4), Certolizumab (1)	5–10 m	5–10 m: 50
			Ctrl, *n* = 20	20	M: 55.2								
Sokol et al.	France	2008	CD, *n* = 21	–	–	Ileocolonic resection	6 m	Endoscopic recurrence	Rutgeerts score	–	*Lactobacillus johnsonii* LA1, Placebo	6 m	6 m: 61.9
Neut et al.	France	2002	CD, *n* = 61	36.1	R: 19–68	Ileocolic resection	3 m, 1 y	Endoscopic recurrence	Rutgeerts score	Cefoxitin (13) at the time of surgery	No medication	3 m, 1 year	3 m: 42.9
			Ctrl, *n* = 10	–	M: 62								1 years: 65
Total			CD, *n* = 709 Ctrl, *n* = 98				3–18 m						33.3–73.7

CD, Crohn's disease; Ctrl, control; M, median; R, range; m, month; y, year; 5-ASA, 5-aminosalicylic acid; AZA, azathioprine; 6-MP, mercaptopurine; MTX, methotrexate; TNF, tumor necrosis factor; RR, recurrence rate.
"–": No information provided.

TABLE 2 | Mucosa-associated microbiota of patients with CD from the resection specimen at the time of surgery compared to healthy controls.

Studies	Wright et al.	Dey et al.	Neut et al.	Machiels et al.	Cruz et al.	Total ↑	Total ↓
α-Diversity							
Richness	↓	↓	–	↓	↓	0	4
Diversity	↓	↓	–	↓	↓	0	4
β-Diversity	D	D	–	D	D	D = 4	
PHYLUM							
Bacteroidetes	↓	↓				0	2
Proteobacteria	↑	↑		↑		3	0
FAMILY							
Bacteriodaceae	↓	↓				0	2
Clostridiaceae	↑			↑		2	0
Enterobacteriaceae	↑	↑				2	0
Enterococcaceae	↑			↑		2	0
Lachnospiraceae	↓			↓		0	2
GENERA							
Actinomyces	↑				↑	2	0
Bacteroides	↓	↓	↓		↓	0	4
Bifidobacterium	↑		↓		↓	1	2
Blautia				↓	↓	0	2
Clostridium			↓		↓	0	2
Coprococcus				↓	↓	0	2
Dorea				↓	↓	0	2
Enterococcus	↑		↑		↑	3	0
Eubacterium			↓	↑	↓	1	2
Faecalibacterium	↓			↓	↓	0	3
Fusobacterium	↑	↑			↑	3	0
Lachnobacterium	↓			↓	↓	0	3
Lachnospira	↓			↓	↓	0	3
Lactobacillus	↑				↑	2	0
Odoribacter	↓				↓	0	2
Paraprevotella				↓	↓	0	2
Phascolarctobacterium	↓			↓		0	2
Prevotella			↓	↓		0	2
Ruminococcus	↓	↓	↓	↓	↓	0	5
Streptococcus	↑				↑	2	0
Veillonella	↑				↑	2	0

Only bacteria, for which at least two studies have reported the alterations at different taxonomic levels (phylum, family, and genera), are displayed in the table.
↑ = higher α-diversity or bacteria are more abundant, in CD compared to control; ↓ = lower α-diversity or bacteria are less abundant, in CD compared to control; D = bacterial β-diversity differ between CD patients compared to controls. N = no difference in β-diversity. – = no information provided. Red box represents an increased level, green box represents a decreased level, and empty box represents that no comparison was reported in the included studies.

TABLE 3 | Changes in mucosa-associated microbiota of patients with CD at different time points of follow-up compared to baseline.

Studies	Wright et al.	Sokol et al.	Neut et al.		Machiels et al.	Mondot et al.	Total ↑	Total ↓
Time point (m) / sample	6/M	6–12/M	3/M	12/M	6/M	6/M	3–12/M	
α-Diversity								
Richness	N	N	–	–	–	N	N = 3	
Diversity	N	N	–	–	–	N	N = 3	
β-DIVERSITY	D	D	–	–	N	D	D = 3	
PHYLUM								
Actinobacteria	↓				↓	↓	0	3
Bacteroidetes	↑	↓				↑	2	1
FAMILY								
Bifidobacteriaceae	↓				↓		0	2
Clostridiaceae	↓					↓	0	2
Lachnospiraceae	↑	↑				↑	3	0
Pseudomonadaceae	↓					↓	0	2
Sphingomonadaceae	↓	↓				↓	0	3
Staphylococcaceae					↓	↓	0	2
Streptococcaceae	↓	↓				↓	0	3
GENERA								
Anaerostipes	↑	↑					2	0
Bacteroides	↑		↑	↑			2	0
Bifidobacterium	↓		↑	↑	↓		1	2
Blautia		↑				↑	2	0
Clostridium	↑		↑	↑	↑		3	0
Dialister		↓			↑		1	1
Dorea		↑				↑	2	0
Enterococcus		↓	↑	↑			1	1
Fusobacterium			↑	↑	↑		2	0
Haemophilus	↓	↑					1	1
Lachnoclostridium		↑					1	0
Lactobacillus	↓						0	1
Mesorhizobium		↓				↓	0	2
Prevotella			↑	↑			1	0
Pseudomonas	↓					↓	0	2
Roseburia	↑	↑					2	0
Ruminococcus			↑	↑			1	0
Sphingomonas		↓				↓	0	2
Staphylococcus					↓	↓	0	2
Streptococcus	↓	↓				↓	0	3
Turicibacter	↓	↓				↓	0	3

Bacteria alterations at different taxonomic levels (phylum, family, and genera) are displayed in the table.
↑ = higher α-diversity or bacteria are more abundant, in CD patients following surgery compared to baseline; ↓ = lower α-diversity or bacteria are less abundant, in CD patients following surgery compared to baseline; D = difference in β-diversity between CD patients following surgery compared to baseline. N = no difference in β-diversity. – = no information provided. Red box represents an increased level, green box represents a decreased level, and empty box represents that no comparison was reported in the included studies.
m, month; M, mucosa.

Clostridium, *Dorea*, *Fusobacterium*, *Prevotella*, *Roseburia*, *Ruminococcus*, and *Lachnoclostridium* genera and lower abundances of the *Lactobacillus*, *Mesorhizobium*, *Pseudomonas*, *Sphingomonas*, *Staphylococcus*, *Streptococcus*, and *Turicibacter* genera. Moreover, higher Enterococcaceae and *Fusobacterium* and lower Lachnospiraceae and *Faecalibacterium* were found in both post-operative mucosal and fecal microbiota in CD patients. Changes in *Bifidobacterium*, *Dialister*, *Enterococcus*, and *Haemophilus* were inconsistent among the included studies. A detailed description of gut microbiota alterations at the

phylum, class, order, family, and genus levels in post-operative CD patients is provided in **Supplementary Table 4**. The fecal microbiota profiles of CD patients were assessed before and after surgery in two longitudinal studies. Strömbeck et al. found that the fecal microbiota composition at an early follow-up (3–10 weeks) after resection is similar to that at a 1-year follow-up (25), while Hamilton et al. demonstrated that fecal bacterial communities are associated with the protection from and the occurrence of CD recurrence after surgery (24).

Gut Microbiota Profiles of CD Patients at Post-operative Follow-Ups

The gut recolonization and details of the bacterial diversity and composition of CD patients that had undergone surgical intervention were compared with those of healthy control subjects at different time points during follow-up periods in three studies. Wright et al. reported that ileal specimens that were obtained from CD patients 6 and 18 months post-operatively had decreased alpha diversity compared to the control samples (30). In addition, the microbial composition of mucosal and fecal samples differed significantly between CD patients (at the post-operative follow-up) and healthy control subjects, which was reported in two studies.

A comparison of the gut microbial communities, including taxonomic changes and abundances in post-operative CD case and control subjects, is shown in **Supplementary Table 5**. The fecal microbiota composition in CD patients at the 1-year follow-up only had higher *Ruminococcus gnavus*, *Shigella* spp., and *Escherichia* spp. levels, while the majority of the bacterial taxa were less abundant than those in healthy subjects. Neut et al. identified and quantified specific bacterial populations in the mucosa-associated microbiota in CD patients that had undergone intestinal resection, using a traditional culture-based method; the authors demonstrated that *Bifidobacterium*, *Eubacterium*, and *Ruminococcus* populations were rarely encountered in the CD biopsy specimens collected after ileocolectomy at the 3-months and 1-year follow-ups, compared to the ileocolectomy controls (35). The authors of another study with a larger sample size reported that the mucosal biopsy samples that were obtained 6 and 18 months post-operatively from CD patients differed significantly from those of surgical controls; more specifically, they detected increased Fusobacteria, *Bifidobacteriaceae*, *Enterococcaceae*, *Bifidobacterium*, *Fusobacterium*, and *Trabulsiella* counts (30).

Disease Recurrence-Related Microbiota at the Time of Resection and at Follow-Up

Numerous studies have indicated that distinct gut microbiota profiles at the time of surgery and during the post-operative follow-up are associated with disease recurrence and remission, respectively. As for post-operative endoscopic recurrence-related microbiota, no clear overall conclusion could be drawn from the included studies; however, a number of differences were observed between recurrence cases and those without recurrence when comparing the relative abundance of bacterial taxa (**Table 4**). Bacteria taxa from mucosal biopsies obtained at the time of resection and associated with PR risk were examined in seven studies, while feces-associated microbiota only examined in one study. The counts of the Bacteroidetes and Firmicutes phyla in CD patients with PR decreased, while those of the Actinobacteria, Fusobacteria, and Proteobacteria phyla increased. The counts of the *Erysipelotrichaceae* and *Lachnospiraceae* families in CD patients with PR were significantly lower, while those of the *Enterobacteriaceae* family increased. Several studies reported that the relative abundance of the *Clostridium*, *Corynebacterium*, *Dialister*, *Enterococcus*, *Fusobacterium*, *Lactobacillus*, *Lactococcus*, *Proteus*, *Streptococcus*, and *Veillonella* genera in CD patients with PR increased, while that of the *Alistipes*, *Bacteroides*, *Bifidobacterium*, *Blautia*, *Coprobacillus*, *Dorea*, *Faecalibacterium*, *Gemella*, *Odoribacter*, *Parabacteroides*, *Paraprevotella*, *Ruminococcus*, *Subdoligranulum*, and *Turicibacter* genera decreased. However, the disease recurrence-associated changes in the *Actinomyces* and *Streptococcus* counts were inconsistent among the included studies. Additionally, the microbial communities of fecal samples that were obtained before surgery revealed *Atopobium*, *Corynebacterium*, *Gemella*, and *Rothia* counts in CD patients that developed PR than in those that did not. Nine studies provided detailed microbiota profiles associated with recurrence and remission observed at a post-operative colonoscopy, and three studies investigated the fecal microbiota composition after surgery. The findings regarding the mucosa-associated microbiota at the time of surgery revealed that the patients with PR experienced increases in their Fusobacteria and Proteobacteria counts and decreases in their Bacteroidetes and Firmicutes counts. Moreover, CD patients experiencing PR had decreased *Alistipes*, *Atopobium*, *Bacteroides*, *Bifidobacterium*, *Blautia*, *Dialister*, *Dorea*, *Faecalibacterium*, *Odoribacter*, *Parabacteroides*, *Paraprevotella*, *Ruminococcus*, *Subdoligranulum*, and *Turicibacter* counts and increased *Clostridium*, *Collinsella*, *Coprobacillus*, *Enterococcus*, *Fusobacterium*, *Proteus*, and *Streptococcus* counts. However, there was no consensus among studies on how the *Odoribacter* and *Oscillospira* counts differed in CD patients with PR. Strömbeck et al. detected higher Actinobacteria and lower Alistipes counts in the fecal microbiota of the PR group at their 1-year follow-up; Alistipes was found to correlate negatively with the Rutgeerts score (25). In another study tracking the trends in the fecal microbiota changes in patients with endoscopic recurrence, the relative Fusobacterium and Bifidobacterium abundances increased and decreased, respectively, in 1, 3, and 6 months after surgery compared to that in patients in remission (26). In addition, Hamilton et al. found that bacterial clusters enriched with *Enterobacteriaceae* and *Lachnospiraceae* were associated with an increased risk of disease recurrence and the maintenance of remission, respectively (24).

Predictive Potential of Microbial Factors in the PR of CD

The predictive potential of microbial factors at the time of surgery and at the time of post-operative endoscopic evaluation was further evaluated to guide disease diagnosis and treatment (**Table 5**). However, no consistent differences were detected in

TABLE 4 | Recurrence-associated fecal or mucosal microbiota at the time of resection and post-operative follow up.

Studies	Hamilton et al.		Strömbeck et al.	Wright et al.		Sokol et al.		Dey et al.	Machiels et al.					Cruz et al.		Mondot et al.	Sokol et al.		Keshteli et al.	Laffin et al.	
Time point Sample	6 m (F)	18 m (F)	12 m (F)	6 m (M)	18 m (M)	0 m (M)	6–12 m (M)	0 m (M)	0 m (M)	0 m (F)	1 m (F)	6 m (M)	6 m (F)	0 m (M)	6 m (M)	6 m (M)	0 m (M)	6 m (M)	0 m (M)	0 m (M)	6 m (M)
PHYLUM																					
Actinobacteria			↑																	↑	
Bacteroidetes																	↓	↓	↓	↓	↑
Firmicutes									↓								↓	↓		↓	↓
Fusobacteria												↑	↑						↑		
Proteobacteria					↑		↑												↑	↑	
FAMILY																					
Actinomycetaceae							↓		↓												
Bacteroidaceae																↓			↓	↓	↑
Enterobacteriaceae																			↑	↑	
Enterococcaceae		↑					↑									↑					
Erysipelotrichaceae								↓	↓												
Lachnospiraceae		↓					↓	↓												↓	↓
Peptostreptococcaceae	↓									↓		↑									
GENERA																					
Actinomyces									↓					↑							
Alistipes			↓											↓	↓						
Atopobium					↓					↑											
Bacteroides														↓	↓	↓					
Bifidobacterium			↓										↓		↓		↓	↓			
Blautia							↓							↓							
Clostridium														↑	↑						
Collinsella							↑							↓							
Coprobacillus										↓		↑		↓							
Corynebacterium						↑				↑				↑							
Dialister							↓							↑	↓	↓					
Dorea							↓							↓		↓					
Enterococcus							↑							↑	↑	↑					
Faecalibacterium				↓	↓									↓			↓	↓			
Fusobacterium												↑	↑	↑							
Gemella										↑				↓							
Lachnobacterium					↓																
Lactobacillus														↑							
Lactococcus														↑							
Odoribacter					↓							↑		↓	↓						
Oscillospira					↓							↑									
Parabacteroides														↓	↓						
Paraprevotella					↓							↑		↓							
Proteus				↑	↑									↑							
Ruminococcus						↓	↓		↓					↓	↓	↓					
Streptococcus						↑			↓					↑	↑						
Subdoligranulum														↓	↓						
Turicibacter					↓									↓							

Only bacteria, for which at least two studies have reported the alterations at different taxonomic levels (phylum, family, and genera), are displayed in the table.

↑ = bacteria are more abundant in CD patients with post-operative endoscopic recurrence compared to those in remission; ↓ = bacteria are less abundant in CD patients with post-operative recurrence compared to those in remission. Red box represents an increased level, green box represents a decreased level, and empty box represents that no comparison was reported in the included studies.

m, month; M, mucosa; F, feces.

TABLE 5 | Specific gut microbiota and metabolites as predictor of post-operative endoscopic recurrence at the time of resection and post-operative follow-up.

Studies	Time point (sample)	Model	AUC
Wright et al.	6/18 m (M)	*Proteus*, *Faecalibacterium*, smoking status	0.74 (95% CI 0.69–0.79)
Sokol et al.	0 m (M)	*Corynebacterium*, *Ruminiclostridium 6*	RF: 0.81 (95% CI 0.61–1.00)
Machiels et al.	0 m (M)	*Ralstonia*, *Haemophilus*, *Gemella*, *Phascolarctobacterium*	C5.0: 0.738; RF: 1.00
	0 m (F)	*Coprobacillus*, *unidentified Lachnospiraceae*, *Dorea*	C5.0: 0.79; RF: 0.50
Keshteli et al.	0 m (U)	Levoglucosan, L-DOPA, propylene glycol, ethylmalonate	MCCV: 0.71 (95% CI 0.73–1.00)

m, month; M, mucosa; F, feces; U, urine; AUC, area under the curve; CI, confidence interval; RF, random forest model; MCCV, Monte-Carlo cross validation; levoglucosan, 1,6-anhydro-beta-D-glucose; L-DOPA, L-3,4-dihydroxyphenylalanine.

the counts of specific bacteria, which would allow their use in predicting endoscopic recurrence. Wright et al. reported that microbial analysis of the ileal mucosa, which takes into account the presence of Proteus, abundance of Faecalibacterium, and smoking status, at 6 and/or 18 months post-operatively, is moderately accurate in predicting endoscopic recurrence (30). In addition, increased *Corynebacterium* and decreased *Ruminiclostridium 6* counts at baseline were identified as predictive factors of endoscopic recurrence for CD patients (27). Machiels et al. reported that the *Ralstonia*, *Haemophilus*, *Gemella*, and *Phascolarctobacterium* abundances in resected specimens were good predictors using C5.0 classification tree analyses or random forest models (26). However, the considerable predictive power of *Coprobacillus*, *unidentified Lachnospiraceae*, and *Dorea* obtained from fecal samples before surgery could not be confirmed after validation using the forest model. Furthermore, Keshteli et al. found that the concentrations of urinary 1,6-anhydro-beta-D-glucose, L-3,4-dihydroxyphenylalanine, propylene glycol, and ethylmalonate were related to CD recurrence after ileocolonic resection, with an associated area under the curve value of 0.91 (95% CI: 0.73–1.00) (28).

Microbiota-Based Therapies to Prevent the PR of CD

The recolonization of the intestinal tract by gut microbiota plays a critical role in determining whether there will be post-operative relapse at the resection site. This indicates that interventions that aim at manipulating the microbiome should, in theory, have an integral role in the prevention of PR for CD patients. However, there is a lack of evidence-based recommendations on this topic.

As is shown in **Table 6**, five studies (three comparing metronidazole with a placebo, one comparing ornidazole with a placebo, one comparing ciprofloxacin with a placebo) evaluated the efficacy of antibiotics in preventing post-operative endoscopic or clinical recurrence in CD patients. Based on the available data, nitroimidazole antibiotics (metronidazole, ornidazole) are effective in preventing the clinical and endoscopic PR of CD compared to placebo drugs, which may be the most cost-effective option. In the first study, Rutgeerts et al. demonstrated that 3-month-long metronidazole therapy significantly decreased the severe endoscopic recurrence incidences (13 vs. 43%, $P = 0.02$) in the neoterminal ileum. Additionally, this treatment seemed to delay symptomatic recurrence; however, it was associated with a high incidence of side effects (40). Nevertheless, there were no significant differences between the metronidazole treatment and the placebo treatment in terms of reducing the clinical PR at the 1-year follow-up. In a subsequent study, the same authors found that taking ornidazole at a dose of 1 g/day for a year is effective in preventing PR at the 3-months and 1-year follow-ups, while no significant differences in terms of clinical recurrence were observed at subsequent follow-ups (39). The results of another recent study showed that taking low doses of metronidazole (250 mg three times per day) for 3 months decreases significantly endoscopic PR rates in CD patients within 12 months and is well-tolerated (35). However, Mañosa et al. showed that the risk of endoscopic recurrence is not reduced significantly with the combined use of metronidazole and azathioprine compared to the sole use of azathioprine; however, the use of metronidazole does not worsen azathioprine's safety profile (37). Yet in spite of this, the results of another randomized controlled trial demonstrated that the long-term addition of azathioprine to a post-operative 3-months course of metronidazole is more effective than using metronidazole alone (49). Moreover, a 6-months course of ciprofloxacin is not more effective than using a placebo drug in terms of preventing PR in CD patients who underwent surgery, and a high proportion of patients discontinue their treatment because they cannot tolerate it well (38).

Another method of gut microbiota manipulation is by using a supplement of live and safe microbes that restore the beneficial intestinal microbial flora; in this case, the use of a probiotic formulation may be an appealing alternative. Seven studies examined the effect of probiotics on preventing the PR of CD; however, none of these studies detected a significant effect of probiotics on clinical or endoscopic recurrence (as is shown in **Table 7**). Among the included studies, three investigated the ability of VSL#3 (a mixture of eight different bacterial strains), two evaluated the efficacy of *Lactobacillus johnsonii* LA1, one examined the effect of *Lactobacillus rhamnosus* strain GG, and one observed the potency of Synbiotic 2000 (a cocktail of four probiotics and four prebiotics). Nevertheless, the combination of rifaximin and VSL#3 was efficient in preventing the severe endoscopic recurrence of CD after surgical resection, as reported by Campieri et al. (47).

DISCUSSION

There is limited consensus on the gut microbiota profiles of CD patients at the time of surgical resection and at the post-operative follow-up. Understanding the microbial communities associated

TABLE 6 | Summary of studies of antibiotic to prevent post-operative recurrence in patients with CD.

Study	Year	Group, Number	ABX, Doses	Treatment duration	Recurrence definition	Recurrence score	Follow-up	ER rate (%), *P*-value				CR rate (%), *P*-value			
Glick et al.	2019	TX, $N = 35$ PBO, $N = 35$	Metronidazole 250 mg/tid	3m	ER	Rutgeerts	12 m	12 m: 20 (7/35) 12 m: 54.3 (19/35)	0.01						
Mañosa et al.	2013	TX, $N = 25$ PBO, $N = 25$	Metronidazole 15–20 mg/kg per day	3m	ER, CR	Rutgeerts, HBI	6, 12 m	6 m (PP): 22 (5/23) 6 m (PP): 36 (8/22)	0.23	6 m (ITT): 28 (7/25) 6 m (ITT): 44 (11/25)	0.19	6 m (PP): 0 (0/23) 6 m (PP): 0 (0/22)	NS		
								12 m (PP): 30 (7/23) 12 m (PP): 50 (11/22)	0.15	12 m (ITT):36 (9/25) 12 m (ITT): 56 (14/25)	0.15	12 m (PP): 4 (1/23) 12 m (PP): 9 (2/22)	0.48		
Herfarth et al.	2013	TX, $N = 17$ PBO, $N = 16$	Ciprofloxacin 500 mg/bid	6m	ER, CR	Rutgeerts, HBI	1, 3, 6m	6 m (PP): 42 (3/7) 6m (PP): 55 (5/9)	0.61	6 m (ITT): 65 (11/17) 6m (ITT): 69 (11/16)	0.81	6 m (PP): 22 (2/9) 6m (PP): 20 (2/10)	0.92	6 m (ITT): 12 (2/17) 6m (ITT): 13 (2/16)	0.67
Rutgeerts et al.	2005	TX, $N = 38$ PBO, $N = 40$	Ornidazole 1 g/d	12 m	ER, CR	Rutgeerts, CDAI	3, 12, 24, 36m	3 m: 34.4 (11/32) 3 m: 58.8 (20/34)	0.047			12 m: 7.9 (3/38) 12 m: 37.5 (15/40)	0.005		
								12 m: 53.6 (15/28) 12m: 78.8 (26/33)	0.037			24 m: 29.7 (11/37) 24 m: 45 (18/40)	0.17		
												36 m: 45.9 (17/37) 36 m: 47.5 (19/40)	0.53		
Rutgeerts et al.	1995	TX, $N = 30$ PBO, $N = 30$	Metronidazole 20 mg/kg per day	3 m	ER, CR	Rutgeerts, Symptoms	3, 12, 24, 36m	3 m: 52 (12/23) 3 m: 75 (21/28)	0.09			12 m (PP): 4 (1/23) 12 m (PP): 25 (7/28)	0.044	12 m (ITT): 7 (2/29) 12 m (ITT): 25 (7/28)	0.06
												24 m (PP): 28 (6/23) 24 m (PP): 43 (12/28)	0.171	24 m (ITT): 24 (7/29) 24 m (ITT): 43 (12/28)	0.112
												36 m (PP): 30 (7/23) 36 m (PP): 50 (14/28)	0.13	36 m (ITT): 31 (9/29) 36 m (ITT): 50 (14/28)	0.117
Total		TX, $N = 145$ PBO, $N = 146$		3–12 m			1–36 m								

CD, Crohn's disease; TX, treatment; ASX, antibiotic treatment; PBO, placebo; ER, endoscopic recurrence; CR, clinical recurrence; HBI, Harvey–Bradshaw Index; CDAI, Crohn's Disease Activity Index; ITT, intention-to-treat; PP, per-protocol; m, month; NS, no significance.

TABLE 7 | Summary of studies of probiotics to prevent post-operative recurrence in patients with CD.

Study	Year	Group, Number	Probiotics, Doses	Treatment duration	Recurrence definition	Recurrence score	Follow-up	ER rate (%)	*P*-value	CR rate (%)	*P*-value
Madsen et al.	2020	TX, $N = 58$ PBO, $N = 62$	VSL#3 1.8×10^{12} CFU/d	3 m	ER	Rutgeerts	3 m	9.3 (4/43) 15.7 (8/51)	0.36		
Fedorak et al.	2015	TX, $N = 58$ PBO, $N = 62$	VSL#3 1.8×10^{12} CFU/d	3 m	ER	Rutgeerts	3 m	39.5 (17/43)	0.3		
Chermesh et al.	2007	TX, $N = 20$ PBO, $N = 10$	Synbiotic 2000 NS	-	ER	Rutgeerts	3, 24 m	47.1 (24/51) -	NS	- -	NS
Gossum et al.	2007	TX, $N = 34$ PBO, $N = 36$	LA1 2 g/d (10^{10} CFU/d)	3 m	ER, CR	Rutgeerts, CDAI	3 m	ITT: 46.4 (13/28) ITT: 29.6 (8/27) PP: 19 (5/27) PP: 9 (2/22)	0.158 0.054	15 (4/27) 13.5 (3/22)	0.91
Marteau et al.	2006	TX, $N = 48$ PBO, $N = 50$	LA1 4×10^9 CFU/d	6 m	ER, CR	Rutgeerts, CDAI	6 m	ITT: 49 (21/43) ITT: 64 (30/47) PP: 49 (17/35) PP: 63 (27/43)	0.15 0.21	9.3 (4/43) 6.3 (3/47)	0.45
Prantera et al.	2002	TX, $N = 23$ PBO, $N = 22$	Lactobacillus GG 1.2×10^{10} CFU/d	12 m	ER, CR	Rutgeerts, CDAI	12 m	60 (9/15) 35.3 (6/17)	0.297	16.6 (3/18) 10 (2/20)	0.45
Campieri et al.	2000	TX, $N = 20$ PBO, $N = 20$	VSL#3 6 g/d (3×10^{11} CFU/d)	9 m	ER	Rutgeerts	12 m	20 (4/20) 40 (8/20)	0.15		
Total		TX, $N = 261$ PBO, $N = 262$		3–12 m			3–24 m				

CD, Crohn's disease; TX, treatment; PBO, placebo; ER, endoscopic recurrence; CR, clinical recurrence; CDAI, Crohn's Disease Activity Index; ITT, intention-to-treat; PP, per-protocol; m, month; NS, no significance.

with PR has the potential of improving the therapeutic options for CD patients that have undergone intestinal resection.

In this systematic review, we initially evaluated whether CD patients had a distinct microbiota composition at the time of surgery compared to healthy controls. As has been shown in previous studies, the majority of the studies included in this review suggested that the ileal mucosa-associated microbiota in CD patients exhibited reduced bacterial richness and diversity, while there was a clustering of samples with statistically significant differences between CD patients and healthy controls. Only one study from Machiels et al. simultaneously analyzed preoperative mucosal and fecal microbiota and found that CD patients had distinct characteristics of mucosal and fecal microbiota before surgery, which further confirmed that fecal and mucosal microbiota constitute different ecological environments (26). Although not even a single bacterial taxon had consistently altered counts across all included studies, we identified bacterial taxa obtained from surgical specimens that allowed us to discriminate between CD patients and healthy subjects, in several studies. Among the bacterial taxa reported to have altered relative abundances in CD cases, the Bacteroidetes and Firmicutes phyla were significantly less represented, while the Proteobacteria phylum was significantly more represented in mucosal microbiota, which corroborates the fecal microbiota findings. In addition, the expansion of Fusobacteria, a putative aggressive phylum, was observed in the surgical biopsies. A number of factors could influence the bacterial populations following ileocolonic resection, including substantial catabolic stress, retrograde flow of colonic contents, inflammatory changes involved in intestinal wound healing, and altered immune function (50, 51). At the time of post-operative endoscopy, the population of Actinobacteria, a proteolytic bacterial phylum with the capacity to invade and exacerbate inflammation, was depleted in patients with CD; however, the phylum Fusobacteria maintained its higher relative abundance, while the alterations in the phylum Bacteroidetes count were inconsistent. Even so, higher abundance of Enterococcaceae and *Fusobacterium* and lower abundance of Lachnospiraceae and *Faecalibacterium* existed in both post-operative mucosal and fecal microbiota in CD patients.

The counts of many known gut pathogens (*Enterococcus*, *Escherichia*, *Fusobacterium*, *Streptococcus*, *Trabulsiella*, and *Veillonella*) increased in the inflamed resection specimens, whereas the counts of essential types of butyrate and other short-chain fatty acid (SCFA)-producing bacteria were observed, such as *Faecalibacterium*, *Blautia*, *Clostridium*, *Coprococcus*, *Lachnobacterium*, *Lachnospira*, and *Ruminococcus* spp. In addition, baseline samples with enriched facultative anaerobic and oxygen-tolerant bacteria likely reflect active inflammation. The mechanisms through which these mucosa-associated bacteria that are involved in CD affect the intestinal permeability are by increasing the ability of the bacteria to adhere to the intestinal epithelium, inducing inflammatory responses by regulating the expression of inflammatory genes, restricting epithelial cell growth and differentiation by restricting energy sources, promoting invasion by pathogenic bacteria by destroying the intestinal mucus, weakening the

anti-inflammatory functions by altering regulatory T cell differentiation, and influencing IBD-related genetic risk variants by altering the abundance of gut microbiota (52–58).

Numerous studies have documented that alterations in gut microbial profiles at the time of surgery or at the post-operative follow-up are linked to the post-operative disease course in CD patients. As fecal and mucosal-associated microbiota constitute different ecological environments, they have different alteration trends (59). Notably, the presence of mucosal bacterial genera, such as *Bacteroides*, *Prevotella*, and *Parabacteroides*, which are associated with saccharolytic metabolism, has been correlated with increased remission compared to the presence of bacterial genera, such as *Enterococcus* and *Veillonella*, which are associated with fermentation and lactic acid production. However, no specific bacterial taxa are consistently different between CD patients with or without PR in any of the included studies. Among the bacteria reported to have increased relative abundance in CD patients with PR, several genera have previously been associated with triggering host inflammatory responses (26, 60, 61). Conversely, several commensal bacteria with lower relative abundance are known to exert an anti-inflammatory effect by decreasing proinflammatory colonic pro-inflammatory cytokine synthesis and inducing anti-inflammatory cytokine secretion (33). In addition, patients with recurrent CD retain microbiota that favors proteolytic-fueled fermentation and lactic acid production, while CD patients in remission retain a predominantly saccharolytic and SCFA-producing microbiota (32). Moreover, altered bacteria involved in hydrogen sulfide production or a specific enzymatic machinery associated with the metabolism of bile acids are involved in the post-operative disease course of CD patients (31, 62, 63). Furthermore, changes in the ecology of depleted SCFA-producing bacteria may permit the expansion of pathogenic bacteria through luminal environmental perturbation (24). To date, no study has evaluated the role of the metabolomic profiling of gut microbiota at the time of surgery or at the post-operative follow-up in the identification of metabolites that may be associated with CD recurrence after intestinal resection.

As there were differences between CD patients with PR and those without in terms of their gut microbiota alterations, there may be a window of opportunity for microbial biomarkers to predict and monitor the post-operative disease outcome. Moreover, equipping clinicians with the prognostic biomarkers that will allow them to identify patients more likely to experience PR will reduce the duration of the drug treatment that is unlikely to be beneficial. However, the number of published studies investigating the involvement of gut microbiota both in monitoring the post-operative disease progression and in assessing the response of CD patients to a treatment is surprisingly low. In this review, we identified only four studies that provide information on the potential of gut microbiota to predict the PR of CD, but their results were too heterogeneous to allow us to reach confident conclusions regarding a microbial biomarker. Among the most discriminative features, the high abundance of bacteria from the Proteobacteria phylum (e.g., *Proteus* and *Ralstonia*) as well as in *Ruminiclostridium gnavus* (Gammaproteobacteria) and *Corynebacterium*, and the reduced abundance of several members of the Firmicutes phylum, particularly the *Lachnospiraceae* and the *Ruminococcaceae* families (e.g., *Faecalibacterium*, *Gemella*, *Phascolarctobacterium*, *Coprobacillus*, *unidentified Lachnospiraceae*, and *Dorea*), were predictive of endoscopic PR. Furthermore, adding the usual clinical risk factors (e.g., smoking status) in the prediction model may improve its diagnostic efficiency and accuracy regarding the gut microbiota of interest. As reported by Keshteli et al., distinctive urinary metabolomic profiling associated with Bacteroidales and Gammaproteobacteria has the potential to be used as a biomarker for the identification of CD patients who develop endoscopic disease recurrence after ileocolonic resection (28). The currently available studies are insufficient in elucidating the prevalence, diversity, distribution, and function of the identified bacteria associated with PR. Further research is required to confirm and define the sensitivity and specificity for recurrence or remission of such bacterial profiles using larger patient cohorts and more targeted bacterial analysis. Moreover, functional analyses are required to characterize the phylogenetic alterations at the gene expression level.

Various pharmaceutical treatments have been developed in an attempt to prevent or delay potential recurrence and subsequent surgery. However, the strategy directly targeted to gut microbiota disturbance is currently quite limited. While studies have attempted to utilize antibiotics or probiotics in an effort to prevent the PR of CD, their results are non-conclusive. Antibiotics can potentially ameliorate the microbial environment of patients suffering from IBD, both by decreasing the counts of pro-inflammatory bacteria and by increasing those of beneficial ones, throughout the intestinal lumen (64). Funayama et al. reported that antibacterial treatment was useful in post-operative CD patients whose assessments were complicated by bacterial overgrowth (65). Nitroimidazole antibiotics have been proven to be effective in preventing the clinical (RR: 0.23; 95% CI: 0.09–0.57; NNT: 4) and endoscopic (RR: 0.44; 95% CI: 0.26–0.74; NNT: 4) PR of CD, but the high rate of patients that are intolerant to them and their questionable long-term effects beyond the end of the treatment preclude their widespread use. Recent promising data on the activity of the non-absorbable antibiotic rifaximin (which is generally well-tolerated) in CD patients suggest that assessment of this agent in reducing post-operative recurrence is warranted. Probiotic supplements have been used successfully in the prevention of pouchitis and the maintenance of remission in active ulcerative colitis; nevertheless, the present review demonstrates that studies to date have failed to identify any benefit of probiotic supplement administration in preventing the PR of CD (66–69). Intriguingly, Campieri et al. reported that the combination of a non-absorbable antibiotic (rifaximin) and a highly bacterial concentrated probiotic (VSL#3) is efficient in preventing the severe endoscopic recurrence of CD (47). However, none of the studies examined in the present review investigated the therapeutic mechanisms of either antibiotics or probiotics on gut microbiota modification. Several mechanisms mediate the therapeutic action of antibiotics and probiotics, such as the inhibition of pathobionts, increase in beneficial bacteria, modification of bacterial metabolites, regulation of immunity, improvement in the mucosal barrier, or absorption

of toxic substances (70–75). The outcomes of microbial-based treatments indicate that the possibility of using combination-based strategies, such as the early post-operative use of antibiotics to prevent pathogenic recolonization followed by maintenance with the use of probiotics to establish a durable anti-inflammatory post-operative microflora, may yield the greatest benefit with the least risk of disease recurrence in CD patients. Unfortunately, the efficacy of fecal microbiota transplants in preventing the PR of CD remains unknown.

There are some limitations to this review that warrant discussion. It is well-known that several factors may exert an influence on the composition of gut microbiota, including geographic, cultural, demographic, dietary, and preoperative and post-operative medications differences, which may explain some of the observed discrepancies among the different studies (76, 77). In addition, other risk factors associated with disease recurrence failed to be addressed in this review, which perhaps explain some of the discrepancies between the results. Furthermore, the included studies consisted of 10 retrospective studies and two prospective studies, which might be one possible reason for the inconsistencies. Lastly, differences in the methodology used, such as specimen type, sample storage methods, DNA extraction methods, primers targeting different regions, bioinformatic pipelines, and reference databases used, may explain the heterogeneous results observed in the examined studies.

CONCLUSION

In this systematic review, we characterized the mucosa-associated microbiota at the time of surgery and the profiles of the bacteria that recolonized the intestinal tract following resection. Additionally, we highlighted specific bacterial taxa, the counts of which either increased or decreased and that are associated with the endoscopic PR of CD. Although consistent recurrence-associated gut microbiota with predictive value could not be identified from the examined studies, a few microbial predictors were suggested. CD patients with PR tend to gain pathogenic bacteria with a pro-inflammatory effect and lose SCFA-producing bacteria. The gut microbiota manipulation through the administration of either antibiotics or probiotics may not offer a promising alternative in the prevention of PR in patients with CD. Future research should focus on investigating differences in the function and composition of the gut microbiota associated with PR and post-operative remission. Additionally, the use of larger patient cohorts is recommended to confirm the sensitivity and specificity of bacterial profiles with predictive value. Furthermore, effective microbial-based therapies based on an individual patient's microbial profile that are used to prevent PR and can be administered for prolonged time periods with acceptable side effects are urgently awaited.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Materials**, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

MC guarantor of the article. MC and XZ designed the study. XZ wrote the manuscript. ZT and XL collected the data. MZ, NL, and SX analyzed the data. RM, ZZ, and RF revised the manuscript. All authors approved the final version.

FUNDING

This work was supported by The Leona M. & Harry B. Helmsley Charitable Trust Grant (2019PG-CD018).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2020.615858/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Medicine

CASE REPORT
published: 06 April 2021
doi: 10.3389/fmed.2021.553110

Case Report: CMV Infection and Same Mechanism-Originated Intestinal Inflammation Compatible With Bowel/Crohn's Disease Is Suggested in ATP4A Mutated-Driven Gastric Neuroendocrine Tumors

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OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Gastroenterology, a section of the journal Frontiers in Medicine

Received: 10 September 2020
Accepted: 08 February 2021
Published: 06 April 2021

Citation:
Calvete O, Reyes J and Benítez J (2021) Case Report: CMV Infection and Same Mechanism-Originated Intestinal Inflammation Compatible With Bowel/Crohn's Disease Is Suggested in ATP4A Mutated-Driven Gastric Neuroendocrine Tumors. Front. Med. 8:553110. doi: 10.3389/fmed.2021.553110

Mutations in the ATP4A proton pump prevent gastric acidification and explain the chronic autoimmune gastritis scenario that conducts the gastric neuroendocrine tumor (gNET) formation. Here, we wanted to investigate the co-occurrence cytomegalovirus (CMV) infection and intestinal inflammation that presented all members of a family affected with gNET and carrying an *ATP4A* mutation. Intestinal inflammation persisted after CMV eradication and anemia treatment. The inflammation was compatible with a ileitis/Crohn's disease and was originated by the same autoimmune mechanism described in the tumorigenesis of gNETS. The same secondary disease but no the CMV infection was observed in all members affected with gNET and carrying the ATP4A mutation. Our results suggest that the *ATP4A* malfunction not only explained gNETs but also the co-occurring disease and opportunistic infections, which allowed to link autoimmune pathologies and gNETs in a unique mechanism. Our results open a new window to better understand not only gastric neoplasms formation but the co-occurring autoimmune disorders and the inflammatory mechanism that compose a premalignant scenario for other tumor formation. Our findings are important since contribute to describe the genetic landscape of the Inflammatory Bowel/Crohn's disease and alert clinicians to monitor patients with gastric neoplasms mediated by achlorhydria mechanisms for concomitant secondary pathologies.

Keywords: gastric neuroendocrine tumor, ATP4A, co-occurring autoimmune disorders, cytomegalovirus, inflammatory bowel disease, Crohn's disease

INTRODUCTION

Type I gastric neuroendocrine tumors (gNETs) arise from enterochromaffine-like (ECL) cells in patients with autoimmune chronic atrophic gastritis and give rise to hypergastrinemia and parietal cell (PC) atrophy that leads to gastric hyperplasia and achlorhydria, respectively. However, we described a homozygous mutation in the *ATP4A* gene (p.R703C) that prevented gastric

acidification and was the main effector of progression of the gNETs. This contrasts with the classical model, where hypergastrinemia leads to achlorhydria (1). A knock-in (KI) mouse model for this *ATP4A* mutation was constructed in order to perform functional studies (2). This model confirmed the relation of the candidate gene with achlorhydria and gNET development, and served us to better understand the relation between impaired capability to export protons across the plasma membrane of PCs and tumor progression. We observed that the ATP4A$^{p.R703C}$ mutation drove gastric achlorhydria, but also would impair the acid–base balance within PCs, affecting mitochondrial biogenesis and activating ROS signaling, which triggers caspase-3-mediated apoptosis of parietal cells (3). Recently, we studied a second family affected with gNETs and other autoimmune pathologies (hypothyroidism and rheumatoid arthritis). A cumulative effect of two mutations in a digenic model (*ATP4A* and *PTH1R* genes) explained the genetic landscape underlying the gNETs in this family. In addition, the *PTH1R* gene is involved in the regulation of Ca2+ metabolism and this explained the associated hypothyroidism and rheumatoid arthritis (4). Here, we present the monitoring results of the first family with the atypical and aggressive gNET caused by the ATP4A$^{p.R703C}$ mutation (1) and the recent re-evaluation of the altered clinical parameters not involved in the gastric pathology.

MATERIALS AND METHODS

Patients

A consanguineous family (the parents were cousins) from Majorca Island (Spain) with ten siblings was previously evaluated (1). Five of them were diagnosed (age of onset around 30 years in average) with type I gastric NETs (II-1, II-3, II-7, II-8, II-9). Three (II-1, II-3, and II-7) showed nodal infiltration and one (II-3) had a synchronous focus of gastric adenocarcinoma without nodal infiltration (T1b N0) (**Figure 1A**). All patients were treated with total gastrectomy, were negative in *MEN1* gene mutation studies by Sanger sequencing and for *H. pylori*. Both parents (I-1 and I-2) and siblings II-3, II-7 and II-9 were selected for WES, which uncovered the ATP4A mutation (in heterozygosis in parents and in homozygosis in affected siblings). Blood samples from the remaining healthy individuals in the family were also obtained for further study of the segregation of the variants (**Figure 1A**). Role of the ATP4A mutation was later validated in a KI mouse model (2). Informed consent was obtained for all patients. Serological and biochemical parameters were obtained from routine serum/blood tests.

Immunohistochemistry Studies

Formalin-fixed paraffin-embedded (FFPE) tissue samples were obtained from monitoring biopsies of the proband. FFPE blocks were cut into 5-μm-thick sections and stained with Hematoxylin and Eosin (H&E) for light microscopy examination. FFPE blocks were cut into 5-l m-thick sections for immunohistochemistry (IHC) studies. Inflammation of the ileum was tested with anti-cytokeratin 7 antibody from DAKO (ref: M7018). Mitochondrial activity in the inflamed area was tested with anti-Pyruvate Carboxylase antibody from NOVUS Biologicals (Ref: NBP1-49536) following the manufacturers' instructions.

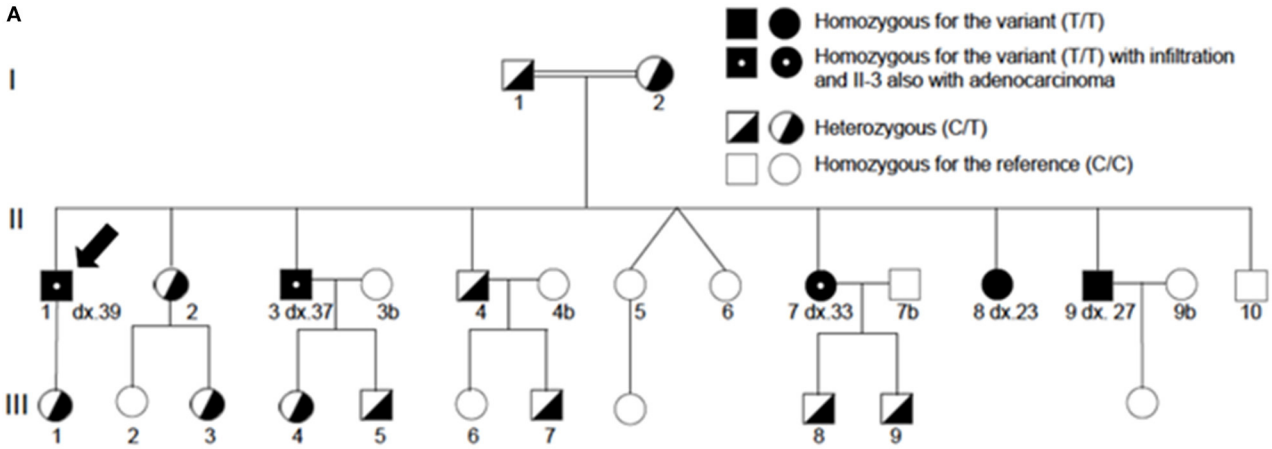
CASE PRESENTATION

A consanguineous family with gNETs was previously studied. The proband of this study (**Figure 1A**) was diagnosed in 2007 (dx.39) with four well-differentiated foci of gNETs of 1.7, 1.0, 0.9, and 0.7 cm in size that were infiltrating the submucosal layer (pT1b). The tumors were immunoreactive for the general neuroendocrine markers but also had a component with glandular growth, which was morphologically classified as an intestinal type adenocarcinoma (G1/4) (5). Perineural and lymph node invasion (size: 0.4 cm) was also reported for this patient. Total gastrectomy was performed to prevent adenocarcinoma metastasis.

RESULTS AND DISCUSION

In 2017, of this patient revealed persistent acute anemia and an intestinal inflammatory process (**Figure 1B**). Endoscopy of the small intestine revealed several aphthous ulcers and Haematoxylin and eosin (H&E) staining confirmed atrophic and flattened microvilli with reduction of goblet cells, microvilli oedema, cryptitis, and inflammatory infiltrate (**Figure 2A**). Cytomegalovirus (CMV), which is an opportunistic infection for weakened/immunosuppressed tissue, was also PCR-positive in a CMV culture on the ileal mucosa biopsy, which could explain the observed inflammation (**Figure 1B**). No other relevant clinical findings were reported after physical examination. However, HIV infection and other causes of immunosuppression studied were negative. The patient was standard treated with a 14-day cycle of intravenous ganciclovir followed by a 14-day cycle of oral ganciclovir. In the 2018 evaluation, biochemical and serological monitoring studies revealed persistent anemia and inflammation without traces of CMV infection (**Figure 1B**). Evaluation of the biopsy still showed aphthous ulcers in the small intestine and inflammatory infiltrate (**Figure 2B**). The patient was also treated with intravenous iron, to which he responded positively and restored serum ferritin levels. However, at the most recent monitoring (2019 evaluation), calprotectin and fibrinogen were still altered (**Figure 1B**), which suggested an intestinal inflammation compatible with ileitis/Crohn's disease. H&E evaluation confirmed the intestinal inflammation (**Figures 3A,B**). Thus, intestinal inflammation was independent of the CMV infection in the patient. Functional imaging using Tc-99m-tektrotyd-SPECT, which binds to receptors specific for neuroendocrine lesions in the distal ileum (6), showed increased absorption in ileum and peri-ileal lymph nodes that was in agreement with premalignant neuroendocrine lesions in the intestine, similar to those previously observed in the stomach (data not shown).

In order to test a putative relation and possible metaplasia or metastasis from the previously diagnosed gNET, we stained the 2019 biopsy with anti-Chromogranin A antibody, which typically stains hyperplasia of neuroendocrine cells and was found to



B

gNET — 2007 Total gastrectomy — Ganciclovir treatment — Intravenous Fe treatment

Pathological process	Clinic Value	Reference values	2017	2018	2019
Anaemia	Red blood cells	[4.50-6 uL]	3.29	3.06	4.36
	Haemoglobin	[13-17.5 g/dL]	10.9	10.9	12.6
	Haematocrit	[40-53%]	33	33.4	36.7
	Ferritin	[20-275 ng/mL]	25	15	113
Inflammation	Sedimentation rate	[1-20 mm/h]	24	25	33
	C reactive protein	[0-0.50 mg/dL]	1.76	1.44	1.35
Disease Biomarker	Fibrinogen *	[150-500 mg/dL]	430	492	503
	Gastrin	[100-150 ng/L]	< 10 ng/L	< 10 ng/L	< 10 ng/L
	Calprotectin**	[<50 ug/g]	NT	688	877
	Celiac		NT	Negative	NT
	Cytomegalovirus º		Positive	Negative	Negative

* compatible with malignant progression

** Intestinal inflammation compatible with Ileitis/Crohn's disease

º Opportunistic infection for weakened/immunosuppressed tissue

NT: Not tested

CMV infection

Pernicious anaemia

Atrophic Inflammation and ulceration

FIGURE 1 | (A) Pedigree of studied family with complete segregation of the ATP4A$^{p.R703C}$ mutation (C: Reference allele for the position; T: Alternative allele for the position). Black arrow shows the proband evaluated in this study. **(B)** Serologic information of proband patient regarding anemia, inflammation and disease markers measured in 2017, 2018, and 2019. CMV infection was eradicated after ganciclovir treatment. Anemia markers become partially restored after Fe treatment. Atrophic inflammation persisted in the 2019 evaluation.

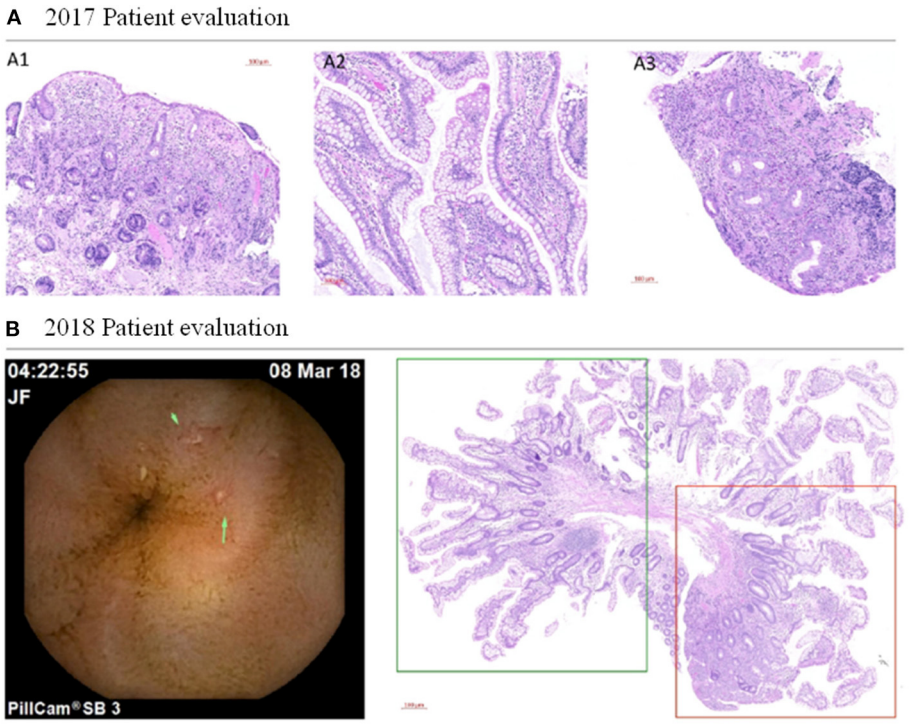


FIGURE 2 | Clinicopathologic evaluation of the patient. **(A)** Histopathologic evaluation of the patient's 2017 biopsy. Intestine biopsy revealed atrophic and flattened microvilli with reduction of goblet cells (A1), microvilli oedema and cryptitis (A2), and inflammatory infiltrate (A3). Scale bar: 100 μm. **(B)** Histopathologic evaluation of the patient's 2018 biopsy. Left panel: The endoscopy uncovered aphthous ulcers (green arrows) in the small intestine even without cytomegalovirus infection. Right panel: haematoxylin and eosin staining of biopsied tissue shows moderate microvilli atrophy in the normal intestinal tissue of the patient (boxed in green) and inflammation process (boxed in red). Scale bar: 500 μm.

be increased in gastric tissue of this patient (1). No increased staining was observed in the 2019 biopsy of the intestine, suggesting a primary inflammation in the intestine unrelated to the previously diagnosed gNET.

Immunohistochemistry studies were performed to further characterize the inflammation. The 2019 biopsy was stained with anti-cytokeratin 7 antibody (CK7) biomarker following epithelial inflammatory neoplasms guidelines for expression patterns (7). Multiple anti-CK7 positive foci were observed for this patient (**Figure 3C**), which indicates inflammatory bowel disease-associated dysplasia, either ulcerative colitis or Crohn's disease (8). On the other hand, the origin of the gNET tumor of this patient was related to an impaired acid-base balance, which was altering mitochondrial function in the stomach (3). To test the involvement of this same mechanism in the new inflammation process, we stained the 2019 biopsy with anti-Pyruvate Carboxylase antibody and compared it to a healthy intestine (**Figure 3D**). Reduction of staining in the patient's sample corresponds to the lack of mitochondrial function, as we previously observed in the stomach (3). Finally, to evaluate if this observation was specifically found in this patient or whether it was a common trait in all members of the family affected with gNETs, we conducted a non-invasive serological evaluation of patients II.3, II.7, II.8, and II.9 (all gastrectomized) (**Table 1**). A high sedimentation rate was observed in the gNET patients of the family, which suggested an inflammatory process for these individuals as well. Calprotectin was highly increased in all individuals from the family affected with gNETs, mimicking the described monitoring studies of the proband patient. No traces of CMV infection were observed in these patients. Most relevant biochemical parameters were also tested in healthy siblings of the family without gNETs disease as control. In average, normal values were found for C reactive protein (0.183 C mg/dL), ferritin (47.6 ng/mL), and Sedimentation rate (15.5 mm/h) parameters.

In summary, a concurrent inflammatory disease was observed in all patients of the family first diagnosed with an aggressive ATP4A-mutated gNET. Anemia, calprotectin and anti-CK7 staining were in agreement with progression of ileitis-associated neoplasia compatible with inflammatory bowel and Crohn's diseases (8). Autoimmune and inflammatory manifestations occur frequently in patients with primary immunodeficiencies (9). In particular, concomitant gastric and small intestinal inflammatory disorders have been described including celiac disease and more extensive collagenous inflammatory disease (10). In addition, there is a described higher incidence of e.g., inflammatory bowel disease or gastric cancer in patients with Common variable immunodeficiency. However, the associated genetic pathogenesis still remains ambiguous (11). Our results are important since contribute to describe the genetic landscape of these clinical associations. In this

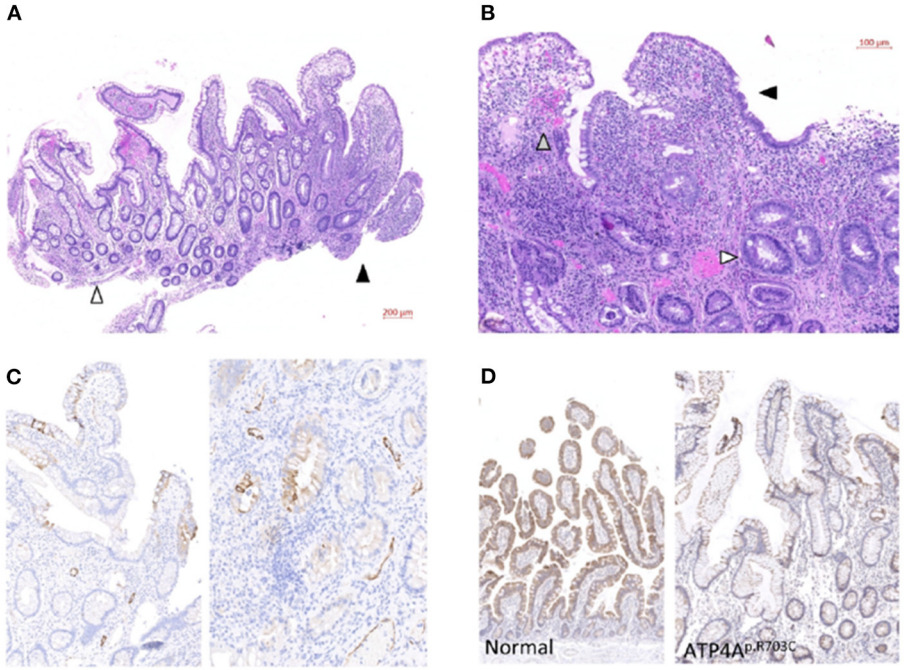


FIGURE 3 | Clinicopathologic evaluation of the patient. **(A)** Representative H&E staining of 2019 ileum biopsy. White arrowhead: moderate microvilli atrophy in normal intestinal tissue of the patient. Black arrowhead: severe inflammation process in a biopsied aphthous ulcer. Scale bar: 200 μm. **(B)** Detailed evaluation of inflamed area shows atrophic and flattened microvilli with strong reduction of goblet cells (black arrowhead) and oedema and cryptitis (gray arrowhead). Reactive glands (white arrowhead) and inflammatory infiltrate with abundant neutrophils and eosinophils are observed in the whole mucosa. Scale bar: 100 μm. **(C)** Two different representative areas of the 2019 patient intestine biopsy stained with anti-cytokeratin7 antibody. Scale bar: 100 μm. **(D)** Representative immunohistochemical staining with anti-Pyruvate Carboxylase antibody of a normal intestine (left) and the 2019 patient biopsy (right). Reduced staining signal in the patient correlates with altered mitochondrial function. Scale bar: 100 μm.

TABLE 1 | Pre and post Fe-treatment serology evaluation of the other members of the family affected with gastric neuroendocrine tumors.

Patient	**II.3**		**II.7**		**II.8**		**II.9***	
Fe-treatment	**Pre**	**Post**	**Pre**	**Post**	**Pre**	**Post**	**Pre**	**Post**
Red blood cells [4.50–6 uL]	5.07	5.19	3.76	4.52	3.6	3.6	5.13	NT
Hemoglobin[13–17.5 g/dL]	15.3	14.9	8.9	13.5	11.3	11.3	12.5	13.7
Haematocrit [40–53%]	45.9	46.8	27.8	40.6	35.5	35.5	38.8	42
Ferritin [20–275 ng/mL]	12	54	2	NT	9	70	NT	5
Sedimentation rate [1–20 mm/hour]	13	16	35	NT	5	49	NT	33
C-reactive protein [0–0.50 mg/dL]	0.07	0.03	<0.10	NT	<0.10	0.03	NT	1.44
Fibrinogen [150–500 mg/dL]	319	349	333	NT	284	341	NT	NT
Calprotectin [<50 ug/g]	NT	140	NT	NT	NT	246	NT	NT

*NT: Not tested; *No Fe treatment.*
Reference values are showed in brackets.

work, the inflammation in the intestine was observed as a primary disease and not derived from the gastric neoplasm. Moreover, the same mitochondria-malfunction mechanism responsible for the autoimmune-originated gastric neoplasm in the stomach was also observed in the inflamed intestinal tissue, suggesting that the same impaired acid-base balance mechanism caused by the *ATP4A* mutation might also explain the second inflammatory disease. Study of the other gNETs members of the family also revealed inflammatory disease, which is in agreement with the co-occurrence in all patients of the family carrying the same mutation. In addition, this prospective study is in agreement with the observed autoimmune pathologies concurrent with the gNETs in the second family, where the cumulative effect of two mutations in the *ATP4A* and *PTH1R* genes explained the genetic landscape of the gastric disease and the concomitant autoimmune hypothyroidism and rheumatoid arthritis (4). Thus, our results underscore the important role of *ATP4A* mutations in gNET progression, but also of other concurrent pathologies that must be monitored in these patients to prevent malignant transformation in other tissues.

Finally, CMV infection is usually described in immunodepressed patients and contributes to inflammation. However, no cause of immunosuppression other than the ATP4A mutation was found for this patient. In addition, CMV infection in the proband was not observed in the other members of the family with inflammation, which progressed in the patient even after CMV eradication (**Figure 1B**). Thus, our data suggest a secondary colonization of the virus not involved in the inflammatory progression but colonizing the injured intestinal

tissue. Importantly, secondary opportunistic *H. pylori* infection, which classically contributes to achlorhydria and chronic gastritis, was suggested in patients with gastric achlorhydria (3). Therefore, our results suggest a second pathologic event in the studied family.

The exact molecular mechanism of this histological transformation or the prognostic implications is still unclear. Till then it might be prudent to follow up these patients to assess for the relapse of inflammatory bowel disease as well as for dysplasia surveillance. Perspective follow-up based in mild-moderate ileitis in the context of Crohn's disease guidelines is being carried out for these patients. Evolutionary control is carried out with analytics and periodic termination of calprotectin in feces. Likewise, when the diarrhea symptoms become more intense, we have performed treatment with short courses of oral budesonide as indicated for microscopic colitis (12), with a clear improvement in the symptoms.

CONCLUSION

Our results are important since patients with ATP4A mutations or achlorhydria-mediated gNETs have been observed to have secondary pathologies and infections that co-occur with the gastric neoplasm. Long-standing inflammatory bowel disease and CMV infection are premalignant conditions and pose an increased risk of colorectal adenocarcinoma (13) and gastrointestinal neoplasms (14). Therefore, our findings should alert clinicians to monitor patients with gastric neoplasms mediated by mechanisms involving achlorhydria. Further studies in other families affected with gNETs or chronic atrophic gastritis must be performed in order to describe the whole spectrum of concurrent pathologies but these patients must be monitored and careful attention must be paid to other inflammatory diseases that may trigger new severe pathologies.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

Written informed consent was obtained from the individuals for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

OC and JR obtained the data collection and performed the data analysis and interpretation. OC and JB supervised the study and draft the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by JB's lab and is partially funded by Instituto de Salud Carlos III, cofunded by European Regional Development Fund (ERDF) grant (PI16/00440). OC was granted by H2020 BRIDGES project (634935).

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Medicine

ORIGINAL RESEARCH
published: 28 April 2021
doi: 10.3389/fmed.2021.621337

Association of Serum Immunoglobulins Levels With Specific Disease Phenotypes of Crohn's Disease: A Multicenter Analysis in China

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Specialty section:
This article was submitted to Gastroenterology, a section of the journal Frontiers in Medicine

Received: 26 October 2020
Accepted: 29 March 2021
Published: 28 April 2021

Citation:
Song DJ, Shen J, Chen MH, Liu ZJ, Cao Q, Hu PJ, Gao X, Qian JM, Wu KC, Lai LJ and Ran ZH (2021) Association of Serum Immunoglobulins Levels With Specific Disease Phenotypes of Crohn's Disease: A Multicenter Analysis in China. Front. Med. 8:621337. doi: 10.3389/fmed.2021.621337

Background and Aim: Serum immunoglobulins were reported to be associated with clinical characteristics of inflammatory bowel disease. However, whether a difference exists in the serum immunoglobulins levels in patients with Crohn's disease (CD) with different disease location and behavior phenotypes remains unclear. Therefore, this study aimed to explore the associations of serum immunoglobulins levels with specific CD phenotypes.

Methods: Patients with CD having recorded serum immunoglobulins levels were recruited through multicenter collaborative efforts. The associations between serum immunoglobulins levels and distinct phenotypes of CD were evaluated using multiple logistic regression models.

Results: A total of 608 patients with CD were included in the study. Elevated (above the upper limit of normal) serum immunoglobulin G (IgG), IgA, IgM, and IgG4 were identified in 24.5, 17.4, 2.1, and 8.2% of patients, respectively. Elevated serum IgG4 levels negatively correlated with complicated disease behavior [odds ratio (OR) 0.49, 95% confidence interval (CI) 0.26–0.92]. Elevated serum IgG was linked to isolated ileal disease with an OR of 0.37 (95% CI 0.23–0.61). The ORs of isolated ileal disease progressively reduced across increasing quartiles of IgG (P for trend < 0.001). The adjusted ORs of isolated ileal disease for increasing quartiles of IgM were 1.82 (1.07–3.1), 1.92 (1.14–3.24), 1.17 (0.69–1.98), and 1 (P for trend $= 0.008$). Besides, serum IgA and IgG levels significantly correlated with several disease activity indices.

Conclusions: These results suggested that certain serum immunoglobulins were associated with specific disease phenotypes of CD. Further investigations to account for the associations are warranted.

Keywords: Crohn's disease, disease phenotypes, disease activity, serum immunoglobulins, cross-sectional study

INTRODUCTION

Crohn's disease (CD), one subtype of inflammatory bowel disease (IBD), is a chronic, relapsing-remitting inflammatory disorder involving the gastrointestinal tract. The increased prevalence of IBD with time in developing nations has been confirmed (1, 2). The innate and adaptive immune systems are critical to the development of IBD, and the latter is the more proximate driver of tissue damage in patients with IBD (3). The adaptive immune system is composed of B cells, T cells, and regulatory T/B cells. The function of B cells in the etiology of IBD has received increasing attention in recent years (4, 5). B cells are transformed into plasma cells, which synthesize and release immunoglobulin G (IgG), IgA, IgM, IgD, and IgE. The isotypes and subclasses of immunoglobulins have distinct effector functions and represent particular immunologic processes (6). The change in the serum levels of immunoglobulin isotypes has been confirmed and the distribution is unique in different autoimmune diseases, thus confirming the role of certain isotypes of immunoglobulin in disease development (7–9). Serum IgG has been found to provide risk prediction in patients with immunoglobulin A nephropathy, autoimmune hepatitis accompanied by systemic lupus erythematosus (SLE-AIH), and hepatitis B virus-related acute-on-chronic liver failure (HBV-ACLF) (10–12).

Serum immunoglobulins levels have been reported to be associated with distinct clinical characteristics of IBD. Previous studies showed that the levels of serum IgG, IgG1, and IgG4 were significantly increased in ulcerative colitis (UC) compared with CD. Conversely, the serum IgG2 levels were significantly decreased in UC compared with CD (13, 14). In addition, a possible relationship between low serum IgG or IgG1 levels and the need for small bowel resection in patients with IBD has been reported (15). Furthermore, hypergammaglobulinemia defined as elevated IgG levels contributes to distinguishing arthritis from arthralgia in pediatric IBD (16). Patients with IBD having high levels of mucosal and serum IgG4 tend to have severe and extensive lesions (17).

Patients with CD can be further classified according to the Montreal classification, including age of onset, disease behavior, and disease location (18). Complicated disease (stricturing or penetrating behavior) and ileal disease location at diagnosis conferred an increased risk of intestinal resection in CD (19, 20). Thus, patients with CD with specific disease phenotypes may benefit from early intervention. Nevertheless, studies focusing on the associations of serum immunoglobulins levels with different disease location and behavior phenotypes of CD are limited. A clear understanding of the immune mechanisms involved in CD contributes to risk stratification and personalized prevention.

It was hypothesized that the expression of serum immunoglobulins is different in patients with CD with different disease location and behavior phenotypes. Hence, a cross-sectional study was performed to explore the possible associations between serum immunoglobulins levels and phenotypic features of CD.

METHODS

Patient Population

Patients with CD with recorded serum immunoglobulins levels between 2016 and 2018 were recruited from seven tertiary hospitals in China. The diagnosis of CD was performed according to the European Crohn's and Colitis Organization (ECCO) consensus (21). Meanwhile, patients with CD coexisting with other autoimmune diseases were excluded. Finally, the present study comprised 608 individuals who received serum IgG, IgA, IgM, and IgG4 testing.

The study was approved by the Medical Ethics Committee of Renji Hospital, School of Medicine, Shanghai Jiao Tong University.

Clinical Characteristics of CD Patients

The demographic data, Montreal classification of CD, surgical history, history of appendectomy and perianal operation, disease activity, and other laboratory tests such as C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), hemoglobin, platelet (PLT) count, albumin, and prealbumin were collected from each patient. CD phenotypes were evaluated based on the Montreal classification. Complicated disease was defined as B2, B3, and B2B3 (22). Perianal disease consisted of perianal abscess and perianal fistula. Surgical history was bowel resection associated with CD. The disease activity was determined based on the Harvey-Bradshaw index (23).

Analysis of the Levels of Serum IgG, IgA, IgM, and IgG4

As a retrospective study using electronic medical records, the serum immunoglobulins assays used were not controlled. The serum immunoglobulins levels were considered as elevated when they were higher than the upper limit of normal in each hospital. The serum immunoglobulins levels from different centers were normalized based on their distributions for quantitative and correlation analyses (24–26).

Statistical Analysis

All statistical analyses were carried out using SPSS software (version 19). Categorical variables were presented as numbers (percentages) and continuous variables as medians [interquartile

range (IQR)] unless specifically annotated. The normal distribution of continuous data was determined using the Kolmogorov-Smirnov test. Comparisons of percentages between groups were performed using the chi-square or Fisher's exact test. Comparisons between groups for ordered variables were performed using the rank-sum test. Statistical differences for normally distributed data between two groups were analyzed using the Student *t*-test. Non-normally distributed continuous variables were analyzed using non-parametric tests. Spearman's correlation test was applied for data with non-normal distribution. Laboratory data from different hospitals were normalized according to their distribution as mentioned earlier. Principal component analysis (PCA) was performed with the R Statistic program version 4.0.2.

The multivariate logistic regression was used to assess the independent associations between serum immunoglobulins levels and the presence of complicated disease and isolated ileal disease. The serum immunoglobulins levels were also categorized into quartiles to determine the shapes of relationship. These confounders in the models were selected according to their relationship with dependent variables or a change in the effect estimate of more than 10%. Tests for linear trend were conducted by entering the median values of each category as a continuous variable. *P*-values of <0.05 indicated a statistically significant difference.

RESULTS

Patient Characteristics

The baseline characteristics of participants are shown in **Table 1**. Among the total of 608 patients, 68.4% were male and the median age of the included patients was 30.5 years (IQR 25–39 years). Isolated ileal disease was observed in 42.3% of participants. Complicated disease was observed in 60.4% of participants. The PCA analysis for data collected from different centers revealed there was no obvious separation from our centers to other centers (**Supplementary Figure 1**).

Demographic and Clinical Data of Patients in Accordance With the Serum Immunoglobulins Status

Elevated serum IgG, IgA, IgM, and IgG4 were found in 24.5, 17.4, 2.1, and 8.2% of participants, respectively. The univariate analysis was not performed for IgM because only 13 of 608 patients had elevated serum IgM levels. The univariate analysis of demographic and phenotypic characteristics associated with elevated serum IgG, IgA, and IgG4 are listed in **Table 2**. Elevated serum IgG was related to gender ($P = 0.015$), age ($P = 0.011$), disease duration ($P = 0.033$), history of appendectomy ($P = 0.011$), surgical history ($P = 0.001$), disease location ($P < 0.001$), and disease activity ($P = 0.004$). Elevated serum IgA was significantly related to age ($P = 0.009$), age at diagnosis ($P = 0.026$), disease location ($P = 0.003$), disease behavior ($P = 0.007$), and disease activity ($P < 0.001$). Elevated serum IgG4 was related to age ($P < 0.001$), age at diagnosis ($P < 0.001$), disease duration ($P < 0.001$), and complicated disease ($P = 0.006$).

TABLE 1 | Baseline characteristics of the study population.

	All patients ($n = 608$)
Age, y, median (IQR)	30.5 (25–39)
Age at diagnosis, y, median (IQR)	29 (23–37)
Male, *n* (%)	416 (68.4%)
Disease duration (y), *n* (%)	
≤1	179 (29.4%)
1–5	228 (37.5%)
5–10	138 (22.7%)
>10	63 (10.4%)
Disease location, *n* (%)	
L1 ± L4	257 (42.3%)
L2 ± L4	50 (8.2%)
L3 ± L4	301 (49.5%)
Disease behavior, *n* (%)	
B1: Inflammatory	241 (39.64%)
B2: Stricturing	265 (43.58%)
B3: Penetrating	47 (7.73%)
B2B3: Stricturing + Penetrating	55 (9.05%)
Perianal disease, *n* (%)	328 (53.9%)
Complicated disease, *n* (%)	367 (60.4%)
Appendectomy, *n* (%)	51 (8.4%)
Perianal operation, *n* (%)	178 (29.3%)
Surgical history, *n* (%)	100 (16.4%)
Treatment naïve patients	192 (31.6%)
Active/remission	537/71

y, years; IQR, interquartile range.

Independent Association of Serum IgG4 Levels With Complicated Disease

The levels of serum IgG and IgG4 were significantly reduced in CD patients with complicated disease compared with CD patients with inflammatory phenotype (**Figures 1A,D**). However, there was no difference in serum IgA and IgM levels in CD patients with and without complicated disease (**Figures 1B,C**). Univariate analysis revealed that elevated serum IgG4 was inversely related to complicated disease (OR: 0.44, 95% CI: 0.25–0.8) (**Table 3**). Multiple logistic regression was employed to further evaluate the association of serum IgG4 with complicated disease. When serum IgG4 levels were assessed as quartiles, a significantly increased probability of complicated disease was found in patients in quartile 3 (OR: 1.82, 95% CI: 1.11–2.98) and quartiles 1–2 (OR: 1.86, 95% CI: 1.2–2.88) compared with those in quartile 4. For a per-standard deviation (SD) increase in serum IgG4 levels, the OR for complicated disease was 0.74 (0.62–0.89) in the final multivariable model (**Table 3**).

Independent Association of Serum IgG and IgM Levels With Isolated Ileal Disease

In accordance with the results of univariate analysis in **Table 2**, the levels of serum IgG were significantly reduced in CD patients with isolated ileal disease (**Figure 1E**). Similar tendencies were also observed for IgA and IgM (**Figures 1F,G**). However, there

TABLE 2 | Demographic and phenotypic characteristics of cases according to serum total IgG, IgA, and IgG4 status.

	Elevated IgG ($n = 149$)	Normal IgG ($n = 459$)	P	Elevated IgA ($n = 106$)	Normal IgA ($n = 502$)	P	Elevated IgG4 ($n = 50$)	Normal IgG4 ($n = 558$)	*P*
Male	90 (60.4%)	326 (71%)	0.015	66 (62.3%)	350 (69.7%)	0.13	36 (72%)	380 (68.1%)	0.57
Age, Median (IQR)	29 (22–38)	31 (25–39)	0.011	28 (24–34.25)	31 (25–40)	0.009	24 (19–32.25)	31 (25–39)	<0.001
Age at diagnosis, Median (IQR)	27 (21–36)	29 (23–37)	0.067	27 (22–32)	29 (23–38)	0.026	23 (19–31.25)	29 (23–37)	<0.001
Disease duration, Median (IQR)	2 (0.75–6)	3.3 (1–7)	0.033	3 (0.96–7)	3 (1–7)	0.24	1 (0.5–3)	3.5 (1–7)	<0.001
Appendectomy	5 (3.4%)	46 (10%)	0.011	12 (11.3%)	39 (7.8%)	0.23	1 (2%)	50 (9%)	0.15
Perianal operation	42 (28.2%)	136 (29.6%)	0.74	26 (24.5%)	152 (30.3%)	0.24	16 (32%)	162 (29%)	0.66
Surgical history	12 (8.1%)	88 (19.2%)	0.001	15 (14.2%)	85 (16.9%)	0.48	4 (8%)	96 (17.2%)	0.09
Disease Location			<0.001			0.003			0.54
L1 ± L4	31 (20.8%)	226 (49.24%)		30 (28.3%)	227 (45.2%)		24 (48%)	233 (41.76%)	
L2 ± L4	20 (13.4%)	30 (6.53%)		14 (13.2%)	36 (7.2%)		5 (10%)	45 (8.06%)	
L3 ± L4	98 (65.8%)	203 (44.23%)		62 (58.5%)	239 (47.6%)		21 (42%)	280 (50.18%)	
Isolated ileal disease	31 (20.8%)	226 (49.2%)	<0.001	30 (28.3%)	227 (45.2%)	0.001	24 (48%)	233 (41.8%)	0.39
Disease behavior			0.068			0.007			0.049
B1: Inflammatory	69 (46.3%)	172 (37.5%)		39 (36.8%)	202 (40.2%)		29 (58%)	212 (38%)	
B2: Stricturing	51 (34.2%)	214 (46.6%)		38 (35.8%)	227 (45.2%)		14 (28%)	251 (45%)	
B3: Penetrating	14 (9.4%)	33 (7.2%)		11 (10.4%)	36 (7.2%)		3 (6%)	44 (7.9%)	
B2 B3: Stricturing and penetrating	15 (10.1%)	40 (8.7%)		18 (17%)	37 (7.4%)		4 (8%)	51 (9.1%)	
Complicated disease	80 (53.7%)	287 (62.5%)	0.055	67 (63.2%)	300 (59.8%)	0.51	21 (42%)	346 (62%)	0.006
Perianal disease	89 (59.7%)	239 (52.1%)	0.103	64 (60.4%)	264 (52.6%)	0.14	29 (58%)	299 (53.6%)	0.55
Disease activity[a]			0.004			<0.001			0.99

[a]*Comparison of disease activity was performed using Rank-sum test.*

TABLE 3 | Association of serum IgG4 levels with complicated disease.

	Crude	Model 1[a]	Model 2[b]
Elevated serum IgG4	0.44 (0.25–0.8)	0.49 (0.27–0.89)	0.49 (0.26–0.92)
Quartile 1 and quartile 2 of serum IgG4	1.92 (1.29–2.85)	1.82 (1.21–2.74)	1.86 (1.2–2.88)
Quartile 3 of serum IgG4	1.66 (1.05–2.63)	1.59 (1.002–2.52)	1.82 (1.11–2.98)
Quartile 4 of serum IgG4	Reference	Reference	Reference
P for trend	0.001	0.004	0.005
Per-SD increase	0.72 (0.61–0.86)	0.74 (0.62–0.88)	0.74 (0.62–0.89)

Data are odds ratio (95% confidence interval).
[a]*Adjusted for gender and age.*
[b]*Adjusted for gender, age, disease duration, history of appendectomy and intestinal surgery, disease location, and disease activity.*

was no difference in serum IgG4 levels in CD patients with and without isolated ileal disease (**Figure 1H**). Furthermore, the crude and adjusted associations of serum IgG and IgM with isolated ileal disease were analyzed by multivariate logistic regression and are presented in **Tables 4**, **5**, respectively. Elevated serum IgG was negatively related to isolated ileal disease (OR: 0.37, 95% CI: 0.23–0.61). The OR of isolated ileal disease was 1 (95% CI: 0.61–1.63) for quartile 2, 0.55 (95% CI: 0.33–0.92) for quartile 3 and 0.3 (0.165–0.53) for quartile 4 compared with quartile 1 in the final multivariate model (P for trend < 0.001). An SD increase in serum IgG levels was associated with a 45% reduction in the adjusted probability of isolated ileal disease. Besides, the adjusted ORs between decreasing quartiles of serum IgM levels and the presence of isolated ileal disease were as follows: 1, 1.17 (0.69–1.98), 1.92 (1.14–3.24), and 1.82 (1.07–3.1) (P for trend $= 0.008$). An SD increase in serum IgM levels was related to isolated ileal disease with an adjusted OR of 0.74 (95% CI: 0.6–0.91). In all, serum IgG and IgM levels had a negative correlation with isolated ileal disease.

Correlation of Serum IgA and IgG Levels With Disease Activity

Further, the serum immunoglobulins levels in CD patients with different disease activities were compared. The serum IgG levels were significantly lower in CD patients in remission and patients with mild disease compared with patients with severe disease ($P < 0.05$) (**Figure 2A**). The serum IgA levels were significantly decreased in CD patients in remission and patients with mild-to-moderate disease compared with patients with severe disease ($P < 0.05$) (**Figure 2B**). The correlation analysis revealed that serum IgG and IgA levels positively correlated with CRP and ESR levels, and PLT count, but negatively correlated with prealbumin levels ($P < 0.001$) (**Figure 2C**).

DISCUSSION

This study evaluated the associations between serum immunoglobulins levels and complicated disease phenotype,

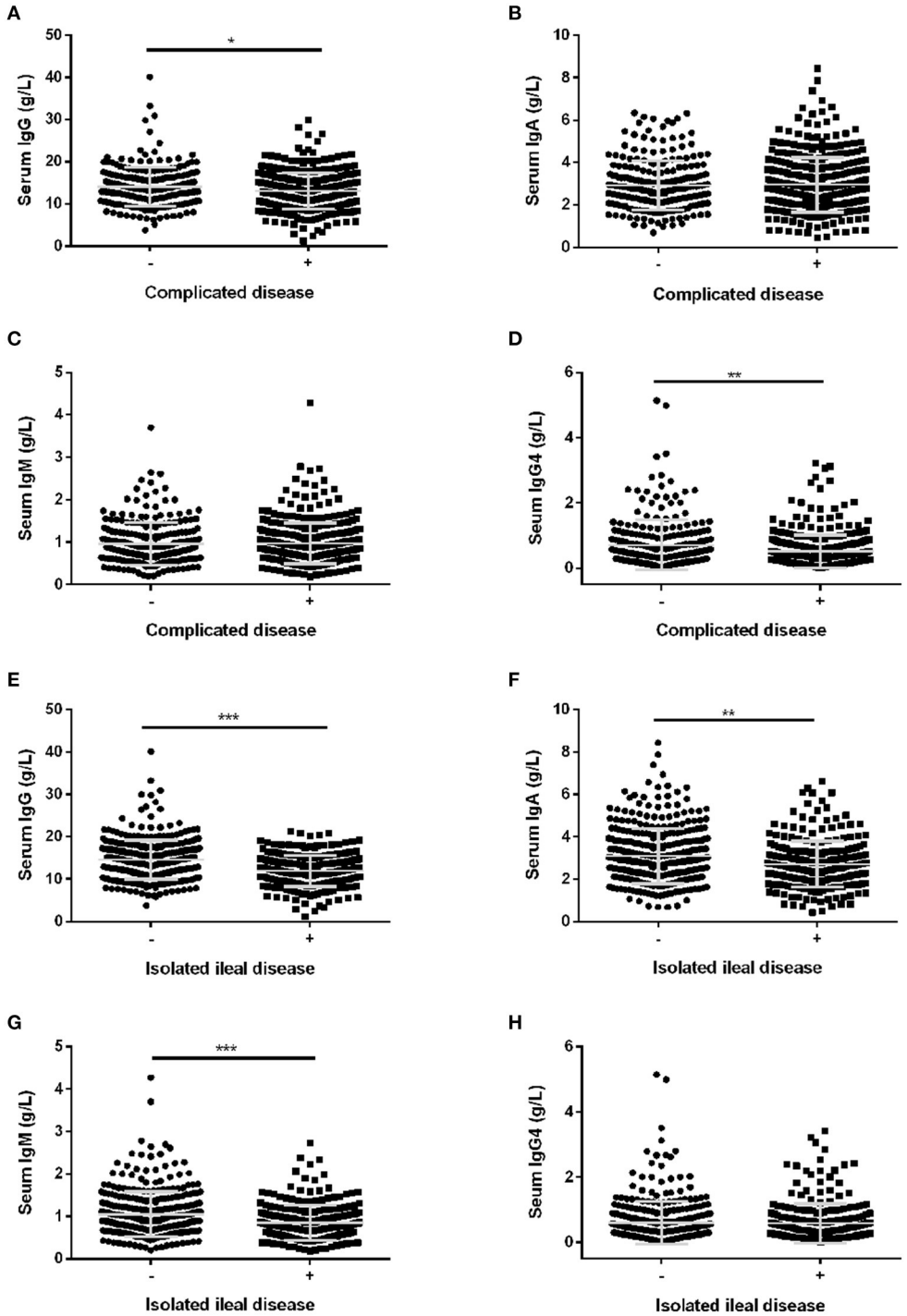


FIGURE 1 | The levels of serum immunoglobulins in CD patients with different disease phenotypes. The levels of serum IgG **(A)**, serum IgA **(B)**, serum IgM **(C)**, and serum IgG4 **(D)** in CD patients with complicated disease. The levels of serum IgG **(E)**, IgA **(F)**, IgM **(G)** and IgG4 **(H)** in CD patients with isolated ileal disease. Bar graphs are presented as mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

isolated ileal disease, and disease activity of CD. The results indicated that serum IgG4 levels inversely correlated with complicated disease behavior. Serum IgG and IgM levels were found to be negatively associated with isolated ileal disease. In addition, serum IgA and IgG levels correlated with the disease activity of patients with CD.

TABLE 4 | Association of serum IgG levels with isolated ileal disease.

Serum IgG	Crude	Model 1[a]	Model 2[b]
Elevated serum IgG	0.27 (0.175–0.42)	0.29 (0.185–0.45)	0.37 (0.23–0.61)
Quartile 1 of serum IgG	Reference	Reference	Reference
Quartile 2 of serum IgG	0.83 (0.53–1.31)	0.835 (0.53–1.32)	1 (0.61–1.63)
Quartile 3 of serum IgG	0.44 (0.28–0.7)	0.46 (0.29–0.74)	0.55 (0.33–0.92)
Quartile 4 of serum IgG	0.2 (0.12–0.33)	0.21 (0.13–0.355)	0.3 (0.165–0.53)
P for trend	<0.001	<0.001	<0.001
Per-SD increase	0.485 (0.4–0.59)	0.5 (0.41–0.61)	0.55 (0.43–0.7)

Data are odds ratio (95% confidence interval).
[a]*Adjusted for gender, age at diagnosis.*
[b]*Adjusted for gender, age at diagnosis, history of appendectomy and intestinal surgery, serum IgA, and IgM, perianal disease, complicated disease, and disease activity.*

TABLE 5 | Association of serum IgM levels with isolated ileal disease.

Serum IgM	Crude	Model 1[a]	Model 2[b]
Quartile 1 of serum IgM	2.97 (1.845–4.78)	2.5 (1.525–4.1)	1.82 (1.07–3.1)
Quartile 2 of serum IgM	2.47 (1.535–3.97)	2.145 (1.32–3.49)	1.92 (1.14–3.24)
Quartile 3 of serum IgM	1.52 (0.94–2.46)	1.4 (0.85–2.28)	1.17 (0.69–1.98)
Quartile 4 of serum IgM	Reference	Reference	Reference
P for trend	<0.001	<0.001	0.008
Per-SD increase	0.62 (0.51–0.75)	0.67 (0.55–0.815)	0.74 (0.6–0.91)

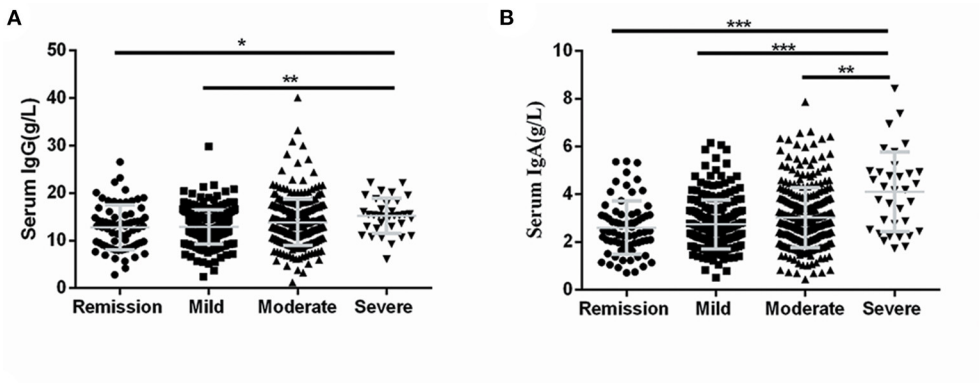
Data are odds ratio (95% confidence interval).
[a]*Adjusted for gender, age at diagnosis.*
[b]*Adjusted for gender, age at diagnosis, history of appendectomy and intestinal surgery, serum IgA and IgG, perianal disease, complicated disease, and disease activity.*

The clinical implications of abnormal serum IgG4 levels in IBD patients has been explored with inconsistent results. A recent study conducted in United States has revealed that IBD patients with low serum IgG4 levels were more likely to experience complicated disease progress such as increased rates of CD-related surgeries and IBD-related hospitalizations as well as increased requirement for biologics, systemic steroids, and antibiotics (27). However, another study conducted in China revealed that elevated serum or mucosal IgG4 levels were associated with more extensive disease in patients with IBD (17). In our study, a negative association between elevated serum IgG4 levels and the presence of complicated disease was observed. Reduced interleukin (IL)-10 production in complicated disease may be responsible for low serum IgG4 levels in complicated disease. The IL-10 production of whole blood cell cultures was significantly reduced in CD patients with stricturing and penetrating phenotype compared with CD patients with inflammatory phenotype (28). Furthermore, IL-10 has been found to enhance IgG4 production by IL-4-stimulated peripheral blood mononuclear cells and was suggested to potentiate both IgG4 switching and growth of IgG4-secreting B cells (29, 30). However, further mechanistic studies are needed to account for this phenomenon.

The present study also found that serum IgG levels negatively correlated with isolated ileal involvement. Signatures of gut microbiota in CD patients with ileal disease were distinct from those in CD patients with colonic and ileocolonic disease. CD patients with isolated ileal disease had stronger microbial dysbiosis (31), which may be an important mechanism to induce the reduced serum IgG levels. Substantial studies have revealed that microbiota and microbial products indeed regulate serum IgG levels, although the precise mechanism is unclear yet. Serum IgG levels were significantly reduced in germ-free mice compared with conventional mice (32). Under homeostatic conditions, a selective subset of gram-negative symbiotic bacteria possesses the ability to disseminate systemically to induce systemic IgG responses, which is dependent on T cells and Toll-like receptor 4 on B cells. And one possible mechanism is that symbiotic bacteria possess additional virulent factors that help them to enter the bloodstream and thereby activate systemic immune response (33). In addition, probiotics supplementation including *Bifidobacterium bifidum*, *Lactobacillus frumenti*, and *Bacillus subtilis* can increase serum IgG levels in hosts such as infants, piglets and rabbits, respectively (34–36). Furthermore, short-chain fatty acids, products of the fermentation of dietary fibers by intestinal microbiota, can augment systemic IgG responses through regulating gene expression for plasma B cell differentiation and increasing cellular metabolism as well as boosting glycolytic activity in B cells (37). However, further investigations are required to explain the exact mechanism underlying the association between serum IgG levels and isolated ileal involvement.

The clinical implications of abnormal serum IgG in different clinical settings are inconsistent. Low serum IgG has been reported to be associated with poor outcomes in patients with sepsis and septic shock (38) as well as patients with immunoglobulin A nephropathy (10). However, elevated serum IgG may predict poor prognosis in patients with SLE-AIH (11) and in patients with HBV-ACLF (12). Our cross-sectional study suggested CD patients with isolated ileal disease had lower serum IgG levels. Whether there is a potential link to the study conducted in United States that showing IBD patients with low serum IgG/G1 levels required more small bowel resections (15) remains to be further explored. In addition, more longitudinal studies exploring the clinical significance of abnormal serum IgG levels in patients with IBD are warranted.

IgM has been found to be an anti-inflammatory factor and elevated IgM levels indicated a better immune status (39). It has been reported that reduced plasma IgM levels at the onset of sepsis predicted decreased sepsis survival (40). In our cohort, CD patients with isolated ileal involvement were more likely to have low serum IgM levels. Similar to serum IgG, increasing evidence suggests that serum IgM levels can also be influenced by microbiota. Lysozyme supplementation could alter the composition of gut microbiota and increase serum IgM levels in sows, and correlation coefficients revealed that *Ruminiclostridium 9* significantly positively correlated with serum IgM levels (39). Additionally, a positive relationship between serum IgM levels and *Bacteroidetes/Firmicutes* ratio has been reported in healthy middle-aged people (41). Besides, a positive correlation was also found between total IgM level and the abundance of *Synergistetes* in patients with SLE (42). Furthermore, reduced serum IgM level correlated with the HLA-DRB1*03 allelic variant among patients with SLE and controls



C

Variable	IgG		IgA	
	Spearman's rho	p value	Spearman's rho	p value
CRP	0.226	< 0.001	0.244	< 0.001
ESR	0.39	< 0.001	0.37	< 0.001
PLT	0.23	< 0.001	0.24	< 0.001
Prealbumin	-0.27	< 0.001	-0.22	< 0.001

FIGURE 2 | Serum IgG and IgA levels correlated with disease activity. Serum IgG **(A)** and IgA **(B)** values were significantly increased in severely active Crohn's disease, The spearman coefficient of correlation between serum IgG and IgA and several markers of disease activity **(C)**. The symbols represent values in individual patients. Values are shown as mean ± SD. *P < 0.05, **P <0.01, ***P < 0.001. CRP, C-reactive protein; ESR, erythrocyte sedimentation rate; PLT, platelet count.

(43). These findings suggested that IgM levels might be partially influenced by gut microbiota and genetic factors. As to IBD, HLA*DRB1*04 and DQB1*04 were linked to only small intestine involvement in patients with CD (44). HLA DRB1*0103 was suggested to be associated with colonic disease in IBD (45). Thus, in our study, we speculate that the variation in serum IgM levels in different location phenotypes might be partially due to the alteration of gut microbiota or heredity.

The correlation analysis in our study revealed that serum IgA and IgG levels significantly correlated with CRP, ESR, PLT count and prealbumin level, consistent with a study reporting that serum IgA levels had a positive correlation with several markers of disease activity in CD patients (13). However, another study reported that serum IgG and IgA levels were not associated with disease activity of CD and UC, but serum IgG and IgA levels were significantly increased in patients with pouchitis and cuffitis (46). Previous studies have demonstrated that IgA and IgG coating could identify pathogenic bacteria in IBD (47, 48). Furthermore, the levels of fecal soluble IgA and IgG were positively associated with disease activity in patients with IBD, and the correlation between soluble IgA or IgG in feces and CRP or ESR was stronger than our results (49), probably due to the difference in the type of sample analyzed. In clinical practice, IgG and IgA levels in feces are probably superior to those in serum for identifying the degree of disease activity in CD patients due to its non-invasive nature and stronger correlation.

This study has several limitations. It was cross-sectional in design. Repeated measurements and longitudinal observation of serum immunoglobulins are required to evaluate alteration of serum immunoglobulins levels along with change in disease phenotypes of CD. On the other hand, despite adjustments for some potential confounding factors, the possibility that other factors not included in the study might influence the results could not be ruled out. Smoking, alcohol consumption, and common metabolic abnormalities have been found to be related to serum levels of immunoglobulins in an adult population (50). However, these factors were not included in the present study. In addition, healthy controls were not recruited in this study. Although serum immunoglobulins levels in the background population could not be provided, the results of this study were compared with those of other studies and few differences were observed. Elevated serum IgG was identified in 24.5% of participants in the present cohort, which was comparable with a study that found 23% of pediatric CD patients had elevated serum IgG levels (16). Furthermore, 8.2% of patients in the present study had increased serum IgG4 levels, which was higher than the 6.34% reported in four kinds of autoimmune diseases, including primary Sjogren syndrome, systemic sclerosis, SLE, and primary biliary cirrhosis (9) and lower than the 9.9% reported in IBD patients in Sichuan, China (17). Finally, the normal reference ranges of serum immunoglobulins, as well as other laboratory tests such as CRP, ESR, PLT, and prealbumin from different centers were not consistent. Although these data were normalized before quantitative and correlation analyses, the application of a central laboratory was more perfect.

More prospective longitudinal studies are required to reveal whether the abnormal serum immunoglobulins participate in the disease progress of different phenotypes of CD or just are the consequence of disease development. It has been reported that CD patients with different location phenotypes have different natural history and should be regarded as separate groups. It would be worth exploring whether CD patients with abnormal serum immunoglobulins levels showing unique clinical trajectory. Furthermore, the studies involving the clinical implications of the abnormal serum IgG4 levels in patients with IBD from different races showed different results (17, 27). Thus, whether the results from a region can be transferred to another region is worthy of future research. Collectively, identifying the causality between serum immunoglobulins levels and specific disease phenotypes of CD and the underlying mechanisms may contribute to risk stratification and personalized prevention for patients with CD.

CONCLUSION

In summary, the present study provided a novel insight into the associations of serum immunoglobulins with different phenotypes of CD. Our findings suggested that CD patients with complicated disease phenotype are more likely to have reduced serum IgG4 levels and CD patients with isolated ileal disease are more likely to have low serum IgG and IgM levels. In addition, serum IgA and IgG levels positively correlated with disease activity in patients with CD. Further studies are required to elucidate the pathogenic mechanism linking serum immunoglobulins levels with different phenotypes of CD.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Medical Ethics Committee of Renji Hospital, School of Medicine, Shanghai Jiao Tong University. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

DS: data collection, statistical analysis, and manuscript drafting. JS: study design and critical revision of the manuscript. MC, ZL, QC, PH, XG, JQ, and KW: data collection and review of the article. LL: data collection and statistical analysis. ZR: study design, data interpretation and critical revision of the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by the National Natural Science Foundation of China (Grant No. 81670497, 81770545) and MDT Project of Clinical Research Innovation Foundation, Renji Hospital, School of Medicine, Shanghai Jiao Tong University (PYI-17-003).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2021.621337/full#supplementary-material

Supplementary Figure 1 | Principal components analysis (PCA) plot of clinical data based on centers.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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REVIEW
published: 01 July 2021
doi: 10.3389/fmed.2021.686778

Iron Deficiency Anemia in Inflammatory Bowel Disease: What Do We Know?

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One of the most common extraintestinal manifestations of inflammatory bowel disease is iron deficiency anemia. It is often an untreated condition that significantly impairs patients' quality of life and elevates mortality and morbidity. Although it is often accompanied by mild symptoms (e.g., fatigue, lethargy), it can provoke severe health conditions, such as dyspnea, palpitation, angina, and mental disorders, and increases hospitalization and mortality rate as well. As anemia develops through several pathomechanisms, such as occult bleeding, chronic inflammation, and medicines (e.g., methotrexate), treating anemia effectively requires to manage the underlying pathological changes as well. Based on international publications and data, it is a frequent condition and more frequent in pediatrics. According to Goodhand et al., iron deficiency is present in more than 60% of children, whereas only 14% of them received oral iron therapy. Compared to adult patients, 22% have iron deficiency, and 48% of them received oral and 41% intravenous iron therapy. Miller et al. also highlighted that among young patients iron deficiency anemia is a frequent condition, as almost 50% of the patients were anemic in their cohort. European Crohn's and Colitis Organisation's statements are clear regarding the diagnosis of iron deficiency anemia, and the iron supplementation as well. Third-generation parenteral iron supplementations seem to be safer and more effective than oral iron pills. Oral iron in many cases cannot replace the iron homeostasis as well; furthermore, it can provoke dysbiosis, which can potentially lead to relapse. As a result, we claim that both oral and parenteral should be used more frequently; furthermore, intravenous iron could replace oral medicines as well in certain cases. Despite the fact that iron deficiency anemia is examined by many aspects, further questions can be raised. Can it imply underlying pathological lesions? Are both oral and intravenous iron therapy safe and effective? When and how are they used? We demand that more studies should be conducted regarding these issues.

Keywords: inflammatory bowel disease, iron deficiency anemia, iron supplementation, anemia, parenteral iron supplementation, oral iron supplementation

OPEN ACCESS

Edited by:
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Marmara University, Turkey
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Specialty section:
This article was submitted to Gastroenterology, a section of the journal Frontiers in Medicine

Received: *27 March 2021*
Accepted: *17 May 2021*
Published: *01 July 2021*

Citation:
Resál T, Farkas K and Molnár T (2021) Iron Deficiency Anemia in Inflammatory Bowel Disease: What Do We Know? Front. Med. 8:686778. doi: 10.3389/fmed.2021.686778

INTRODUCTION

Inflammatory bowel disease [IBD: Crohn disease (CD), ulcerative colitis (UC)] is a chronic, immune-mediated disease that impairs patients' quality of life (QoL), and it is associated with many comorbidities. One of the most common concomitant diseases is iron deficiency anemia (IDA), which also worsens the condition of patients and mostly remains untreated (1). It can occur at any

stage of IBD and can be the first symptom of the disease as well. It is often associated with frequent chronic activity, but can also be encountered without clinical signs of activity. In such cases, the IDA diagnosis raises the possibility of asymptomatic, subclinically occurring inflammation and mucosal damage in the presence of long-term chronic activity that damages the condition and function of the intestine (1).

Frequency of IDA

At the time of the diagnosis, the prevalence of IDA in patients younger than 18 years is approximately 41–75% (2), whereas in adult patients it is also high; however, it varies with wide ranges, from 6 to 74% (3). According to a Swedish study, conducted by Sjöberg et al., the prevalence of IDA is almost twice as high in children (55%) as in adult patients (27%) at the time of the diagnosis. Furthermore, they found significant difference as well in the prevalence of IDA among patients with CD, compared to UC, following the first year after the diagnosis. They also found that anemia in CD was more common in colonic engagement, and in UC, extensive inflammation increased the prevalence (4). Eriksson et al. conducted a study to assess the incidence, prevalence, and clinical outcome of anemia in IBD, comparing CD and UC, and they found as well that CD is associated with higher prevalence and a worse outcome regarding the resolution of anemia (5).

Symptoms and Clinical Role

Generally speaking, it should be highlighted that patients with IDA claim to have decreased QoL. In a Spanish study, conducted by García-López et al., it was found that treating IDA improves the QoL, regardless of the symptoms of IBD (6).

As iron plays key role in the function of many cells (e.g., erythrocyte, macrophage), cellular proteins, and enzymes (e.g., cytochromes, myoglobin), the symptoms of IDA vary over a wide range (7, 8). Key symptoms, such as shortness of breath, palpitation, tachycardia, and even angina, occur because of the hypoxemia. As a result of the decreased blood oxygen level, there is a compensatory decrease in intestinal blood flow, which may cause motility disorder, malabsorption, nausea, weight loss, and abdominal pain. Central hypoxia may lead to headache, vertigo, and lethargy, as well as cognitive impairment, and several studies proved that normalizing anemia improves cognitive functions (9–12) (**Table 1**).

Michailidou et al. compared the risk of postoperative complications between anemic and nonanemic patients. In their study consisting of more than 15,000 people, it was found that patients with anemia were more likely to have postoperative complications (e.g., morbidity and mortality rate; undesirable cardiovascular, renal, pulmonary, and wound healing complications; postoperative sepsis and shock) (13).

TABLE 1 | Symptoms of iron deficiency anemia.

Nervous system	Headache, lethargy, vertigo, syncope, cognitive impairment, depression
Cardiovascular system	Palpitation, tachycardia, hypotension, angina, ischemic electrocardiographic signs, cardiac failure
Respiratory system	Shortness of breath
Skin	Paleness, alopecia, cold intolerance
Gastrointestinal symptoms	Anorexia, nausea, motility disturbances, angular stomatitis, glossitis (Plummer–Vinson syndrome)
Immune system	Disorder of the innate and adaptive immune system
Urogenital symptoms	Decreased libido, menstrual disorders
General symptoms	Decreased quality of life, lower physical activity

TABLE 2 | Ethiology of anemia in IBD.

Most common causea of anemia in IBD	- Iron deficiency anemia - Anemia of chronic inflammation - Anemia of mixed origins
Less common causes	- Folic acid/B_{12} deficiency
Rare causes	- Hemolysis - Myelodysplastic syndrome - Aplasia - Protein starvation - Liver disease

Etiology of Anemia in IBD

The most common causes of anemia in IBD are IDA, chronic inflammation, and anemia of mixed origins, whereas B_{12} deficiency and folic acid deficiency (mostly due to medications) belong to the less common causes. In addition, it may also occur because of hemolysis, myelodysplastic syndrome/medication-induced aplasia, protein starvation, and liver disease (e.g., primary sclerosing cholangitis) (14, 15) (**Table 2**).

Pathophysiology of IDA

In IBD, the IDA can develop through several pathomechanisms (16):

I. Intestinal mucosal damage resulting in occult, chronic blood loss

II. Chronic inflammation

a) Reduced iron-absorbing capacity of enterocytes
b) Iron is trapped in macrophages
c) Inhibition of the erythropoietin and the differentiation/proliferation of the erythroid progenitor cells

Cytokines and acute-phase proteins cause changes in iron homeostasis during inflammation. Hepcidin plays the central role in the regulatory process. It is an antimicrobial protein, produced by the liver in case of iron surplus and in inflammation, triggered by interleukin 6 and lipopolysaccharides. Hepcidin binds to the iron-transporting ferroportin receptor and degrades it, which results in decreased iron transport from the enterocytes to the circulation, and causes retention of the iron in the monocytes/macrophages; these processes are enhanced by anti–tumor necrosis factor α. In addition, hepcidin reduces the absorption of the Fe^{2+} from the duodenum, through the inhibition of the DMT1 (divalent metal transporter 1) (17).

Transferrin is the main iron carrier protein, and during inflammation, acute-phase proteins (e.g., α-1 antitrypsin) bind

TABLE 3 | Pathomechanisms of different type of anemias in IBD.

Iron deficiency anemia	Chronic blood loss Reduced absorption of Fe^{2+} (bowel resection, inflammation) Anorexia
Anemia of chronic disease	Iron retention in monocytes/macrophages Reduced absorption of Fe^{2+} (inflammation) Reduced biological half-life of erythrocytes (e.g., erythrophagocytosis) Inhibition of erythropoiesis
Other origin	Vitamin deficiency (B12, folic acid) Drug-induced bone marrow suppression (methotrexate, azathioprine)

to transferrin receptors and inhibit the iron uptake in the erythroid progenitors cells, resulting in reduced differentiation and proliferation (18).

Diagnosis/Differential Diagnosis in Anemia

Anemia and iron homeostasis should be monitored regularly in IBD (depending on activity and the type of the treatment):

- At the time of diagnosis
- During activity—every 3 months
- In remission—every 6 to 12 months

Vitamin B_{12} and folic acid should be monitored every year, in case of presence of risk factors (e.g., resection, pouch, extensive ileal disease) every 3–6 months.

The diagnosis of anemia is assessed by the World Health Organization (WHO) diagnostic criteria, depending on the gender and age of the patients (**Table 3**).

Differential Diagnosis in Anemia

According to the European Crohn's and Colitis Organization (ECCO) recommendations, the following parameters should be monitored if hemoglobin is below normal (**Table 4**): erythrocyte count, serum ferritin, C-reactive protein (CRP) concentration, transferrin saturation, reticulocyte count, erythrocyte width distribution, and mean corpuscular volume. Ferritin is an acute-phase protein produced by the liver, and it is responsible for binding and storing iron in the liver, spleen, and reticuloendothelial system. It is reduced in case of iron deficiency and elevated in inflammation. Hence, in determining the cause of anemia in IBD, it is important to assess disease activity based on disease scoring systems (Crohn's Disease Activity Index and Mayo score) and serum CRP and fecal calprotectin levels. In case of inflammation, transferrin saturation helps in differential diagnosis. Transferrin saturation (accepted normal range = 20–45%) is lower in inflammation, liver disease, malignancy, nephrotic syndrome, and anorexia, whereas it is elevated in iron deficiency and pregnancy (19).

IDA (19):

a) Anemia based on WHO criteria (low hemoglobin and hematocrit). Clinically and endoscopically, no inflammation can be found, CRP level is normal, and serum ferritin is <30 μg/L.
b) Anemia based on WHO criteria. Clinically and/or endoscopically, inflammation can be found, CRP is elevated, and ferritin is <100 μg/L.

TABLE 4 | World Health Organization's anemia criteria.

	Hemoglobin (g/dL)	Hematocrit(%)
Children between 6 months and 5 years	11	33
Children between 5 and 11 years	11.5	34
Children between 12 and 13 years	12	36
Pregnant women	11	36
Women	12	33
Men	13	39

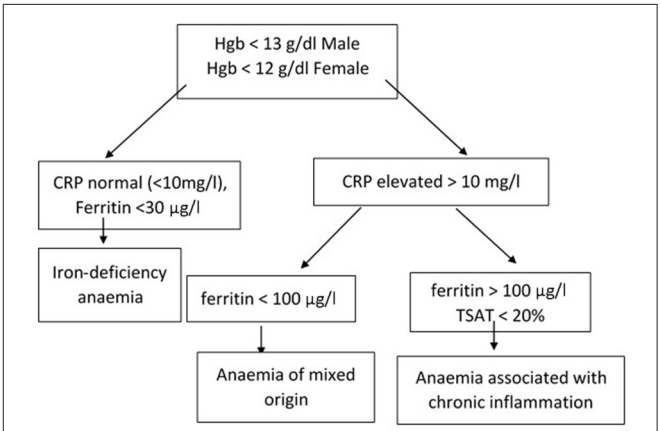

FIGURE 1 | Differential diagnosis of IDA and chronic inflammation–associated iron deficiency.

Anemia associated with chronic inflammation:

- Anemia based on WHO criteria. Clinically and/or endoscopically, inflammation can be found, CRP is elevated, ferritin is >100 μg/L, and transferrin saturation is $<20\%$.

Anemia of mixed origin:

- Anemia based on WHO criteria. Clinically and/or endoscopically, inflammation can be found, CRP is elevated, and ferritin is between 30 and 100 μg/L (**Figure 1**).

Iron Supplementation

Goodhand et al. pointed out how undertreated the IDA is in IBD. In children (88%) and adolescents (83%), the incidence of IDA is much higher compared to that in adults (55%), and only a small proportion of patients received oral (children 13%, adolescents 30%, adults 48%) or parenteral iron supplementation (children 0%, adolescents 30%, adults 41%) (20).

In addition to improving the QoL, the goal of iron supplementation is to normalize hemoglobin, serum ferritin, and transferrin saturation and to refill iron stores (ferritin >100 g/L).

TABLE 5 | Administration of parenteral iron replacement.

Hemoglobin (g/dL)	Body weight <70 kg	Body weight ≥70 kg
10–12 (Female)	1,000 mg	1,500 mg
10–13 (Male)		
7–10	1,500 mg	2,000 mg

Based on the recommendation of the ECCO (19):

Iron supplementation is recommended to all patients with anemia associated with iron disorder. If iron deficiency exists without anemia, iron supplementation requires consideration of the patient's individual clinical status, as there is no evidence in IBD regarding the efficacy of the treatment (19).

Oral iron supplementation is recommended to every patient with IDA with hemoglobin >10 g/dL in case of remission (no clinical/endoscopic activity, normal CRP level). The recommended oral iron intake is 100 mg/day for adults (higher doses are not recommended), and 2–3 mg/kg body weight per day in children. An acceptable therapeutic response is an increase of 2 g/dL in hemoglobin over 4 weeks. If there is intolerance, adverse effects or unsatisfactory therapeutic response is present, intravenous iron therapy is recommended (19).

Parenteral iron supplementation is recommended as a first choice in IDA in case of active IBD (elevated CRP levels and/or clinically active IBD), or hemoglobin level <10 g/dL, or previous intolerance to iron supplementation is present. If the elevation is <2 g/dL in hemoglobin level after 4 weeks' therapy, it is recommended to complete the treatment with Erythropoietin (EPO) stimulant. The required iron intake is estimated based on the body weight and the hemoglobin, as it is more effective in patients with IBD suffering from iron deficiency than the traditional Ganzoni formula (19) (**Table 5**).

Following the resolution of IDA with parenteral iron supplementation, ferritin level is recommended to be maintained above 400 μg/L to prevent short-term recurrence. After successful iron supplementation, patients should be monitored every 3 months in the first year following the correction and every 6–12 months thereafter (including hemoglobin, ferritin, transferrin saturation, CRP). Recurrent anemia may indicate underlying inflammation despite clinical and biochemical remission. The goal of preventive treatment is to keep ferritin and hemoglobin at normal levels. Reinitiation of intravenous iron supplementation is recommended in cases where the ferritin level falls below 100 μg/L or the hemoglobin level is <12–13 g/dL (female/male) (19).

When considering between oral and intravenous iron therapy, the advantages and disadvantages of the therapeutic approaches should be considered as well (21–23).

Oral Iron Supplementation

a. Advantages
   - Low cost
   - Easier to implement in daily practice
   - More accessible
   - Effective in good intestinal absorption

b. Disadvantages

   Compliance issues

   Certain foods reduce iron absorption (e.g., tea, coffee, dairy products, fiber)

   Certain medications reduce iron absorption

   i. Multivitamin/dietary supplements (Ca^{2+}, Zn^{2+}, Cu^{2+})
   ii. Antacids, H_2 blockers, PPI
   iii. Quinolones, tetracyclines

   Dysbiosis

   Dysbiosis induced relapse

   Side effects are more common compared to parenteral iron suppl.

   i. Nausea
   ii. Abdominal pain
   iii. Diarrhea
   iv. Constipation

Parenteral Iron Supplementation

a. Advantages
   - More effective
   - Fast correction of iron homeostasis
   - Safe and well tolerated
   - Fewer side effects
   - Effective in inflammation
   - The condition of the mucosa does not influence the efficacy

b. Disadvantages
   - Higher cost
   - Harder to implement in daily practice
   - Potential risk of iron overload
   - Potential risk of anaphylaxis
   - Possibility of hypophosphatemia

Parenteral Iron Supplementation

Intravenous iron supplementations consist of an Fe^{3+} core and a carbohydrate layer. The side effect profile, clearance, tolerable dose, and duration of the infusion are dependent on the magnitude of the core and quality of the carbohydrate layer. The different generations of intravenous iron supplementation comprised different carbohydrate layers (24).

- First generation—high-molecular-weight iron dextran
- Second generation—low-molecular-weight iron dextran
  a. Ferrous gluconate
  b. Iron sucrose
- Third generation
  a. Ferumoxytol
  b. Iron carboxymaltose
  c. Iron isomaltoside

The disadvantage of the HMWID is the higher probability of anaphylactic reaction/side effects; because of that, it is advised to use higher-generation products. The representatives of the second generation are more efficient with fewer side effects; however, they are not as stable complexes as the representatives of the third-generation preparations; consequently, they can only

be administered in low doses, and so they require frequents visits. Third-generation preparations are much more efficient, with minimal side effect profile; furthermore, they can be implemented easier in the daily practice. These formulations are more stable, so they can be administered in higher doses, resulting in faster correction of the iron homeostasis, and the duration of the infusion is lesser (23, 25).

DISCUSSION

Anemia and IDA are common consequences of IBD in the developed world. Despite that we know how frequent it is, physicians tend to pay less attention to treat it, even though it affects the course of the disease and heavily reduces the patients' QoL. Although modern medicine knows many facts about the pathophysiology of anemia and IDA, and there are many efficient agents in the therapeutic arsenal, it still raises relevant questions. However, the ECCO's recommendations are clear; we would like to highlight that it should be still a matter of individual judgment, and in certain cases, parenteral iron supplementation should be the choice, instead of oral, because of the side effects. Based on international publications and data, as intravenous iron supplementation tends to be more efficient and safe in IBD, we claim that more studies should be conducted regarding third-generation agents and clarify the boundary line in the recommendations. However, to sum up, we demand that both oral and intravenous iron treatments should be more widespread.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

FUNDING

This work was supported by the research grants of the National Research, Development and Innovation Office (Grant ID: 125377, 129266, and 134863), by the National Excellence Programme (20391-3/2018/FEKUSTRAT to KF), by the New National Excellence Program of the Ministry of Human Capacities (UNKP-19-4-SZTE-44, UNKP-20-5-SZTE-161 to KF), Janos Bolyai Research Grant (BO/00598/19/5) and the Géza Hetényi Research Grant (to KF) by the Faculty of Medicine, University of Szeged.

ACKNOWLEDGMENTS

The authors would like to take this opportunity to thank Mariann Rutka MD. Ph.D. and Anita Bálint MD. Ph.D., for suggesting we publish this article in Frontiers in Medicine.

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Conflict of Interest: KF has received speaker's honoraria from AbbVie, Janssen, Ferring, Takeda, and Goodwill Pharma. TM has received speaker's honoraria from MSD, AbbVie, Egis, Goodwill Pharma, Takeda, Pfizer, and Teva.

The remaining author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Medicine

ORIGINAL RESEARCH
published: 09 July 2021
doi: 10.3389/fmed.2021.662488

Body Mass Index and Disease Activity Are Associated With Moderate to Severe Disability in Crohn's Disease: A Cross-Sectional Study in Shanghai

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Specialty section:
This article was submitted to Gastroenterology, a section of the journal Frontiers in Medicine

Received: 01 February 2021
Accepted: 14 June 2021
Published: 09 July 2021

Citation:
Bian D, Jiang Y, Gu Y, He Z, Chen Q, Tang Y, Zhong J and Shi Y (2021) Body Mass Index and Disease Activity Are Associated With Moderate to Severe Disability in Crohn's Disease: A Cross-Sectional Study in Shanghai. Front. Med. 8:662488. doi: 10.3389/fmed.2021.662488

Background: The inflammatory bowel disease disability index (IBD-DI) was used to access body functional consequences and disease burden. However, Chinese population data are considerably limited.

Objective: We aimed to screen for disability in patients with Crohn's disease (CD) and to assess potential associations with clinical parameters as well as indices related to sarcopenia.

Methods: This cross-sectional study includes 146 CD patients from Ruijin Hospital in Shanghai, China. All patients were screened for disability and sarcopenia on the basis of the IBD-DI scale, and the criteria for Asian Working Group for Sarcopenia, respectively. Clinical and demographic variables were collected.

Results: Approximately 52.05% of the subjects suffered from moderate or severe disabilities. The prevalence of sarcopenia (48.68 vs. 31.43%, $P = 0.043$), Patient-Generated Subjective Global Assessment score or PG-SGA≥4 (39.47 vs. 17.14%, $P = 0.003$), and high-level C- reactive protein (27.63 vs. 11.43%, $P = 0.021$) were higher in patients with moderate to severe disability than in those without to minimal disability. By multivariate regression modeling, the following were identified as independent factors related to moderate to severe disability: disease activity (OR:10.47, 95% CI: 2.09–52.42) and body mass index (BMI) (OR:4.11, 95% CI: 1.80–9.38).

Conclusions: Disability is common in CD patients. Our study showed that moderate to severe disability is not directly associated with muscle mass or muscle quantity but is mostly correlated with disease activity as well as BMI. Thus, close monitoring and follow-up should be conducted on patients who are at high risk of disability, and effective measures should be taken, which may be the best way to prevent disability.

Keywords: disability, Crohn's disease, risk factors, disease activity, sarcopenia, body mass index

INTRODUCTION

The prevalence of inflammatory bowel disease (IBD) has markedly increased in mainland China (1). Crohn's disease (CD) is a chronic inflammatory bowel disorder that may affect any part of the digestive tract, causing abdominal pain and diarrhea, subsequently leading to functional disability and life-threatening complications (2). CD patients are characterized by their lifelong disease, marked by episodes of remission and relapse (3). Thus, living with CD may negatively affect the physical, psychological, social, and familial quality of life (4–6). Numerous disease-related questionnaires have been developed to evaluate patient-reported outcomes in CD (7–10). Nonetheless, the majority of CD studies focus on health-related quality of life (such as the inflammatory bowel disease questionnaire) assessments, a subjective assessment about the limitations caused by the disease with high uncertainty. Disability assessment determines function loss in an individual and the social cost of CD.

The International Classification of Functioning, Disability, and Health (ICF) of the World Health Organization defines disability as an objective measure of loss of functioning and impairment in patient activity (11). The Inflammatory Bowel Disease Disability Index (IBD-DI) was developed (12) and updated in 2017 (13). The IBD-DI includes body functions, body structures, as well as activity and participation. Studies confirmed that the IBD-DI was associated with gender, clinical disease activity, and disease duration in the West (14–16). However, our literature review revealed that studies are rarely conducted on conditions and clinical results related to disability in IBD patients. This situation has been exacerbated by the allocation of greater funding for basic research instead of clinical studies. Moreover, in CD, risk factors, disease features, economic conditions, cultural background, and medical systems do not completely overlap between the East and the West. Therefore, more research on disability needs to be conducted on the Chinese population.

Inflammatory bowel diseases are often associated with malnutrition and significant alteration in body composition (17). Bryant et al. found that muscle depletion among IBD patients may affect the quality of life (18). Norman et al. suggested that malnutrition-related factors can reduce the quality of life in IBD patients (19). None of the available studies assessed the relationship between disability and body composition.

Liu et al. (20) reported that a large proportion of Chinese IBD patients experience impaired health-related quality of life. Nonetheless, considerably few studies have focused on disability and risk factors in Chinese CD patients. Thus, this study aimed to evaluate disability prevalence among CD patients, evaluated the correlation between disability and body composition, as well as the risk factors that may be associated with a CD-related disability.

MATERIALS AND METHODS

Study Participants

In this cross-sectional study, CD outpatients ($n = 197$) under anti-TNF-α therapy were included at Ruijin Hospital in Shanghai from February 2019 to June 2019. All CD patients were included in the study, independent of occupational activity, gender, race, or social class. Specifically, these participants were diagnosed with CD by their treating physicians and met the criteria established by the Inflammatory Bowel Disease Group of the Chinese Society of Gastroenterology. The following exclusion criteria were applied: patients with metabolic diseases (e.g., diabetes, hyperthyroidism) whose related indices were not well-controlled after hospitalization; patients with gastrointestinal tumors or tumors at other sites; patients with impaired water and mineral metabolism caused by cortical hormone therapy; and patients with metal implants (such as cardiac pacemakers) that could influence bioelectrical impedance results. Fifty-one patients were not included for missing values in the variables of body composition, BMI or others (**Figure 1**). The final sample was comprised of 146 participants.

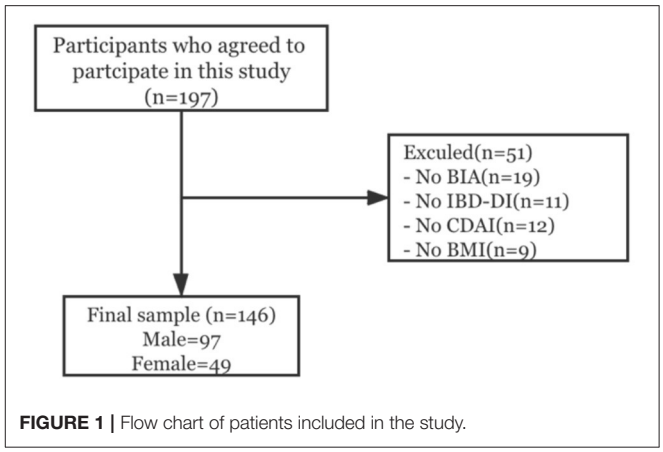

FIGURE 1 | Flow chart of patients included in the study.

The participants were informed of the study objective before the experiments started. Informed consent was obtained from each participant, who also consented to the publication of relevant data. This study was approved by the Ethics and Research Committee of Ruijin Hospital.

Before the start of the study, a pilot study consisting of 15 participants was conducted as a preliminary investigation and to pretest the survey instrument and subsequently adjust the scale. All researchers involved in this study were trained together. The samples were described using a structured questionnaire, including sociodemographic data and lifestyle.

The following information was collected: (a) sociodemographic data, including age, gender, height, weight, and smoking and drinking habits; (b) disease characteristics, including disease duration and disease activity assessed by Crohn's disease activity index (CDAI). Patients with CDAI $\leq$150 were considered in clinical remission, whereas those with CDAI $>$150 were considered in the active phase.

Body Composition Measurement

We assessed body composition by multifrequency bioelectrical impedance analysis using the InBody S10 body water analyzer (InBody Korea). We also evaluated the total fat mass, body fat percentage, total muscle mass, skeletal muscle mass, and

the muscle/fat ratio of the patients. This analyzer processes 30 impedance measurements by using six different frequencies (1, 5, 50, 250, 500, 1,000 kHz) in each of five segments of the body (right arm, left arm, trunk, right leg, and left leg) and 15 reactance measurements by using tetrapolar 8-point tactile electrodes at three different frequencies (5, 50, and 250 kHz) in each of the aforementioned segments.

The appendicular skeletal muscle mass (ASM) was measured as the sum of the muscle mass from the four limbs, and the ASM index (kg/m^2) was calculated. Loss of skeletal muscle mass was determined based on the criteria set by the Asian Working Group for Sarcopenia (2019) (21): ASMI <7.0 kg/m^2 for men and <5.7 kg/m^2 for women.

Handgrip Strength Evaluation

Measurement was performed with the participant in the following position: seated on an armless chair, with feet supported on the floor, hips and knees flexed at 90°, arms parallel to the body, elbows flexed at 90°, and forearms and wrists in a neutral position. The dominant side was measured three times at 1-min intervals, with verbal stimulation applied. The results were expressed in kilogram-force (kg), and the mean of the three measures was used. Muscle weakness was determined based on the criteria set by the Asian Working Group for Sarcopenia (21): handgrip strength <28 kg for men, and <18 kg for women.

Nutritional Assessment

Patient-Generated Subjective Global Assessment (PG-SGA) was recommended as a nutritional assessment scale for IBD patients by the Inflammatory Bowel Disease Group of the Chinese Society of Gastroenterology. The PG-SGA assessment included (i) body weight, (ii) dietary intake, (iii) symptoms, (iv) movement and body function, (v) relationship between disease and nutritional requirement, (vi) metabolism requirement, and (vii) physical examination. According to the criteria, the scores indicated the following: 0–1, good nutritional status; 2–3, light malnutrition requiring nutritional education and/or nutritional intervention; 4–8, moderate malnutrition requiring nutritional intervention; and ≥9, severe malnutrition requiring symptom improvement and nutritional therapy. Patients with scores ≥4 were defined as having moderate or severe malnutrition. Those with scores<4 were enrolled in the low PG-SGA group.

Inflammatory Bowel Disease Disability Index (IBD-DI)

CD-related disability was assessed based on the IBD-DI (13). The original IBD-DI comprises 19 items capturing five aspects of the ICF categories, including general health, body function (sleep/energy, affect, body image, pain, diarrhea, body mass index, and weight loss), body structure (presence of blood in the stool, arthralgia/arthritis), participation activity (regulating defecation, looking after own health, interpersonal activities, and work/education), and environmental factors (exacerbating effect or medication, food, family, and health care professional). A simplified version of the IBD-DI, which contains 14 items, was validated in 2017. All items were rescaled to range from 0 to 4. Binary items were coded as "0" for "no" and "4" for "yes." BMI > 18.5 was assigned a score of "0," whereas BMI < 18.5 was assigned a score of "4." The total score ranged from 0 to 100 using the following formula: score × 100/(p × 4), where p represents the number of answered items. The total scores and their corresponding classifications were as follows: 0–20 (no disability), 20–35 (mild disability), 35–50 (moderate disability), and 50–100 (severe disability).

Statistical Analysis

The data were analyzed using SPSS version 22.0. $P < 0.05$ indicated statistical significance. For normally distributed continuous data, the mean (standard deviation [SD]) was used as the measure of central location, whereas for continuous data with non-normal distribution, the median (interquartile range) was used. These characteristics were compared between the moderate to severe disability group and the no disability and minimal disability group by using Pearson's chi-square or Fisher's exact test to compare proportions. The odds ratio (OR) and its 95% confidence interval (CI) were calculated to determine the association of risk factors with moderate or severe disabilities. Statistically significant variables in the univariate analyses were then included in a multivariate regression model to identify the independent risk factors of moderate to severe disability by backward elimination analysis.

RESULTS

Patient Characteristics

Table 1 presents the demographics and baseline characteristics of the population. All patients who completed the study allowed the use of their administrative data, which were thus included in this study. The mean age of the participants was 38.89 ± 10.68, 66.43% were male, 52.05% had moderate or severe disabilities, 28.76% had moderate or severe malnutrition (PG-SGA≥4), and 40.41% had sarcopenia. The average duration of IBD was 5.59 y. The disease was considered clinically in remission in 120 patients. The IBD disability index for all CD patients was 33.62 ± 9.63 (0–61).

Table 2 showed baseline characteristics of activity and remission phase participants of the CD patients. The active phase subjects had a higher CRP level (80.77 vs. 6.67%, $P = 0.012$), moderate to severe disability (92.31 vs. 43.33%, $P < 0.001$), PG-SGA≥4 (69.23 vs. 20.00%, $P < 0.001$) compared to the remission phase. The active phase subjects were also more frequently disability and sarcopenia compared with patients with the remission phase.

General Characteristics of CD Patients With Moderate to Severe Disability vs. Without or Minimal Disability

Table 3 lists the body composition, sociodemographic factors, age, nutritional-biochemical indexes, nutritional status of the patients by their disability status. The moderate to severe disability group had lower values for body weight, BMI, BFM, ASMI, and handgrip strength, compared with the group without to minimal disability ($P < 0.05$). The moderate to severe disability group had higher CDAI and PG-SGA scores, compared with the

TABLE 1 | Characteristics of the study participants.

Characteristics	Total ($n = 146$) Mean ± SD	*N* (%)
Age, years	31.58 ± 10.00	
Gender		
Male		97 (66.43%)
Female		49 (33.56%)
BMI. Kg/m^2	19.67 ± 3.37	
Smoking		12 (8.22%)
Drinking		1 (0.68%)
Duration of disease (years)	4.99 ± 4.91	
IBD-DI		
Moderate to severe disability		76 (52.05%)
Without or minimal disability		70 (47.95%)
Malnutrition		
PG-SGA≥4		42 (28.76%)
PG-SGA<4		104 (71.23%)
Sarcopenia		
Yes		41 (28.08%)
No		105 (71.92%)
Disease activity		
Active phase		26 (17.81%)
Remission phase		120 (82.19%)
High level CRP, ≥10 mg/L		29 (19.86%)
Hemoglobin, g/L	127.82 ± 19.60	
Albumin, g/L	41.10 ± 5.58	

without to minimal disability group ($P < 0.05$). No significant differences in age, duration of disease, and hemoglobin levels were found between the moderate to severe disability group and the without to minimal disability group ($P > 0.05$). The prevalence of sarcopenia (48.68 vs. 31.43%, $P = 0.043$), PG-SGA≥4 (39.47 vs. 17.14%, $P = 0.003$), and high-level CRP (27.63 vs. 11.43%, $P = 0.021$) were higher in the moderate to severe disability group than in the without to minimal disability group.

Correlations Between the IBD-DI and Clinical Variables

In CD patients, the variables that were significantly correlated with the IBD-DI were BMI, CDAI, PG-SGA, ASMI, handgrip strength, and CRP (**Figure 2**).

Risk Factors Associated With Moderate to Severe Disability

In univariate regression models, disability was significantly associated with BMI (OR: 4.60, 95% CI: 2.28–9.30), sex (OR: 2.25, 95% CI: 1.13–4.47), CDAI (OR: 15.69, 95% CI: 3.54–69.42), BMI (OR: 4.60, 95% CI: 2.28–9.30), sarcopenia (OR: 3.40, 95% CI: 1.59–7.72), and CRP (OR:1.31, 95% CI: 1.05–1.64) (**Table 4**).

By multivariate analysis, the BMI (OR:4.11, 95% CI: 1.80–9.38) and disease activity (OR:10.47, 95% CI: 2.09–52.42) were identified as the most significant factors predicting disability.

TABLE 2 | Activity phase differences in the prevalence of sarcopenia and disability in CD patients.

	Activity ($n = 26$)	Remission ($n = 120$)	*P*
Anthropometric indicators			
Age, y	33.47 ± 10.09	31.72 ± 11.85	0.452
Sex			0.247
Male	14 (53.85%)	83 (69.17%)	
Female	12 (46.15%)	37 (30.83%)	
BMI, kg/m^2	18.37 ± 2.58	19.96 ± 3.47	0.027*
Biochemical indexes			
High level CRP, ≥10 mg/L	21 (80.77%)	8 (6.67%)	0.012*
Hemoglobin, g/L	110.35 ± 22.41	131.60 ± 16.77	0.000*
Albumin, g/L	35.35 ± 4.39	42.54 ± 4.79	0.000*
Body composition			
BFM, kg	10.21 ± 5.83	9.27 ± 5.92	0.948
ASMI, kg/m^2	6.39 ± 1.07	7.03 ± 1.13	0.010*
FFM, kg	44.71 ± 8.91	49.81 ± 9.73	0.083
TBW, kg	31.63 ± 5.86	34.18 ± 7.65	0.135
VFA, cm^2	31.63 ± 29.50	36.58 ± 28.94	0.433
PBF, %	14.60 ± 7.40	16.42 ± 7.95	0.338
BCM, kg	28.90 ± 5.64	31.74 ± 7.03	0.087
Handgrip strength, kg	27.60 ± 8.27	32.32 ± 9.69	0.023*
Nutritional assessment			
PG-SGA≥4	18 (69.23%)	24 (20.00%)	0.000*
PG-SGA<4	8 (30.77%)	96 (80.00%)	
IBD-DI			
Moderate to severe disability	24 (92.31%)	52 (43.33%)	0.000*
Without to minimal disability	2 (7.69%)	68 (56.67%)	
Sarcopenia			
Yes	13 (50.00%)	28 (23.33%)	0.008*
No	13 (50.00%)	92 (76.67%)	

Data are presented as mean ± standard deviation (median) or as stated.
BFM, body fat mass; VFA, visceral fat area; PBF, percent body fat; BCM, body cell mass; CRP, Creactive protein; BMI, body mass index; ASMI, appendicular skeletal muscle mass index; FFMI, fat free mass index; FFM, fat free mass; PG-SGA, Patient-Generated Subjective Global Assessment; CDAI, Crohn's disease activity index.
*p-value from a t-test for independent samples or a chi-square test, depending on the type of variable. *p < 0.05. Continuous variables (mean ± SD) and categorical variables (percentage).*

DISCUSSION

In the past 20 years, CD has markedly increased and become a common disease of the digestive system in China (22). About 70–80% of CD patients may suffer from intestinal obstruction, abdominal abscess, intestinal perforation, and other complications. CD patients may receive surgery, high medical costs. A complicated disease course increases disability, including serious damage to the physical and mental health of patients, a decline in the quality of life (23), and work efficiency (24–26), and even depression (27). In the current study, we confirmed CD in the active phase to be significantly associated with disability.

We used the scale developed by Gower-Rousseau et al., which consisted of 14 items. The later Korean, Portuguese, and Prevost

TABLE 3 | General characteristics of CD patients with moderate to severe disability vs. Without to minimal disability.

	Moderate to severe disability ($n = 76$)	Without to disability ($n = 70$)	*P*
Anthropometric indicators			
Age, y	34.27 ± 11.31	31.67 ± 10.05	0.236
Sex, female, (%)	36 (47.37%)	20 (28.57%)	0.027*
Height, cm	165.26 ± 8.91	170.89 ± 9.40	0.001*
Body weight, kg	51.67 ± 12.31	61.79 ± 12.84	0.000*
BMI, kg/m^2	19.56 ± 3.22	21.68 ± 3.06	0.001*
Duration of disease, >5 y	29 (38.16%)	27 (38.57%)	0.547
CDAI index	115.58 ± 75.93	60.23 ± 48.21	0.000*
Biochemical indexes			
Hemoglobin, g/L	124.87 ± 20.54	131.01 ± 18.13	0.058
Albumin, g/L	40.07 ± 5.54	42.20 ± 5.45	0.021*
High-level CRP, ≥10 mg/L	21 (27.63%)	8 (11.43%)	0.021*
Nutritional assessment			
PG-SGA≥4	30 (39.47%)	12 (17.14%)	0.003*
PG-SGA<4	46 (60.52%)	58 (82.86%)	
Body composition			
ASMI, kg/m^2	6.59 ± 1.07	7.27 ± 1.12	0.000*
FFM, kg/m^2	43.29 ± 8.57	48.93 ± 10.79	0.001*
BFM, kg/m^2	8.77 ± 5.59	11.46 ± 5.82	0.000*
VFA, cm^2	28.88 ± 27.74	43.19 ± 28.69	0.003*
WC, cm	67.97 ± 8.68	74.21 ± 9.03	0.000*
AC, cm	25.02 ± 3.28	27.40 ± 3.71	0.000*
BCM, L	28.73 ± 6.11	33.57 ± 6.77	0.000*
TBW, kg	31.84 ± 6.41	35.76 ± 7.85	0.002*
PBF, %	14.95 ± 6.96	17.16 ± 8.53	0.126
Handgrip strength, kg	28.61 ± 9.30	34.61 ± 8.96	0.000*
Sarcopenia			
Yes	37 (48.68%)	22 (31.43%)	0.043*
No	39 (51.32%)	48 (68.57%)	

Data are presented as mean ± standard deviation (median) or as stated.
CRP, C-reactive protein; BMI, body mass index; ASMI, appendicular skeletal muscle mass index; FFMI, fat-free mass index; FFM, fat-free mass; BFM, body fat mass; PG-SGA, Patient-Generated Subjective Global Assessment; VFA, visceral-fat area; WC, waist circumference; AC, arm circumference; CDAI, Crohn's disease activity index.
*p-value from a t-test for independent samples or a chi-square test, depending on the type of variable. *p < 0.05. Continuous variables (mean ± SD) and categorical variables (percentage).*

TABLE 4 | Risk factors associated with moderate to severe disability for all CD patients.

Variables	Univariate analysis		Multivariate analysis	
	OR (95%CI)	*p*	OR (95%CI)	*p*
Sex				
Male	1		1	
Female	2.25 (1.13–4.47)	0.021*	1.64 (0.70–3.82)	0.254
Duration of disease				
<5years	1			
≥5years	0.98 (0.50–1.91)	0.959		
Disease activity				
Remission	1		1	
Active	15.69 (3.54–69.42)	<0.001*	10.47 (2.09–52.42)	0.004*
BMI				
≥18.5 kg/m^2	1		1	
<18.5 kg/m^2	4.60 (2.28–9.30)	<0.001*	4.11 (1.80–9.38)	0.001*
Sarcopenia				
No	1		1	
Yes	3.40 (1.59–7.72)	0.002*	1.29 (0.47–3.53)	0.620
Malnutrition				
No	1		1	
Yes	3.15 (1.46 to 6.83)	0.004*	1.79 (0.69 to 4.66)	0.233
CRP				
≥10 mg/L	1		1	
<10 mg/L	1.31 (1.05 to 1.64)	0.017*	1.11 (0.85 to 1.45)	0.444
Albumin				
≥35 g/L	1		1	
<35 g/L	2.44 (0.94 to 6.35)	0.065	1.39 (0.41 to 4.74)	0.604

Data are presented as mean ± standard deviation (median) or as stated.
Univariate analysis: binary logistic regression analysis unadjusted; Multivariate analysis: After univariate analysis, all variables with P < 0.15 were considered in subsequent multivariate analyses.
CRP, C-reactive protein; BMI, body mass index; PG-SGA, Patient-Generated Subjective Global Assessment; CDAI, Crohn's disease activity index; Malnutrition, PG-SGA≥4.
**p < 0.05.*

versions containing 14 items showed good reliability and validity. We determined the average IBD-DI score, which was 33.62 ± 9.63, similar results were observed in the Gower-Rousseau study. In the current study, 52.05% of the CD patients had a moderate to severe disability, whose results were similar to those reported by Marinelli et al. (28) and Yoon et al. (23). Notably, the results of the multivariate analysis indicated that disease activity was an independent risk factor for disability in patients with CD (OR:10.47, 95%CI:2.09–52.42). In a validation study from Australia (29), disability was significantly correlated with CDAI, and lBD-Q. A study among Koreans showed that disability in patients with IBD was correlated with disease activity and poor quality of life (23, 29). A meta-analysis found a significant correlation between disease activity and disability in IBD patients (30). All of these observations confirmed that disease activity is an important factor affecting disability. Therefore, effective treatment measures should be applied to reduce the degree of disease activity in patients.

BMI was another independent predictor for CD patients with a moderate to severe disability in this study (OR:4.11, 95 CI: 1.80–9.38). The patients with moderate to severe disability had lower BMI than those without or with minimal disability (19.56 ± 3.22 vs. 21.68 ± 3.06, $P = 0.001$). However, this was the first study to concurrently demonstrate that BMI was significantly associated with disability. A Portugal study reported that BMI was related to the quality of life in IBD patients; moreover, the effect of BMI on the psychological and physical quality of life was mediated via the mechanisms of body image (31). An Israel study enrolled 100 IBD patients and found that a lower BMI was associated with a more severe disease course (32). A

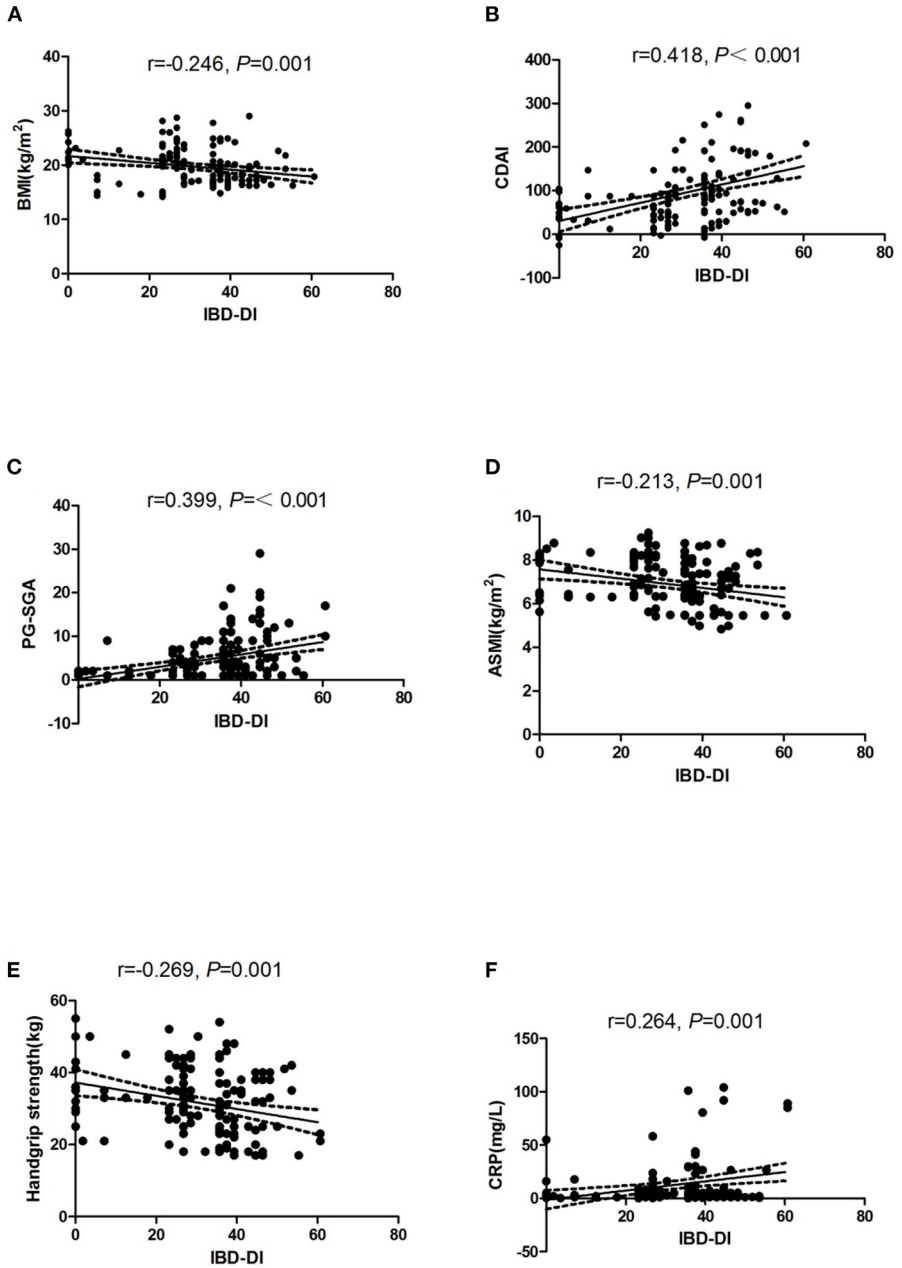

FIGURE 2 | Dispersion graphs depicting correlations between IBD-DI and BMI **(A)**, CDAI **(B)**, PG-SGA **(C)**, ASMI **(D)**, Handgrip strength **(E)**, and CRP **(F)**. *P*-values on each graph were calculated for all CD patients. *r* indicates the Spearman's rank correlation coefficient. IBD-DI, inflammatory bowel disease disability index; BMI, body mass index; CDAI, Crohn's disease activity index; PG-SGA, Patient-Generated Subjective Global Assessment; ASMI, appendicular skeletal muscle mass index; CRP, C-reactive protein.

case-control study revealed that women with ulcerative colitis exhibited decreased lower limb strength and mobility limitations, which were associated with BMI (33). Besideds, a systematic review indicated that BMI was normally lower in CD patients, and regular medical therapy could not improve BMI in these patients (34). Our previous study (35) demonstrated that the dietary structure of IBD patients was unreasonable, characterized by insufficient intake of energy and protein; in addition, lack of physical activity can lead to body muscle depletion. Wardle et al. (36) found an increased prevalence of disordered eating behavior in CD and a greater prevalence of binge eating, food craving, low mood, and high anxiety. Moreover, Chan et al. (27) reported that symptoms of anxiety and depression were independently associated with IBD-related disability. Arigo et al. (37) suggested

that fear and anxiety surrounding gastrointestinal symptoms can lead to disordered eating practices of a restrictive nature. In a French survey (38), nearly half of the subjects reported that the disease had changed the pleasure of eating, with only a quarter of the patients eating a normal diet during relapse. Thus, reduced dietary intake and disordered eating behavior can potentially lower BMI and aggravate disability which may associated with anxiety and depression.

Studies on the association between IBD-related disability and muscle-related sarcopenia have rarely, if ever, been reported. A previous study determined that the prevalence of sarcopenia among adult Chinese patients with CD was 60% (39). In the current study, the prevalence rates of sarcopenia were lower than the previously reported rate in Chinese patients. The discrepancies were most likely attributable to significant differences in patient selection and the methods used. We found that the muscle mass in remission was significantly higher than that in activity, which is consistent with our previous findings. Notably, patients with moderate to severe disability had lower sarcopenia-related index than patients with without to minimal disability (ASMI: 6.91 ± 1.12 vs. 7.59 ± 0.97, $P = 0.002$, handgrip strength: 28.61 ± 9.30 vs. 34.61 ± 8.96, $p < 0.001$). Research has shown that sarcopenia is a progressive and generalized syndrome characterized by the loss of skeletal muscle mass and muscle strength with adverse outcomes, such as frailty, poor quality of life, and mortality (40, 41). The pathogenesis of muscle wasting includes several elements, such as aging, systemic inflammation, mitochondrial dysfunction, increased proteolysis, decreased proteosynthesis, and insulin resistance (40). Cravo et al. reported that reduced lower muscle attenuation seemed to be associated with more severe phenotypes in patients with CD (42). These conditions may aggravate disability in CD patients. Univariate analysis showed that moderate to severe disability was significantly associated with CD-related sarcopenia; however, multivariate analysis revealed no such finding.

In the univariate analysis, moderate to severe disability was found in 2.25 times more female than male CD patients. This finding is consistent with the studies conducted among French (13) and Spanish (14) subjects, which also found a higher IBD-DI in female than male subjects. By contrast, the current study did not find the same result after multivariate analysis. Similarly, a Dutch study observed no link between disability and sex (43). In addition, the data indicated that patients in the moderate to severe disability group had higher CRP levels than those in the without to minimal disability group (27.63 vs. 11.43%, $P = 0.021$). However, this finding was not supported by the multivariate analysis. Nonetheless, close attention to the clinical CRP score should be given attention.

In China, CD patients have poor access to IBD treatment centers and specialist doctors. Moreover, doctors focus more on disease treatment and remission than the quality of life and disability in CD patients (44). Although an increasing number of studies have reported that disability in CD can affect their work efficiency and psychological status, reports on Chinese-related populations were limited. Thus, CD patients in China must monitor the conditions of their disability during clinical treatment. Moreover, prompt corrective measures have to be undertaken to alleviate body mass depletion and maintain remission, consequently improving the quality of life and reducing disability. A multidisciplinary assessment of patients with CD is always encouraged, and nutrition strategies should always be suitable to the needs of the patient.

We also identified several limitations. First, this study is a single-center cross-sectional study. This research sample size was small and the size of the cohort was only defined by the number of consecutive outpatients during the sample collection. Selection bias could not be excluded and not all Chinese CD patients could be represented, considering the sample; moreover, the correlation between patient disability and demographic and disease characteristics at a certain time node does not imply causality. Second, selection bias could not be avoided because not all patients who were surveyed were willing to participate. Third, a cross-sectional protocol provides limited evidence concerning changes over time, which would be better assessed by a future longitudinal study. Furthermore, multicenter, larger sample clinical observational research, longitudinal follow-up and intervention studies would be required to determine the reversal of disability for the successful management of disease activity and BMI.

CONCLUSIONS

We demonstrated that disability is strongly related to disease activity and body mass in CD patients. In the management of the disease, not only the regular progression of the disease but also disability and BMI should be investigated during the regular treatment of clinical outpatients. In conclusion, close monitoring and follow-up should be conducted for patients with a high risk of disability, and effective measures should be adopted, which may be the best approach to preventing disability and helping patients return to normal life. This would also allow for a novel multidisciplinary approach based on collaborative efforts between gastroenterologists, nurses, nutritionists, and psychologists to improve the quality of life in general.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics and Research Committee of Ruijin Hospital. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

DB, JZ, and YS: conceptualization. DB, YG, YJ, ZH, and YT: investigation. DB, JZ, and YS: methodology. YS: funding acquisition. DB and YJ: data curation. DB: writing—original draft

preparation. YG, ZH, JZ, and YS: writing—review and editing. YT, JZ, and YS: project administration. JZ and YS: supervision. QC and ZH: validation. All authors have read and agreed to the published version of the manuscript.

ACKNOWLEDGMENTS

We would like to thank all of the participants and investigators for their support and cooperation.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Medicine

REVIEW
published: 16 July 2021
doi: 10.3389/fmed.2021.681907

Thiopurines in Inflammatory Bowel Disease. How to Optimize Thiopurines in the Biologic Era?

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OPEN ACCESS

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Specialty section:
This article was submitted to Gastroenterology, a section of the journal Frontiers in Medicine

Received: *17 March 2021*
Accepted: *23 June 2021*
Published: *16 July 2021*

Citation:
Gargallo-Puyuelo CJ, Laredo V and Gomollón F (2021) Thiopurines in Inflammatory Bowel Disease. How to Optimize Thiopurines in the Biologic Era? Front. Med. 8:681907. doi: 10.3389/fmed.2021.681907

Thiopurines have been a cornerstone in the treatment of inflammatory bowel disease (IBD). Although they have been used for more than 50 years, there are still some unsolved issues about their efficacy and, also, some safety concerns, mainly the risk of myelosuppression and life-threatening lymphoproliferative disorders. Furthermore, the development of biological therapy raises the question whether there is still a role for thiopurines in the IBD treatment algorithm. On the other hand, limited cost and wide availability make thiopurines a reasonable option in settings of limited resources and increasing prevalence of IBD. In fact, there is a growing interest in optimizing thiopurine therapy, since pharmacogenomic findings suggest that a personalized approach based on the genotyping of some molecules involved in its metabolism could be useful to prevent side effects. Polymorphisms of thiopurine methyltransferase enzyme (TPMT) that result in low enzymatic activity have been associated with an increased risk of myelotoxicity, especially in Caucasians; however, in Asians it is assumed that the variants of nudix hydrolase 15 (NUDT15) are more relevant in the development of toxicity. Age is also important, since in elderly patients the risk of complications seems to be increased. Moreover, the primo-infection of Epstein Barr virus and cytomegalovirus under thiopurine treatment has been associated with severe lymphoproliferative disorders. In addition to assessing individual characteristics that may influence thiopurines treatment outcomes, this review also discusses other strategies to optimize the therapy. Low-dose thiopurines combined with allopurinol can be used in hypermethylators and in thiopurine-related hepatotoxicity. The measurement of metabolites could be useful to assess compliance, identify patients at risk of adverse events and also facilitating the management of refractory patients. Thioguanine is also a rescue therapy in patients with toxicity related to conventional thiopurine therapy. Finally, the current indications for thiopurines in monotherapy or in combination with biologics, as well as the optimal duration of treatment, are also reviewed.

Keywords: thiopurines, inflammatory bowel disease, pharmacogenomics, toxicity, indications, optimize

INTRODUCTION

Inflammatory bowel disease (IBD) includes mainly two chronic disorders affecting the gastrointestinal tract, Crohn's disease (CD) and ulcerative colitis (UC), and it has a worldwide distribution (1). The medical treatment is based on 5-aminosalicylates, corticosteroids, immunomodulators (thiopurines and methotrexate) and biologics (2, 3).

Thiopurines (azathioprine, mercaptopurine and thioguanine) are antimetabolites of purines which have been a cornerstone in the treatment of IBD for more than 50 years (4). In spite of that, there is still lack of evidence about its efficacy in some scenarios. Firstly, guidelines do not recommend using thiopurines (TP) as induction therapy (2, 3, 5–7). In CD most evidence comes from studies comparing azathioprine (AZA) and placebo (8); and there is only one randomized controlled trial comparing AZA and biologic therapy (infliximab), concluding that AZA was inferior (9). Although the quality of the studies has been questioned, the evidence for AZA as induction therapy in UC is also absent (7, 10). In maintaining of remission, there are Cochrane reviews of randomized controlled trials for both, CD and UC, demonstrating a superiority of AZA against placebo; however, the quality of evidence is again low, especially in UC (11, 12). Another common indication is the prevention of post-surgical relapse, but despite the fact that it seems to be superior to placebo, there is a wide heterogeneity in the designs of studies and in one small randomized trial comparing AZA with biologics (adalimumab) there were no differences in efficacy between both treatments while in other study adalimumab was superior (5, 13, 14). Finally, the evidence supporting combination therapy of AZA with biologics relays mainly on two prospective trials in which combination therapy was superior to monotherapy in CD and UC (9, 15). Despite the superiority, the appropriate duration of combination therapy is still unknown (5, 7).

In addition to unsolved efficacy issues, safety concerns may also limit the use of TP in clinical practice. The rate of adverse events is up to 25% in some studies and nearly 20% of patients have to discontinue the treatment (16). Some strategies, as periodic blood tests, determination of genetic polymorphisms and metabolites measurement, are useful to decrease the risk of some side effects such as myelotoxicity, but it cannot be prevented in all cases and it can occur at any time of the treatment (17). Although it could be a serious adverse event, the risk of death due to myelotoxicity is relatively low (1%) (18). Other uncommon life-threatening hematological conditions, such as hemophagocytic lymphohistiocytosis (HLH) and other lymphoproliferative disorders have also been associated with TP (19). Pancreatitis and hepatotoxicity are other limiting side effects related to TP (20, 21).

Despite efficacy issues, toxicity risks and the availability of other therapeutic options, there are some arguments in favor of optimizing TP. Firstly, the epidemiological evolution of IBD is changing and pediatric onset of the disease is becoming more common in some Western countries (1, 22), which implies a longer evolution of the disease in these patients and, probably, the need for different IBD treatments during their life; especially considering that the course of the disease seems to be more aggressive (23). In spite of the availability of many therapeutic options for IBD, there are still some refractory patients who will eventually need surgery (24); therefore, optimizing medical treatment before escalation seems a reasonable option. Furthermore, due to the increasing prevalence of IBD in Western countries, the number of patients in IBD units and, consequently, the treatment-related costs are expected to increase (1, 25). TP are cheaper compared to biologics; in fact, in some countries, before the biologics, the cost of drugs represented 25% of the IBD care cost and, after them, the cost has increased from 30 to 70% (26). Finally, the overall efficacy of TP has been demonstrated for many years and, in general, patients who respond to these drugs tend to maintain a long remission (4, 27). Clinical experience with TP also helps to manage most side effects, and those that are life-threatening are uncommon.

HOW TO IMPROVE EFFICIENCY OF THIOPURINES?

Using the Treatment in Selected Patients Depending on Individual Characteristics

Pharmacogenomics

AZA is a prodrug and, after a non-enzymatic change, 88% of it is converted into mercaptopurine (MP), that can be metabolized through different pathways into another active and inactive metabolites, as shown in **Figure 1** (27, 28). The thiopurine methyltransferase enzyme (TPMT) methylates MP into methylmercaptopurine (MMP), an inactive metabolite associated with some adverse events, mainly hepatotoxicity. MP can also be oxidized by xanthine oxidase into thiouric acid (TUA), another inactive degradation product. However, MP can be converted by hypoxanthine-guanine phosphoribosyltransferase (HPRT) into thiosine monophosphate (TIMP), which can also be transformed by 5- inosine monophosphate dehydrogenase (5-IMPDH) into thioguanine monophosphate (TGMP) and, then, into thioguanine diphosphate (TGDP) and triphosphate (TGTP) (28). Thioguanine (TG) is also metabolized by HPRT into TGMP. TGMP, TGDP and TGTP are thioguanine nucleotides (TGNs) and the active metabolites of AZA, responsible for the efficacy and myelotoxicity of TP. These nucleotides antagonize the endogenous purines and incorporate into cellular RNA-DNA, inhibiting cellular proliferation. Other mechanism of action includes inhibition of Rac1 activation with costimulation of CD28 leading to T cell apoptosis (29). The main action of TPMT enzyme is to methylate MP, TG, TIMP and TGMP; so they become inactive products, TGNs synthesis decreases and, subsequently, TP are less effective (30). Efficacy and side effects are consequence of a tight balance between the pathways that activate and inactive TP and a wide inter-individual variability has been described in this setting (31).

Currently, more and more studies highlight the role of pharmacogenomics in optimizing TP (31–33). Polymorphisms of TPMT, nudix hydrolase 15 (NUDT15), alpha-ketoglutarate dependent dioxygenase (FOT), class II HLA and inosine triphosphate pyrophosphatase (ITPA) have been associated with an increased risk of adverse events (**Table 1**).

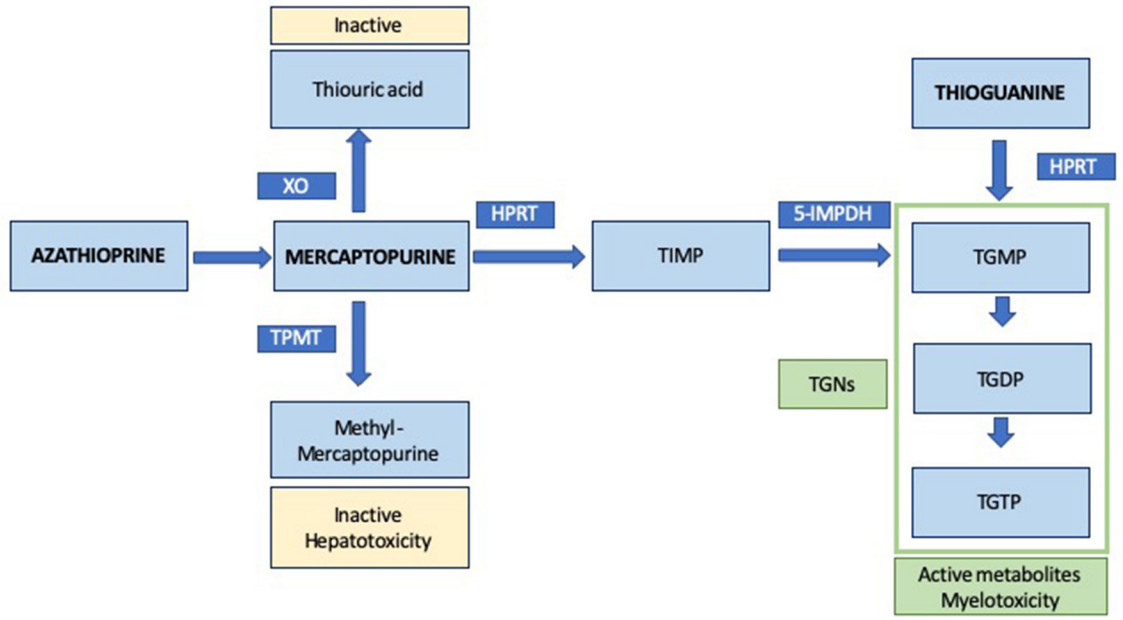

FIGURE 1 | Simplified metabolism of thiopurines. XO, xanthine oxidase; TPMT, thiopurine methyltransferase; HPRT, hypoxanthine-guanine phosphoribosyltransferase; 5-IMPDH, 5- inosine monophosphate dehydrogenase; TIMP, thiosine monophosphate; TGNs, thioguanine nucleotides; TGMP, thioguanine monophosphate; TGDP, thioguanine diphosphate; TGTP, thioguanine triphosphate.

TABLE 1 | Most important genetic variants associated with thiopurine toxicity.

Genetic variant	Functional consequence	Clinical consequence
TPMT*2 (rs1800462) TPMT*3C (rs1142345) TPMT*3A: contains *3B (rs1800460) and *3C (rs1142345)	Low TMPT enzymatic activity	Risk of myelotoxicity
NUDT15 p.Arg139Cys or c415C>T (rs116855232)	Low NUDT15 enzymatic activity	Risk of myelotoxicity
NUDT15 p.Val18_Val19insGlyVal allele	Low NUDT15 enzymatic activity	Risk of myelotoxicity
Class II HLA polymorphism (rs2647087)	Unclear	Risk of pancreatitis
ITPA 94C > A (rs1127354)	Low enzymatic activity	Inconclusive data about increased risk of side effects
FTO Ala134Thr (rs79206939)	Low enzymatic activity	Leukopenia

Thiopurine Methyltransferase

Patients with increased TPMT activity are called "hypermethylators" or "non-responders" because they mainly produce methylated inactivated products of AZA with very low amounts of active metabolites (34). On the other hand, patients with low-activity in both alleles of TPMT gene mainly produce active metabolites by increasing IMPDH pathway and are at risk of severe adverse events, especially myelotoxicity. The TPMT alleles can be classified into functional (*1) or non-functional (*2, *3A, *3B, *3C, *4) (35). There are many different polymorphisms associated with low TPMT activity but the most important are TPMT*2, TPMT*3A and TPMT*3C, as they represent 60–95% deficient alleles in most populations (31). Depending on genotype, patients could be classified into homozygous of high activity alleles (two or more functional alleles), heterozygous with intermediate activity (one functional allele and one non-functional) and homozygous with low activity (two non-functional alleles). In Caucasians, 0.3% are homozygous for low activity alleles, 11% are heterozygous and 89% are homozygous for high activity alleles (36). Based on the genotype, a full dose of TP is recommended in high activity homozygous, a 50% dose-reduction in heterozygotes, and avoiding treatment in low activity homozygous (35, 37). TPMT activity can be measured using phenotype in red blood cells or genotype, based on the analysis of polymorphisms associated with low TPMT activity (38). Genotype appears to be superior to phenotype, as it better identifies heterozygous with similar rates by misclassifying TPMT defectives (39). On the other hand, one advantage of TPMT phenotype, rather than genotype, is that within one genotype there can be significant

variation in phenotypes, allowing for further individualization of dosing.

Despite the theoretical utility of TPMT genotyping, the TOPIC trial did not find an advantage of this strategy to reduce the incidence of myelotoxicity (37), except in heterozygotes with high-risk variants of TPMT, while the TARGET trial did not find a benefit in this subgroup of patients (40). The correct management of heterozygous is also controversial. Studies suggest that 30–60% of patients with intermediate TPMT activity will develop myelotoxicity under full dose of thiopurines, therefore the guidelines recommend a dose-reduction in heterozygotes; however, if we guide treatment only by TPMT activity, 40% of patients will be undertreated (31, 32). Furthermore, only 25% of myelosuppression can be explained by a TPMT deficiency (41).

The benefit of routinely testing TPMT is unclear (42). Some societies, such as the *American Gastroenterological Association* (43) and the *British Society of Gastroenterology* (6), recommend testing TPMT routinely before starting TP, but with low quality of evidence, while others, such as the *Spanish Working Group on Crohn's and Ulcerative Colitis* (GETECCU) or the *European Crohn's and Colitis Organization* (ECCO) support this recommendation, but suggest that is not essential before starting treatment (3, 5). Regarding the cost of routine TPMT testing, some authors suggest that it is cost-effective (33, 38) and others conclude that genotyping is a cost-neutral strategy (44). If available, it seems reasonable to test TPMT before starting TP; however, it may also depend on the prevalence of homozygous and heterozygous in each population (18). Moreover, periodic blood tests are mandatory throughout treatment because the risk of myelotoxicity does not disappear (42).

Nudix Hydrolase 15

The incidence of leukopenia under TP therapy ranges from 3% in Caucasians (18) to 40% in Koreans (45), probably due to polymorphisms in genes responsible for TP metabolism (46). TPMT polymorphisms are less common in Asians than in Caucasians (47); even when leukopenia rates are higher, therefore genotyping TPMT in Asians is not so useful (48). Moreover, even in Caucasians, only a small part of myelotoxicity can be explain by TPMT polymorphisms (49), implying that other genes could play an important role in the development of toxicity. Recent studies suggest that genes like NUDT15 and FTO are associated with some cases of myelotoxicity, especially in Asians, although this association has also been identified in Caucasians (50).

Despite of the fact that the mechanism of action of NUDT15 is not well understood, it is probably responsible for the inactivation of TGNs (49), participating in the degradation of TGTP into TGMP, avoiding the incorporation of thioguanine nucleotides into cellular DNA and reducing some effects of TP (51). NUDT15 variants associated with low enzymatic activity do not change the total amount of TGNs but modify the ratio of TGTP and TGMP (49); therefore monitoring the levels of TGTP and TG integrated into the DNA could be useful to adjust the dose of TP in patients with NUDT15 deficiency (52). Although measurement of individual 6TGNs–6TGMP, 6TGDP, and 6TGTP- would be preferable as it is mentioned, these assays are not widely available.

Patients with genetic variants of NUDT15 resulting in low activity are at risk of developing toxicity, even those with intermediate activity. In Koreans, there is a variation in NUDT15 (rs116855232, mainly called p.Arg139Cys or c415C>T) that has a 89.4% sensitivity and 93.2% specificity for TP leukopenia (53). Studies suggest that virtually all patients homozygous for p.Arg139Cys will develop severe leukopenia; however, other diplotypes could also result in low enzymatic activity. Therefore, testing for p.Arg139Cys could be useful to avoid the treatment in homozygous; however, if we want to find the ideal initial dose of TP for each patient, the study of diplotypes may be necessary (54). In this way, the p.Val18_Val19insGlyVal allele is another variant of NUDT15 associated with low enzymatic activity (49). Homozygous or compound heterozygous for high-risk variants of NUDT15 are more likely to develop toxicity than those carrying intermediate or normal activity diplotypes. There are many possible combinations of these alleles and the clinical consequences can be very different, so the application of NUDT15 activity in the TP algorithm is challenging.

In Japan almost 25% of population carry one copy of the NUDT15 risk alleles and 2% are homozygous or compound heterozygous (52). The ethnic distribution of p.Arg139Cys varies from 9.8% in East Asians to 0.2% in Europeans (55). Clinical relevance also varies between populations; for example, in Chinese the risk variants of NUDT15 are supposed to be one of the most relevant in the development of toxicity (51); however, in Guatemalans these variants are not so important (49). These results suggest that ethnicity is probably one of the most important risk factors for developing toxicity under TP treatment.

A randomized controlled trial in Koreans analyzed the influence of genotyping NUDT15 before starting treatment (56). In heterozygous, they used 50 mg of AZA and, in homozygous, the treatment with TP was avoided. The rest of patients (wild type and controls) followed a stepped dose strategy. In this study, genotyping before starting treatment decreased the risk of toxicity (HR = 0.37; 95% CI: 0.18–0.77; $p = 0.008$). These results encouraged the authors to propose a similar algorithm to manage TP therapy more efficiently based on NUDT15 genotyping. They strongly recommend avoiding TP in NUDT15 homozygous.

Other Genetic Polymorphisms

In Asians, a polymorphism of FTO gene (rs79206939 or Ala134Thr) has been associated with a 65% decrease in its enzymatic activity and an increased risk of leukopenia (56, 57), while another polymorphism of this gene (rs16952570 CC genotype) could have a protective impact on the risk of myelotoxicity (58). Moreover, the class II HLA rs2647087 polymorphism has been associated with an increased risk of pancreatitis (59, 60). In homozygous (C/C) the risk is significantly higher (14.63%), than in heterozygous (A/C) (4.25%) or wild type (A/A) (0.53%); therefore, some authors recommend avoiding TP in homozygous and, if possible, also in heterozygous (60).

As previously mentioned, the inhibition of Rac1 activation leads to T-cell apoptosis. The Rac 1 rs34932801 polymorphism has been associated with a poor response to thiopurine therapy (61). There are also inconclusive data regarding inosine triphosphate pyrophosphatase (ITPA) polymorphisms [94C > A (rs1127354) and IVS2 + 21A>C] resulting in low enzymatic activity leading to increased 6-TGN levels and thiopurine-related toxicity (31, 62, 63).

Other Personal Risks

Age

Since, in some studies, up to 20% of patients are diagnosed of IBD at age 60 or older, new treatments improve survival and the aging of population, an increase in the number of elderly patients with IBD is expected (64). In this subgroup of patients there is concern about starting treatment with immunomodulators and biologics due to the theoretical increased risk of neoplasms, adverse events and drug interactions (65). In fact, Parian et al. found that patients older than 65 years could take an average of nine chronic drugs and, in 40% of them, there was a potential interaction between IBD therapy and chronic treatment (66).

In a recent study, patients who started TP over 60 years of age had significantly more adverse events (43.4 vs. 29.7%; $p < 0.01$), infections (3.6 vs. 2.0%; $p < 0.001$) neoplasms (1.5 vs. 0.2%; $p < 0.001$), myelotoxicity (14 vs. 7.6%, $p < 0.01$), hepatotoxicity (9 vs. 4.7%, $p < 0.001$) and digestive intolerance (12.3 vs. 10%; $p = 0.002$), than the younger ones (65). In another study, the risk of malignancy and mortality was higher in elderly patients treated with TP than in those with anti-TNF therapy (H= 3.017; 95% CI: 1.050–8.666; $p = 0.0403$ and HR= 3.682; 95% CI: 1.192–11.377; $p = 0.0235$, for malignancy and mortality, respectively) (67). Regarding neoplasms, in the prospective observational CESAME study, the rate of urinary tract cancer in patients receiving TP was 0.48/1,000 patient-years (68). Male sex (HR = 3.98, $p = 0.04$) and age over 65 years (HR = 13.26, $p = 0.0001$) were the main risk factors.

Viral Infections (Epstein-Barr Virus and Cytomegalovirus Serological Status)

The primo-infection of Epstein-Barr virus (EBV) in young patients treated with TP has been associated with an increased risk of HLH (69). There are also some case reports about the relationship between cytomegalovirus (CMV) and HLH or severe primo-infection in patients under thiopurine therapy (70, 71). In a recent study, exposure to TP was an independent risk factor for developing serious viral infections, mainly caused by CMV or EBV (72). After EBV infection, the virus can be reactivated and, under normal conditions, the T-lymphocytes can neutralize it; however, under TP therapy, these lymphocytes are unable to act and an uncontrollable proliferation could lead to the development of complications (69). A Spanish group found that 97.4% of IBD patients were EBV-IgG positive and, among the negative ones, the seroconversion rate was 29.7% during 4 years of follow-up, without differences between young and elderly patients; therefore, EBV serological status should be assessed, regardless of age or sex, in all patients before starting TP (69).

In a meta-analysis, the risk of lymphoma was higher in patients treated with combination therapy than in those with TP o anti-TNF monotherapy (RR= 1.10; 95% CI: 1.03–2.81; $p = 0.039$) (73). It could be explained by an additive effect: TP affect the activity of T-lymphocytes, anti-TNFs decrease the action of natural killers and both help to propagate the lymphoblastoid transformation initiated by EBV (73, 74). In addition, the results of a recent study suggest that the risk of EBV-related lymphoma could be increased in patients with low thiopurine therapy compliance, since under the treatment there is an inhibition of cell replication, but after discontinuing it, an increase in the number of B-cells and lymphoblastoids can led to uncontrollable proliferation (74). Patients with exposure to TP have a five times higher risk of lymphoproliferative disorders than those who are not treated with these drugs; however, the 10-years risk of developing these disorders in young patients is <1% (75). Moreover, in a recent study, the incidence of acute myeloid leukemia and/or myelodysplastic syndrome was 18.7 per 100,000 IBD patients-year (76). The risk was increased in patients with current TP therapy but not in those with previous exposure. Based on the data above, ECCO guidelines recommend assessing EBV serological status before starting immunosuppressive therapy (77) and balancing the risk-benefit and the availability of other compounds we recommend avoiding thiopurines in EBV-IgG negative patients, particularly in young males. Despite the increased risk of lymphoproliferative disorders associated with TP, it is important to note that these drugs have also been associated with a reduction in the incidence of colorectal cancer, due to different mechanisms not only related to its anti-inflammatory effect (78).

Using Low Dose Thiopurine and Allopurinol

Allopurinol is a xanthine oxidase inhibitor and when added to TP increases its metabolism through 5-IMPDH into 6-TGN. LDTA can be useful in "hypermethylators" (79), in which TGN levels are usually <230–400 pmol/8 $\times$ 10^8 and/or 6-MMP levels are over 5,700–6,000 pmol/8 $\times$ 10^8 (80), so that they are at risk of hepatotoxicity and refractoriness to TP (81).

The increase in the levels of active metabolites can lead to myelotoxicity; therefore a 25–50% reduction in the dose of TP has been proposed to prevent it (82). In some studies, the dose of allopurinol ranges from 50 to 100 mg per day (80), however recent evidence suggests a clinical benefit without increasing toxicity using 100 mg (83). Some authors also suggest adjusting the dose of combination therapy based on TGN levels 4 weeks after starting treatment, 4 weeks after any dose change and every 6–12 months (81).

The efficacy of LDTA in non-responders and also in patients with TP-related hepatotoxicity has been demonstrated (81, 83, 84). In a recent randomized clinical trial, clinical response to LDTA was observed from week 2 of therapy, suggesting a faster onset of action, probably due to a rapid increase in TGN levels (83). Allopurinol is usually well-tolerated, with no major side effects, with skin rashes and gastrointestinal symptoms being the

most common. Toxic epidermal necrolysis has been described in Asians (81).

Adjusting Dose of TP Depending on Metabolites

The measurement of TP metabolites (TGN and MMP) can be useful to assess compliance, identify patients at risk of adverse events and also to manage refractory patients as shown in **Table 2** (31); however, the efficacy and toxicity thresholds are still unclear (42).

It has been suggested that the TGN target levels are likely to depend on the situation. When TP are used as monotherapy, TGN levels above 230–235 pmol/8 $\times$ 10^8 RBC have been associated with clinical response, and more than 450 pmol/8 $\times$ 10^8 RBC have been associated with an increased risk of myelotoxicity (5, 85). Regarding mucosal healing, a cutoff level of 397 pmol/8 $\times$ 10^8 RBC has been proposed with high specificity but low sensibility (86.7 and 35.3%, respectively) (86). In Chinese patients, a cutoff point between 180 and 355 pmol/8 $\times$ 10^8 RBC has been associated with remission (87). If the goal is to decrease immunogenicity related to anti-IFX antibodies, TGN levels $\geq$ 120 pmol/8 $\times$ 10^8 RBC appear to be enough to significantly reduce antibodies (88). In other studies, a similar cutoff (105–125 pmol/8 $\times$ 10^8 RBC) was also associated with maintenance of therapeutic IFX levels (89, 90).

High levels of MMP have been associated with thiopurine-related hepatotoxicity (91–93). Moreover, in one study, patients with MMP levels between 3,615 and 5,700 pmol/8 $\times$ 10^8 RBC had a 4-fold risk of hepatotoxicity (85); however, subsequent studies did not confirm this association (21). In fact, in one study almost 90% of patients with high concentration of MMP did not develop hepatotoxicity and, in 40% of patients with hepatotoxicity, MMP levels were below the risk cutoff (21). Therefore, it seems that high levels of MMP should be associated with other alterations, such as hypertransaminasemia, to be considered a marker of hepatotoxicity (94).

The overall benefit of routine metabolite monitoring remains unclear (42), since some studies do not find a benefit (95, 96) while others consider it as a strategy to optimize TP before switching to biologics (91, 97, 98). Data on the cost-effectiveness of this strategy is also insufficient (42).

Using Thioguanine

TG is a thiopurine with a simpler metabolism than AZA or MP. In a single pathway by HPRT, TG is transformed into TGNs, thus the methylated products associated with toxicity are not produced (99). Moreover, an experimental study in mice suggests that the effects of TG do not depend only on lymphocyte inactivation, because TG can be transformed into TGNs by the local action of colonic mucosal cells and colonic microbiota, leading to autophagy and intracellular destruction of bacteria (100). In fact, TGNs appear to accumulate in areas of intestinal inflammation, which explains the faster onset of the effect and could decrease lymphocyte-related myelotoxicity.

In terms of efficacy, ~65% of patients previously treated with AZA or MP, have clinical response with TG (101) and, in one study, in 57% of patients the addition of TG to anti-TNF therapy led to an improvement in the clinical situation (102). Efficacy seems to be similar to LDTA and discontinuation rates due to adverse events do not differ from conventional TP (16, 103). However, the main limitation of TG has been the risk of hepatotoxicity, especially the risk of nodular regenerative hyperplasia, which may be dose-related, since high doses of TG (40 mg/day) have been associated with liver injury (104), whereas studies using lower doses (20 mg/day) did not find a significant risk of this side effect and the efficacy was similar (99, 105–108). Furthermore, in another study the development of biopsy-diagnosed nodular regenerative hyperplasia was not associated with significant clinical consequences in most patients (109).

INDICATIONS OF THIOPURINES IN IBD IN THE BIOLOGIC ERA

Thiopurines as Monotherapy

Evidence in Crohn's Disease

TP as monotherapy seem to be inefficient for induction of clinical remission in active luminal CD and more recent clinical guidelines suggest against their use for this indication based on very low-quality evidence. Five placebo-controlled studies involving 380 patients have evaluated TP in this indication using validated outcomes measures (CDAI $<$ 150 or HBI $\leq$ 3). The use of concomitant steroids was allowed in four of them. The pooled analysis (intention-to-treat basis) showed no differences between TP and placebo for induction of remission (48 vs. 37%, RR= 1.23; 95% CI: 0.97–1.55) (110–114). Three trials have evaluated clinical response (not using validate outcomes measures) and no differences were found between TP and placebo (RR= 1.87, 95% CI: 0.44–7.96). Heterogeneity was serious in this case (I^2 = 69%) and imprecision very serious due to sparse data and wide confidence intervals; being the quality of evidence very low for this outcome (115–117).

However, effectiveness of TP as maintenance treatment for steroid-dependent luminal CD has been consistently proven. A 2015 Cochrane systematic review, which included six trials published between 1971 and 2013, showed that AZA was superior to placebo for the maintenance of remission (73% of patients treated with AZA remained in remission compared to 62% of those who were treated with placebo, RR= 1.19; 95% CI: 1.05–1.34). Probably these figures underestimate the efficacy because the meta-analysis included studies with infra-therapeutic doses ($<$2 mg/kg/day), but also is important to note that the number of patients included was modest (489) and follow up was limited (6–18 months) (11). The effect of TP on fistula healing in complex perianal CD has been reported in RCT in very few patients and, therefore, there is no evidence that support its use as monotherapy in this scenario. Very interesting is a recent large-scale study whose results suggest a re-evaluation of the place for TP monotherapy in the maintenance treatment algorithm in CD. *Stournaras et al.* study assessed the long-term effectiveness of TP monotherapy with the intention of maintaining medically induced remission in 11,928 patients (4,968 UC, 6,960 CD). TP were effective, without the need for escalation to biologic therapy

TABLE 2 | Thiopurine monitoring based on metabolites.

6-TGN	6-MMP	Cause	Consequences	Recommendation
Normal or high	Low	Therapeutic dose	Control of disease activity	Continue therapy
		Refractoriness if absence of response	No control of disease activity	Change therapy
High	High	Overdose	Myelotoxicity Hepatotoxicity	Reduce dose
		Refractoriness if absence of response	No control of disease activity	Change therapy
High	Low	Low TPMT activity	Risk of myelotoxicity	Reduce dose
		Refractoriness if absence of response	No control of disease activity	Change therapy
		Response	Control of disease activity	Continue monitoring
Low	High	Hypermethylators	No control of disease activity Hepatotoxicity	Reduce dose (25–50%) and add allopurinol
Low	Low	Underdose	No control of disease activity	Increase dose
		No compliance		Assess adherence

6-TGN levels: low (<230–235 pmol/8 × 10^8), high (> 450 pmol/8 × 10^8), normal (230–450 pmol/8 × 10^8).
6-MMP levels: high (>5,700 pmol/8 × 10^8), low (<5,700 pmol/8 × 10^8).

or need for surgery, in both UC and CD, but its efficacy was significantly lower in CD patients than in UC patients (34.2 vs. 52.7%) (118). The data summarized by Verstockt et al. on the Leuven hospital experience are less hopeful. Among 780 patients included with CD, only a small proportion of patients (7.5%, 59 patients) continued TP monotherapy till final follow-up (median of 13 years), suggesting that even in this widely accepted indication its long-term role is limited (119).

Another common use of TP is prevention of post-surgical relapse in CD. There is moderate certainty evidence that AZA and MP are superior to placebo. According to a very recent systematic review and meta-analysis (2019), after a follow-up of 12–36 months, 51% of patients treated with AZA/MP relapsed compared to 64% of patients with placebo (RR= 0.79; 95% CI 0.67–0.92; 408 participants; three studies; IR = 0%). Compared to anti-TNF drugs, TP seem to be inferior in this scenario but quality of evidence is very low. Cochrane review shows that after a follow-up of 12–24 months, 43% of participants treated with AZA clinically relapsed vs. 14% of patients in the anti-TNF group (RR= 2.89, 95% CI 1.50–5.57, 139 participants, three studies, IR = 0%) (120).

Finally, a question that has been raised in the last decade is if the early introduction of TP could modify disease course. Two studies have evaluated this point: the AZTEC and the RAPID trials (8, 121). RAPID trial compared early AZA use to classical step-up therapy in patients with risk factors for serious CD, and the AZTEC trial compared AZA with placebo up to week 76 at inducing sustained steroid-free remission in recently diagnosed uncomplicated CD. Both studies showed no effect of early AZA, which seems to argue against its early use. However, there are some caveats, including discrepancies in disease severity between groups and outcome definitions. Interestingly, in the RAPID trial, early AZA was associated with a significant reduction in new perianal fistula and a *post hoc* analysis of the AZTEC showed significantly lower rate of moderate to severe CD relapse with early AZA therapy (12 vs. 30%). Hence, the data are not completely clear on the effects of the timing of TP initiation, but delaying initiation until irreversible complications is unlikely to maximize their benefit.

Evidence in Ulcerative Colitis

In a meta-analysis that compared TP with placebo and/or salicylates in induction of remission in UC flare, differences were not found (122). Conversely, some observational studies have reported remission rates up to 65% (CI 95%: 55–75%) which suggest a possible efficacy in this indication. There is solid evidence about the fact that TP requires a minimum of time to obtain efficacy (at least 1 month, in most cases more than two). Because of that, such a long latency is not acceptable when patients have a flare; current guidelines do not recommend the use of TP monotherapy as inductors of remission in UC flare (123).

Maintenance of clinical remission after a mild/moderate flare in patients with steroid-dependent/steroid-resistant UC is one of the main indications of TP. Its efficacy in this scenario has been evaluated by two meta-analysis. Gisbert et al. meta-analysis reported 60% of efficacy in controlled trials with a NNT of 5 (6 RCT included) and a 76% of efficacy in uncontrolled studies (overall OR = 2.56, 95% CI: 1.51–5.3) (122). Cochrane Institute meta-analysis that included 4 RCT with 232 patients concluded that patients treated with AZA have a lower rate of failure compared to placebo (44 vs. 65%, respectively, RR = 0.68; IC 95%: 0.54–0.86) (12). It is necessary to mention the only high quality randomized controlled trial available that compared AZA and mesalazine in steroid-dependent patients, showing that AZA is significantly more effective (53 vs. 21%; OR = 4.78; 95% CI: 1.57–14.5) to induce clinical and endoscopic remission and to avoid steroid requirements in the first 6 months after the flare than mesalazine (124). Additionally, probably the efficacy of TP in this trial is underestimated because it lasted only 6 months, and efficacy would not be seen in some slow TP responders. In summary, numerous observational good quality studies that include many patients followed during very long periods confirm that TP are globally effective in UC, even more than in CD (118). However, the adverse effects of TP and the efficacy and safety

of mesalazine make it the choice in many patients. In steroid-dependent patients, the superiority of TP is obvious. Also, we want to mention the first RCT that compared the efficacy of infliximab monotherapy, AZA monotherapy and combination of both drugs for UC (SUCCESS trial) (15). *Panaccione et al.* showed that in anti-TNF naïve patients with moderate-severe UC, the rates of steroid-free remission were significantly higher in patients with combination therapy than either agent alone (combo 39.7% vs. infliximab 22% vs AZA 23%). Mucosal healing at week 16 was also significantly higher in combo group (62.8%) and infliximab alone group (54.6%) than in patients receiving AZA alone (36.8%).

Regarding the use of TP as maintenance treatment after severe flare, if patients were on TP treatment when severe flare occurs, subsequent maintenance with TP monotherapy after remission is very ineffective. In naïve TP patients, TP may reduce the rate of colectomy in the mid-term after severe UC flare controlled with intravenous cyclosporine, but the rate of colectomy remains very high (at least 33% a year) (125). Because of that, a more aggressive strategy by using anti-TNF drugs is more adequate to reduce the rate of colectomies to a maximum.

Evidence About TP Monotherapy Withdrawal

Whether TP can be safely interrupted in patients after achieving deep remission is a challenging question in daily practice. Patients and physicians have concerns about the long-term safety of these drugs. Seven RCT, three with placebo controlled, assessed the rate of relapse after immunomodulator withdrawal compared to continued therapy (126), but the total number of patients included in these trials was low (334 patients with CD and 67 with UC) and the follow-up period not very long (ranged from 10 months to 2 years). In the single study of UC patients, there were not significant differences between both strategies. However, a recent meta-analysis of these trials shows a significantly higher relapse rate after stopping immunomodulators compared to ongoing therapy (RR= 1.85, 95% CI: 1.44–2.38, $P < 0.001$, without between-study heterogeneity). In addition, at least three observational studies have analyzed this item. Relapse rates were also higher after withdrawal TP monotherapy (126). It should be noted that many of the studies on TP withdrawal are prior to biological era when alternatives were almost non-existent. In short, although evidence suggests that the relapse rates after TP monotherapy withdrawal is higher, the question that arises is whether it compensates with the long-term toxicity that they can cause in some patients. The most feared adverse event of TP is the occurrence of a lymphoma. The absolute risk is extremely low, but the risk at 2.5-fold and its result is devastating (73). Moreover, there are other risks associated with TP use undoubtedly more common as non-melanoma skin cancers. Interestingly, the risk seems to be proportional to duration of use and decreases on cessation of TP (127). Therefore, a balance of risk-benefit must be carried out individually with each patient, especially since there are other alternatives that are safer in the long-term and more effective, although more expensive. It is important not to forget that recent reports suggest that the risk of lymphoma is comparable for TP and anti-TNF drugs. Embarking on TP treatment is a long journey and clinicians should discuss with patients and decided on a case-by-case basis. If there is one undoubted thing, it is that regular monitoring should be provided to both, in patients continuing TP in the long term and in patients after TP withdrawal.

Authors Comments

Sometimes it is difficult to interpret the available evidence and to apply it to a specific patient. IBD specialists have been using TP for over 50 years and scientific evidence have demonstrated TP efficacy and effectiveness in the maintenance treatment of both CD and UC. In fact, population-based long-term observational studies (118) suggest that many patients may benefit from these drugs. However, TP are applicable only in a proportion of patients, because around a 25% of them have a limiting toxicity that prevents their use. Furthermore, they are only effective in a variable proportion of those who tolerate them. In fact, after a while only a small proportion of the patients, in whom they have been used, continue to be treated with TP. In addition, the risk of toxicity is real and potentially serious, including the possibility of hematological and cutaneous neoplasms. This risk does not disappear over time, and it can affect especially patients over 60 years of age, which in the immediate future will be a very important proportion in IBD patients (128). On the other hand, we have more and more alternatives, that although they are more expensive, present fewer risks than TP. Therefore, although there is still a group of patients in which TP monotherapy is a good option, it seems that TP role is going to become more and more limited, especially if the price of the alternatives decreases.

Combination Therapy (TP Plus Biologics)

Evidence

Post hoc analysis of initial registration trials did not show differences in outcomes stratified by baseline TP treatment. However, in 2010 was published the SONIC trial which included 508 patients naïve to both anti-TNF drugs and TP with moderate to severe CD. Results of this trial showed the superiority of combination treatment (TP plus infliximab) compared to infliximab or AZA monotherapy in achieving steroid free clinical remission (56.8% vs. 44.4 and 30%, respectively) and mucosal healing (43.9% vs. 30 and 16.5%, respectively) at week 26 (9). The rates of adverse events were similar in the three arms and rather, there were significantly lower rates of serious adverse events in those patients that received combination therapy (RR= 0.56; 95% CI: 0.32–0.97). Later, the subsequent UC SUCCESS trial employed a similar design than SONIC trial but in naïve UC patients. Results of UC SUCCESS trial were also in favor of combination therapy. Combo-therapy was more effective than either agent alone in inducing clinical remission at week 16 and more effective than AZA monotherapy in reaching mucosal healing (62.8 vs. 36.8%, $p = 0.001$). However, there was no significant difference in the rates of mucosal healing observed with combination therapy vs. infliximab monotherapy (62.8 vs. 54.6%; $p = 0.295$) (15). In both trials, AZA co-therapy dramatically reduced the formation of anti-infliximab antibodies (in SONIC 0.9 vs. 14.6% and in SUCCESS 3 vs. 19%). SONIC trial also showed an increase in IFX median trough concentrations at week 30 in the combination arm (3.5

vs. 1.6 micgr/ml, $p < 0.001$). Conversely, for adalimumab and azathioprine, the DIAMOND trial (only 176 patients with CD) showed rates of clinical remission similar between monotherapy and combination therapy and although the rate of mucosa healing at 26 weeks was superior in combo group, this benefit was not sustained at 1 year (129). Of note, the dose of AZA used in this trial was lower than the usual dose used in CD patients (25–100 mg/day instead of 2–2.5 mg/kg/day).

Based on pharmacokinetic data of these trials the hypothesis emerged that infliximab is more immunogenic than adalimumab and the addition of immunosuppressive therapy confers more benefit what was reflected as higher drugs level. This hypothesis was supported by the prospective observational UK PANTS study that showed formation of anti-drug antibody was more frequent with infliximab than with adalimumab and was decreased by combination therapy (immunomodulator drug and anti-TNF) (130). Curiously, although the absolute risk of anti-drug antibody was lower with adalimumab, the relative risk reduction with the concomitant use of immunosuppression was similar for both anti-TNF drugs. Combination therapy has also been shown to raise adalimumab levels, which itself is associated with higher rates of clinical and endoscopic remission. Notably, last year (2020) was published the work of *Targownik and colleagues* that included 11,244 Canadian patients and used data from four population level health care databases (131). Authors showed that use of a concomitant immunomodulator (TP or methotrexate) at the time of anti-TNF initiation (infliximab or adalimumab) was associated with significantly reduction in the likelihood of treatment failure. The choice of immunomodulator did not show a significant effect in CD, but better outcomes were seen with AZA than methotrexate in UC. This study supports that benefits of combination therapy seen in RCT seem to extend to the real-world setting. It also supports the idea that adding an immunomodulator in adalimumab initiation can improve clinical outcome in the medium and long term.

Another commonly encountered scenario in clinical practice is patients who have failed or have had an inadequate response to TP and in whom anti-TNF therapy is started. No RCT has directly compared whether in such cases TP maintenance in combination with the anti-TNF would carry additional benefits in terms of efficacy. A *post-hoc* analysis of RCTs has shown no added benefit (132). However, immunogenicity should be considered and, in the absence of direct evidence, an individualized approach should be considered.

A new role of TP may be in case of switching anti-TNF. The addition of a TP is an effective method of managing secondary loss of response. A large retrospective study (2017) showed that the addition of an immunomodulator resulted in disappearance of antidrug antibody in 77% of patients with a subsequent increased of drug concentration and recapture of clinical response (133). Current reactive therapeutic drug monitoring-based algorithms propose that patients with secondary loss of response to anti-TNF with high titer of antidrug antibody should switch to another anti-TNF agent (134). Current evidence shows that these patients have more risk of developing antibody and secondary loss of response to a subsequent anti-TNF (135, 136). This effect seems to be able to be mitigated by the addition of a TP as demonstrated Robblin et al. in their RCT (included 90 patients with immune-mediated loss of response to a first anti-TNF in monotherapy) (137).

Authors Comments

Evidence shows that combination therapy (TP plus anti-TNF) is superior to monotherapy in treating naïve CD and UC patients, mainly due to the effect of TP on immunogenicity but also partly due to an additive immunosuppressive effect. However, before starting combination therapy, a patient-stratified risk of combination therapy-related serious adverse events (special attention to the risk of lymphomas and cutaneous neoplasms with long-term therapies and infections) must be done and a regular monitoring should be provided to these patients. Emerging data suggest that an "optimize anti-TNF monotherapy" using proactive monitoring drug levels to ensure adequate circulating anti-TNF concentrations is associated with higher rates of clinical and endoscopic remission (138, 139). This strategy may obviate in the future the need for combination therapy, but a pragmatic trial comparing both strategies has not yet taken place. In any way, in patients in whom monotherapy is chosen, we think that a proactive drug monitoring strategy is advisable. Moreover, testing patients for HLA-DQA1 * 05, which according to recent evidence is associated with an increased risk of development of antibodies against anti-TNF drug, might help physicians decide if patients should be treated with anti-TNF alone or combination therapy (140).

Maintenance After Combination Therapy

When a patient starts combined treatment there is always a concern about how long the patients should take both treatments. Current clinical guidelines suggest maintenance with the same biologic agent in monotherapy after achieving remission with combination therapy (anti-TNF plus TP) (3, 123). There are conflicting data as to whether continuing TP beyond a certain time provides additional clinical benefit. One study has shown that, in most cases, immunization occurs during the first 12 months of anti-TNF, suggesting that to lengthen the combo-therapy further in many patients may be no necessary (141). A recent meta-analysis including a total of 186 patients with IBD in remission on combination therapy with TP plus anti-TNF (infliximab or adalimumab) analyzed the relapse rate after TP stopping (126). No statistically significant difference was observed between the groups (RR = 1.30, 95% CI: 0.81–2.08, $p = 0.269$; $I^2 = 0.0\%$, $p = 0.641$). Van Assche et al. suggested that AZA can be withdrawn after 6 months of remission on combination infliximab/AZA but many patients were on AZA for > 6 months prior to AZA withdrawal and continuation of combination therapy was associated with lower levels of serum C reactive protein and higher infliximab trough levels (142). In the study of Roblin et al., three strategies were compared: continuing with both treatments, stopping TP and decreasing TP dose (90). There were no clinical significantly differences between the strategies, but authors concluded that reducing the dose of TP was associated globally (considering levels of infliximab, antidrug antibodies and unfavorable evolution) with a better outcome

than treatment stopping. In combination therapy, a reduced dose of TP may reduce the production of neutralizing anti-TNF antibodies, thereby providing a lower chance of developing adverse events. Although TP withdrawal from combination regimen carries a higher risk of anti-drug antibody formation, their effect on clinical outcomes may take longer than a year to become apparent. Meta-analysis of nine studies on adalimumab by Chalhoub et al. (after data included were re-analyzed) did not reveal any differences in maintenance clinical remission (RR= 1.01, 95% CI: 0.91–1.13) between combo and monotherapy (3, 143). In the ADHERE cohort, an open label extension study of patients included in CHARM study on adalimumab, the rates of clinical remission were similar in patients with and without concomitant immunomodulators at baseline after 3 year of follow up (144). In addition, several observational studies have investigated the risk of relapse in IBD patients who received combination treatment followed by discontinuation of immunomodulator. Two French retrospective observational studies have documented fewer flares, fewer perianal complications and fewer switching with combination therapy for > 2 years (145, 146). Two studies of CD and one of UC reported that a shorter duration of combo-treatment was associated with an increased risk of treatment failure after withdrawal of TP. The thresholds were 27, 6 and 9 months, respectively (145, 147, 148). Another recent observational study also shows that long combo-therapy (>12 months) was not more efficacious than short combination (149).

Authors Comments

TP withdrawal from combination with biologics after clinical remission remains a preferred approach of long-term treatment to avoid toxicity, but balancing between adverse drug effects and disease progression is unavoidable in patients with severe inflammation and complications. In severe IBD, advantages of combination therapy can outweigh the risk of lymphoma and severe infection, but in patients with mild/moderate IBD, the risk/benefit ratio is clearly less favorable. Predictive factors of relapse and evidence of deep remission should be included in the risk/benefit analysis prior to therapy withdrawal. The need for a RCT to facilitate decision making about the exact timing and optimal group of patients to discontinue therapy is clear. Recently, SPARE trial, that addresses this issue, has been completed and its results will be known soon.

CONCLUSIONS

Thiopurines are still useful for maintenance of remission in steroid-dependence, prevent post-surgical relapse and improve the outcomes of biologic therapy. The key is to select patients properly based on personal characteristics and the course of the disease. Moreover, as explained above, many strategies are available to improve the efficacy of these drugs and to prevent adverse events.

AUTHOR CONTRIBUTIONS

FG conceived the idea of the review, coordinated, supervised, and edited the manuscript. CG-P and VL performed the literature review and wrote the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by the Institute for Health Research Aragón, IIS Aragón.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Medicine

ORIGINAL RESEARCH
published: 26 August 2021
doi: 10.3389/fmed.2021.663920

Association of Serum Neuron-Specific Enolase and C-Reactive Protein With Disease Location and Endoscopic Inflammation Degree in Patients With Crohn's Disease

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Specialty section:
This article was submitted to Gastroenterology, a section of the journal Frontiers in Medicine

Received: *04 February 2021*
Accepted: *26 July 2021*
Published: *26 August 2021*

Citation:
Yang R-X, Song W-J, Wu Z-Q, Goyal H and Xu H-G (2021) Association of Serum Neuron-Specific Enolase and C-Reactive Protein With Disease Location and Endoscopic Inflammation Degree in Patients With Crohn's Disease. Front. Med. 8:663920. doi: 10.3389/fmed.2021.663920

Objective: The objective of this study was to explore the association between serum markers neuron-specific enolase (NSE) and C-reactive protein (CRP) with intestinal lesion location and degree of inflammation in patients with Crohn's disease (CD).

Design: The levels of serum NSE, CRP, and fecal calprotectin (FC) in patients with CD were analyzed retrospectively. The severity of inflammatory lesions in the intestinal wall was accessed using the Simple Endoscopic Score for Crohn's disease (SES-CD).

Results: The levels of NSE in patients with CD were higher than those of healthy individuals (14.87 vs. 12.68 ng/ml, $P < 0.001$). The levels of CRP in patients with CD were higher than those of healthy individuals (12.30 vs. 3.40 mg/l, $P < 0.001$). The FC levels in patients with CD were higher than those of patients with non-inflammatory bowel disease (1,143.90 vs. 114.21 μg/g, $P < 0.05$). The levels of NSE in CD with ileal lesions and simultaneous ileal and colon lesions were significantly higher than those in patients with CD with colonic lesions. However, the CRP was higher in patients with colonic lesions than those with ileal lesions. The levels of NSE in patients with severe inflammation were higher than those in patients with moderate inflammation (15.95 vs. 13.89 ng/ml, $P < 0.05$). Similarly, the NSE levels in patients with CD with severe inflammation were higher than those in patients with CD with mild inflammation (15.95 vs. 13.53 ng/mL, $P < 0.05$). The levels of CRP in severe inflammation were higher than those in moderate inflammation (29.80 vs. 19.60 mg/l, $P < 0.05$). In addition, the CRP levels in severe inflammation were higher than those in mild inflammation (29.80 vs. 5.86 mg/l, $P < 0.05$). ROC curve analysis showed that when NSE was combined with CRP for distinguishing between patients with CD and those without CD, sensitivity increased to 80.41%, specificity increased to 74.66%, and a highest AUC was equal to 0.843.

Conclusion: Our study shows that serum NSE and CRP can be used to assess the severity of CD as well as the location of intestinal involvement. Therefore, NSE and CRP could be used as the non-invasive tests in detecting the location and severity of disease in patients with CD in daily routine practice.

Keywords: NSE, CRP, crohn's disease, IBD, diagnosis

INTRODUCTION

Crohn's disease (CD) is a chronic inflammatory condition with transmural involvement of the gastrointestinal tract (GIT), a form of inflammatory bowel disease (IBD) (1, 2). CD can involve ileal (30%), ileocolonic (40%), and colon (30%) cases, unlike ulcerative colitis (UC) (3). Chronic active inflammation can lead to recurrent ulcers, intestinal stenosis, intestinal perforation, abdominal abscess, anal fistula, and colorectal cancer (4–6). Endoscopy is currently the main method to diagnose and estimate the disease severity, but a combination of clinical, laboratorial, endoscopic, and histological and sometimes radiological methods is required.

Multiple biomarkers such as serum calprotectin, albumin, and C-reactive protein (CRP) have been studied to estimate the disease severity in CD (7). Many studies have recently shown that fecal calprotectin (FC) can be used as a non-invasive biomarker of disease activity (8–10). However, FC might not be increased in patients with CD who predominantly have ileal involvement (11). In addition, the pretreatment process of stool samples is complicated, which will lead to low test repeatability.

Neuron-specific enolase (NSE) is a specific neuronal marker of differentiation. NSE is a protein localized in the cytoplasm of the neurons, red blood cells and platelets, etc. (12). It is involved in the glycolytic energy metabolism in the neurons and is often released from neurons as a response to injury. The levels of NSE have been found to be elevated in the many conditions including traumatic brain injury, post-cardiac arrest, and lung cancer. In fact, NSE is currently the most reliable tumor biomarker in the diagnosis, prognosis, and follow-up of small cell lung cancer (13). Busikova-Malenovska et al. (14) reported the localization of NSE in the neurons of the intestinal wall of five patients with CD using monoclonal antibodies against human-NSE. Here, we aimed to investigate the levels of NSE in patients with CD and further to evaluate their application in the diagnosis and disease severity.

CRP is an acute-phase biomarker with increased levels in some inflammatory conditions. CRP has also been used to differentiate functional bowel disorders such as irritable bowel syndrome from CD (15). However, studies have produced inconsistent results regarding the elevation in CRP levels in patients with active CD. Florin et al. found that 10% of active patients with CD (defined by CDAI >200) had normal CRP values. They further analyzed that patients with active disease but normal CRP levels had significantly more isolated ileal disease (16). No patients in active colonic CD had normal CRP levels. Another study found that elevated high-sensitivity CRP (hs-CRP) was associated with ileal (43.2%), colonic (70%), and ileocolonic (72.6%) diseases. The mean hs-CRP was also significantly higher in ileocolonic/colonic when compared to ileal disease (17). However, other studies did not find a significant association between CRP and disease location (18, 19).

In the present study, we have retrospectively explored the relationship of serum biomarkers NSE and CRP with the CD intestinal lesion location and degree of inflammation in an effort to provide a basis for their use in the diagnosis and treatment of CD.

MATERIALS AND METHODS

Ethical Consideration

Ethics approval was obtained from the ethics committee of the First Affiliated Hospital of Nanjing Medical University (Protocol # 2020-SR-010, 23 March 2020). The study protocol conforms to the ethical guidelines of the 2013 Declaration of Helsinki as reflected in a prior approval by the institution's human research committee. Due to the retrospective nature of the study, informed consent was waived.

Study Populations

The study cohort consisted of newly diagnosed patients with CD who were followed up at the First Affiliated Hospital of Nanjing Medical University between January 1, 2013, and October 31, 2018. The CD was diagnosed on the basis of patients' clinical manifestations, abdominal imaging, endoscopy findings, and pathology results. Exclusion criteria for both patients and healthy people included (1) those who take non-steroidal anti-inflammatory drugs and proton pump inhibitors, (2) alcoholics, (3) people with autoimmune diseases, (4) those who had a history of gastrointestinal surgery, (5) those with a history of liver and kidney diseases and a history of cardiovascular and cerebrovascular diseases, (6) pregnant women, and (7) cancer patients.

Description of Variables

Patients' information including demographics, clinical presentation, duration of the disease, disease location, presence of perianal lesions, and serum levels of NSE were collected. The severity of inflammatory lesions in the intestinal wall was accessed using the Simple Endoscopic Score for Crohn's Disease (SES-CD) (20).

Collection of Samples and Test Interpretation

A fasting (for 12–14 h) venous blood sample (around 3 ml) was collected from the subjects in vacuum inert separation gel conduit on the day before endoscopy. The upper serum

was collected after centrifugation for 5 min at 3,000 r/min (centrifugal radius 17.49 cm). The CRP levels were assessed using a BN-II-specific protein analyzer and its matching reagents. The NSE levels were assessed using a Roche cobas e 602 electrochemiluminescence immunossay instrument and its matching reagents; the intra-/inter-assay CVs for NSE measurements were very small. All tests were strictly operated according to the instrument and reagent instructions. Each batch of tests was tested for low-value and high-value quality control. The FC was assessed with the standard enzyme-linked immunosorbent assay (ELISA) method produced by Bühlmann, Switzerland.

Statistical Analysis

The IBM SPSS 27.0 and R version 3.6.3 software were used for the statistical analysis. The skewness coefficient and kurtosis coefficient are calculated to judge the distribution of data. The continuous variables which were non-normally distributed were expressed as the median and range. Differences in characteristics between groups were analyzed using the Kruskal–Wallis test with Dunn *post hoc* tests (for continuous variables, R package FSA), and the Bonferroni-adjusted *P*-value was reported. Dunn's Kruskal–Wallis multiple comparisons and *P*-values adjusted with the Bonferroni method were used for the comparison between the two groups. The receiver operating characteristic (ROC) curve was established to evaluate the NSE and/or CRP levels to distinguish the patients with and without CD. The ROC curves were plotted using the "pROC" package by R. In this analysis, patients with (case, encoded as 1) and without (control, encoded as 0) CD were considered as response and the NSE and/or CRP levels were predictors. A binormal smoothing was performed as the ROC approximation method. To analyze the relationship between different inflammation degrees, different lesion locations, NSE, and CRP, univariate multivariate multinomial logit regression analysis was performed. In this model, the different inflammation degrees and different lesion locations were considered as dependent variables, while the levels of NSE and CRP were considered as independent variables.

RESULTS

Patients

From January 2013 to December 2018, a total of 291 confirmed cases of CD were included in the study. The control group consisted of 292 healthy people and 64 patients with non-inflammatory bowel disease (6 patients with intestinal obstruction, 16 patients with irritable bowel syndrome, 11 patients with intestinal polyps, and 31 patients without gastrointestinal disease). Two hundred and ninety-one patients with CD comprising 182 males aged 14–81 years (mean age 32.14 ± 13.27 years) and 109 females 14–77 years of age (mean age 43.25 ± 13.59 years) were included in the analysis. The data for 292 healthy individuals (182 males, mean age 33.45 ± 8.76 years and 110 females, mean age 38.72 ± 13.44 years) were collected. We also collected data for 64 patients with non-inflammatory bowel disease comprising 29 males (mean age 50.86 ± 18.90 years) and 35 females (mean age 51.46 ± 18.98 years) as the control during the same period. The main characteristics of the patients with CD are summarized in **Table 1**, as per the Montreal classification. The two groups did not show any significant differences with respect to age and gender.

TABLE 1 | Baseline characteristics of patients with CD at the time of endoscopy.

	Overall cohort (*n* = 291)
Sex (M/F)	182/109
Age (years), mean (range)	36.3 (14–81)
Weight change, *N* (%)	
Without weight change	204 (70)
Weight loss	87 (30)
Symptoms, *N* (%)	
Diarrhea only	51 (17)
Abdominal pain only	98 (34)
Diarrhea and abdominal pain	111 (38)
Others	31 (11)
Age (years), *N* (%)	
A1	7 (2.4)
A2	178 (61.2)
A3	106 (36.4)
Disease behavior, *N* (%)	
B1	113 (39)
B2	65 (22)
B3	113 (39)
CD location, *N* (%)	
L1	104 (36)
L2	56 (19)
L3	117 (40)
L4	14 (5)
History of perianal lesions, *N* (%)	102 (35)
Crissum abscess	50 (17)
Anal fistula	34 (12)
Others	18 (6)

CD, Crohn's disease; F, female; M, male.

The average CV for NSE measurements was 3.1% in 2018. A total of 245 patients had levels of serum CRP, and 53 patients underwent testing for FC at the time of admission. The NSE levels in patients with CD were higher than those in healthy individuals [14.87(6.10–51.66) ng/ml vs. 12.68(3.73–25.20) ng/ml] ($Z = -7.030$, $P < 0.001$). The CRP levels in patients with CD were higher than those in healthy individuals [12.30(0.16–112.00) mg/l vs. 3.40(0.22–24.80) mg/l] ($Z = -8.429$, $P < 0.001$). The FC levels in patients with CD were higher than those in patients with non-inflammatory bowel disease [1143.90(16.32–3403.64) μg/g vs. 114.21(3.15–5497.78) μg/g] ($Z = -2.245$, $P < 0.05$).

Levels of NSE and CRP Based on the Intestinal Location of Crohn's Disease

Levels of NSE in CD with ileal lesions were higher than those in patients with CD with colon lesions [15.48(6.10–31.00) ng/ml vs. 12.61(7.10–26.95) ng/ml] ($Z = -3.103$, $P = 0.012$). The NSE levels in patients with ileocolonic lesions were higher than

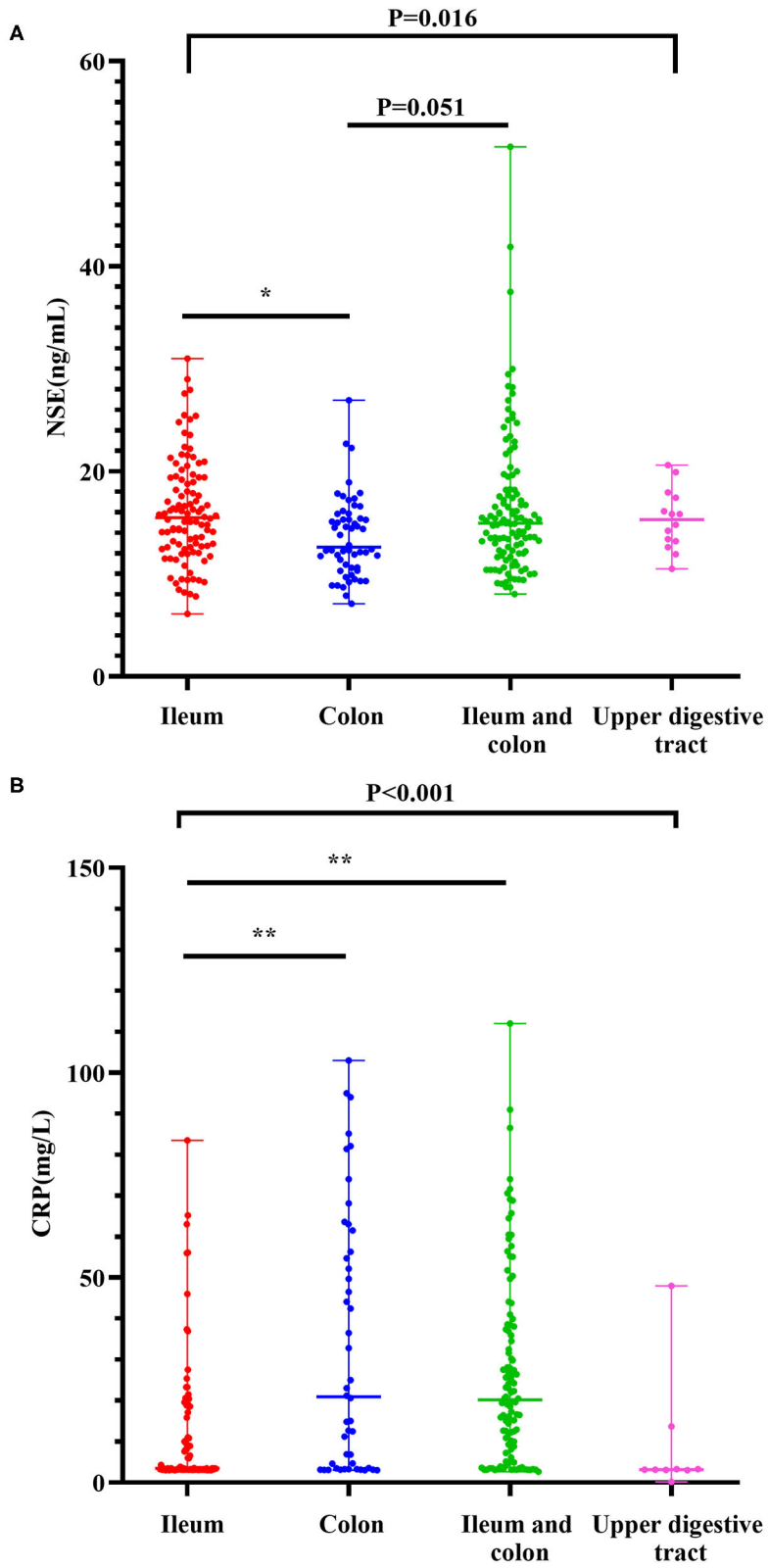

FIGURE 1 | Levels of NSE as per the phenotype of Crohn's disease **(A)**. Levels of CRP as per the phenotype of Crohn's disease **(B)**. The boxplots show median, upper, and lower quartiles of the data; the whiskers indicate the 95% confidence interval of the values (*$P < 0.05$ and **$P < 0.001$).

TABLE 2 | Association between different inflammation degrees, different lesion locations and NSE and CRP levels.

		log2(NSE)		log2(CRP)	
		OR (95% CI)	***P***	**OR (95% CI)**	***P***
Lesion location					
	Colon/ileal lesion	0.32 (0.15–0.69)	<0.001	1.70 (1.34–2.16)	<0.001
	Ileocolonic lesion/ileal lesion	1.01 (0.57–1.79)	0.96	1.70 (1.40–2.07)	<0.001
	Upper gastrointestinal lesion/ileal lesion	0.90 (0.27–3.04)	0.87	0.64 (0.36–1.11)	0.11
Degree of inflammation					
	Moderate inflammation/mild inflammation	1.35 (0.50–3.63)	0.55	1.28 (0.95–1.72)	0.10
	Severe inflammation/mild inflammation	3.57 (1.36–9.39)	0.01	1.59 (1.17–2.17)	<0.001

those with only colonic lesions [14.94(8.04–51.66) ng/ml vs. 12.61(7.10–26.95) ng/mL] ($Z = -2.620$, $P = 0.051$) (**Figure 1A**). The result of the multinomial logit model showed that between colonic lesions in patients with CD and ileal lesions in patients with CD, the increased NSE level was more common in patients with ileal lesions (OR = 0.32, $P < 0.001$), see in **Table 2**.

The CRP levels in colonic patients with CD were higher than those in ileal patients with CD [20.90(3.01–103.00) mg/l vs. 3.44(3.01–83.50) mg/l] ($Z = -4.251$, $P < 0.001$). In addition, the levels of CRP in ileocolonic patients with CD were higher than those in ileal patients with CD [20.15(2.66–112.00) mg/l vs. 3.44(3.01–83.50) mg/l] ($Z = -5.577$, $P < 0.001$) (**Figure 1B**). The results of the multinomial logit model showed that between colonic lesions in patients with CD and ileal lesions in patients with CD, the increased CRP level was more common in patients with colon lesions (OR = 1.70, $P < 0.001$); between ileocolonic lesions in patients with CD and ileal lesions in patients with CD, the increased CRP level was more common in patients with ileocolonic lesions (OR = 1.70, $P < 0.001$) (see **Table 2**).

The Association of the Levels of NSE and CRP With SES-CD

The levels of NSE in patients with severe inflammation were higher than in patients with moderate inflammation [15.95(8.04–51.66) ng/ml vs. 13.89(6.10–41.90) ng/ml] ($Z = -3.068$, $P = 0.006$). Similarly, the levels of NSE in patients with CD with severe inflammation were higher than those in patients with mild inflammation [15.95(8.04–51.66) ng/ml vs. 13.53(7.80–23.77) ng/ml] ($Z = -2.590$, $P = 0.029$) (see **Figure 2A**). In addition, the results of the multinomial logit model showed that between patients with CD with severe inflammation and patients with CD with mild inflammation, the increased NSE level was more common in patients with severe inflammation (OR = 3.57, $P < 0.05$), see in **Table 2**.

The levels of CRP in severe inflammation were higher than those in moderate inflammation [29.80(3.01–103.00) mg/l vs. 19.60(2.66–91.00) mg/l] ($Z = -1.942$, $P = 0.156$). The levels of CRP in severe inflammation were higher than those in mild inflammation [29.80(3.01–103.00) mg/l vs. 5.86(3.10–112.00) mg/l] ($Z = -2.916$, $P = 0.011$) (see in **Figure 2B**). The results of the multinomial logit model showed that between patients with CD with severe inflammation and patients with CD with mild inflammation, the increased CRP level was more common in patients with severe inflammation (OR = 1.59, $P < 0.001$) (see in **Table 2**).

Receiver-Operating Characteristic Analysis

Using ROC analysis, the optimal cutoff point for the serum NSE level in order to distinguish patients with CD from those without CD was 15.06 ng/ml, with a sensitivity of 48.98%, a specificity of 82.88%, and the highest area under the curve (AUC) equal to 0.676 (0.629–0.723, $P < 0.001$). The optimal cutoff point for the serum CRP level in order to distinguish patients with CD from those without CD was 7.93 mg/l, with a sensitivity of 56.73%, a specificity of 89.73%, and a highest AUC equal to 0.711 (0.664–0.758, $P < 0.001$). The optimal cutoff point for FC in order to distinguish patients with CD from those without CD was 752.40 μg/g, with a sensitivity of 73.58%, a specificity of 79.69%, an accuracy of 76.92%, and the highest AUC equal to 0.785 (0.700–0.871, $P < 0.001$). However, if the current agreed standard normal value of FC (50.00 μg/g) is used as the cutoff point, the sensitivity was 88.68%, the specificity was 34.38%, and the accuracy was only 58.97%. ROC curve analysis showed that when NSE was combined with CRP for distinguishing between patients with CD and those without CD, sensitivity increased to 80.41%, specificity increased to 74.66%, and the highest AUC was equal to 0.843 (0.808–0.878, $P < 0.001$) (**Table 3**, **Figure 3**), and spearman rank correlation showed that there was no correlation between NSE and CRP($r = 0.025$, $P = 0.567$).

DISCUSSION

In this study, we found that levels of NSE and CRP in patients with CD were significantly higher than those of healthy people. Neuroproliferation has been shown to be a feature of CD with the use of NSE (21). However, our study is the first clinical study to evaluate the utility of NSE in daily routine practice in patients with CD. In addition, our study shows that high CRP levels could be useful as an adjunct in the diagnosis of CD, which is consistent with the results of most previous studies.

We further performed the analysis based on the intestinal location of the disease. We found that levels of NSE in patients

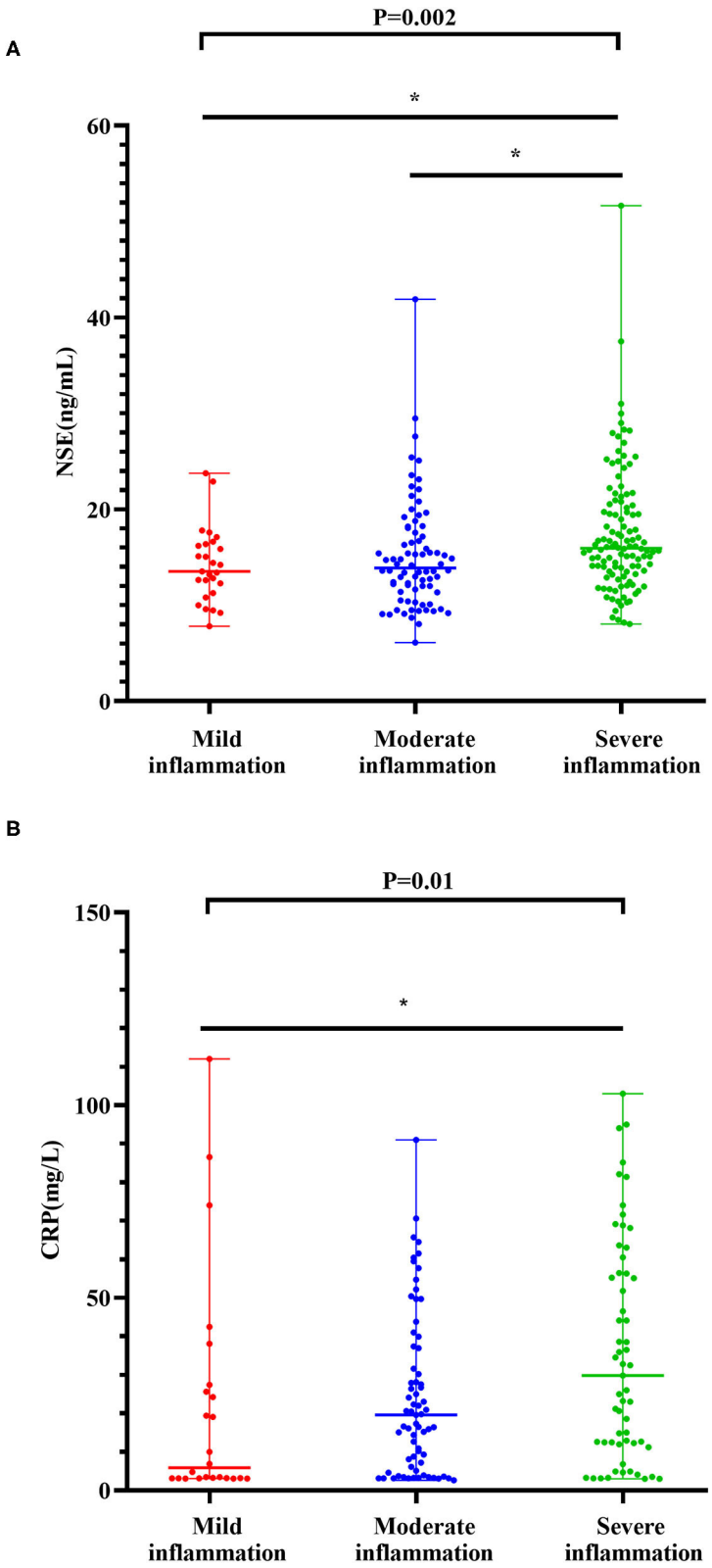

FIGURE 2 | Levels of NSE with degrees of inflammation in the CD cohort **(A)**. Levels of CRP with degrees of inflammation in the CD cohort **(B)**. The boxplots show median, upper, and lower quartiles of the data; the whiskers indicate the 95% confidence interval of the values (*$P < 0.05$ and **$P < 0.001$).

TABLE 3 | Sen, Spe, Acc, PPV, and NPV for serum NSE, CRP, and FC in discriminating between patients with CD and healthy people.

	Sen (%)	Spe (%)	Acc (%)	PPV (%)	NPV (%)
NSE (in ng/mL cutoff 15.06)	48.98	82.88	67.41	70.59	65.94
CRP (in mg/L cutoff 7.93)	56.73	89.73	74.67	82.25	71.20
FC (in μg/g cutoff 752.40)	73.58	79.69	76.92	75.00	78.46
NSE+CRP	80.41	74.66	77.28	72.69	81.95

CD, Crohn's disease; NSE, neuron-specific enolase; CRP, C-reactive protein; FC, fecal calprotectin; Sen, sensitivity; Spe, specificity; Acc, accuracy; PPV, positive predictive value; NPV, negative predictive value.

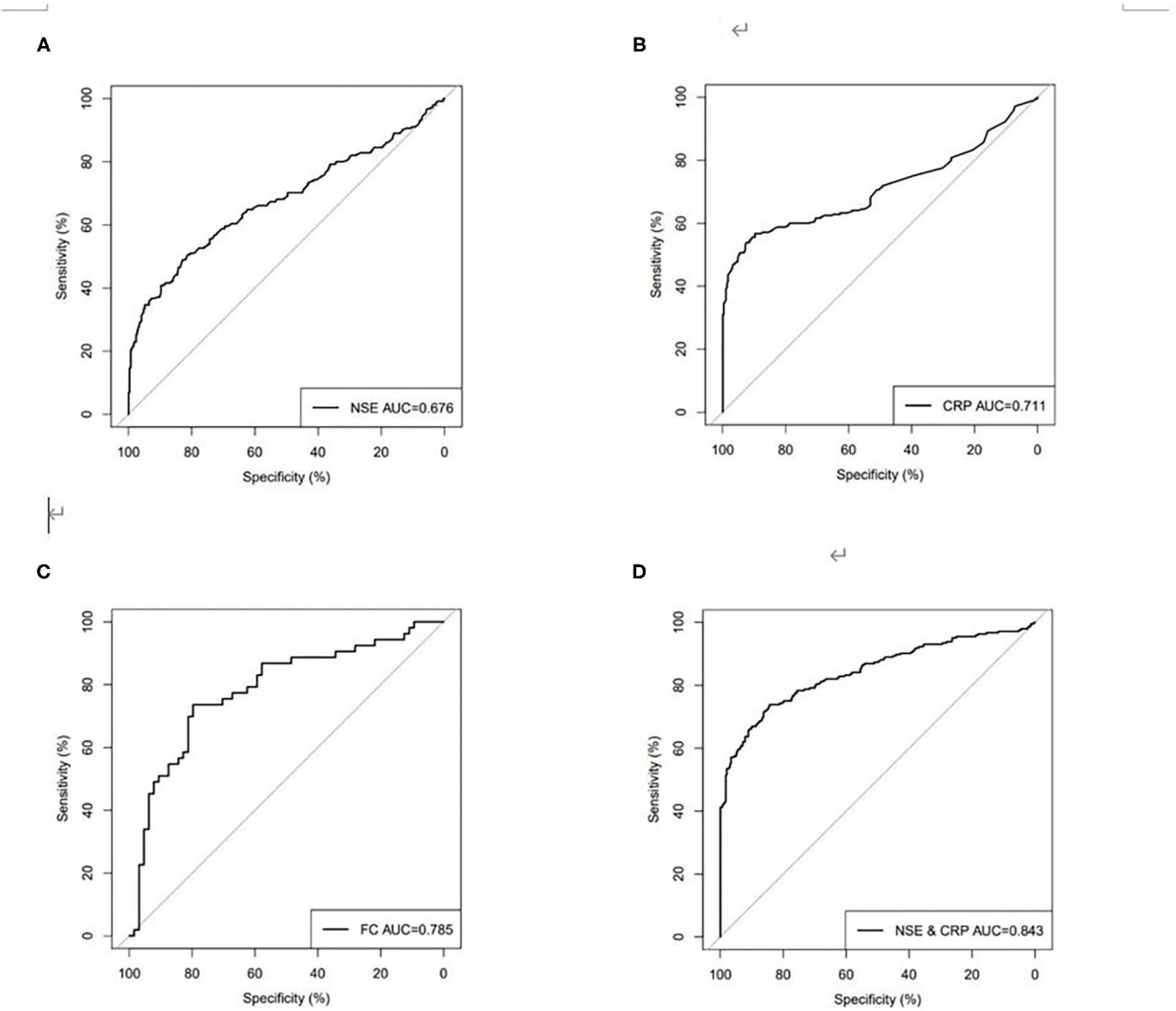

FIGURE 3 | Receiver operating characteristics (ROC) curves for **(A)** NSE, **(B)** CRP, **(C)** FC, **(D)** combined NSE and CRP for discriminating between patients with CD and healthy people.

with CD with ileal lesions were higher than those in patients with CD with colon lesions. NSE levels in patients with ileocolonic lesions were higher than those with only colonic lesions. Most studies have shown that serological markers such as CRP can aid in diagnosing CD and have refined the diagnosis to the site of the damage (22). The mechanism of the different concentrations of NSE in different damaged digestive tract sites is not well understood. We suspect that this difference may be due to the uneven distribution of intestinal neurons in the ileum and colon. Some relevant studies have revealed similar neuronal and

ganglionic densities and neurochemical profiles in the human distal colon and rectum (23). However, the comparison of neuronal and ganglionic densities and neurochemical profiles in the human ileum and colon have not been reported.

Bourgonje et al. (24) recently reported that inflammatory biomarkers [serum amyloid A (SAA), Eotaxin-1, IL-6, IL-8, IL-17A, and TNF-α] showed better prediction of IBD disease activity than routine measures (CRP, fecal calprotectin, and HBI/SCCAI scores) and demonstrated that the combination of SAA, IL-6, IL-8, and Eotaxin-1 could reliably predict endoscopic disease activity in IBD, which contributed to establishing an immunology-based prediction model for the endoscopic mucosal status in IBD. In our study, in addition to the abovementioned diagnostic significance of NSE and CRP in the different parts of the digestive tract, we also found that levels of NSE and CRP varied with the amount of inflammation in the intestines. Patients with CD with severe inflammation have higher levels of NSE and CRP than patients with moderate and mild inflammation. Therefore, NSE and CRP may be of added value as inflammatory biomarkers in monitoring disease activity in CD. Studies have shown that high CRP levels often indicate poor prognosis and increased incidence of complications in patients with CD (25). However, higher inflammatory biomarker levels were not observed in some patients who did not get symptomatic relief (26), which indicated that the degree of inflammation does not fully reflect the prognosis of the disease. Margolis et al. (27) found that the severity of intestinal inflammation is associated with the density of the enteric innervation in mice. The authors postulated that the abnormalities in enteric nervous system development might contribute to the pathogenesis of IBD. There have been a number of previous clinical reports of increased numbers of enteric neurons in the inflamed regions of the bowel in IBD (28, 29), as well as studies that have shown an association of neuronal genes (such as LRRK2 and Ninjurin2) with the increased risk of IBD (30, 31). However, it is not yet clear how enteric neuronal density affects the severity of intestinal inflammation. It has been postulated that effectors of both innate and acquired immunity express receptors for neurotransmitters/neuromodulators. Some immune cells express nicotinic receptor subunits and that nicotinic stimulation is anti-inflammatory, which probably accounts for intestinal anti-inflammatory properties of vagus nerve stimulation (32, 33). Moreover, this is the first study reporting the increase in the serum NSE concentration with an increase in intestinal inflammation in patients with CD, laying the foundation for use of NSE for CD diagnosis.

Determination of FC levels has been used as a good screening method in the diagnosis as well for the evaluation of acute exacerbation of CD (9). Calprotectin is a major protein found in the cytosol of inflammatory cells (34). FC is an effective biomarker in the diagnosis and treatment of patients with CD. However, the collection of fecal samples is not easy to standardize, which affects the detection results. Moreover, the detection process is cumbersome and time-consuming, and there are many influencing factors in the detection process, so the results vary quite greatly. Our results have shown that the combined use of NSE and CRP is comparable to FC on the accuracy of CD diagnosis. Moreover, the collection of blood samples is easy to be standardized, and the detection process of NSE and CRP is automated, so the detection results have better repeatability and reproducibility and easier to carry out in clinical practice.

We would also like to note that our study has a small sample size, an inherent shortcoming of retrospective cohort analysis. Not all patients in the CD cohort underwent serum NSE and CRP testing at the same time. Only 53 patients underwent FC testing, as this test was not available in our hospital before 2018. However, the results of our study are very important, as we have presented NSE as a potential marker for diagnosis of CD as well as its utility in the determination of the degree of intestinal inflammation. We have tried to expand the armamentarium of the previously available biomarkers that reflect the degree of inflammation.

Crohn's disease imposes a huge financial burden on the affected patients and requires early diagnosis and aggressive management to reduce complications. Although endoscopy is a traditional invasive method of evaluation of the diagnosis and monitoring of the disease, the majority of the patients can avoid the use of endoscopy with the help of effective biomarkers (35, 36). Therefore, strategies to select the patients with CD who would benefit most from endoscopy are of current interest. Serum biomarkers provide a convenient, rapid, and non-invasive method for clinical diagnosis and disease activity monitoring. Our study shows that serum NSE and CRP can be used to assess the severity of CD as well as the location of intestinal involvement. Therefore, NSE and CRP could be used as non-invasive tests to detect the location and severity of disease in patients with CD in daily routine practice. In addition, this study will pave the way for further prospective studies for NSE use as a non-invasive biomarker for the diagnosis and disease monitoring in patients with CD.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics approval was obtained from the ethics committee of the First Affiliated Hospital of Nanjing Medical University (Protocol # 2020-SR-010, 23 March 2020). The patients/participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

FUNDING

This study was supported by the Natural Science Foundation of Jiangsu Province of China (BK20181492), the National Key Clinical Department of Laboratory Medicine of China in Nanjing, the Key Laboratory for Laboratory Medicine of Jiangsu Province (ZDXKB2016005), and the Priority Academic Program Development of Jiangsu Higher Education Institutions.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Medicine

ORIGINAL RESEARCH
published: 27 August 2021
doi: 10.3389/fmed.2021.656493

A Convolutional Neural Network Deep Learning Model Trained on CD Ulcers Images Accurately Identifies NSAID Ulcers

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OPEN ACCESS

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Specialty section:
This article was submitted to Gastroenterology, a section of the journal Frontiers in Medicine

Received: 20 January 2021
Accepted: 10 August 2021
Published: 27 August 2021

Citation:
Klang E, Kopylov U, Mortensen B, Damholt A, Soffer S, Barash Y, Konen E, Grinman A, Yehuda RM, Buckley M, Shanahan F, Eliakim R and Ben-Horin S (2021) A Convolutional Neural Network Deep Learning Model Trained on CD Ulcers Images Accurately Identifies NSAID Ulcers. Front. Med. 8:656493. doi: 10.3389/fmed.2021.656493

Background and Study Aims: Deep learning (DL) for video capsule endoscopy (VCE) is an emerging research field. It has shown high accuracy for the detection of Crohn's disease (CD) ulcers. Non-steroidal anti-inflammatory drugs (NSAIDS) are commonly used medications. In the small bowel, NSAIDs may cause a variety of gastrointestinal adverse events including NSAID-induced ulcers. These ulcers are the most important differential diagnosis for small bowel ulcers in patients evaluated for suspected CD. We evaluated a DL network that was trained using CD VCE ulcer images and evaluated its performance for NSAID ulcers.

Patients and Methods: The network was trained using CD ulcers and normal mucosa from a large image bank created from VCE of diagnosed CD patients. NSAIDs-induced enteropathy images were extracted from the prospective Bifidobacterium breve (Blf95) trial dataset. All images were acquired from studies performed using PillCam SBIII. The area under the receiver operating curve (AUC) was used as a metric. We compared the network's AUC for detecting NSAID ulcers to that of detecting CD ulcers.

Results: Overall, the CD training dataset included 17,640 CE images. The NSAIDs testing dataset included 1,605 CE images. The DL network exhibited an AUC of 0.97 (95% CI 0.97–0.98) for identifying images with NSAID mucosal ulcers. The diagnostic accuracy was similar to that obtained for CD related ulcers (AUC 0.94–0.99).

Conclusions: A network trained on VCE CD ulcers similarly identified NSAID findings. As deep learning is transforming gastrointestinal endoscopy, this result should be taken into consideration in the future design and analysis of VCE deep learning applications.

Keywords: deep learning, capsule endoscopy, Crohn disease, anti-inflammatory agents, non-steroidal, NSAID

INTRODUCTION

Non-steroidal anti-inflammatory drugs (NSAIDS) are among the most commonly used medications in the world, very frequently purchased over the counter. Gastrointestinal adverse events are common with these medications and involve both upper and lower gastrointestinal tract (1). In the small bowel, NSAIDs may cause a variety of mucosal lesions, including mucosal breaks, mucosal edema, diaphragm-like strictures and ulcers that may resemble intestinal inflammatory lesions in patients with Crohn's disease (2). The prevalence of such small bowel abnormalities among NSAIDs users was reported to be as high as 75% in studies that utilized video capsule endoscopy (VCE) for evaluation of the small bowel (3–6).

NSAID-induced enteropathy is the most important differential diagnosis for small bowel ulcers in patients evaluated for suspected Crohn's disease (CD); abstinence from NSAIDs is recommended before performance of VCE, usually for at least 4 weeks (7–9). As NSAID-induced enteropathy is associated with a significant risk of gastrointestinal bleeding (10), several therapeutic agents such as misoprostol and probiotics were evaluated for mucosal protection in NSAIDs users (11, 12).

In recent years, deep learning is becoming frequently used in multiple areas of medicine including radiology, ophthalmology, oncology and gastroenterology (13–16). Convolutional neural network (CNN) techniques demonstrated excellent accuracy for detection of various endoscopic pathologies (16), and we have previously demonstrated their high accuracy for automated identification and classification of VCE-detected small bowel ulcers in CD patients (17, 18). It is expected that DL research for VCE will continue to emerge and will influence clinical practice, ultimately shortening required physicians' time for reading VCE films or possibly abolishing this need altogether. An overview of DL and CNN is presented in the methods section.

NSAIDS-induced enteropathy is commonly encountered in VCE. DL applications for VCE are being rapidly introduced into research and clinics. Most of CNN are trained using images of CD ulcers. However, the ability of DL to identify findings related to NSAID enteropathy have not yet been evaluated.

We evaluated a DL network for the detection of NSAID ulcers on VCE images. Since training a DL network requires a large number of images, and as NSAID enteropathy ulcers are morphologically similar to CD ulcers, we have used a CD ulcers dataset for training the model. Then the machine was solely tested on NSAIDs ulcers dataset that was extracted from a clinical trial in which healthy individuals received NSAID and underwent VCE.

PATIENTS/MATERIAL AND METHODS

Overview of Deep Learning Algorithms for Image Analysis

Deep learning (DL), an emerging technology which has gained much interest in the past decade. It is considered a sub-type of artificial intelligence (AI) (13, 14). AI describes algorithms which perform tasks that usually require "human cognitive".

DL is almost synonymous with artificial neural networks (ANNs). The structure of ANNs may be considered as a cluster of interconnected linear regression units. This cluster of regression units are joined using non-linear activation functions to create sequential neural layers. Similar to linear regression, each neuron in the network has multiple inputs which are termed weights. These inputs are the outputs of neurons in the previous layer (**Figure 1**). ANNs may contain many "hidden"/"deep" layers, hence the term "deep learning".

The process of training and ANN is essentially tweaking of all the network's weights to achieve an optimized network. Given data, an optimized network will produce a desired prediction. For instance, the network can be given VCE images with either ulcers or normal mucosa. An optimized network could satisfactory differentiate between these two types of images.

Convolutional neural networks (CNN) are a sub-type of ANN designed for image analysis. These algorithms are a very similar to classic ANNs. CNNs were specifically built to recognize repeating patterns in data. Since images contain many repeated patterns, CNNs are optimal for this task.

The CNN is inputted with an image (for example a VCE image), which is an array of pixels. The array is propagated along the deep layers of the CNN until a final neuron outputs a classification prediction (i.e., "ulcer"/"no ulcer"). Each CNN neuron corresponds to a small matrix (i.e., 3X3, 5X5) of weights. The uniqueness of CNN is that these small matrixes are propagated along the image. Thus, each matrix is "shared" across all the regions of the image. This matrix sharing is what allows the pattern recognition of CNNs (**Figure 2**).

In the past few years, CNNs are being rapidly introduced into healthcare. Since these algorithms are specifically designed for image analysis, the major involved fields are those dealing with medical images, such as radiology images, endoscopy images, fundoscopy, ECG, etc.

Study Design

We previously published results of a CNN trained on VCE images of CD patients (17). In the current study, we retrospectively evaluated this CNN for its accuracy in detecting NSAIDs ulcers on VCE images. Thus, the network was solely trained on CD ulcers and solely tested on NSAIDs ulcers.

The network was trained using CD ulcer and normal mucosa images sourced from a repository of VCE diagnosed CD patients as previously described (17). All images were acquired from studies performed using PillCam SBIII (Medtronic Ltd, Dublin, Ireland) and reviewed with Rapid 9 (Medtronic Ltd, Dublin, Ireland) capsule reading software.

NSAIDs induced enteropathy images were extracted from VCEs that were performed during the Bifidobacterium breve (BIf95) randomized controlled trial published by Mortensen at al. (11); the methodology and results of the study are covered in detail elsewhere (11). Briefly, this was a single-site, double-blind, parallel group study that enrolled 75 heathy volunteers given acetylsalicylic acid (ASA) (300 mg) daily for 6 weeks. The participants were randomized to groups given oral capsules of Bif95 or placebo for 6 weeks. Small-intestinal damage was analyzed by serial VCEs for the duration of the study. All VCEs

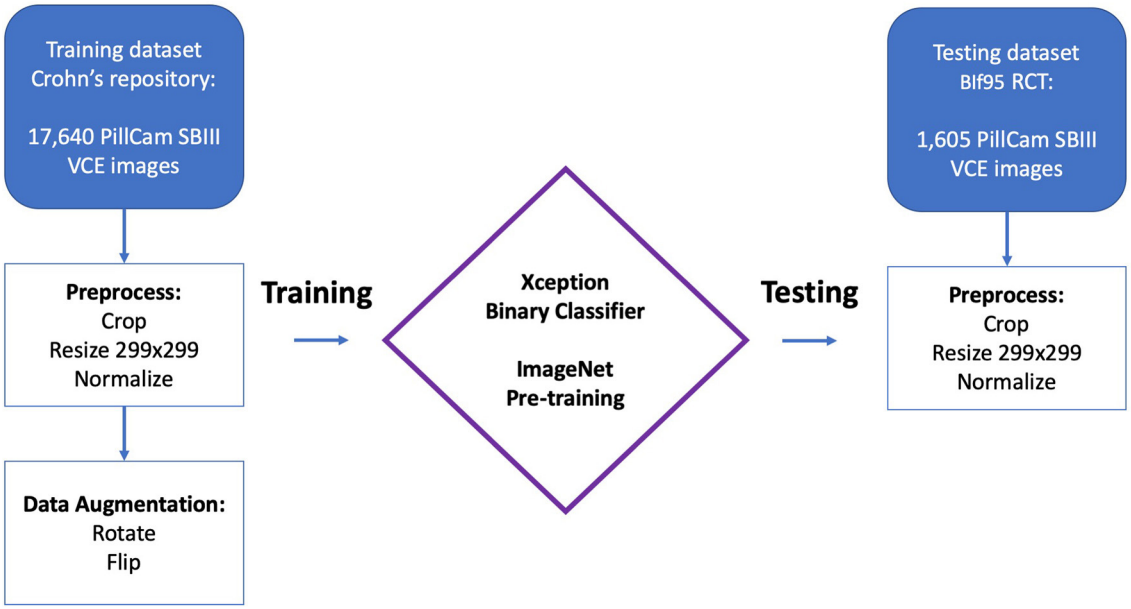

FIGURE 1 | Schematic representation of an artificial neural network (ANN). The network is composed by many linear regression units, termed neurons. These units are structured in sequential layers. Outputs of neurons from the previous layer are the inputs of the neurons in the next layer. The connection between the layers are termed weights, which are similar to the weights of a linear regression unit. Data, such as an image, is propagated through the network, and a final prediction is outputted. An optimized network will produce a desired prediction for each data entry.

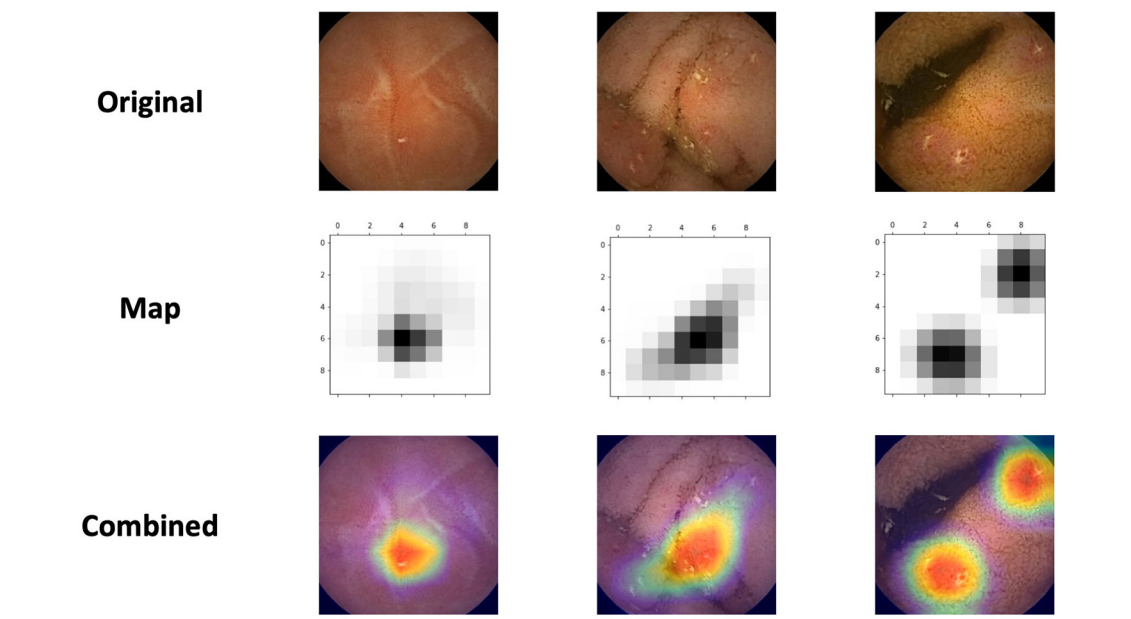

FIGURE 2 | Convolutional neural networks (CNN) are a sub-type of artificial neural networks (ANN). CNNs, are unique in their capacity to identify repeating patterns. Thus, they are ideal for image analysis, as images contain many repeating patterns. CNNs are composed from many small matrixes which are a collection of weights. These small matrices are shared across the array of pixels which constitutes an image.

were performed using PillCam SBIII (Medtronic Ltd, Dublin, Ireland) and reviewed with Rapid 9 (Medtronic Ltd, Dublin, Ireland) capsule reading software.

For the purpose of the current study, we only selected the VCEs of patients in the placebo arm, i.e. ASA 300 mg for six weeks with placebo and discarded those who received ASA

with Bif95. All studies were reviewed in consensus by VCE experts (UK, AG, RE) and images containing mucosal ulcers were extracted.

Software and Hardware

The models were written in Python (ver. 3.7) utilizing the open-source Keras (ver. 2.1.5) library and the open-source TensorFlow (ver. 1.5.0) library as backend. Models ran on an Intel i7 CPU and two GeForce GTX 1080ti Graphics Cards.

CNN Model

As previously described (17), an Xception CNN (19) was trained to classify capsule images into either images of normal mucosa or images with CD mucosal ulcers. The network's weights were initialized using weights from the 1.2 million everyday color images of ImageNet (20).

Preprocessing of capsule images included cropping of images' borders and legends. Images were then resized into a 299 × 299 matrix and pixels were normalized into the range 0–1 by dividing by 255 (17).

The following hyper-parameters were used for training the network: 2 epochs; batch size 8; Adam optimization with a learning rate of 10^{-4}. Softmax was used as the output activation function.

Class Activation Maps

We used class activation maps (CAM) to analyze which parts of a given image led the network's decisions. This method is helpful for understanding the decision process of the CNN. Of particular interest, we wanted to investigate whether the network identifies NSAIDs ulcers as CD ulcers.

For this purpose, we've applied Gradient-weighted Class Activation Mapping (Grad-CAM). This algorithm uses the gradients of the target label, flowing into the final convolutional layer, to highlight the important regions in the image (21).

Metrics

We assessed the networks performance for detecting images containing NSAIDs mucosal ulcers.

Receiver operating curves (ROC) were plotted for the network's results by varying the operating threshold. The model's metrics included area under the curve (AUC), sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV), and F1 score. All metrics were computed for the Youden's index and for fixed specificities of 90, 95 and 99%.

Bootstrapping validations (1,000 bootstrap resamples) were used to calculate 95% confidence intervals (CI) for the metrics.

RESULTS

Study Cohort

Overall, the CD training dataset included 17,640 CE images from 49 patients; 7,391 images with mucosal ulcers and 10,249 images of normal mucosa. Out of 10,249 normal images, 3,577 originated from patients with normal CE and 6,672 from patients with CD (17).

The NSAIDs testing dataset included 1,605 CE images (11). This included 980 images of NSAIDs ulcers and 625 of normal mucosa.

CNN Results

The network's prediction time for the 1,605 images in the testing dataset was 8.9 s. This yields an average of 5.5 ms per single image. For a VCE film with 10,000 images, this amounts to 55 s.

Figure 3 presents the network's activation heatmaps for NSAID ulcers. As can be seen in the heatmaps, a network trained on CD ulcers, clearly detected NSAID ulcers.

The receiver operating characteristic curve for predicting NSAIDs ulcers is presented in **Figure 4**. The network exhibited an AUC of 0.97 (95% CI 0.97–0.98) for identifying images with NSAID mucosal ulcers. This is comparable to the previous results for CD patients, with AUCs of 0.94–0.99.

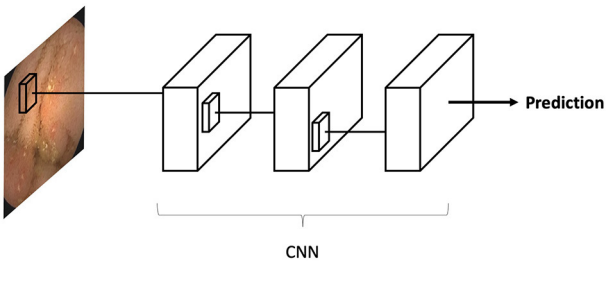

FIGURE 3 | Three examples of the network's activation heatmaps for video capsule endoscopy images of NSAID ulcers.

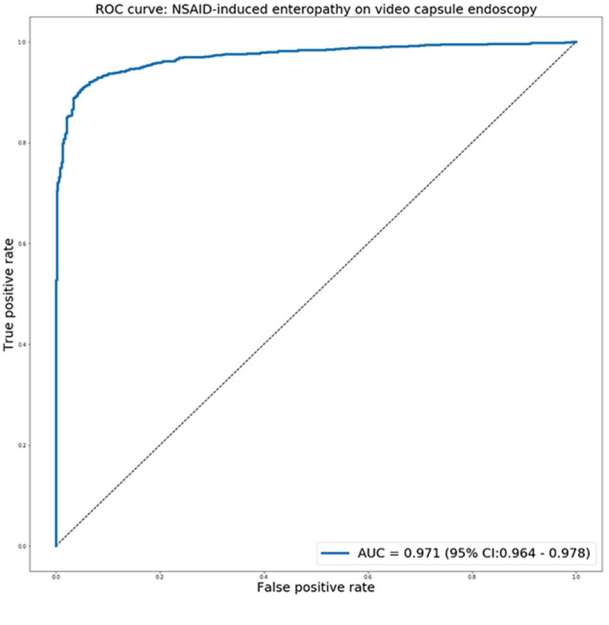

FIGURE 4 | The receiver operating characteristic curve for predicting NSAIDs ulcers using the deep learning network.

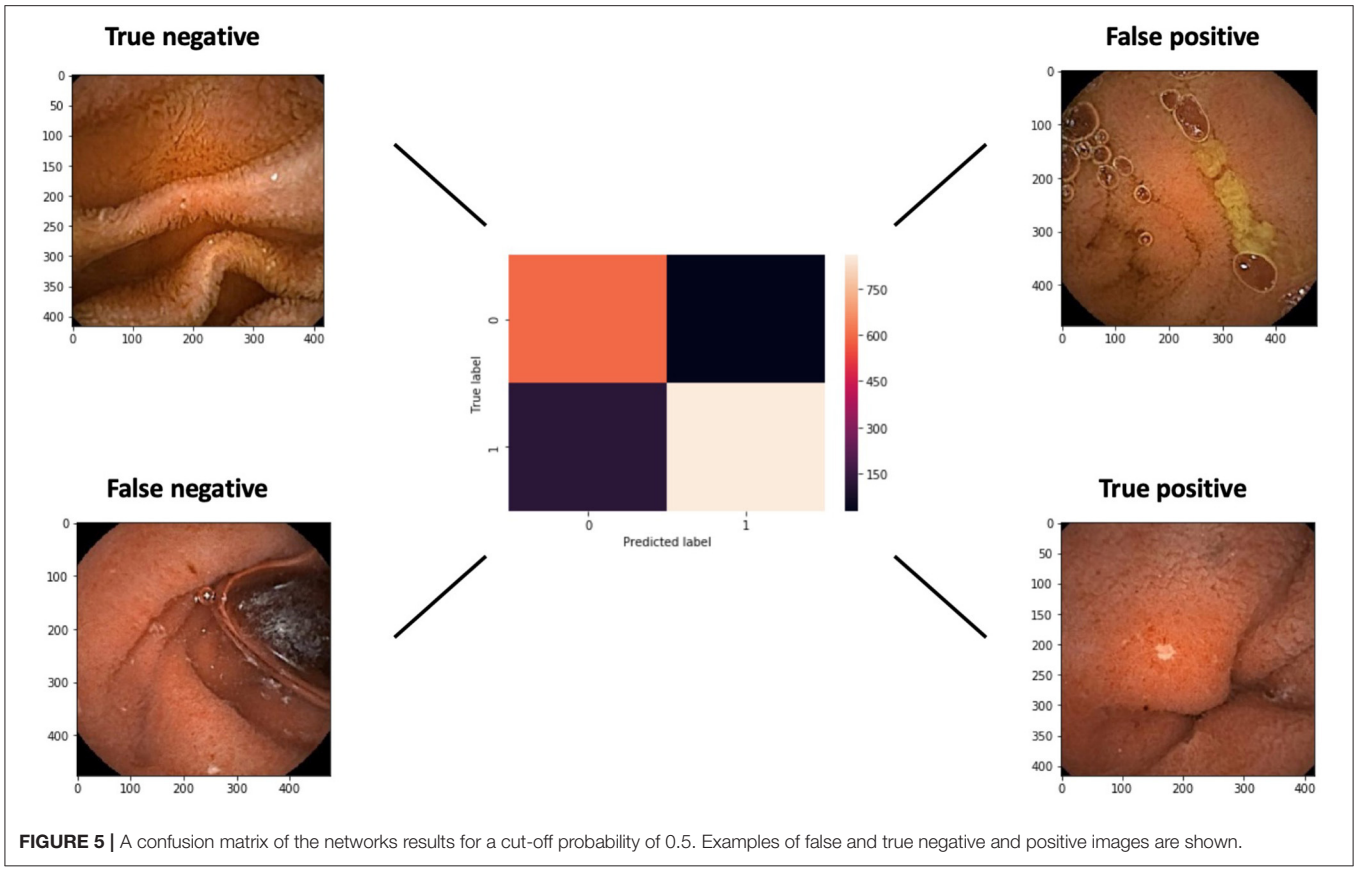

FIGURE 5 | A confusion matrix of the networks results for a cut-off probability of 0.5. Examples of false and true negative and positive images are shown.

TABLE 1 | Metrics of the deep learning network for detecting NSAID-enteropathy.

Fixed specificity	Sensitivity	Specificity	PPV	NPV	F1
Youden's index	0.92 (95% CI: 0.90–0.94)	0.95 (95% CI: 0.94–0.97)	0.97 (95% CI: 0.95–0.98)	0.89 (95% CI: 0.86–0.91)	0.94 (95% CI: 0.93–0.95)
90%	0.94 (95% CI: 0.92–0.95)	0.90	0.93 (95% CI: 0.92–0.95)	0.90 (95% CI: 0.88–0.92)	0.94 (95% CI: 0.92–0.95)
95%	0.92 (95% CI: 0.91–0.94)	0.95	0.97 (95% CI: 0.95–0.98)	0.89 (95% CI: 0.86–0.91)	0.94 (95% CI: 0.93–0.95)
99%	0.85 (95% CI: 0.82–0.87)	0.99	0.99 (95% CI: 0.98–1.00)	0.80 (95% CI: 0.78–0.83)	0.91 (95% CI: 0.90–0.93)

Metrics (Sensitivity, Specificity, PPV, NPV, F1 score) of the deep learning network probabilities for detecting NSAID-enteropathy in video capsule endoscopy (VCE) images. The different metrics were computed for Youden's index and for fixed specificities of 90, 95, and 99%.

A confusion matrix with examples of true and false, negative and positive, images is presented in **Figure 5**. The false negative example exhibits an ulcer at the edge of the image.

For Youden's index, the network showed sensitivity 92% and specificity 95%. For all specificities cut-off values, a high F1 score of >0.90 was shown (**Table 1**).

DISCUSSION

In the past few years, there has been a surge of publications related to the use of artificial intelligence in gastroenterology and gastrointestinal endoscopy. Several studies have demonstrated the impressive results of detecting ulcers in VCE (16). In recent studies we have found that AI can accurately identify CD ulcers and also rank their severity (17, 18). However, small bowel ulcers are not specific to Crohn's disease, and may be caused by various other etiologies.

NSAID induced enteropathy is a common complication, potentially afflicting >50% of chronic NSAID users and may be detected even in occasional and low-dose NSAID users. NSAID-induced small bowel ulcers are a common cause of occult small bowel bleeding. As NSAID-induced ulcers are morphologically similar to those of CD, this entity should be considered as a potential differential diagnosis for suspected CD.

In order to train a neural network, a large number of images are needed (14). The construction of NSAID ulcers dataset is a complex endeavor. It is also methodologically challenging to generate a 'clean' dataset of NSAID-induced lesions as other mimicking etiologies are often hard to exclude in real-practice patients. The present study utilized a unique dataset from a clinical trial in which healthy individuals received NSAID

and underwent VCE, thereby overcoming the aforementioned methodological constraints. Using healthy individuals, who deliberately ingested aspirin to induce enteric ulcerations as part of a clinical trial is a strong methodological advantage of the present study allowing validated etio-morpholgical comparison with CD ulcers. This stands in contrast with any such future attempt using clinically accrued patients, in whom ascribing ulcers to aspirin or NSAIDs etiology cannot be definitively done in most cases.

The DL network, trained using a large dataset of CD ulcers, achieved an AUC of 0.97 for detecting NSAID-induced ulcers. This prediction capability is similar to the AUC (0.94–0.99) of the network for detecting CD ulcers (17). Our findings corroborate the morphological similarity of CD and NSAID-induced ulcers. Moreover, the network was very fast and efficient in detection of the ulcers, arguably much faster than the expected reading time of a human reader.

Our neural network has proved to be efficient in identifying NSAID induced ulcers; however, it is important to take into account that this system is trained solely on CD ulcers and thereby will identify both types of ulcers as the same entity. The current algorithm will be able to identify ulcers in general and will not be able to specifically determine whether it is a NSAID or a CD induced ulcer. Recently, DL has been implemented in the clinic for GI applications (22). When using this tool, physicians should be aware of the interactions between various pathologies as well as the limitations of AI.

This study has several limitations. This is a retrospective analysis of a prospectively collected data. Prospective interventional studies are needed to evaluate the retrospective results to show real-world usefulness. The study includes a limited number of images, particularly for NSAID induced ulcers. Moreover, DL is a rapidly emerging field, and new algorithms are continuously developed. In this work we've used the Xception model since it was used in a previous related work (17). Yet, this model has several limitations: 1. Xception CNN model network is compatible only with the TensorFlow backend, 2. Xception require 299 × 299 pixel inputs, 3. Xception image weights are between 90 and 100MB. Other models may show better results. Lastly, future projects should prospectively investigate networks in real-life scenarios.

The described method presents a classification model for still VCE images. Future studies should expand this method for entire video analysis to provide a wholistic prediction and to replicate clinical scores such as the Lewis or the CDEIS scores. Moreover, clinical data could be integrated into the final layers of the neural network to augment the visual data.

CONCLUSION

A network trained on VCE CD ulcers similarly identified NSAID enteropathy findings. As DL is transforming gastrointestinal endoscopy, this result should be taken into consideration in the future design and analysis of VCE deep learning applications.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Cork University. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

EK had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. UK, EK, and SB-H: concept and design. EK, UK, BM, AD, SS, YB, EK, AG, RY, MB, FS, RE, and SB-H: acquisition, analysis or interpretation of data, critical revision of the manuscript for important intellectual content, and administrative, technical, or material support. UK, EK, and SS: drafting of the manuscript. EK: algorithm design and statistical analysis. UK, EK, SB-H, and RE: supervision. All authors contributed to the article and approved the submitted version.

FUNDING

The original study was funded by CHR Hansen.

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Conflict of Interest: UK: speaker and advisory fees- ABBVIE, Jannsen, MSD, Takeda, Medtronic. Research support- Janssen Takeda Medtronic. SB-H: speaker and advisory fees for Takeda, Abbvie, Janssen, Celltrion, GSK, Pfizer and research support from Takeda, Celltrion, Janssen, and Abbvie. SB-H: received advisory and/or research support from Abbvie, MSD, Janssen, Celltrion, Takeda, GSK, Pfizer. RE: received advisory and/or research support from Abbvie, Janssen, Takeda and Medtronic. FS: co-founder of Alimentary Health Ltd, 4D pharma Cork and Atlantia Food Clinical Trials and has been scientific adviser to Kaleido Biosciences Ltd.

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frontiers in Medicine

ORIGINAL RESEARCH
published: 27 August 2021
doi: 10.3389/fmed.2021.644003

Running Behind "POPO"—Impact of Predictors of Poor Outcome for Treatment Stratification in Pediatric Crohn's Disease

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Background and Aims: Intensifying therapy for Paediatric Crohn's Disease (CD) by early use of immunomodulators and biologics has been proposed for cases in which predictors of poor outcome (POPO) were present. We investigated therapy stratifying potential comparing POPO-positive and -negative CD patients from CEDATA-GPGE®, a German-Austrian Registry for Paediatric Inflammatory Bowel disease.

Methods: CD patients (1–18 years) registered in CEDATA-GPGE® (2004–2018) within 3 months of diagnosis and at least two follow-up visits were included. Disease course and treatments over time were analysed regarding positivity of POPO criteria and test statistical properties.

Results: 709/1084 patients included had at least one POPO criterion (65.4%): 177 patients (16.3%) had persistent disease (POPO2), 581 (53.6%) extensive disease (POPO3), 21 (1.9%) severe growth retardation POPO4, 47 (4.3%) stricturing/penetrating disease (POPO6) and 122 (11.3%) perianal disease (POPO7). Patients with persistent disease differed significantly in lack of sustained remission >1 year (Odd Ratio (OR) 1.49 [1.07–2.07], $p = 0.02$), patients with initial growth failure in growth failure at end of observation (OR 51.16 [19.89–131.62], $p < 0.0001$), patients with stricturing and penetrating disease as well as perianal disease in need for surgery (OR 17.76 [9.39–33.58], $p < 0.001$; OR 2.56 [1.58–4.15], $p < 0.001$, respectively). Positive Predictive Value for lack of sustained remission was >60% for patients with initial growth failure, persistent or stricturing/penetrating disease.

Conclusion: Predictors of poor outcome with complicated courses of disease were common in CEDATA-GPGE®. An early intensified approach for paediatric CD patients with POPO-positivity (POPO2-4, 6-7) should be considered, because they have an increased risk to fare poorly.

Keywords: pediatric Crohn's disease, outcome parameters, predictors of a poor prognosis, patient registry, big data

OPEN ACCESS

Edited by:
Barbara Dorottya Lovasz, Semmelweis University, Hungary

Reviewed by:
Johanna Caroline Escher, Erasmus Medical Center, Netherlands
Katalin Müller, University of Pécs, Hungary

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Specialty section:
This article was submitted to Gastroenterology, a section of the journal Frontiers in Medicine

Received: *19 December 2020*
Accepted: *05 August 2021*
Published: *27 August 2021*

Citation:
de Laffolie J, Zimmer K-P, Sohrabi K and Hauer AC (2021) Running Behind "POPO"—Impact of Predictors of Poor Outcome for Treatment Stratification in Pediatric Crohn's Disease. Front. Med. 8:644003. doi: 10.3389/fmed.2021.644003

INTRODUCTION

Medical management in paediatric Crohn's Disease (CD) still consists primarily of a step-up approach, with immunomodulators or biologics often being used only after other options have failed. Although these medications may induce and maintain remission with catch-up growth (1), this frequently failed to compensate for already acquired growth retardation (1–4). Optimization of therapeutic strategies for and identification of high-risk patients who require intensified therapy earlier is therefore mandatory.

The consensus guidelines of ESPGHAN/ECCO therefore describe predictors of poor outcome (POPO) as means for risk stratification (2). These are deep colonic ulcerations on endoscopy (POPO1), persistent severe disease despite adequate induction therapy (POPO2), extensive disease (POPO3), marked growth retardation [<-2.5 Height Standard Deviation Score (SDS); POPO4], severe osteoporosis (POPO5), stricturing and penetrating disease at onset (POPO6) and also perianal disease (POPO7). Whether these predictors may be used for definite risk stratification in paediatric CD, however, has not been substantiated to date by large scale data.

In 2004, CEDATA-GPGE® was founded as Registry of the Society for Paediatric Gastroenterology and Nutrition of German speaking countries (GPGE), enrolling paediatric Inflammatory Bowel Disease (IBD) patients in Germany and Austria. The main objective of this observational study was to evaluate the POPO potential for risk stratification by characterising and comparing disease course and treatment of POPO-positive vs. -negative patients from CEDATA-GPGE® and to describe "POPO" criteria test statistical properties.

PATIENTS AND METHODS

All CD patients (eligible 1–18 years of age, diagnosis in one of the participating centres) registered between 2004 and 2018 in CEDATA-GPGE® were included if documented in the registry within 3 months of diagnosis (to avoid recall bias) and at least two follow up visits, independent of therapeutic strategy chosen. Patients with delayed diagnostic workup (initial workup not completed within 3 months of diagnosis in the registry) were excluded. CEDATA-GPGE® is a prospective, multicentre registry for paediatric IBD in German speaking countries approved by ethic committees of all participating centres (5). Data entry is encouraged at least every 6 months by means of a secure and easy-to-use online registry environment (**eSupplementary Example Figures**). Diagnosis was based on Porto criteria (6) and while deep colonic ulcerations (POPO1) and osteoporosis (POPO5) had not been recorded systematically, the remaining 5 of 7 POPO criteria were evaluated in all patients, POPO3 extensive disease was approximated by L3+-L4a/b (**Table 1**). POPOs were considered positive when present at diagnosis, outcome measures were considered outcomes when they appeared at any point in time after diagnosis (for survival analysis, the first 4 weeks were eliminated from outcomes). For purposes of cohort homogeneity, patients with disease onset <1 year of age were excluded.

A sensitivity analysis for the exclusion of younger patients in our main findings was conducted to evaluate if exclusion of patients with <1 or 2 years of age would change results. Enrolled patients were divided into two groups: Patients fulfilling at least one criterion were **POPO-positive,** and those without fulfilling any criterion **POPO-negative**. Both groups were compared in the context of basic characteristics such as age and gender, disease activity (Paediatric Crohn's Disease Activity Index PCDAI) (6) assessment of diagnostic latency (time from onset of symptoms to diagnosis as described by the patients or parents), disease presentation and course: Growth failure (Height < −2.5 SDS, **Table 1**) and failure to gain weight (history of failure to gain weight, failure to thrive) were documented as presenting symptoms, severe growth retardation was one of the predictors to be investigated and growth failure was again used as an endpoint toward which predictive measurements were evaluated. Therapy over a maximum of 5 years was analysed additionally, with restriction criteria to azathioprine as an immunomodulatory and infliximab as a biologic agent. Since accelerated step-up treatment strategy has to be decided earlier than at 1 year, we defined lack of response to adequate induction therapy at 3 months as "persistent disease." Adequate induction therapy was defined as documented acceptable therapies for induction of paediatric CD like exclusive enteral nutrition, systemic steroids

TABLE 1 | "POPO" groups and patients' characteristics.

POPO	Description	Definition in Registry
"POPO"-2	Persistent disease	- on physician general assessment † - at 12 weeks after diagnosis ‡ - under recommended treatments §
"POPO"-3	Extensive disease	- L3 Paris classification with/without upper gastrointestinal tract involvement
"POPO"-4	Severe growth retardation	- body height <-2.5 SDS ¶
"POPO"-6	Stricturing/penetrating disease	- radiologic/endoscopic assessment
"POPO"-7	Perianal disease	- excluding simple tags and fissures, clinical assessment (7)

† *Physician general assessment: By 4-item assessment scale (remission – mild – moderate – severe disease activity, levels 3–4) used in other large registries (8).*
‡ *12 weeks after inclusion: Time point for relevant information on early disease course.*
§*Recommended treatments: Exclusive enteral nutrition, steroids, 5-ASA, methotrexate, azathioprine/6-mercaptopurin, infliximab.*
¶*Body height <-2.5 SDS, according to reference values from the "KIGGs Study," a representative nationwide health survey (9).*

Abbreviations: CD, Crohn's disease; IBD, inflammatory bowel disease; PCDAI, pediatric crohn's disease activity index; POPO, predictors of poor outcome; CEDATA, registry for pediatric inflammatory bowel disease; GPGE, gesellschaft für pädiatrische gastroenterologie und ernährung; PPV, positive predictive value; SDS, standard deviation score; NPV, negative predictive value; EIM, extraintestinal manifestation.

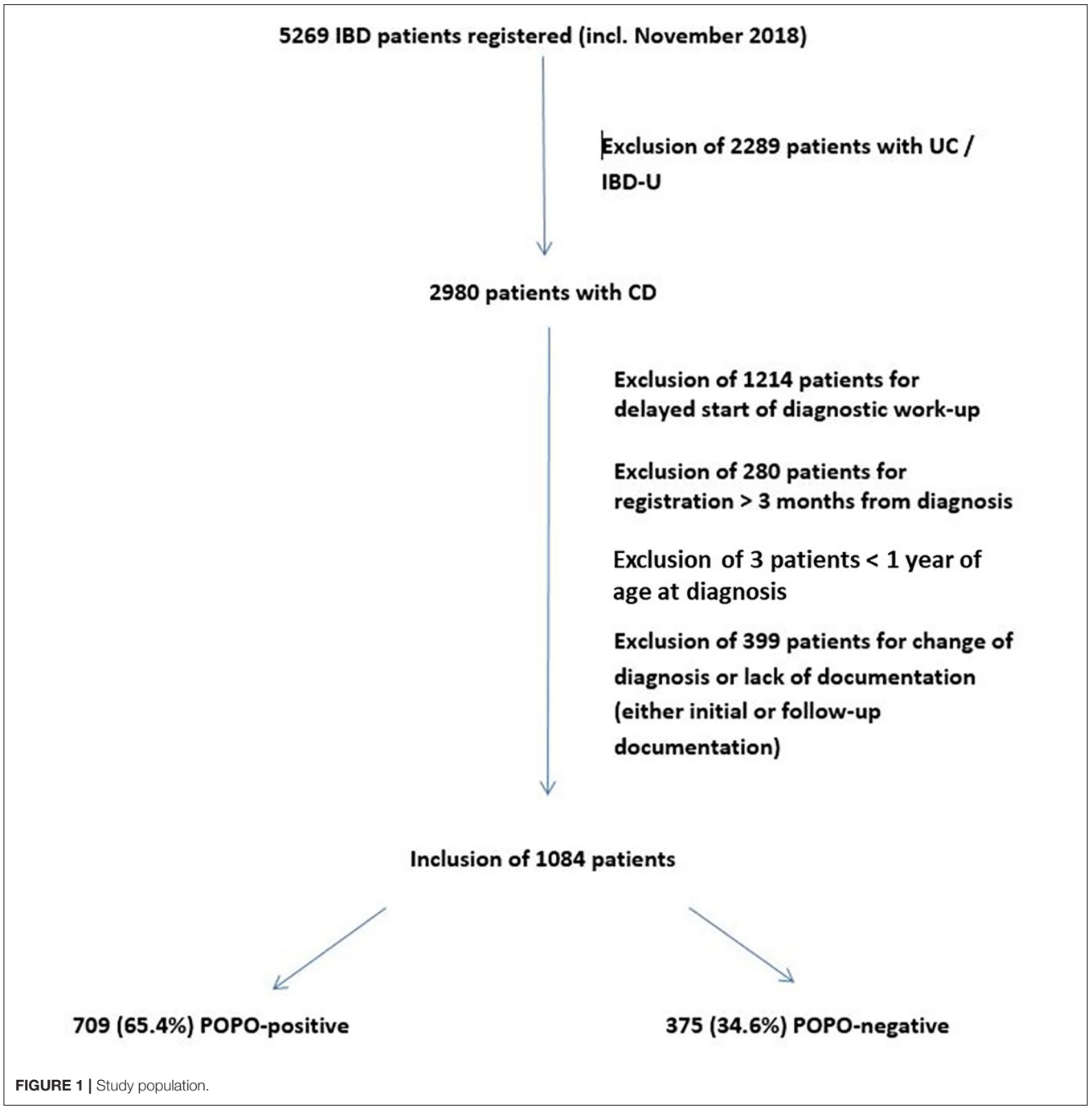

FIGURE 1 | Study population.

or infliximab/Adalimumab. Patients between 1 and 6 years received subgroup analysis (very early onset IBD).

For each POPO criterion sensitivity, specificity, positive and negative predictive values [PPV and NPV, respectively (resp.)] including 95% confidence intervals for odds ratios were calculated with regard to the variety of outcome parameters. These were extraintestinal manifestations (EIM), as recorded in the online registry (uveitis, arthritis, skin manifestations, hepatobiliary involvement, others), lack of sustained remission for >1 year (sustained remission represented by at least two physician global assessments indicating full remission at least 365 days apart without intermittent inflammatory activity or intensified therapy), presence of abscess, fistula or stenosis (including perianal fistula), surgery (both as recorded in the registry by the treating physician) and growth failure at the end of observation (body height < -2.5 SDS). We also analysed differences of negative outcomes comparing early vs. late ($<=$ 3 vs. >3 months from diagnosis, dichotomized for analysis) start of azathioprine or infliximab. This cut off was chosen according to relevant literature in the field.

To address longterm outcome survival analysis for all predictor positive groups vs. negative groups was performed (Kaplan Meyer Survival Curve, Log Rank Analysis, Cox Regression Analysis where follow-up time differed). Events within the first four weeks after diagnosis were eliminated from Kaplan Meyer and Log Rank Analysis.

Statistical analysis was performed on the SAS Version 9.4 (SAS-Institute©, North Carolina) and R (CRAN, Vers 3.6.3). Fisher exact-test, chi-square-and Wilcoxon tests were used for comparison variables between groups. The *p*-value for statistical significance was defined as <0.05.

RESULTS

Baseline Characteristics

Of 5,269 IBD patients (age 0–18 years) registered in "CEDATA-GPGE®" from 2004 to 2018, 2,980 patients were diagnosed with CD and 1,084 were included in the study (**Figure 1**). 50 (4.6%) of them were between 1 and 6 years of age. The sensitivity analysis on the lower age margin showed no difference between the exclusion at 1 or 2 years of age.

The median follow-up was 10 visits [interquartile range (IQR) 5–15 visits, max 94 visits] over a time period of a median of 769 days (IQR 275–1,595 days, max 3,570 days). In 709 patients (65.4%; 70% between 1 and 6 years) at least one POPO-criterion was found. 177 (16.3%; 45.7% between 1–6 years) patients had persistent severe disease despite recommended induction therapy (POPO2). 581 patients (53.6%; 68.6% between 1 and 6 years) showed extensive disease (POPO3), 21 (1.9%) had severe growth retardation (POPO4) and 47 (4.3%) stricturing or penetrating disease (POPO6). Perianal disease (POPO7) was recorded in 122 patients (11.3%; **Figure 2**). While patients between 1 and 6 years of age were significantly more often POPO2- and−3-positive than the older age group, only few of them were POPO4-, POPO-6, and POPO-7-positive, resp.

POPO-Positive vs. POPO- Negative Groups

Characteristics

POPO-positive and -negative patients did not differ significantly in age and gender, and just reached significance regarding PCDAI at diagnosis (23.3 vs. 19.3, $p = 0.05$) and diagnostic latency (8.9

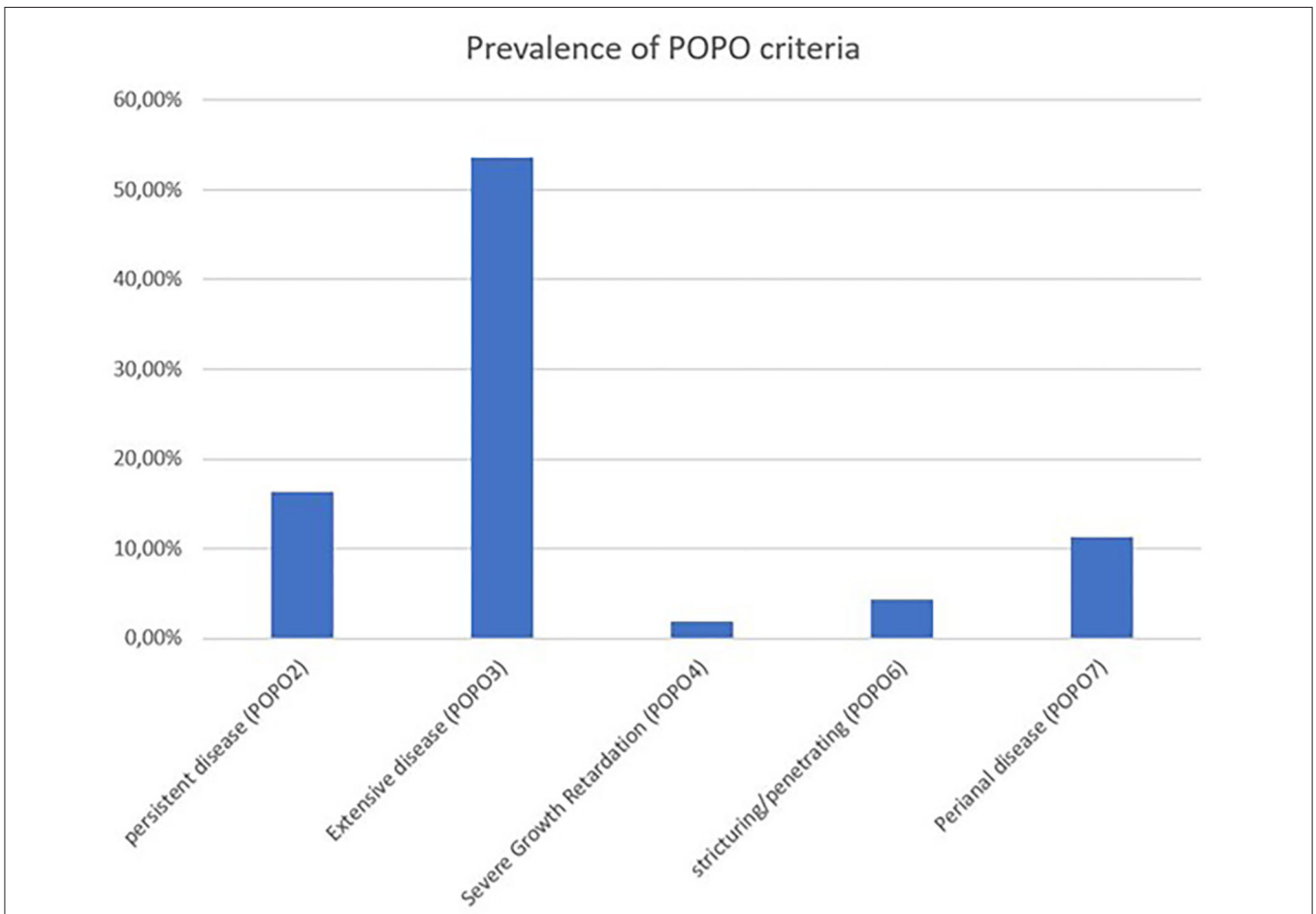

FIGURE 2 | Prevalence of POPO criteria. Prevalence of POPO criteria at initial diagnosis of paediatric CD patients from CEDATA GPGE registry.

TABLE 2 | Characteristics of POPO*-positive and POPO-negative patient groups.

Characteristics	POPO positive ($n = 709$)	POPO negative ($n = 375$)	*P*
Male	419 (59.1%)	217 (57.9%)	0.70
Mean age at diagnosis (years ± standard deviation SD)	12.4 ± 3.2	12.9 ± 3.1	0.06
Mean diagnostic latency (months ± SD)	8.9 ± 12.6 ($n = 687$)	7.9 ± 12 ($n = 361$)	**0.05**
PCDAI*, ** at diagnosis mean ± SD	23.3 ± 13.1 ($n = 313$)	19.3 ± 13.6 ($n = 171$)	**0.05**
Azathioprine	528 (74.5%)	260 (69.3%)	0.07
Infliximab	154 (21.7%)	76 (20.3%)	0.6
mean duration of follow-up (months ± SD)	36.00 ± 28.4	34.05 ± 28.0	0.28

*POPO, predictors of poor outcome; **PCDAI, paediatric crohn's disease acitivity index; SD, standard deviation. The bold values indicates $p < 0.05$.

vs. 7.9 months; $p < 0.05$; **Table 2**, missing values did not differ significantly between groups).

Symptoms at Diagnosis

On diagnosis, POPO-positive patients had significantly reduced appetite and limitation in activities in comparison to POPO-negative patients ($p = 0.04$; $p = 0.03$, resp., **Supplementary Table e3**), but did not differ significantly regarding all other presenting symptoms evaluated (abdominal pain, diarrhoea, blood in stool, failure to gain weight, growth failure, fever, anaemia, loss of appetite, EIM; **Supplementary Table e3**).

Analysis per POPO-Group: POPO2-Positive

(persistent disease) patients had a significantly higher risk for lack of sustained remission >1 year (OR 1.49 [1.07–2.07], $p = 0.02$), with a PPV of 61.02% (**Table 3**; **Figure 3**). A majority of patients with initial severe growth retardation (**POPO4 positive**) continued to have growth failure, differing significantly in comparison to POPO-negative patients (OR 51.16 [19.89–131.62], $p < 0.0001$). The PPV for lack of sustained remission was 61.9% (**Table 3**). Patients with stricturing or penetrating disease behaviour (**POPO6-positive**) had significantly less treatment with azathioprine than POPO negative patients (0.54 [0.3–0.98]; $p < 0.04$) and EIM (OR 0.33 [0.17–0.66], $p = 0.001$). They developed abscess, fistula, or stenosis significantly more often (OR 276.31 [37.82–2018.97], $p < 0.0001$) and also had surgery significantly more often (OR 17.76 [9.39–33.58], $p < 0.001$; **Table 3**; **Supplementary Table e4**; **Figure 3**). PPV for lack of sustained remission was 63.83% (**Table 3**). **POPO7-positive** patients (perianal disease) had significantly increased risks for abscess, fistula, or stenosis (OR 7.36 [4.93–11.0], $p < 0.0001$) and surgery (OR 2.56 [1.58–4.15], $p < 0.001$; **Table 3**; **Supplementary Table e4**; **Figure 3**). Specificity and negative predictive value toward negative outcomes were high in case of POPO2, POPO4, POPO6 and POPO7 (**Supplementary Table e2**).

Therapy

Analysis of therapy according to POPO-positivity revealed POPO-positive patients being treated significantly more often with infliximab (11.1 vs. 7.3%; $p = 0.047$, **Supplementary Table e1**) than POPO-negative patients in the first year from diagnosis. There were no significant differences regarding infliximab treatment in the second year, nor regarding azathioprine therapy in the first two years from diagnosis (64.4 vs. 61.5%; **Supplementary Table e1**) and beyond.

Survival Analysis

In search for the most relevant predictor, the group of patients who did not ever reach steroid free remission over 1 year stood out (**Figure 4**). This group of children showed a significantly decreased event free survival over 200 weeks toward surgery, EIM and abscess, fistula and stenosis, a predictive value that was not reached by another one of the initially proposed criteria.

DISCUSSION

A modern approach to CD should be influenced by the patient's underlying prognosis and there are several outcomes aimed for with CD: Remission, in adults commonly graded by the Crohn's disease activity index (10) or Harvey Bradshaw index scoring system (8), is one such outcome. Other outcomes include the avoidance of surgery, endoscopic mucosal remission, and reduction of long-term bowel damage. Whether additional analysis of genotypes, antimicrobial serologies, ileal gene expression, and faecal microbiota might be used for predicting a complicated CD course in children has been addressed recently by Kugathasan et al. in a multicentre inception cohort study. Specific bacteria, i.e., *Ruminococcus* and *Veillonella*, were found to be implicated in stricturing and penetrating complications, resp., and the signature of upregulated ileal genes controlling extracellular matrix production associated with stricturing disease, thus supporting the usefulness of risk stratification at diagnosis (11).

We therefore aimed at analysing POPO longitudinally by using data from the CEDATA-GPGE® set, the second largest prospective registry of paediatric IBD worldwide, and, as is mostly true with registries, with this bringing in an additional value in its own, reflecting daily life clinical care (12). To ensure data quality, which might have been impaired due to the registry's long term nature and potential selection bias with only tertiary centres participating, we tried to minimise recall bias of initial presentation and missing of early events, in that only patients registered within 3 months of diagnosis were analysed.

In our study we found two thirds of all patients, and 70% of those younger than 6 years, were POPO-positive, in contrast

TABLE 3 | POPO-positivity and predictive properties.

POPO2 Follow-up Avg. 32.67 +- 26.6 months Endpoint	**Sensitivity**	**Specificity**	**PPV**	**NPV**	**Odds Ratio [95%CI]**	***p*-value compared to POPO neg.**
Surgery	16/118 (13.56%)	805/966 (83.33%)	16/177 (9.04%)	805/907 (88.97%)	0.78 [0.45–1.36]	0.39
Growth failure	7/39 (17.95%)	875/1,045 (83.73%)	7/177 (3.95%)	875/907 (96.47%)	1.13 [0.49–2.59]	0.78
Abscess, fistula, stenosis	30/194 (15.46%)	743/890 (83.48%)	30/177 (16.95%)	743/907 (81.92%)	0.92 [0.6–1.42]	0.72
Lack of sustained remission	108/573 (18.85%)	442/511 (86.5%)	108/177 (61.02%)	442/907 (48.73%)	1.49 [1.07–2.07]	**0.02**
Escalate to Azathioprine	137/788 (17.39%)	256/296 (86.49%)	137/177 (77.40%)	256/907 (28.22%)	1.35 [0.92–1.97]	0.12
Escalate to Infliximab	40/230 (17.39%)	717/854 (83.96%)	40/177 (22.60%)	717/907 (79.05%)	1.10 [0.75–1.62]	0.62
EIM	92/507 (18.15%)	492/577 (85.27%)	92/177 (51.98%)	492/907 (54.24%)	1.28 [0.93–1.77]	0.13
POPO3 Follow-up Avg. 36.65 +- 28.6 months Endpoint	**Sensitivity**	**Specificity**	**PPV**	**NPV**	**Odds Ratio [95%CI]**	***p*-value compared to POPO neg.**
Surgery	70/118 (59.32%)	455/966 (47.10%)	70/581 (12.05%)	455/503 (90.46%)	1.3 [0.88-1.91]	0.19
Growth failure	19/39 (48.72%)	483/1,045 (46.22%)	19/581 (3.27%)	483/503 (96.02%)	0.82 [0.43–1.55]	0.53
Abscess, fistula, stenosis	115/194 (59.28%)	424/890 (47.64 %)	115/581 (19.79%)	424/503 (84.29%)	1.32 [0.96–1.82]	0.08
Lack of sustained remission	310/573 (54.1%)	240/511 (46.97%)	310/581 (53.36%)	240/503 (47.71%)	1.04 [0.82–1.33]	0.72
Escalate to Azathioprine	430/788 (54.57%)	145/296 (48.99%)	430/581 (74.01%)	145/503 (28.83%)	1.15 [0.88–1.51]	0.30
Escalate to Infliximab	125/230 (54.35%)	398/854 (46.60%)	125/581 (21.51%)	398/503 (79.13%)	1.04 [0.76–1.39]	0.80
EIM	280/507 (55.23%)	276/577 (47.83%)	280/581 (48.19%)	276/503 (54.87%)	1.13 [0.89–1.44]	0.32
POPO4 Follow-up Avg. 32.43 +- 27.1 months Endpoint	**Sensitivity**	**Specificity**	**PPV**	**NPV**	**Odds Ratio [95%CI]**	***p*-value compared to POPO neg.**
Surgery	5/118 (4.24%)	950/966 (98.34%)	5/21 (23.81%)	950/1,063 (89.37%)	2.63 [0.94–7.31]	0.05
Growth failure	12/39 (30.77%)	1,036/1,045 (99.14%)	12/21 (57.14%)	1,036/1,063 (97.46%)	51.16 [19.89–131.62]	**<0.0001**
Abscess, fistula, stenosis	7/194 (3.61%)	876/890 (98.42%)	7/21 (33.33%)	876/1,063 (82.41%)	2.34 [0.93–5.88]	0.06
Lack of sustained remission	13/573 (2.27%)	503/511 (98.43%)	13/21 (61.9%)	503/1,063 (47.3%)	1.46 [0.6–3.55]	0.40
Escalate to Azathioprine	15/788 (1.90%)	290/296 (97.97%)	15/21 (71.43%)	290/1,063 (27.28%)	0.94 [0.36–2.44]	0.9
Escalate to Infliximab	6/230 (2.61%)	839/854 (11.71%)	6/21 (28.57%)	839/1,063 (78.93%)	1.50 [0.57–3.91]	0.41
EIM	14/507 (2.76%)	570/577 (98.7%)	14/21 (66.66%)	570/1,063 (53.62%)	2.31 [0.93–5.78]	0.07
POPO6 Follow-up Avg. 21.8 +- 21.9 months Endpoint	**Sensitivity**	**Specificity**	**PPV**	**NPV**	**Odds Ratio [95%CI]**	***p*-value compared to POPO neg.**
Surgery	16/118 (13.56%)	935/966 (96.79%)	16/47 (34.04%)	935/1,037 (90.16%)	17.76 [9.39–33.58]	**<0.001**
Growth failure	1/39 (2.56%)	999/1,045 (95.60%)	1/47 (2.13%)	999/1,037 (96.33%)	0.57 [0.08–4.25]	0.58
Abscess. fistula. stenosis	46/194 (23.71%)	889/890 (99.89%)	46/47 (97.87%)	889/1,037 (85.73%)	276.31 [37.82–2018.97]	**<0.0001**
Lack of sustained remission	30/573 (5.23%)	494/511 (96.67%)	30/47 (63.83%)	494/1,037 (47.64%)	1.61 [0.87–2.95]	0.12
Escalate to Azathioprine	28/788 (3.55 %)	277/296 (93.58%)	28/47 (59.57%)	277/1,037 (26.71%)	0.54 [0.3–0.98]	**0.04**
Escalate to Infliximab	13/230 (5.65%)	820/854 (96.02%)	13/47 (27.66%)	820/1,037 (79.07%)	1.44 [0.75–2.79]	0.27
EIM	11/507 (2.17%)	541/577 (93.76%)	11/47 (23.40%)	541/1,037 (9.64%)	0.33 [0.17–0.66]	**0.001**
POPO7 Follow-up Avg. 36.7 +- 29.0 months Endpoint	**Sensitivity**	**Specificity**	**PPV**	**NPV**	**Odds Ratio [95%CI]**	***p*-value compared to POPO neg.**
Surgery	26/118 (22.03%)	870/966 (90.06%)	26/122 (21.31%)	870/962 (90.44%)	2.56 [1.58–4.15]	**<0.001**
Growth failure	4/39 (10.26%)	927/1,045 (88.71%)	4/122 (3.28%)	927/962 (96.36%)	0.9 [0.32–2.57]	0.84
Abscess. fistula. stenosis	57/194 (29.38%)	833/890 (93.6%)	57/122 (46.72%)	833/962 (86.59 %)	7.36 [4.93–11.0]	**<0.0001**
Lack of sustained remission	64/573 (11.17%)	453/511 (88.65%)	64/122 (52.46%)	453/962 (47.09%)	0.98 [0.67–1.43]	0.93
Escalate to Azathioprine	90/788 (11.42%)	264/296 (89.19%)	90/122 (73.77%)	264/962 (27.44%)	1.06 [0.69–1.63]	0.78
Escalate to Infliximab	34/230 (14.78%)	766/854 (89.70%)	34/122 (27.87%)	766/962 (79.63%)	1.51 [0.99–2.31]	0.06 (0.056)
EIM	59/507 (11.64%)	514/577 (89.08%)	59/122 (48.36%)	514/962 (53.43%)	1.07 [0.74–1.57]	0.71

The bold values indicates $p < 0.05$.

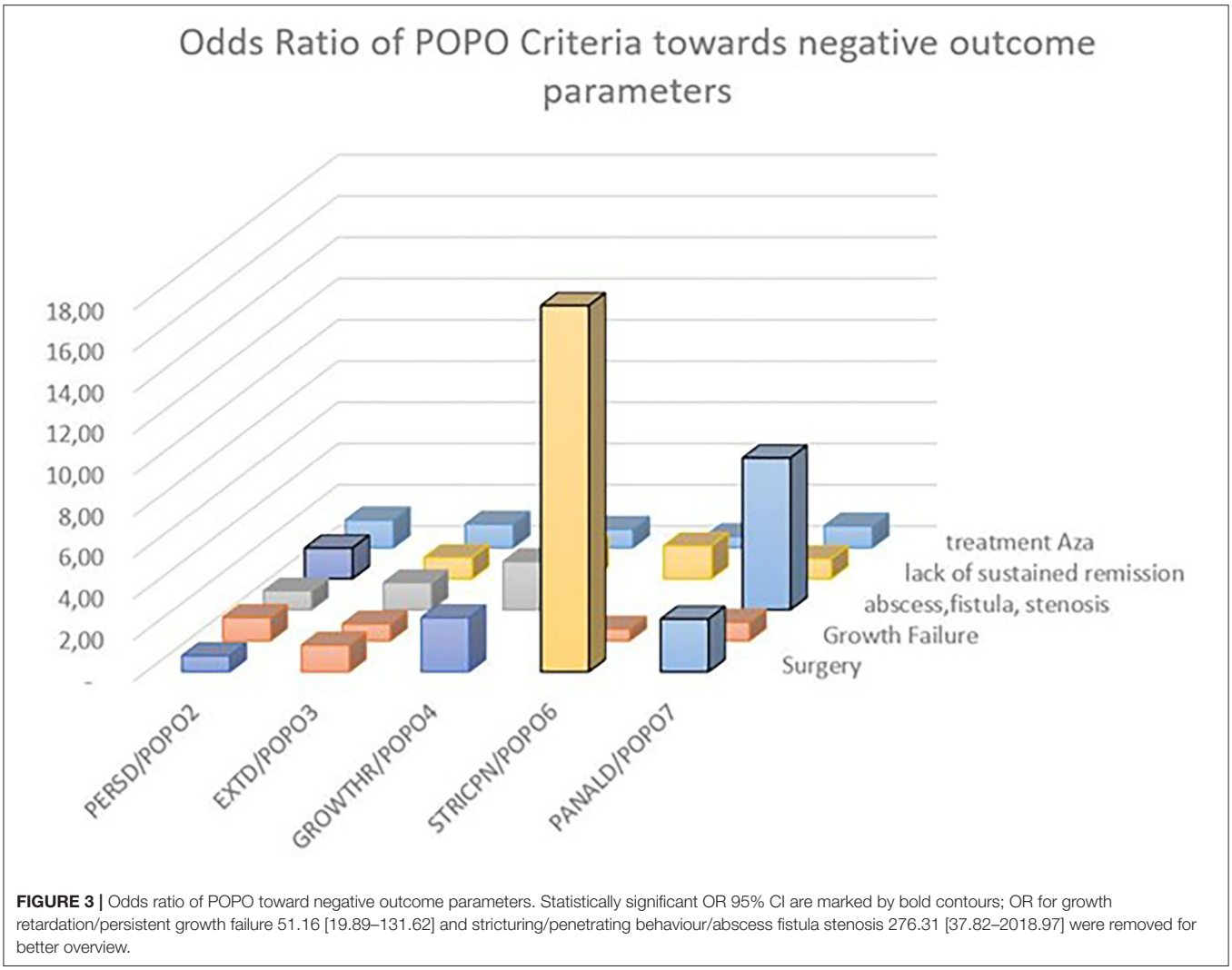

FIGURE 3 | Odds ratio of POPO toward negative outcome parameters. Statistically significant OR 95% CI are marked by bold contours; OR for growth retardation/persistent growth failure 51.16 [19.89–131.62] and stricturing/penetrating behaviour/abscess fistula stenosis 276.31 [37.82–2018.97] were removed for better overview.

to observations from a French population-based cohort study with complicated disease behaviour in only 31% of paediatric CD patients at diagnosis (13). We are thus confident that our cohort is suitable for testing the predictive properties of POPO criteria. POPO-positive patients were only slightly younger than POPO-negative patients, and the age difference of only a few months might not be clinically relevant (14, 15). The higher PCDAI at diagnosis could indicate a more severe disease course, as shown for at least the first year after diagnosis in a report from Hungary (16). However, we did not analyse initial PCDAI separately as a predictor for long term outcome, because, in accordance with published data, its preliminary analysis did not yield a highly significant effect (16).

More than 16% of our patients had persistent severe disease despite adequate induction therapy (POPO2-positive) while the rates for similar patient groups were reported as only 2.1% after 1 year (PCDAI >31) in a Hungarian registry (16) and 5% by Dubner et al. (17). These differences could be explained by other observational periods and disease activity scores. Since an accelerated step-uptreatment strategy has to be decided earlier than at 1 year, we defined lack of response to induction therapy at 3 months as "persistent disease." This definition meets the demand for an early predictive parameter more adequately.

With almost 54% of patients suffering extensive disease (POPO3-positive) our findings are in keeping with own previous reports (3) and data from the EUROKIDS-Registry (17, 18), showing the majority of paediatric CD patients to have pancolonic involvement. However, POPO3-, as well as POPO2-positivity, were recorded significantly more often in children between 1 and 6 years of age than in older children, thus serving as further risk predictors in addition to their classification as high-risk patients based on young age (19).

Less than 2% of study patients were POPO4-positive, i.e., presented with severe growth retardation (< −2.5 SDS), in significant contrast with data from the EPIMAD registry which pointed to growth retardation (< = −2 SDS) in 9.5% of patients (25 of 261) (20) and reports on incidences of growth failure as high as 15–40% (21). The variability of these incidences might be explained in part by the use of different reference systems and cut off values.

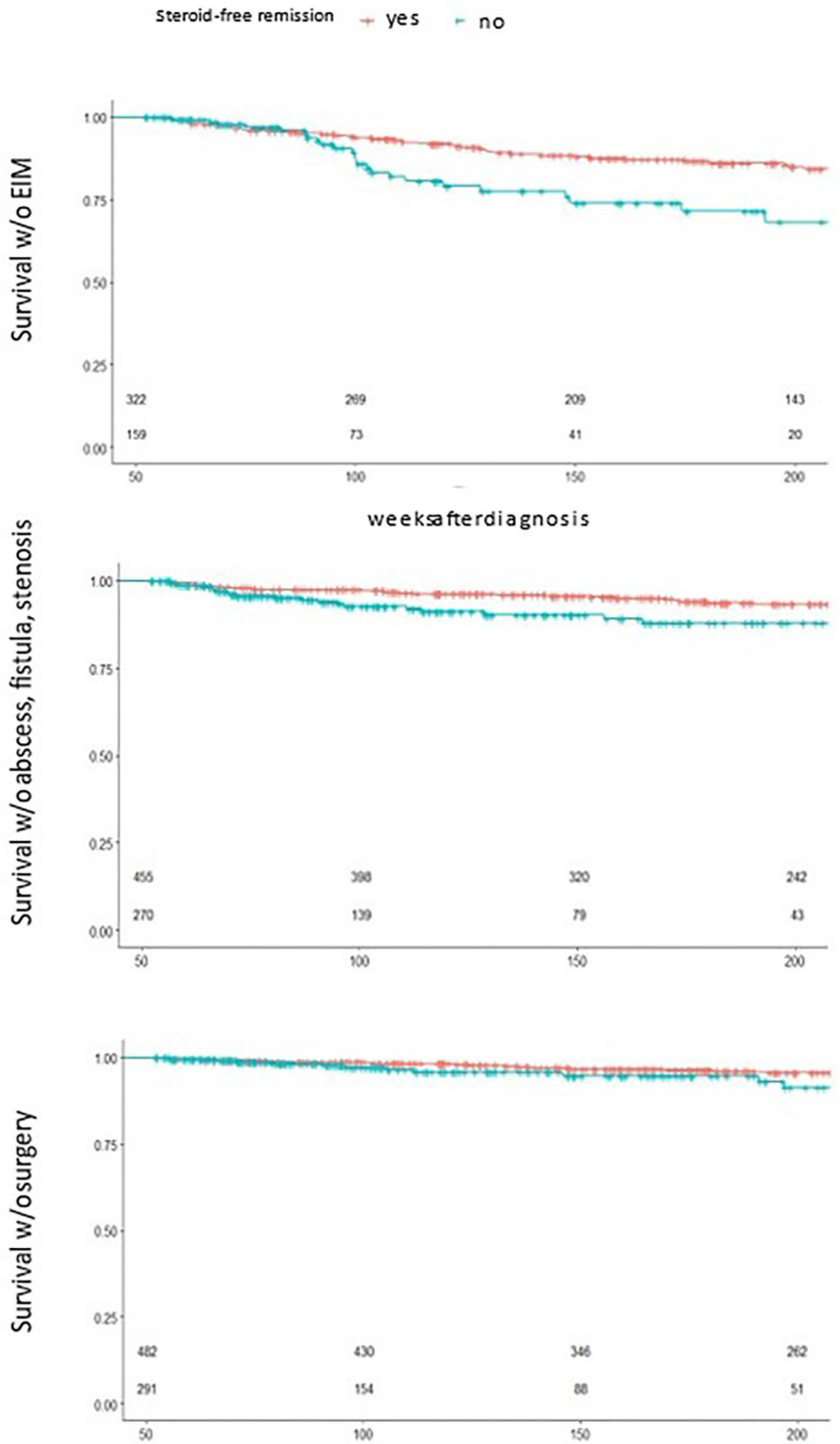

FIGURE 4 | Survival analysis by 1 year steroid free remission. Statistically significant reduced event free survival for children who did not reach 1 year steroid free survival toward EIM(top), abscess, fistula and stenosis (middle) and surgery (bottom), small numbers n patients under risk.

We found that severe growth retardation at diagnosis had an extremely high PPV for persisting growth failure. Because growth retardation in paediatric CD reflects contributing pathogenetic factors such as malnutrition, anorexia and inflammation (22) and intensified therapy with early use of biologics can lead to increased catch up growth (23–25), our data substantiates even further the necessity for more aggressive treatment at an early stage. POPO-4-positive patients had also very high PPVs for lack of sustained remission >1 year and EIM, relevant negative outcome parameters contributing substantially to long-term morbidity (19, 26). In retrospective studies, an increased risk for surgery was associated with lower weight Z-scores (27). Since Z-score deviations in juvenile patients may have causes other than CD we chose a stricter cut-off for this study.

4.3% of the cohort studied had stricturing or penetrating disease (POPO-6-positive), in contrast to reports from France and Scotland, with 29 and 9% of patients showing B2/B3 behaviour at diagnosis, resp., (18, 28) and even higher rates in the Swiss IBD Cohort Study Group (29). These data reflect disease complications over time rather than at diagnosis. In fact, a retrospective analysis of small bowel imaging within 30 days of diagnosis in more than 200 paediatric CD patients showed the majority of increased surgical risk to occur in the first year after diagnosis, with a peak percentage as high as 17% (30). The patients we observed must thus certainly be classified as high-risk patients: Not only did they present with a severe complication at an early phase, but, once again in accordance with reports of others (17, 22–24), we found their risks for developing abscess, fistula or stenosis as well as surgery to be significantly higher, and significantly, the PPV for lack of sustained remission >1 year was highest of all POPO-groups.

This was also true for patients with perianal disease (POPO-7-positive), thus supporting the notion of perianal disease at diagnosis as a strong predictor of unfavourable outcome (19). The influence of perianal disease on developing abscess, fistula or stenosis has so far yielded conflicting results in the literature, with reports on an increased risk (OR 3.5 [1.98–6.20]) (15), not confirmed by others (31). However, a recent study from the Swiss IBD Cohort Study Group revealed higher rates of intestinal complications, including those in the anal region, particularly in paediatric CD patients. In our cohort we also found very high ORs for developing abscess, fistula or stenosis (OR 7.36 [4.93–11.0]) as well as raised ORs for later surgery (2.56 [1.58–4.15]), thus further substantiating POPO-7-positivity as a valuable clinical predictor of complicated disease.

Limitations of our study include that other therapeutics strategies were not included, that a substantial number of patients had a followup of <2 years and that we did not collect information on ethnicity, mucosal healing or biosamples. Another problem is group size especially for subgroups. Also, in evaluating the role of medication and outcome, very short time scales cannot be analysed in an observational dataset. We hope to address this by a different model in a following study. The one main limitation of this study is that it is observational. One might also argue that our registry is based on patient data from large regional referral centres, which may not allow extrapolation to the real paediatric IBD population in Germany and Austria. We would, however, go so far as to suggest that this is one of the strengths of our analysis: CEDATA is a multicentre registry with only tertiary centres and IBD specialists participating. We therefore trust the registered data of this carefully selected and large study cohort with patients exclusively diagnosed according to the Porto Criteria to be robust enough to address the risk stratifying potential of the POPO criteria as suggested in the respective guidelines for the first time.

The long observational period of almost 15 years, with a median follow-up of >2 years and 10 visits documented consecutively, was a very important criterion for this research project, since it enabled us to investigate treatment over time with a continuous change in prescription of azathioprine and infliximab, the immunomodulatory and anti-TNF-agents mainly used in our countries.

CONCLUSION

This large "real-life" data set showed predictors of poor outcome in paediatric CD to be common. While neither presenting symptoms nor initial disease activity scores were suitable candidates for treatment stratification, the predictors showed significantly increased risks of relevant complications and negative outcomes. Patients with persistent disease at 3 months were prone to fail in reaching sustained remission. A patient who did not respond satisfactorily to initial treatment had a >60% risk of missing this important target. Patients who failed to reach sustained remission carried a significantly increased risk for all complications monitored, therefore sufficient disease control is a mandatory target. Patients with extensive disease had a higher risk of developing abscess, fistula, or stenosis.

Since the group with severe growth retardation showed a very highly increased risk of remaining in growth failure to the end of observation, with two thirds of these patients not reaching sustained remission during follow-up, this should also be a good reason for beginning early and aggressive therapy. The same applies to patients with stricturing or penetrating disease behaviour and with perianal disease, who showed significant increases in fistula, abscess and stenosis development and surgery risk. The problem in how far a "therapeutic window of opportunity" in early CD might be clearly identified and made use of by "treat-to-target" has been addressed only very recently. The intention in this research project was to establish biologics for patients with currently known high-risk factors and also predictors of poor outcomes, suggesting a combination of "phenotype at diagnosis" and "comportment follow-up classification" in the year following diagnosis (32).

SUMMARY

Stratifying therapy for Pediatric Crohn's Disease requires predictors for negative long-term prognosis. The patient registry data presented demonstrate the value and characteristics of these criteria for the first time in a large real-world pediatric data set.

DATA AVAILABILITY STATEMENT

The datasets presented in this article are not readily available because; Dataset is part of CEDATA GPGE Registry, cannot be uploaded or transfered, aggregated data is part of the article, additional data is available on request. Requests to access the datasets should be directed to studienzentrale@paediat.med.uni-giessen.de.

ETHICS STATEMENT

Approval for all centers involved was obtained. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

JL performed the research, wrote the first draft, and contributed in editing. K-PZ supervised the manuscript and gave valuable insights during editing and analysis. KS provided IT guidance and supported analysis. AH edited analysis, further developed the manuscript, and coordinated the process. All authors contributed to the article and approved the submitted version.

FUNDING

This study was funded by Justus Liebig University, the Medical University of Graz and the Rudolf-Chaudoire-Wissenschaftsförderung für Schweiz, Deutschland und Österreich. CEDATA-GPGE® is funded by donations from abbvie, Dr Falk pharma, vifor, Ein Herz für Kinder, Takeda.

ACKNOWLEDGMENTS

The authors thank C. Wendt, H. Gurmai, Y. Kho and T. Weidenhausen for their help in preparing and analysing data. The authors also acknowledge S. Cantez' help with preparing the manuscript. The authors thank Prof David Wilson for his assistance in addressing important issues for the manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2021.644003/full#supplementary-material

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frontiers in Medicine

MINI REVIEW
published: 20 September 2021
doi: 10.3389/fmed.2021.723719

Co-pathogens in Periodontitis and Inflammatory Bowel Disease

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Localized inflammatory lesions in one area of the body may affect other distant organs through various modes of transmission thus initiating secondary inflammatory infections. Periodontal disease (PD) and inflammatory bowel disease (IBD) have been shown to coexist. Periodontitis is a multifactorial inflammatory disease, and dental plaque is considered to be the initial risk factor. Individuals with genetic susceptibility are more likely to develop periodontitis when exposed to external stimuli. IBD is affected by host genetics, immunoregulation, daily diet, and the gut microbiota, and its risk factors appear to be shared with those of PD. However, the key etiologies of both diseases remain unclear, thus hindering the exploration of possible links between IBD and PD. Recent studies and systematic reviews have focused on evidence-based statistics of the prevalence and clinical manifestations of both diseases, but discussions of the microbial etiological correlation between periodontitis and intestinal inflammation are scarce. Here, we summarize the potential common pathogenic microorganisms that may serve as bridges between the two diseases. Studies have shown that invasive microorganisms such as *Porphyromonas gingivalis*, *Fusobacterium nucleatum*, *Klebsiella* spp. and *Campylobacter* spp. play key roles in the comorbidity of PD and IBD.

Keywords: periodontitis, inflammatory bowel disease, periodontal disease, bacteria, microbiota, intestine

OPEN ACCESS

Edited by:
Mariann Rutka,
University of Szeged, Hungary

Reviewed by:
Mario Romeo,
University of Campania Luigi Vanvitelli, Italy
Moris Sangineto,
Università di Foggia, Italy

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Specialty section:
This article was submitted to Gastroenterology, a section of the journal Frontiers in Medicine

Received: *11 June 2021*
Accepted: *27 August 2021*
Published: *20 September 2021*

Citation:
Cai Z, Zhu T, Liu F, Zhuang Z and Zhao L (2021) Co-pathogens in Periodontitis and Inflammatory Bowel Disease. Front. Med. 8:723719. doi: 10.3389/fmed.2021.723719

INTRODUCTION

What Is Periodontal Disease?

PD is an inflammatory disease that affects the periodontium and alveolar bone. Changes in the gingiva such as swelling and redness are often the earliest signs (1). If untreated, gingivitis can progressively deteriorate, leading to attachment loss, formation of periodontal pockets and alveolar bone loss, which are known as periodontitis (2). Severe periodontitis can result in loose or missing teeth and is reported to be a major cause of tooth loss in adults (3). However, the pathogenesis of periodontal disease remains uncertain. Both dental plaque and the balance between host immune responses and the microbiota are important initiating factors (4). PD is also associated with various systemic diseases, including diabetes mellitus (5), atherosclerosis (6), and adverse pregnancy outcomes (7), which affect each other bidirectionally. However, the relationship between periodontitis and inflammatory bowel disease (IBD) is unclear, and the precise mechanisms must be clarified.

What Is IBD?

IBD is characterized by chronic recurrent intestinal inflammation and consists mainly of ulcerative colitis (UC) and Crohn's disease (CD). IBD morbidity has increased dramatically from the

twentieth century (8), and its pathogenesis remains unclear. Studies suggest that environmental factors, genetic susceptibility, the intestinal microbiota, and immune responses are involved in IBD development (9, 10). Among these factors, intestinal microorganisms play key roles in IBD occurrence and progression (11). Varied metabolites are also involved in the changes in the intestinal ecosystem (12).

Evidence-Based Association Between IBD and PD

Meta-analyses from evidence-based medicine have integrated several observational studies demonstrating that patients with periodontitis or IBD had an increased risk of also having the other disease, with a pooled odds ratio of 3–5 (13–15). Furthermore, a few cohort studies indicated that patients with IBD tended to have a higher risk of developing PD than did those without IBD (16). Patients with periodontitis also had greater risks of developing UC than did the controls (17, 18). Retrospective and cohort studies have confirmed a bidirectional association between IBD and PD. However, questions remain regarding how the two diseases interact with each other and what are their microbial causes and common risk factors.

Roles of Microorganisms in PD and IBD

Studies have found no specific microbial pathogens for either periodontitis or IBD. Epidemiological statistics, clinical symptoms, and risk factors imply that some correlations, such as the common suspicious microorganisms, exist between periodontitis and IBD (15–17, 19–22). In the oral cavity, microbes gather and propagate at subgingival gaps, forming dental plaques (23, 24), which act as a pathogenic arsenal that can produce antigens to invade the gingival mucosa in the deep periodontal pockets. Furthermore, these pathogens can interfere with host immune defenses to exacerbate inflammation. For example, the red complex (*Porphyromonas gingivalis*, *Treponema denticola* and *Tannerella forsythus*) has been strongly associated with periodontitis (25). Research has demonstrated a correlation between *Porphyromonas gingivalis* infections (26) and a deficiency of Toll-like receptors, which decreases the strength of the innate immune system (27) and leads to an imbalance between invasive pathogens and host defenses in the periodontal tissues. This disrupts the homeostasis, leading to inflammation in the oral cavity.

Humans share many common gut microbes, such as Firmicutes and Bacteroidetes (28, 29). From infancy to adulthood, the resident intestinal microorganisms evolve and ultimately attain relative stability, signifying the maturity of the host's intestinal ecological system (28). The dynamic and relatively constant microbiota contributes to building intestinal homeostasis and shapes a biological barrier in the alimentary tract. Conversely, dysbiosis in the gastrointestinal tract induces inflammation in susceptible hosts (30). Fecal microbiota transplantation alleviates UC symptoms in patients with UC, suggesting that alteration of the intestinal microbes affects the outcomes of IBD (31).

CORRELATION BETWEEN PERIODONTITIS AND IBD

Prevalence and Comorbidity

Several studies have focused on the increasing comorbidity of periodontitis and IBD (15–17). These clinical evidence-based studies can be divided into two categories: (1) patients with IBD who have a higher risk of developing periodontitis and (2) patients with periodontitis who have a higher risk of developing IBD. One cross-sectional study of 1,297 patients reported a 30% higher risk of periodontitis in patients with IBD than in controls without IBD (32). Another investigation showed that patients with IBD had deeper periodontal pockets, less clinical attachment, and more severe gingival bleeding compared with those of the healthy controls (33). Retrospective statistics from the National Health Insurance Research Database in Taiwan revealed that patients with periodontitis had a higher risk of IBD than did matched controls without periodontitis (17). Additionally, patients with periodontitis had a greater risk of subsequent UC than did the controls (17). In treatment, some pharmacotherapies for CD may protect patients against periodontitis, suggesting a relationship between IBD and PD (16).

Microbiological Associations Between Periodontitis and IBD

Compositions of normal bacteria, opportunistic pathogens, pathogenic microorganisms, and probiotics affect the microecological balance. An imbalanced microecology can further lead to disease (34). Oral microbes can be transmitted to the gut *via* the gastrointestinal tract (35). This provides the basis for translocation of pathogenic bacteria from the oral cavity to the gut, thus disrupting the intestinal homeostasis. One hypothesis suggests that suspicious pathogens promote the co-occurrence of PD and IBD. First, periodontal pathobionts migrate from the oral cavity to the gut and lead to dysbiosis of the intestinal ecology. Second, the disordered state of the gut microbiota triggers an intestinal immune response, which manifests as intestinal and systemic inflammation leading to the occurrence or aggravation of periodontitis. Finally, dysbiosis of either the oral or intestinal microbiota can be initial causes of PD or IBD. Subsequent alterations, including microorganisms, virulence factors, harmful metabolites, and other proinflammatory factors, can spread between both the intestines and the oral cavity *via* the circulatory system. **Figure 1** shows the bidirectional reinforcement cycle in the deterioration of PD and IBD. However, no specific pathogenic strain has been identified. We propose several PD- and IBD-associated microorganisms that may be key causative agents for a higher risk of IBD and PD comorbidity.

Porphyromonas Gingivalis

Porphyromonas gingivalis is considered a critical pathogen in periodontitis (19) as well as a risk factor for enteric inflammation *via* altering the microbiota composition and metabolite profiles (20). Mice who were orally administered *P. gingivalis* exhibited increased levels of amino acid metabolism,

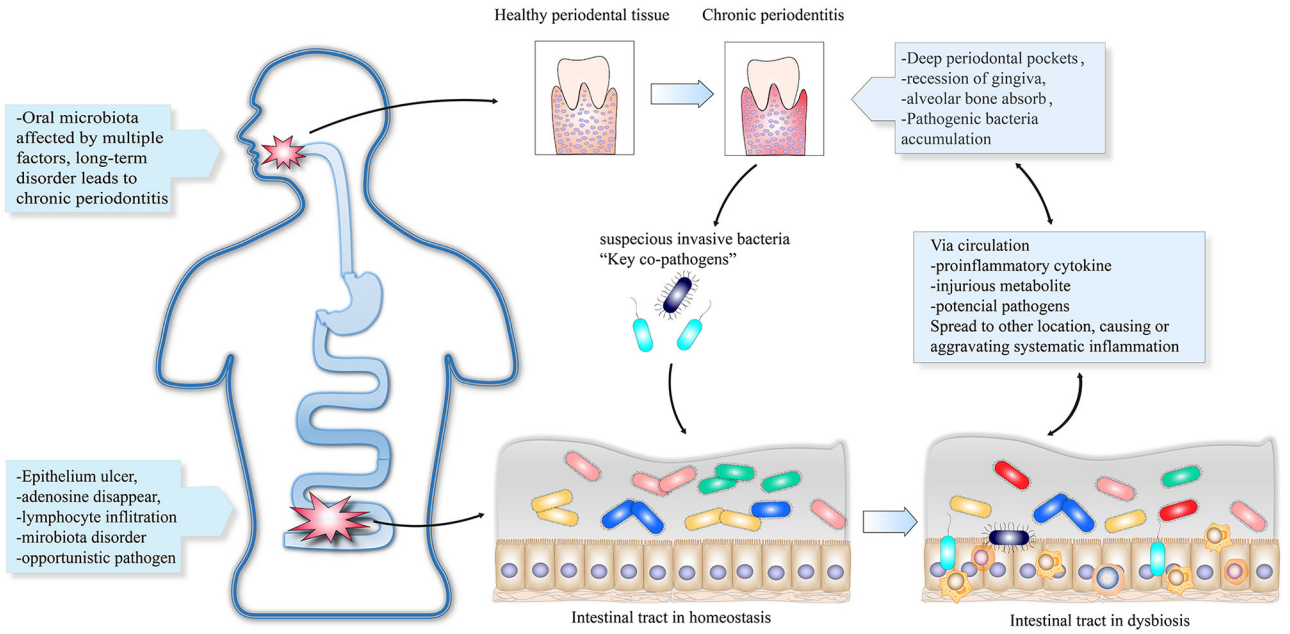

FIGURE 1 | The oral cavity and intestinal tract can contain initial lesions infected with suspicious invasive bacteria that lead to localized dysbiosis. The key pathogens can travel through the digestive tract to other parts of the body and trigger or aggravate inflammatory diseases. Other proinflammatory cytokines and harmful metabolites can be transmitted *via* the circulatory system to initiate inflammation.

including biosynthesis of phenylalanine, glutamine, tyrosine, and tryptophan in the gut and serum, implying that oral administration of *P. gingivalis* induced alterations of the gut microbiota composition and metabolites (20). Jia et al. (36) found that *P. gingivalis* upregulated the Th17-associated transcription factor, RoRγt, and increased the IL-17 and IL-6 levels. Conversely, *P. gingivalis* downregulates the expression of Treg transcription factor Foxp3, TGF-β, and IL-10 *via* the TLR4 pathway. These authors revealed the relationship between *P. gingivalis* and IBD through a dextran sodium sulfate (DSS)-induced IBD mouse model in which *P. gingivalis* activated CD4+ T cells and exacerbated colitis by upregulating the Th17/Treg ratio *via* the JAK-STAT signaling pathway (36). Tsuzuno et al. (37) found a significant exacerbation of colitis in DSS-induced mice administered *P. gingivalis*. These authors reported that *P. gingivalis* reduced the tight junction proteins by decreasing zonula occludens-1 (ZO-1) levels in intestinal epithelial cells, which in turn disrupted the intestinal barrier function. Additionally, They found that *Prevotella intermedia* (38) and *Fusobacterium nucleatum* (39), which are considered periodontopathic bacteria, also exacerbated colitis (37). Although the exacerbation was to a lesser extent than that of *P. gingivalis*, and the exacerbation mechanism did not occur *via* disruption of the tight junctions of the intestinal epithelial barrier, *P. intermedia* and *F. nucleatum* may act as links between periodontitis and IBD and have a synergistic effect in intestinal inflammation (37).

Klebsiella spp.

Klebsiella spp. are oral pathogens that ectopically colonize the gut and induce dysbiosis and inflammation (21, 22). Atarashi et al. (21) gavaged gnotobiotic mice with saliva from patients with CD and analyzed the microbial differences between the saliva and mouse feces *via* 16S rRNA sequencing. It was found that *Klebsiella pneumoniae 2H7* can ectopically colonize the intestines *via* the oral cavity and significantly induce Th1 cell responses, suggesting that some oral pathogenic bacteria can exacerbate intestinal diseases. Kitamoto et al. (40) found that *Klebsiella* and *Enterobacter* spp. triggered colitis in mice with ligature-induced periodontitis (41). Periodontitis can trigger and exacerbate intestinal inflammation *via* the direct microbial pathway and the indirect immunological pathway. To verify the direct pathway, Kitamoto et al. used mouse models of ligature-induced periodontitis and DSS-induced colitis and found that the colitis was more severe in the ligature+DSS model than in the DSS-alone group. High-throughput sequencing technology was used to determine the significantly different *Klebsiella* and *Enterobacter* spp. (40). The bacteria were isolated, cultured, and tested in germ-free $Il10^{-/-}$ mice and specific pathogen-free mice. The oral pathogenic *Klebsiella* and *Enterobacter* spp. were transferred and colonized the intestines through the digestive tract *via* the direct pathway, thus stimulating macrophages to secrete IL-1β *via* caspase-11-mediated inflammasomes to induce colitis (40). To confirm the indirect pathway, Kitamoto et al. used Kaede fluorescent protein mice to track T-cell migration (42) and found that orally primed T cells migrated to the colonic lamina propria only in the ligature + DSS-induced model mice (40). Neither ligature nor DSS alone resulted in the same phenotype. Thus, the reactive Th17 cells were activated immunologically by oral pathogens and migrated from the mouth to the intestines *via* the lymph nodes, where they were

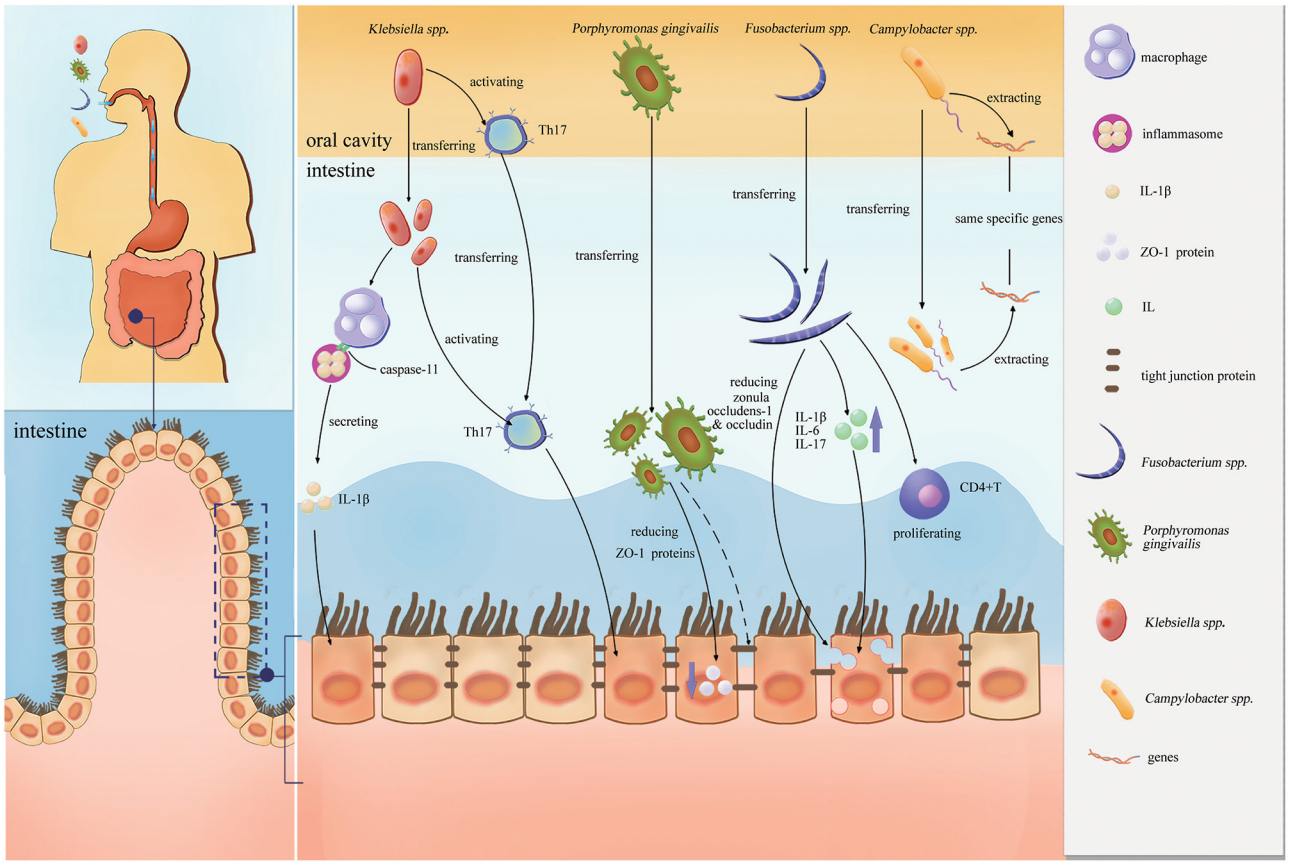

FIGURE 2 | Proinflammatory effects of the four pathobionts (*Porphyromonas gingivalis*, *Klebsiella* spp., *Fusobacterium* spp., and *Campylobacter* spp.) in the intestines.

activated by homologous pathogens from the oral cavity, leading to intestinal inflammation (40).

Fusobacterium spp.

Fusobacterium nucleatum plays an important role in dental plaque and periodontitis formation (39). This microorganism also resides in the intestinal tract and is associated with IBD, especially in patients with UC (43, 44). Huh and Roh (45) analyzed longitudinal metagenomic data from the integrative Human Microbiome Project (iHMP) and revealed that *F. nucleatum* may be associated with early intestinal dysbiosis and could serve as a biomarker for detecting IBD. Strauss et al. (46) found that *Fusobacterium* spp. could be isolated from 63.6% of patients with gastrointestinal disease compared with 26.5% of healthy controls ($P = 0.01$). *Fusobacterium nucleatum* strains derived from inflamed biopsy tissue from patients with IBD were more invasive than those isolated from healthy tissue from patients with IBD or from controls ($P < 0.05$), revealing that *F. nucleatum* in the oral cavity may be a source of highly invasive bacteria in IBD (46). Liu et al. (47) reported that *F. nucleatum* can exacerbate IBD by damaging epithelial integrity and increasing permeability by regulating expression of the tight junction proteins zonula occludens-1 and occludin. It also promotes secretion of cytokines such as IL-1β, IL-6, and IL-17 and induces CD4(+) T-cell proliferation by activating the STAT3 signaling pathway.

Campylobacter spp.

Campylobacter spp. are present in the oral cavity and alimentary tract of IBD patients and are considered causative agents of oral and intestinal diseases (48, 49). Hsu et al. (50) sequenced the genome biology of *Campylobacter showae* and identified the functions of type IV secretion machinery and S-layer proteins in invasive strains. These authors compared the strains isolated from the gut with those from oral supragingival plaques and found that they shared the same specific genes, indicating a similar potential virulence and pathogenic pathway in initiating periodontitis and IBD (50). Furthermore, *Campylobacter concisus* genes isolated from the same patients' gut and oral environments were similar, indicating relocation of oral microbes to the intestinal tract and the role of periodontopathogens in gastrointestinal disease (51).

Figure 2 shows the proinflammatory effects of these four pathobionts (*Porphyromonas gingivalis*, *Klebsiella* spp., *Fusobacterium* spp., and *Campylobacter* spp.) in the intestines.

DISCUSSION

Periodontitis and IBD exhibit a high risk of co-occurrence and are potentially correlated. Studying diseases with interactions is challenging, and an inadequate understanding of IBD and PD further hinder this research. Dysbiosis of the microbiota is a common feature of both diseases. Dysbiosis of the oral microflora can trigger PD, while dysbiosis of the gut microflora contributes to IBD. Multiple risk factors, including dietary intake, smoking habits, oral hygiene, and host genetic susceptibility also influence oral and intestinal homeostasis (52, 53).

We emphasize the critical roles of suspicious pathobionts in both diseases; however, host immune factors are equally important. Susceptible individuals are more likely to experience dysbiosis and immune inflammatory responses triggered by pathogenic microorganisms. Immune cells and inflammatory cytokines can be transferred from areas of the body to other distant organs *via* the blood circulation and trigger subsequent diseases (39, 54). Cytokine expressions can be measured as observational indicators to evaluate the conditions of patients with periodontitis and IBD. Higher IL-1β, IL-4, IL-6, IL-10, and IL-21 expressions were detected in gingival tissues in patients with active IBD compared with those of patients in remission. Proinflammatory cytokine expressions are also positively correlated with disease severity scores (55). The IL-17/IL-23 axis appears to play critical roles in IBD and periodontitis by inducing and regulating the innate immune response to the tissues and pathogens (56, 57). A clinical control trial showed that periodontitis patients with concurrent IBD treated with anti-tumor necrosis factor alpha (anti-TNF-α) therapy had a higher probability of healing than did those managed without anti-TNF-α therapy (58). Transcriptomic analysis of periodontitis identified a special upregulated gene, pleckstrin, which was overexpressed in patients with UC and other chronic inflammatory diseases, supporting the hypothesis of a network between periodontitis and IBD (59).

Conversely, a cohort study by Yin et al. (60) reported an inverse relationship between poor oral health and IBD owing to the hygiene hypothesis (early poor oral hygiene can better induce immune tolerance in the gut, resulting in a lower IBD incidence). However, this may not be contradictory to the increased comorbidity of PD and IBD. Poor oral hygiene differs from periodontitis. Studies have shown that poor oral conditions can lead to a build-up of dental plaque, which in turn leads to gingivitis, but only a portion of gingivitis progresses to periodontitis (61, 62). Thus, poor oral hygiene is a risk factor for periodontitis rather than a direct cause. Yin et al. reported that poor oral hygiene in early childhood may induce immune tolerance, which leads to a lower probability of developing IBD in adulthood. This is distinct from our discussion of the increased risk of IBD in adults with poor oral hygiene and PD. If the hygiene hypothesis is true, individuals with good oral hygiene in early childhood (who do not develop an appropriate immune tolerance) may have a higher chance of developing IBD if they have poorer oral hygiene in adulthood.

CONCLUSIONS

We emphasize the following four points:

1. Periodontitis and IBD have an increased probability of coexisting.
2. Multiple common features suggest a possible bidirectional relationship between periodontitis and IBD.
3. Certain invasive microorganisms (e.g., *Porphyromonas gingivalis*, *Fusobacterium nucleatum*, *Klebsiella* spp., and *Campylobacter* spp.) may play key roles in the comorbidity of PD and IBD.
4. Potential pathogenic microorganisms, immune responses, and other risk factors contribute to the link between PD and IBD, reinforcing the bidirectional cycle in the deterioration of the two diseases.

Increasing evidence implies a correlation between IBD and periodontitis. Patients with IBD can exhibit extraintestinal oral manifestations associated with PD, which can occur before intestinal inflammation and suggest the existence or risk of IBD (63). The severity and risk of developing periodontitis are higher for patients with IBD compared with those of people without IBD (15, 64). Likewise, patients with periodontitis have a higher prevalence of IBD (17, 18). Researchers should evaluate the oral cavity and the intestines simultaneously to more purposefully seek possible co-pathogens in the comorbidity of IBD and PD. This will provide a biomarker for diagnosing both IBD and PD. For patients with only the clinical manifestations of either IBD or PD, this biomarker may remind clinicians of the higher risk of comorbidity of both diseases for purposes of prevention. In the therapeutic field, it may provide target pathogens for the treatment of PD and IBD.

AUTHOR CONTRIBUTIONS

ZC and LZ contributed to conception and design. ZC, TZ, FL, and ZZ contributed to drafting and figure. ZC, TZ, FL, ZZ, and LZ contributed to manuscript revisions. All authors contributed to the article and approved the submitted version.

FUNDING

This study was supported by a research grant from West China Hospital of Stomatology (LCYJ2019-4).

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frontiers in Medicine

ORIGINAL RESEARCH
published: 29 September 2021
doi: 10.3389/fmed.2021.725512

Gastroenterologists Adherence to Tumor Necrosis Factor Antagonist Combination Therapy in Inflammatory Bowel Disease

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Introduction: Tumor necrosis factor antagonists (anti-TNF) therapies are used for the management of moderate to severe inflammatory bowel disease (IBD). Anti-TNF combination therapy, with immunomodulators, has been shown to reduce immunogenicity, especially for infliximab, improve treatment success rate and patient outcomes. We evaluated factors associated with gastroenterologists adherence to anti-TNF combination therapy.

Methods: A retrospective cohort study was performed to evaluate the adherence of gastroenterologists ($n = 14$), at an inflammatory bowel disease center, to anti-TNF combination therapy. Records of patients who received Infliximab ($n = 137$) or adalimumab ($n = 152$) were obtained and their ordering physicians' data was analyzed. Gastroenterologists were divided into six groups according to their age and interest in IBD. The baseline characteristics of their patients were also obtained.

Results: The proportion of patients on combination therapy in the young gastroenterologists group was higher than those in the senior gastroenterologists group for both infliximab (83.2 vs. 55.6%, respectively, $P < 0.001$) and adalimumab (59 vs. 30.8%, respectively, $P < 0.001$). Gastroenterologists with interest in inflammatory bowel disease (IBD interest group) had also more proportion of patients on adalimumab combination therapy compared to gastroenterologists with no interest in IBD (non-IBD interest group) (61.7 vs. 35.2%, respectively, $P < 0.001$). Gastroenterologists who were both young and have interest in IBD had more proportion of patients on combination therapy than those who were senior or had no interest in IBD for both infliximab (89.4 vs. 63.4%, respectively, $P < 0.001$) and adalimumab (75.9 vs. 33%, $P < 0.001$). The IBD interest group was also requesting more antidrug antibody level tests than those in the non-IBD interest group (41.4 vs. 12.3 tests, respectively, $P < 0.001$).

Conclusion: Young gastroenterologists are more likely to prescribe anti-TNF infliximab and adalimumab combination therapy than senior gastroenterologists. In addition, gastroenterologists with IBD interest are more likely to prescribe adalimumab combination therapy than gastroenterologists with no IBD interest. Moreover, young

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Edited by:
Barbara Dorottya Lovasz, Semmelweis University, Hungary

Reviewed by:
Petra Golovics, Hungarian Defense Forces Health Center, Hungary
Aron Vincze, University of Pécs, Hungary

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Specialty section:
This article was submitted to Gastroenterology, a section of the journal Frontiers in Medicine

Received: *15 June 2021*
Accepted: *30 August 2021*
Published: *29 September 2021*

Citation:
Abdullah I, Alhendi G, Alhadab A, Alasfour H and Shehab M (2021) Gastroenterologists Adherence to Tumor Necrosis Factor Antagonist Combination Therapy in Inflammatory Bowel Disease. Front. Med. 8:725512. doi: 10.3389/fmed.2021.725512

gastroenterologists who have interest in IBD are more likely to prescribe both infliximab and adalimumab combination therapy than senior gastroenterologists or those with no IBD interest. In addition, gastroenterologists with IBD interest requested more anti-TNF serum drug concentrations and antidrug antibody level tests than those with no IBD interest.

Keywords: IBD, anti-TNF, combination, gastroenterologist, age, interest

INTRODUCTION

Tumor necrosis factor antagonists (anti-TNF) therapies are considered to be an effective treatment for moderate-to-severe inflammatory bowel disease (IBD) (1–4). However, about one-third of patients treated with anti-TNF therapies may experience primary non-response (PNR), manifested by lack of response during induction therapy (5, 6). Moreover, about half of patients with initial response may experience secondary loss of response (SLR) to anti-TNF, by losing treatment effect during the maintenance of remission (6). Immunogenicity, the formation of antidrug antibodies, is known as one of the most common causes of loss of response to anti-TNF (6, 7). The concurrent administration of an immunomodulator with an anti-TNF, combination therapy, has been associated with improvement in the pharmacokinetics of anti-TNF, by decreasing antidrug antibodies and increasing serum drug concentrations, that potentially results in higher rates of clinical remission and lower rates of immunogenicity.

Based on the available evidence (SONIC and UC-SUCCESS trials), both American Gastroenterology Association (AGA) and European Crohn's and Colitis Organization (ECCO) guidelines support the use of infliximab combination therapy over infliximab monotherapy, in inducing and maintaining clinical remission in patients with moderate to severe IBD (1, 3, 8, 9). Whereas, for adalimumab, ECCO guidelines suggest against the use of combination therapy over monotherapy in patients with Crohn's disease (CD) (3). This recommendation was based on DIAMOND trial and two meta-analyses (Kopylov et al. and Chalhoub et al.), which showed that adalimumab combination therapy is associated with limited impact on maintenance of clinical remission or response (10–12). While AGA guidelines support the use of adalimumab combination therapy over monotherapy, especially for patients who have developed loss of response to anti-TNF (1, 2). A recent study done by Roblin et al., showed that combination therapy can result in better clinical outcomes without clinical failure or unfavorable pharmacokinetics at 24 months in patients with IBD who experienced an immune-mediated loss of response with first anti-TNF (13). Moreover, Kuwait local health authority allows physicians to follow any international recognized guidelines, commonly practiced ECCO or AGA guidelines, in regard to IBD management. We did this study to evaluate the adherence of our gastroenterologists to anti-TNF combination therapy and understand factors that can be associated with their adherence.

MATERIALS AND METHODS

A retrospective cohort study was conducted at Mubarak Al-Kabeer Hospital, an inflammatory bowel disease center, to measure gastroenterologists adherence to anti-TNF combination therapy in inflammatory bowel disease (IBD). Infliximab and adalimumab prescription records along with infliximab and adalimumab antidrug antibody levels were collected. The data was collected retrospectively using patients' electronic medical records from July 22nd, 2018 until February 7th, 2020.

In addition, C-reactive protein (CRP), serum albumin levels and stool fecal calprotectin levels that were performed within 7 days of serum drug concentration/antidrug antibody collections, were also obtained. Trough serum drug concentration was performed either reactively (e.g., treatment failure) or proactively (e.g., at week 14 for infliximab) in all patients' cohort, who were studied, according to individual gastroenterologist practice. Moreover, patients' age, body mass index (BMI), extent of the disease, and steroid use were also collected.

This study was performed and reported in accordance with Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) guidelines (14). Ethical approval was obtained by the standing committee for coordination of health and medical research at the Ministry of Health in Kuwait (IRB 2020/1410).

Study Definitions

Patients on anti-TNF combination therapy are those who received adalimumab or infliximab, with an immunomodulator such as azathioprine, 6-mercaptopurine or methotrexate. Patients who received adalimumab or infliximab alone were considered to be on monotherapy.

We divided our analysis into 6 groups. Gastroenterologists who have interest in inflammatory bowel disease (IBD) were classified as IBD interest group (group A) while those who had no interest in IBD were classified as non-IBD interest group (group B). Moreover, gastroenterologists who were younger than 45 years of age were classified as young gastroenterologists (group C) and those who were 45 years or older were classified as senior gastroenterologists (group D). In addition, gastroenterologists who were both younger than 45 years old and had interest in IBD were called young IBD group (group E) while gastroenterologists who were either more than 45 years of age and have IBD interest or had no interest in IBD regardless of their age were called non-young IBD group (group F).

Outcomes

The primary outcome was the association between gastroenterologist age (≥45, or, <45) or inflammatory bowel disease (IBD) interest and the proportion of patients on combination therapy vs. monotherapy for both infliximab and adalimumab. Moreover, the combined effect of age and interest in IBD on the percentage of patients on combination therapy for infliximab or adalimumab was examined.

Statistical Analysis

The statistical analysis was performed with the SPSS Statistics Version 27.0. Armonk, NY: IBM Corp. Chi square test was used to examine the association between the categorical groups. We tested the association between gastroenterologists' interest and age, at Haya Alhabib Gastroenterology Center, and the use of anti-TNF combination therapy compared to monotherapy.

T-test analysis was conducted for parametric data to calculate the correlation between the number of tests performed among variables in the categorical groups with a 95% confidence interval for the mean difference between the groups. Descriptive analyses were performed to calculate frequencies and proportions within the groups. Two-tailed statistical significance level was used throughout the analysis and set to $\alpha = 0.05$ for all associations.

RESULTS

Demographics

The study included 14 gastroenterologists at Haya Alhabib Gastroenterology Center, of which, 4 (28.5%) have interest in inflammatory bowel disease (IBD). Of all the gastroenterologists, 5 (35.7%) of them were equal or above the age of 45 years at the end of the study period. Only one gastroenterologist was above 45 years old and has interest in IBD. The total number of patients included in the study is 289, of which 137 (47.4%) patients were on infliximab [33 (24%) monotherapy and 104 (75.9%) combination therapy] and 152 (52.6%) patients on adalimumab [77 (50.6%) monotherapy and 75 (49.3%) combination therapy] (**Table 1**). In all groups, the median disease duration was 10 years, and the median anti-TNF therapy use was 4 years (**Table 2**).

Outcomes

Among patients on infliximab, 83.2% of the patients were on combination therapy in the young gastroenterologists' group

TABLE 1 | Sample description.

Variable	Gastroenterologists characteristics (*n* = 14)	Total patients in the study sample (*n* = 289)	*P*-value
Age group (*n*%)			
≥45	5 (35.7%)	88 (30.4%)	
<45	9 (64.28)	201 (69.5%)	
Subspecialty (*n*%)			
Interest in IBD	4 (28.5%)	166 (57.4%)	
No interest in IBD	10 (71.4%)	123 (42.5%)	
Anti-TNF drug (*n*%)	Young gastroenterologists	Senior gastroenterologists	
Infliximab (*n* = 137)			
Monotherapy (*n* = 33)	17 (16.8%)	16 (44.4%)	
Combination therapy (*n*-104)	84 (83.2%)	20 (55.6%)	*P*-value† <0.001
Total*	101 (100%)	36 (100%)	
Adalimumab (*n* = 152)			
Monotherapy (*n* = 77)	41 (41%)	59 (59%)	
Combination therapy (*n* = 75)	77 (50.7%)	16 (30.8%)	*P*-value† <0.001
Total*	100 (100%)	52 (100%)	
Anti-TNF drug (*n*%)	IBD interest	No IBD interest	
Infliximab			
Monotherapy	17 (20%)	16 (30.7%)	
Combination therapy	68 (80%)	36 (69.2%)	*P*-value† = 0.15
Total*	85 (100%)	52 (100%)	
Adalimumab			
Monotherapy	31 (38.2%)	46 (64.7%)	
Combination therapy	50 (61.7%)	25 (35.2%)	*P*-value† < 0.001
Total*	81	71	

IBD, Inflammatory bowel disease.
**Total number of patients in the group.*
†*Chi-square test.*

TABLE 2 | Patient characteristics of each group.

	Group A *n* = 166	Group B *n* = 123	Group C *n* = 201	Group D *n* = 88	Group E *n* = 124	Group F *n* = 165
Age* (years)						
Mean	29.0	31.5	30.0	30.0	29.3	30.7
Sex, *n* (%)						
Male	88 (53.0%)	67 (54.5%)	108 (53.7%)	47 (53.4%)	69 (55.6%)	86 (52.1%)
Female	78 (47.0%)	56 (45.5%)	93 (46.2%)	41 (46.6%)	55 (44.4%)	79 (47.9%)
Body mass index (BMI)						
Median	23.7	23.9	23.7	23.6	23.8	23.8
Disease extent, *n* (%)						
Ulcerative colitis (UC)	74	55	90	40	55	74
E1: ulcerative proctitis	7 (9.5%)	5 (9.1%)	9 (10.0%)	3 (7.5%)	5 (9.1%)	7 (9.5%)
E2: left sided colitis	22 (29.7%)	16 (29.1%)	27 (30.0%)	12 (30.0%)	17 (30.9%)	22 (29.7%)
E3: extensive colitis	45 (60.8%)	34 (61.8%)	54 (60.0%)	25 (62.5%)	33 (60.0%)	45 (60.8%)
Crohn's disease (CD)	92	68	111	48	69	91
L1: ileal	41 (44.6%)	30 (44.1%)	50 (45.0%)	22 (45.8%)	31 (44.9%)	41 (45.1%)
L2: colonic	9 (9.8%)	7 (10.3%)	11 (9.9%)	5 (10.4%)	7 (10.1%)	10 (10.9%)
L3: ileocolonic	37 (40.2%)	27 (39.7%)	45 (40.5%)	19 (39.6%)	28 (40.6%)	36 (39.6%)
L4: upper gastrointestinal	5 (5.4%)	4 (5.9%)	5 (4.5%)	2 (4.2%)	3 (4.3%)	4 (4.4%)
B1: inflammatory	41 (44.6%)	31 (45.6%)	50 (45.0%)	21 (43.8%)	31 (44.9%)	42 (46.1%)
B2: stricturing	23 (25.0%)	17 (25.0%)	28 (25.2%)	12 (25.0%)	17 (24.6%)	22 (24.2%)
B3: penetrating	28 (30.4%)	20 (29.4%)	33 (29.7%)	15 (31.3%)	21 (30.4%)	27 (29.7%)
Median disease duration (years)	10.2	10.7	10.2	10.1	10.2	10.2
Median anti-TNF therapy duration (years)	4.1	4.6	4.1	4.3	4.1	4.2
CRP, mg/L (median)	6.1	11.3	7.6	7.0	6.1	9.5
Albumin, g/L (median)	40.0	40.0	40.0	40.0	40.0	40.0
Median stool fecal calprotectin ug/g	112.0	124.0	111.0	115.0	110.0	126.0
Steroid use, *n* (%)	16 (9.6%)	15 (12.1%)	26 (12.3%)	5 (5.6%)	15 (12.1%)	16 (9.7%)
Anti-infliximab antibody serum levels, (AU/ml)						
Median	10.7	25.7	13.3	15.3	9.3	17.9
Anti-adalimumab antibody serum levels, (AU/ml)						
Median	7.8	7.4	8.2	6.7	8.0	7.3

Group A: IBD interest group, group B: non-IBD interest group, group C: young gastroenterologists, group D: senior gastroenterologists, group E: young IBD group, group F: non-young IBD group.

**Age at the time of sampling.*

(group C) compared to 55.6% in the senior gastroenterologists group (group D) ($P < 0.001$; **Figure 1**; **Supplementary Table 1**). Similarly, more patients were found to be on adalimumab combination therapy in the young gastroenterologists' group (group C) than those in the senior gastroenterologists' group (group D) (59, 30.8%, respectively) ($P < 0.001$; **Figure 2**; **Supplementary Table 2**). In addition, 80% of patients in the IBD interest group (group A) were on infliximab combination therapy compared to 69.2% of patients in the non-IBD interest group (group B). However, the difference between the two groups was not statistically significant ($P = 0.153$; **Figure 1**; **Supplementary Table 3**). Conversely, 61.7% of patients on adalimumab therapy in the IBD interest group (group A) were on combination therapy as opposed to 35.2% of patients in the non-IBD interest group (group B) ($P < 0.001$; **Figure 2**; **Supplementary Table 4**).

In the young IBD group (group E) 89.4% of the patients were on infliximab combination therapy compared to 63.4% of patients in the non-young IBD group (group F) ($P < 0.001$; **Figure 1**). Likewise, 75.9% of patients in the young IBD group (group E) were on adalimumab combination therapy compared to 33% of patients in the non-young IBD group (group F) ($P < 0.001$; **Figure 2**).

Regarding anti-TNF serum drug concentrations and antidrug antibody tests, gastroenterologists in the IBD interest group (group A) requested an average of 41.4 tests while gastroenterologists in the non-IBD interest group (group B) requested an average of 12.3 tests ($P < 0.001$, 95% CI 20.1–40.4; **Figure 3**; **Table 3**; **Supplementary Table 5**). In addition, young gastroenterologists group (group C) requested an average of 22.3 tests compared to an average of 17.6 tests by senio gastroenterologists group (group D) ($P = 0.615$; **Figure 3**; **Table 3**).

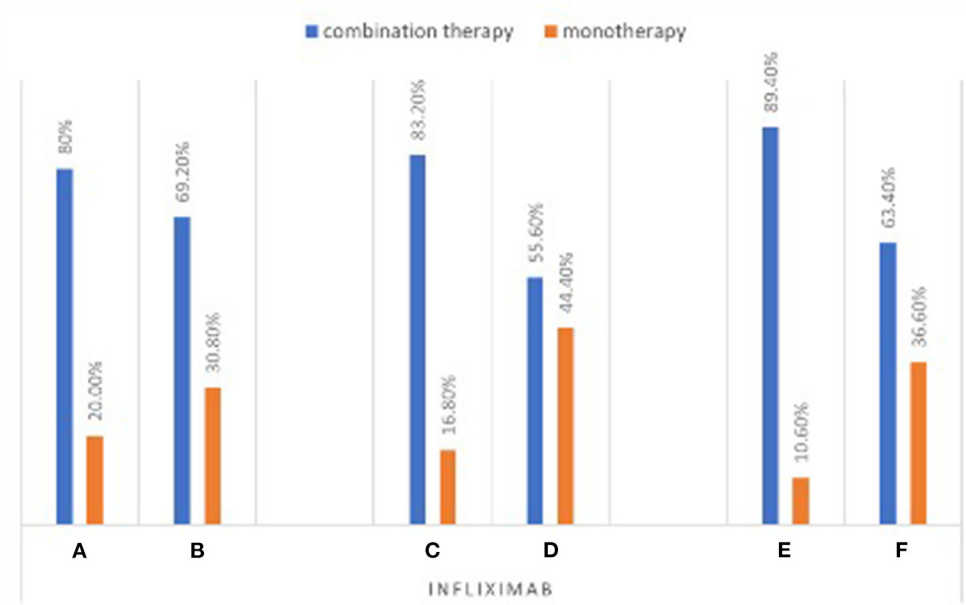

FIGURE 1 | Percentage of patients on infliximab combination therapy stratified by test groups.

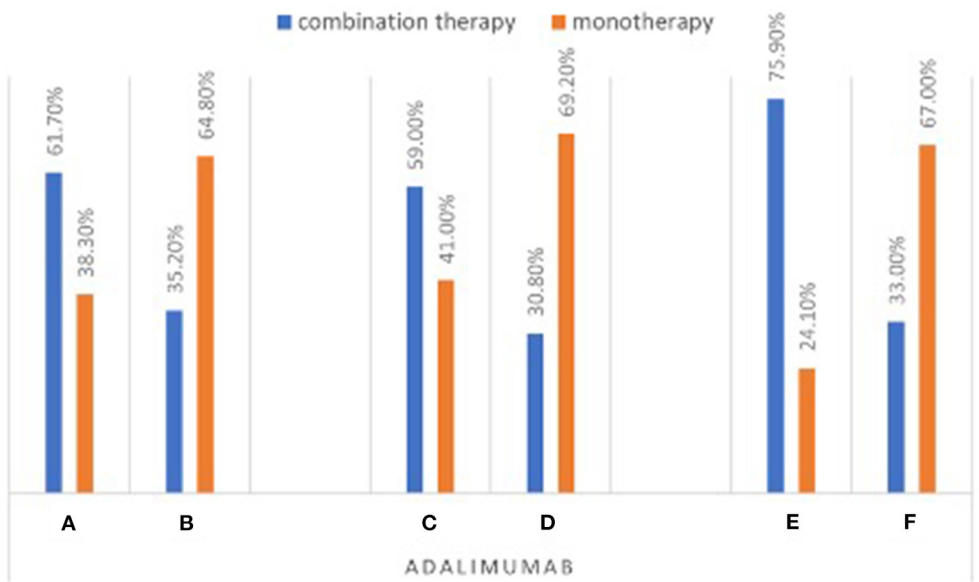

FIGURE 2 | Percentage of patients on adalimumab combination therapy stratified by test groups.

DISCUSSION

A retrospective cohort study which included 14 gastroenterologists at Haya Alhabib Gastroenterology Center, was conducted to assess the adherence to tumor necrosis factor antagonist (anti-TNF) combination therapy in patients with IBD. Two main factors were found to influence the gastroenterologists' adherence to combination therapy, which were their age and interest in IBD. The study showed that the age of the treating gastroenterologist may have some influence, as the gastroenterologists aged 45 or less, appeared to be more adherent to the combination therapy.

The second factor that played a role in the commitment to combination therapy was the gastroenterologist's interest in IBD, which was observed more with IBD subspecialized gastroenterologists, significantly with adalimumab. Reactive

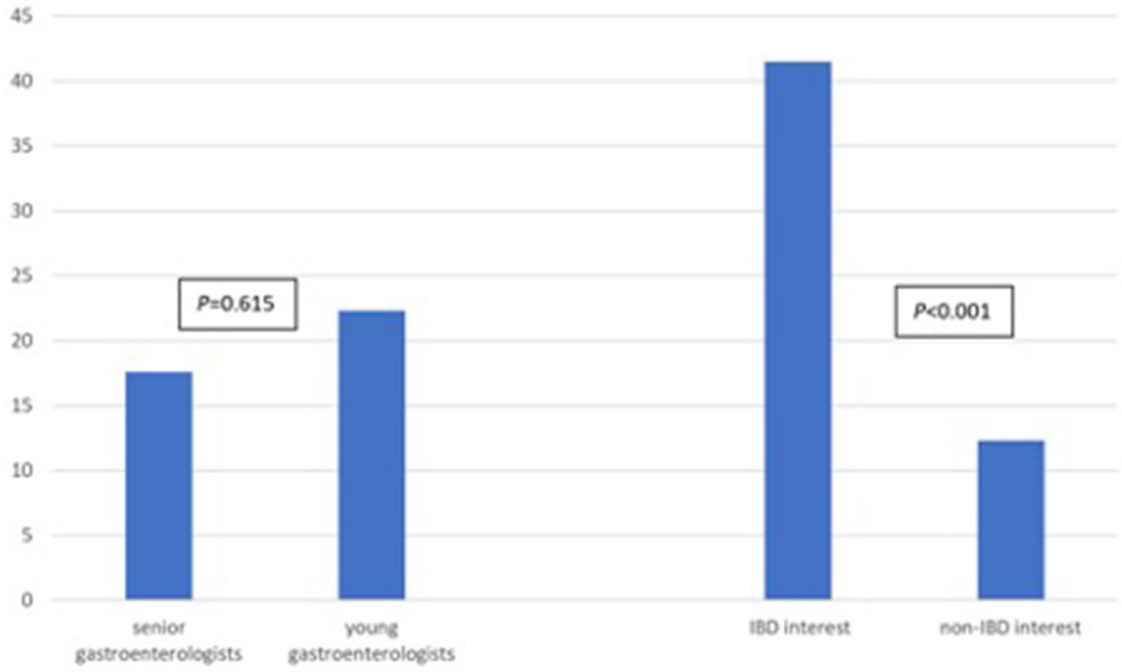

FIGURE 3 | Mean number of tests requested gastroenterologists in the senior vs. young group and in the IBD-interest vs. non-IBD interest group.

TABLE 3 | *t*-Test, average number of anti-TNF serum drug concentrations and antidrug antibody level tests and requested by young gastroenterologists vs. senior gastroenterologists and in the IBD interest group vs. non-IBD interest group.

	Senior gastroenterologists	Young gastroenterologists	*P*-value
Mean number of tests	17.6	22.3	0.615
	IBD interest	**non-IBD interest**	
Mean number of tests	41.5	12.3	<0.001

drug monitoring, which is the evaluation of serum drug concentration and antidrug antibody level in active or poorly controlled disease, is commonly performed more frequently by IBD subspecialized gastroenterologists. An Italian multicenter prospective observational study by Scribanoet et al. reported that IBD subspecialized gastroenterologists had a greater adherence to combination therapy for patients with ulcerative colitis (UC) flare, when compared to general gastroenterologists (15). Also, there is a survey study by Grossberg et al., included 606 physicians in the United States of America (USA), showed that academic gastroenterologists and physicians who were seeing more patients with IBD, have utilized therapeutic drug monitoring (TDM) reactively more frequently (16).

TDM is performed to guide the use of anti-TNF by assessing the drug serum concentrations and antidrug antibodies levels. It can be performed at any point of therapy, whether as reactive monitoring, or as routine proactive monitoring provided to patients in remission (17). There is currently insufficient evidence to recommend the routine use of proactive TDM to improve clinical outcomes when compared to routine care for IBD patients in clinical remission under anti-TNF treatment (17). This is based on 2 RCTs, by Vande Casteele et al. and D'Haens et al., with a total of 359 patients treated with infliximab in combination with an immunomodulator, had shown no difference in clinical remission between clinically-based dosing and concentration-based dosing (proactive TDM) groups. However, the concentration-based dosing groups had fewer relapses during follow-up (18, 19). A recent retrospective study, by Papamichael et al., showed that the proactive TDM of infliximab was associated with more favorable therapeutic outcomes and fewer IBD-related hospitalizations and surgery compared to reactive TDM alone (20). Moreover, a systematic review, by Strand et al., emphasized the role of TDM of anti-TNF therapy (21). This might be helpful in guiding the physicians in improving anti-TNF therapy management and achieving better clinical outcomes.

Our study is the first study in Kuwait to evaluate the adherence to anti-TNF combination therapy amongst gastroenterologists. It emphasizes the importance of gastroenterologists' interest in IBD when treating such patients. It also encourages

gastroenterologists to be updated and follow recently published IBD guidelines. On the other hand, there are some limitations to our study. It is a retrospective single center study with possible confounders and bias. For example. The lack of data regarding proactive vs. reactive TDM testing, and discontinuation of anti-TNF combination therapy, due to side effects or absence of antibodies, were possible confounders. In addition, clinical and endoscopic remission targets were not assessed. However, objective inflammatory markers (such as serum CRP levels, stool fecal calprotectin levels and steroid use) were obtained. Finally, the small number of physicians in the IBD interest group could have influenced our results.

To sum up, young gastroenterologists (<45 years of age) are more likely to prescribe anti-TNF (infliximab and adalimumab) combination therapy than senior gastroenterologists. In addition, Gastroenterologists with IBD interest are more likely to prescribe adalimumab combination therapy than gastroenterologists with no IBD interest. However, no significant difference was found for prescribing infliximab combination therapy. Moreover, young gastroenterologists who have interest in IBD are more likely to prescribe both infliximab and adalimumab combination therapy than those who were either senior gastroenterologists with IBD interest or those with no IBD interest regardless of their age. In addition, gastroenterologists with IBD interest requested more anti-TNF serum drug concentrations and antidrug antibody level tests than those with no IBD interest. In conclusion, physicians with IBD interest or who are young, are more likely to follow IBD guidelines.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by standing committee for coordination of health and medical research at the Ministry of Health in Kuwait (IRB 2020/1410). Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

IA: analysis and interpretation of data and drafting of the manuscript. GA and AA: acquisition of data and drafting of the manuscript. HA: drafting of the manuscript. MS: drafting of the manuscript, critical revision of the manuscript for important intellectual content, statistical analysis, and study supervision. All authors contributed to the article and approved the submitted version.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2021.725512/full#supplementary-material

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