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EDITORIAL

Present state and future challenges in pediatric abdominal pain therapeutics research: Looking beyond the forest

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Abstract

At the present time, it is nearly impossible to treat pediatric functional gastrointestinal disorders associated with pain in an evidence based fashion. This is due to the overall lack of controlled studies and, even more importantly, the complexity of the contributors to disease phenotype which are not controlled or accounted for in most therapeutic trials. In this manuscript, we review the challenges of defining entry criteria, controlling for the large number of biopsychosocial factors which may effect outcomes, and understanding pharmacokinetic and pharmacodynamic factors when designing therapeutic trials for abdominal pain in children. We also review the current state of pediatric abdominal pain therapeutics and discuss trial design considerations as we move forward.

Key words: Pharmacogenomics; Functional dyspepsia; Abdominal pain; Irritable bowel syndrome; Therapeutic trials; Pharmacokinetics

Core tip: For abdominal pain therapeutics research to fulfill the promise of personalized medicine, there is a need to standardize trial entry criteria including validating Rome criteria as predictors of response. There is also a need to embrace complexity and recognize and control for the large number of biologic, psychologic, social, and pharmacologic factors which define each patient and may affect drug response. This approach will allow us not only to understand what treatments work for the population at large, but for the individual patient in front of us.

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TEXT

Personalized medicine involves tailoring treatment to the specific characteristics, needs, and preferences of the individual patient[1]. At its heart, it's the "right drug at the right dose at the right time"[2]. The personalized medicine movement is primarily predicated on two things: (1) that an evidence base exists to support certain treatment options at the level of the population; and (2) that individual variation in patient characteristics, needs, and preferences can be identified in such a way as to allow evidence-based treatment to be optimally tailored. In a sense, personalized medicine focuses on the individual patient within the context of the population. While this is ideal, combining the best of evidence-based research with the best of clinical application, this is not the current reality for many conditions, including pediatric functional gastrointestinal disorders (FGIDs) associated with pain.

At the present time, it is nearly impossible to treat pediatric FGIDs associated with pain in an evidence based fashion. This is due to the overall lack of controlled studies and, even more importantly, the complexity of the contributors to disease phenotype which are not controlled or accounted for in most therapeutic trials. Each patient has a unique set of characteristics which must be understood when delivering an intervention. However, our individual patient care is largely informed by aggregate group data. Furthermore, studies are generally designed to minimize variation within the sample and thus may bear little relationship to actual patients seen in the clinical setting[3]. Group-wise analyses disguise substantial individual variation relevant to treatment. In short, we are unable to see the trees for the forest.

Diagnostic challenges also contribute to the familiar problems experienced by academic investigators and drug developers who conduct drug studies of FGIDs[4,5]. Principal among these are poorly understood etiologies for this very heterogenous group of conditions, poor diagnostic agreement in the classification of patients, incompletely validated criteria on which to subtype patients, and the fluctuating nature of symptoms[6-8]. Absent a clear understanding of the physiological, psychological, and behavioral elements that define this condition and reliable biomarkers that can facilitate patient subtyping[9,10], studies will invariably introduce uncertainty in their findings by pooling patients with putatively different underlying mechanisms of disease.

Collectively, these challenges lead to underpowered studies with variable response rates, large placebo effects, and limited relevance to the average pediatric patient with abdominal pain that is encountered in clinical practice. The role of this editorial is to lay out the relevant history and present state of therapeutics in pediatric abdominal pain research, as well as to identify existing challenges that will need to be considered and addressed to move the field forward toward the personalized medicine ideal in the future.

ROME CRITERIA AS THE ENTRY CRITERIA FOR THERAPEUTIC TRIALS

In 1999, the pediatric Rome Ⅱ committee established the first diagnostic criteria for FGIDs in children. These criteria were consensus-based and modeled after previous work (Rome Ⅰ) in adults. In 2006, the pediatric criteria were revised by the Rome Ⅲ committee. The Rome criteria define four FGIDs related to abdominal pain in children: Functional dyspepsia, irritable bowel syndrome, abdominal migraine, and childhood functional abdominal pain/syndrome[11] (Table 1). The majority of children and adolescents with chronic abdominal pain or discomfort meet criteria for an FGID[12,13]. At present, these Rome diagnoses form the entry criteria for nearly all pediatric abdominal pain therapeutic trials; however, the criteria have remained consensus-based and have never been completely validated nor shown to predict treatment response in clinical trials.

Utilizing the criteria in clinical practice or therapeutic trials is associated with some significant obstacles, including inherent ambiguity, inconsistent application, and differences in symptom reports between the patient and their parents or care givers. Although they seem to be "face valid", the Rome criteria lack a necessary degree of precision that translates to confusion on the part of patients, parents, and providers. For example, what is discomfort? Does the word discomfort mean nausea, bloating, or something else? Does it mean the same thing to parents as to the child? Do symptoms associated with discomfort result from different pathophysiologic processes or respond differently to treatments than does overt pain? In another example, an important criterion used to differentiate FD from IBS in the Rome criteria is relief with defecation which was defined in IBS as greater than 25% of the time but undefined in FD. Does having relief with a stool 5% or 10% of the time exclude FD? What happens when parents and children disagree about the percent time that defecation relieves pain? These are two relatively straightforward examples, but the criteria are open to interpretation in many areas. As suggested in the above examples, variance also is created by who provides the history and in what fashion. Utilizing standardized questionnaires, there is only fair to moderate agreement in diagnosis between symptom reports obtained from the patient and those obtained from their parent or guardian[13,14]. Agreement is low between the evaluating physician and either the patient or parent[13,14]. Further, responses on a standardized questionnaire also have low agreement to a daily symptom diary[14]. Perhaps due to the ambiguity, criteria are inconsistently applied. It has been shown that

Table 1 Criteria for functional gastrointestinal disorders related to pain in children

Functional dyspepsia[1]
Must include all of the following
Persistent or recurrent pain or discomfort centered in the upper abdomen
Pain or discomfort not relieved by defecation or associated with onset of a change in stool frequency or form
Irritable bowel syndrome[1]
Must include all of the following
Abdominal pain or discomfort associated with 2 or more of the following at least 25% of the time
Improved by defecation
Onset associated with a change in stool frequency
Onset associated with a change in stool form
Abdominal migraine
Must include all of the following
Paroxysmal episodes of intense, acute periumbilical pain lasting at least one hour
Intervening periods of usual health lasting at least weeks
Pain interferes with normal activity
The pain is associated with at least two of the following
Anorexia
Nausea
Vomiting
Headache
Photophobia
Pallor
Criteria must be fulfilled at least two times in the preceeding 12 mo
Childhood functional abdominal pain[1]
Must include all of the following
Episodic or continuous abdominal pain
Does not meet criteria for another FGID
Childhood functional abdominal pain syndrome
Must include childhood FAP and at least 25% of the time with at least one of the following
Some loss of daily function
Additional somatic complaints such as headache, limb pain, or difficulty sleeping

All require that there be no evidence of an inflammatory, anatomic, metabolic, or neoplastic process that explains the symptoms. [1]Criteria fulfilled at least once per week for at least 12 mo. FGIDs: Functional gastrointestinal disorders; FAP: Functional abdominal pain.

pediatric gastroenterologists only have fair to moderate agreement regarding FGID diagnosis even when presented identical clinical vignettes[15]. This problem may not be strictly limited to the pediatric population either. In adult FD, which has more specifically defined criteria, adherence to Rome criteria for inclusion was found in only 54% of clinical research trials[16].

Another challenge to utilizing Rome criteria in practice is that there is heterogeneity within diagnoses. Pediatric criteria, unlike the adult Rome criteria, do not recognize subtypes within either FD or IBS. In adult FD, there are two recognized subtypes: (1) postprandial distress syndrome (PDS), characterized by postprandial fullness or early satiety; and (2) epigastric pain syndrome, characterized by epigastric pain or burning unrelated to meals. These are not recognized in the pediatric criteria, although there is some evidence that PDS is associated with increased mucosal mast cells, anxiety, and depression in pediatric FD[17]. These are associations which may affect therapeutic responses to a given treatment. Likewise, IBS in adults may present with primarily constipation, diarrhea, or alternating symptoms; these patterns also have been described in children/adolescents, but are not specified as subtypes within the Rome criteria[18]. Ostensibly pediatric patients with primarily constipation would respond differently to an agent that affects motility than a patient with primarily diarrhea.

There also may be overlap between FGIDs, or between FGIDs and other conditions such as chronic nausea, GERD, or bladder dysfunction, which may influence treatment response. Nausea has been reported to be a commonly associated symptom in children with chronic abdominal pain which spans FGIDs[19]. Nausea frequency is associated with poor school and social functioning, and also predicts social disability; thus, nausea may be a symptom that could affect therapeutic response independent of the FGID category. In adults, significant overlap has been reported between FD and IBS, with overlap associated with more severe symptoms and increased psychological dysfunction[20,21]. Significant overlap has also been reported between FD and IBS in children, although some investigators report no overlap[12,13]. This represents another area of variability in application of the Rome criteria, as some investigators diagnose FD/IBS overlap while other investigators default to IBS when symptoms of both are present.

Finally, the Rome FGIDs were meant to be positive diagnoses in that they do not require organic disease to be ruled out *per se*; however, the criteria contain the requirement that there be no inflammatory, anatomic, metabolic, or neoplastic process to explain the

symptoms. Pediatric gastroenterologists vary significantly in what testing they believe is necessary to rule out biochemical or structural causes of abdominal pain[22]. Additionally, it has also been shown that the Rome Ⅲ criteria are not specific enough to rule out organic diseases and that alarm symptoms do not differentiate between organic and non-organic disease[23]. Taken together, the above issues may at least partly explain why only 39% of pediatric gastroenterologists report using the Rome criteria in their clinical practice[22].

Ultimately, there is a need to fully validate the Rome criteria in children as well as evaluate which symptoms naturally cluster together as has been done in adult populations. Even more importantly, current diagnostic categories (or other symptom complexes) and symptom variability within a diagnostic category need to be assessed for their ability to predict treatment response.

IMPLICATIONS OF THE BIOPSYCHOSOCIAL MODEL

Beyond issues of diagnostic classification, the development of abdominal pain itself is now widely considered to be a complex process. Chronic abdominal pain is believed to arise through multiple pathways that include biological (physiological, genetics), psychological (emotional, behavioral), and social (relational, environmental) factors which interact with one another. Each of these factors may contribute to initiation or maintenance of pain, as well as frequency, duration, and intensity of pain and other related symptoms. Consequently, children may arrive at the same result through different pathways. In addition, the process is dynamic and different processes may contribute to symptoms generation to varying degrees at different time points.

Patients may arrive at the same symptom complex by very divergent pathophysiologic processes including dysmotility, visceral hypersensitivity, inflammation, and alteration of the microbiome. FD for example has been associated with visceral hypersensitivity, as well as a wide variety of electromechanical disturbances including disorders of gastric emptying and accommodation, gastric dysrhythmias, and antroduodenal dysmotility[24]. FD has also been associated with inflammation, specifically mast cell accumulation in the antrum and eosinophil accumulation in the duodenum[25]. Similarly, IBS may result from visceral hypersensitivity, motility disturbances, low grade inflammation, and/or an altered intestinal microbiome[26]. Psychologic disturbances frequently co-exist and interact with other pathophysiologic factors. In addition, each patient has an environment which may interact with other pathohysiologic mechanisms and affect disease presentation and response to therapy over time.

Psychological functioning can be a significant source of patient variability, which cuts across all FGIDs. Psychological measures demonstrate clustering in children with FGIDs related to abdominal pain. In one study, approximately half of pediatric patients ith FGIDs showed no significant emotional, behavioral, or social disturbances, while 35%-45% demonstrated elevations only in anxiety scores and the remaining 13% demonstrated broad-based psychological problems[27]. In another study, pediatric patients with FGIDs demonstrated distinct patterns of pain and adaptation, with adaptation affecting clinical outcome[28].

Lastly, pain is influenced by a number of other broad social and environmental factors including interpersonal relationships, the natural environment, diet, and sleep. While some of these triggers appear common across individuals, there is likely variability among individuals on others[29]. For example, some patients have clear associations between inflammation, pain and the pollen count. These patients may respond to a treatment regimen differently during a time of high pollen exposure as compared to low exposure or differently than individuals unaffected by the pollen count[30].

Ultimately, the complexity of pediatric abdominal pain, and FGIDs specifically, creates significant challenges in controlling for disease variables in therapeutic trials. To date, this issue has largely been addressed through restrictive eligibility criteria, to reduce variability, or "noise", on the front end. However, the biopsychosocial model suggests that we may need to embrace individual variability, rather than control for it, in order to move closer to the personalized medicine ideal. In practice, this means that more liberal inclusion criteria combined with use of alternative research designs and/or more advanced statistical modeling approaches may be needed to support advancement in knowledge and care. We must assess each participant as a complex biopsychosocial system to understand how that system (*i.e.*, the whole person) affects treatment outcome and how that system rearranges in response to a treatment. This offers the hope of being able to determine which factors within the system predict the treatment that would be most effective for an individual patient.

CLINICAL PHARMACOLOGY

Historically, the lack of adequate pediatric drug trials, in general, could be blamed on several factors: (1) questions surrounding the ethics of conducting drug studies in children; (2) concerns related to the disproportionate financial investment required to support studies in a population which comprises a relatively small market share; and (3) assertions that pediatric studies are logistically more complex than comparable adult studies. Fortunately, academicians and regulators have come to appreciate that routinely administering medications to children when they have not been evaluated in this population is not ethically defensible[31,32]. Subsequently, United States legislation has prompted a major push to evaluate drugs in children, shifting discussions from "if" these studies should be performed to "when" and "how" they should be conducted[33]. With the general debate out

of the way, well-intentioned investigators are revealing additional challenges nested in the conduct of pediatric drug studies for FGIDs. Published studies continue to suffer from problems that can be loosely characterized as pharmacokinetic, pharmacodynamic, and diagnostic in nature.

Inadequacies in dosing regimen selection are perhaps the most straightforward to address. Since most academic investigations reflect repurposing of approved drugs there exists, at a minimum, some pharmacokinetic data that can be used to guide dosing decisions. Physiologically-based pharmacokinetic modelling and simulation strategies which take into consideration known maturational changes in the anatomy and physiology of major organs of disposition can provide a reasonable starting point from which to base dose selection[34]. The caveat is that this approach requires some estimation of desired exposure targets which, when unknown, often reflect adult exposures that have demonstrated safety. Notably, there is evidence to suggest that a priori modelling predictions do not always reflect observed pharmacokinetic profiles[35], which is why modelling exercises should be followed by confirmatory pharmacokinetic studies in advance of, or concurrent with, outcome based trials.

Importantly, the paradigm of "one and done" with respect to pediatric pharmacokinetic studies may not be a realistic option in FGIDs where marked variability in the genetic constitution, co-morbidities, and co-administered medications abound. Each of these factors can alter the pharmacokinetic profile of the drug in question to a different extent. However, a number of strategies can be integrated into the design of pediatric drug trials to enhance our understanding of the dose-exposure-response profile for selected treatment regimens. These include classical pharmacokinetic sampling in a small subset of patients, sparse pharmacokinetic sampling over a broader range of patients coupled with population pharmacokinetic analyses, and scavenged pharmacokinetic sampling or opportunistic clinical sampling similarly employing population-based analytical approaches. Though the latter strategies offer less than robust pharmacokinetic parameter estimates, they are only minimally labor intensive (as compared to classical pharmacokinetic studies) and are increasingly being accepted by regulatory agencies[36,37].

Additional pharmacokinetic considerations surround reliable drug delivery. A troublesome consequence of the failure of big Pharma to integrate children early in the drug development process is the lack of age-appropriate dosage formulations[38]. If the only available formulation is a solid oral dosage form, titrating the dose for children of varying weights can be problematic. Consequently, clinical trial outcomes that are not examined in the context of the weight-adjusted dose each participant received may miss important relationships between dose and response[39]. One must also consider the extrapolation of the research findings to the clinical practice setting. Data from a subset of children capable of swallowing the solid oral dosage form is of mixed utility in a pediatric practice setting where extemporaneous compounding may be undertaken to accommodate the needs of younger patients. Extemporaneous manipulation of the drug must be anticipated and the consequence on its relative bioavailability carefully assessed. Without these data, the fate of the molecule in the patient will be unknown and the care of the child potentially compromised[38].

Pharmacodynamic challenges reflected in drug studies of pediatric FGIDs center around adequately capturing unbiased outcomes[6]. As the need for objective biomarkers of treatment response is self-evident, and the current challenges related to subjective outcome measures have been thoughtfully addressed by others[7,10,40], we only reference the problem here to suggest that guidance documents authored by the United States Food and Drug Administration may offer a reasonable starting point for discussions related to the design and utilization of instruments developed to collect patient reported outcomes (PRO)[41,42]. These documents discuss the Agency's views on the adequacy of PRO measurement tools in the context of their characteristics, conceptual framework, content validity, criterion validity, ability to detect change, and suitability in special populations (*e.g.*, children, cognitively impaired, nonverbal, non-native language speakers). They also discusses the integration of these tools into clinical trials including protocol design considerations and statistical considerations nested in the analysis of PRO data[41,42]. We recognize that pediatric labeling is not a forethought for many academicians when designing FGIDs trials but the guidance offers insight into how a body of individuals tasked with the responsibility for determining the efficacy and safety of medicines view these instruments.

Ultimately, drug pharmacokinetics and pharmacodynamics and how these interact with genetics and disease processes must be considered if we are to gain a firm understanding of how to treat individual patients. Once we have selected the right drug for the right patient, pharmacokinetics and pharmacodynamics will allow us to fullfil the last step in a personalized medicine approach, the right dose at the right time.

CURRENT STATE OF ABDOMINAL PAIN THERAPEUTICS

Therapeutic management strategies for the treatment of functional gastrointestinal disorders share a common limitation with the sizeable majority of drugs on the market; namely, insufficient clinical evidence for their use in children. A Cochrane review published in 2002, and updated in 2008, identified a mere 6 trials evaluating drug-based interventions in children with recurrent abdominal pain or irritable bowel syndrome[4,43]. Importantly, only one-half of these trials were randomized and controlled. Our own more recent

Table 2 Chronological list of placebo controlled drug trials for functional gastrointestinal disorders related to abdominal pain in children and adolescents, including summary of treatment, sample size, and outcome

Ref.	Treatment	Diagnosis	Sample size (enrolled/completed)	Superior to placebo for pain relief
Symon and Russell[44] (1995)	Pitotifen	Abdominal migraine	16/14	Yes
Kline *et al*[45] (2001)	Enteric coated peppermint oil capsules	Irritable bowel syndrome	50/42	Yes
See *et al*[46] (2001)	Famotidine	Abdominal pain and dyspepsia	25/25	Yes
Friesen *et al*[47] (2004)	Montelukast	FD with duodenal eosinophilia	40/37	Yes
Bahar *et al*[48] (2008)	Amitriptyline	Irritable bowel syndrome	35/33	Yes
Sadeghian *et al*[49] (2008)	Cyproheptadine	Functional abdominal pain	36/28	Yes
Saps *et al*[50] (2009)	Amitriptyline	FD, irritable bowel syndrome, and functional abdominal pain	90/83	No
Pourmoghaddas *et al*[51] (2014)	Mebeverine	Functional abdominal pain	115/87	No

search of the literature revealed a total of 8 placebo-controlled drug trials for abdominal pain related FGIDs in children/adolescents, indicating that we have not made much progress in the past 7 years (see Table 2 for a summary)[44-51]. The clinical implication surrounding this paucity of well-controlled studies is limited empiric support for even basic prescribing decisions that clinicians make when managing pediatric patients with FGIDs.

Importantly, even within the existing literature, there are inconsistent findings that yield further confusion in applying results to clinical care. Comparison of the two separate trials of amitriptyline demonstrates some of the issues with regard to pediatric trials for FGIDs[48,50]; one trial demonstrated efficacy while the other did not. In adults, amitriptyline has been shown to be efficacious in those with diarrhea-predominant IBS[52]. One of the two pediatric trials for amitriptyline evaluated only participants with diarrhea-predominant IBS and demonstrated efficacy[48]. In contrast, the other pediatric trial evaluated both diarrhea- and constipation-predominant IBS patients, and also included patients with functional abdominal pain and FD, and did not demonstrate efficacy[50]. In addition to the much greater heterogeneity of the sample in the second study, there also was a trend for patients with IBS to be over-represented in the placebo group. The authors did analyze by specific FGID, but were likely underpowered to detect differences; descriptive statistics by group were not provided to allow the reader to evaluate effect size in this case. Thus, the lack of demonstrated efficacy for pain relief may have been due to patient selection, both in terms of inclusion criteria and randomization inequity. Another difference between the two studies may have been the dosing regimen. The per weight dosing likely varied significantly among patients within and across studies, although data on this was not presented nor analyzed as a covariate in the analysis. Furthermore, neither study determined amitriptyline concentrations in the blood to assess exposure. It is possible that success, or a lack thereof, was - at least in part - the result of differential dosing and/or exposure across patients.

Further, there have been almost no studies that have looked at layering of treatments. Although not the focus of this commentary, in addition to the drug trials noted above, there have been 6 controlled trials of probiotics, 4 controlled trials of fiber supplements, and a number of trials of a variety of psychological interventions (with various degrees of control), all mainly done in isolation of, or without regard for, other treatments. Layering of treatments is an important avenue to explore given the many potential contributors to symptoms in a given patient as previously discussed. It seems intuitive that identifying as many potential pain contributors in a given patient and addressing each simultaneously with a treatment specific to the contributor would be the most efficacious approach. However, this remains to be fully proven, as limited data exists. One example of existing evidence for layered treatment would be combining montelukast with biofeedback-assisted relaxation training (BART) in FD associated with duodenal eosinophilia. In the first study, montelukast was shown to be efficacious in this patient group in a double-blind, placebo-controlled crossover trial[47]. Then, in a second study, a separate group of patients was randomized to receive medication alone or in combination with BART. The combined, or layered, treatment resulted in more rapid resolution of pain and associated disability than seen for medication alone[52].

Finally, clinical trials for pediatric FGIDs have largely utilized traditional research designs that analyze data in the aggregate, which serves only to identify evidence at the level of the population without regard for individual characteristics, needs, or preferences. Specifically, within the drug trials identified by our group, only 3 utilized a cross-over design, only 1 evaluated potential biomarkers of response, only 1 utilized multivariate analysis of response predictors with anxiety as a co-variable, and only 1 assessed drug exposure utilizing pharmacokinetics. While there may be a role for

population-level evidence in creating a solid foundation from which individualization of treatment can occur, this cannot be the only approach if we are to learn how to effectively tailor those interventions for the benefit of our clinical patients.

WHERE DO WE GO FROM HERE?

First and foremost, we need to study drugs being used to treat abdominal pain in children. The approach must recognize that this is a multifactorial disease and that each patient has a unique biopsychosocial profile that may affect treatment response. The most direct way to account for this participant-to-participant variability is to have the participant serve as their own control, either through utilizing cross-over designs (when the intervention has limited durability) or through single subject designs. Ultimately, trials need to be of sufficient sample size and comprehensive in collecting data regarding biologic, psychologic, and social/environmental variables such that all of these potential factors can be assessed to determine individual characteristics or profiles that predict response. Trials, particularly early ones, also must consider pharmacokinetics and pharmacodynamics to assess relationships between drug dosing, drug exposure, and clinical response. Generalizability depends on controlling variables in the analysis rather than at entry through overly strict entry criteria.

Given our current state of understanding, investigations of medicines for abdominal pain may benefit from new approaches to trial design, including: (1) adaptive sample size re-estimation which can account for inaccurate assumptions of variance that can affect a studies power[53]; (2) enrichment designs that can limit the enrollment of participants with characteristics that may preclude the detection of a drug effect (*e.g.*, rapidly waxing and waning symptoms, non-adherence)[54]; and (3) n-of-1 trials which can better characterize individual treatment effects and potentially improve trial efficiency while reducing the necessary sample size[55]. Adaptive study designs involve prospectively planned opportunity for modification of one or more specified aspects of the study design and hypotheses based on analysis of interim data. These designs allow the flexibility to alter the direction when it becomes clear that a particular intervention is effective such that a second treatment could be layered on. Conversely, these designs allow an intervention to be dropped when there is sufficient data to indicate that the treatment is ineffective. Single subject designs allow for fewer participants but require more frequent observation to ensure that change occurs only with the intervention. Such designs are more economical and allow researchers to tease out what specific treatments are most effective. These designs allow more rigorous study of combination treatments with different treatments phased in over time and also permit determination of the impact of dose escalation.

CONCLUSION

There can be value in preserving the traditional group aggregate approach, and there certainly remains room for the completion of high quality controlled clinical trials in the treatment of pediatric FGIDs. However, we also need to move past tradition to evaluate naturally occurring variation to understand which characteristic or set of characteristics are relevant to outcome. Pragmatically, this means embracing individual variability rather than restricting it *via* stringent sample selection criteria. This means investigating that variability through pharmacokinetic work to better predict exposure and inform dosing in individual patients. This means creatively applying alternative designs to the execution rather than applying research methods that are comfortable or familiar. And, this means learning new analytic strategies or partnering with statisticians familiar with analysis of adaptive designs, small *n* trials, and/or approaches to modeling intraindividual variation. Ultimately, to move forward, we need to understand variability, not control it.

In short, people are complicated and children with chronic abdominal pain are no exception. If we fail to appreciate that complexity in our research designs, we will never really get useful information out of the trials that we do. We will see only the forest, but not the trees within it. While population-based data can provide a place to start and the broad context for treatment, personalized medicine requires going beyond this to take individual characteristics, needs, and preferences into account in providing treatment to an individual. Thinking flexibly in our research, with individual variability in mind, will allow us to not only assess which treatments are efficacious but equally important, to understand when and for whom the treatment works[3]. Then we can determine not only which treatments work for the population at large, but also - and more importantly - for the individual patient in front of us.

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EDITORIAL

Pharmaceutical management of hepatitis B and C in liver and kidney transplant recipients

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Abstract

The combination of hepatitis B immune globulin with entecavir or tenofovir (at least for a certain period of time) seems to be the most reasonable prophylaxis against recurrent hepatitis B after liver transplantation. Entecavir represents an attractive option for treatment of naïve kidney transplant recipients, because of its high efficacy and the low rates of resistance. However antiviral treatment should be individualized in the view of kidney function and the previous resistance. To date, new captivating therapeutic strategies could make interferon-free regimens viable for treatment of hepatitis C virus positive liver transplant recipients. The recent combinations of sofosbuvir with simeprevir or daclatasvir or ledipasvir plus/minus ribavirin have boosted the on treatment and sustained virological response to rates approaching 100% within liver transplant recipients with recurrent chronic hepatitis C (CHC). Preliminary data showed that the second generation direct oral antivirals could result to high treatment rates of recurrent CHC in kidney transplant recipients as well. Ongoing studies will clarify the optimal treatment of recurrent CHC in kidney transplant recipients.

Key words: Viral hepatitis; Hepatitis C recurrence; Hepatitis B; Hepatitis C; Liver transplantation; Kidney transplantation hepatitis B recurrence

Core tip: Emphasis should be placed in the appropriate nucleo(s)tide analog selection for prevention of recurrent hepatitis B virus post liver and kidney transplantation; Second generation direct acting oral antivirals have demonstrated sustained virological response rates approaching 100%, minimal side effects and drug interactions on liver transplant recipients with chronic hepatitis C virus (HCV); Preliminary data showed outstanding response of kidney transplant recipients with chronic HCV to direct acting oral antivirals.

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INTRODUCTION

Treatment of chronic hepatitis B (CHB) and chronic hepatitis C (CHC) infection in patients who have undergone liver or kidney transplantation represents a challenge for physicians. Efforts to develop effective antiviral medications have been robust over the last decade and the data review showed clinical outcomes comparable with that of the non-infected transplant recipients[1-4].

Recurrent hepatitis B post liver transplantation (Table 1)

Potent nucleos(t)ide analogues (NAs) with high-genetic barrier should be given in all patients with hepatitis B virus (HBV) decompensated cirrhosis in order to improve the liver function and achieve undetectable HBV DNA at the time of liver transplantation (LT)[5]. It is known that NAs prevent HBV recurrence at an acceptable level and lead to long-term survival after LT[6]. Furthermore, the combination of NAs with low-dose hepatitis B immune globulin (HBIG) can prevent HBV recurrence in more than 80% of LT recipients on long-term[6]. However, long-term HBIG administration requires additional measurement for hepatitis B surface antibody titers and it has been proven inconvenient and costly. Thus, various HBIG-elimination prophylactic regimes have been tried, resulting in encouraging efficiency results similar to continuing HBIG[7,8].

A systematic review and two studies carried out by our team[9-11] favored the use of HBIG with a high genetic barrier NAs instead of HBIG and lamivudine combined prophylaxis against HBV recurrence after LT[9], suggesting that the maintenance monotherapy with newer NAs [entecavir (ETV) or tenofovir (TDF)] was effective after discontinuation of HBIG prophylaxis[10]. Indeed, the most recent prospective study coming from our group[11] demonstrated that ETV or TDF monoprophylaxis following combination with low-dose HBIG six months post LT was highly effective and safe in twenty-eight cirrhotic patients with undetectable HBV DNA at the time of LT. Nevertheless, the long-term immunosuppressive therapy may cause considerable renal dysfunction, cardiovascular disease and cancer, in the context of HBV recurrence, that account for significant late mortality[12]. Interestingly, telbivudine administration for prophylaxis of HBV recurrence can improve renal function after LT[13,14].

Generally, some form of HBV prophylaxis should be continued indefinitely after LT[15]. Based on our recent review, that summarizes all the available relevant current data, the choice of therapy should be individualized in regards to patient's HBV-DNA levels before LT and the previous exposure to NA(s). LT recipients with a low risk of HBV recurrence (*i.e.*, undetectable HBV DNA levels before LT, which represents the majority of HBV positive candidates), might discontinue HBIG and maintain on long-term oral antiviral therapy[15]. Patients with high risk of HBV recurrence (high pretransplant HBV DNA levels, HIV coinfection and preexisting drug resistance or high risk of noncompliance to antiviral therapy) may need a more careful and close management[16]. Only few studies have considered HBIG-free prophylactic regimens from the first post-operative day; *i.e.*, the administration of newer NAs (ETV or TDF). In keeping with low cost, these studies[9,17] have given encouraging results, but this approach is still challenging and controversial.

Recurrent hepatitis B post kidney transplantation (Table 1)

Prior to the advent of NAs, HBV infection had such a severe negative impact on kidney transplantation (KT)[1], that many centres regarded HBsAg seropositivity as a contraindication for KT. In the era of NAs administration the 5-10 year survival rate of KT recipients with CHB is approaching that of HBsAg negative patients[1,18]. The introduction of NAs represents a major breakthrough in the field of KT accounting for minor liver complications, effective viral load suppression and better patient survival without compromising the kidney allograft outcome[19]. Nevertheless, the management of KT recipients with CHB should take into account the special therapeutic limitations and the features of an ideal regimen of this patient group. The limitations of NA use include nephrotoxicity, reported mostly after adefovir and tenofovir administration, high resistance rates after long term lamivudine use, and allograft function decline following interferon use[19,20].

The data on NA administration in KT recipients are scarce. Usually, KT candidates are started on antivirals before KT and carry on the same regimen post-KT unless liver disease deterioration or resistance accrues. Prophylactic antiviral therapy commenced prior to KT seems to better prevent the HBV related complications post KT[21]. In this case, ETV should be the first line therapy for KT recipients because of the high efficacy and safety profile and the low rates of resistance[20,22]. TDF is proposed as the best choice for cases with creatinine clearance > 60 mL/min, or history of resistance to lamivudine[23], while telbivudine should be considered for CHB patients with low viremia if the aim is the amelioration of glomerular filtration rate[13,24,25].

Recurrent hepatitis C post liver transplantation (Tables 1 and 2)

The rate of HCV recurrence has been extremely high in patients with HCV viremia at the time of liver transplantation, resulting to 70% decompensation (comparing with 10% in other immunocompetent groups), two thirds of graft failure and high death rates[26]. The introduction of direct oral acting antivirals (DAAs) has revolutionized the treatment of patients with

Table 1 Recommendations for the management of hepatitis B and C infection after liver or kidney transplantation

Chronic hepatitis B	Post-liver transplantation	Prophylaxis and treatment	HBIG (for short term) plus NA[1]
	Post-kidney transplantation		NAs[1]
Chronic hepatitis C	Post-liver transplantation	No prophylaxis	Sofosbuvir based regimens or "3D" regimen plus RBV (for genotypes 1 and 4)
	Post-kidney transplantation		Newer direct oral antivirals plus/minus RBV (studies are ongoing)[2]

[1]Frontline analogues are considered entecavir or tenofovir in renal proper doses (consider telbivudine in the presence of renal dysfunction); [2]Interferon is contraindicated due to the high risk of allograft rejection. HBIG: Hepatitis B immune globulin; NAs: Nucleos(t)ide analogues; 3D: Paritaprevir (plus ritonavir)/ombitasvir/dasabuvir for genotype 1 and paritaprevir (plus ritonavir)/ombitasvir for genotype 4; RBV: Ribavirin.

CHC and represents a major breakthrough especially for difficult to treat populations, involving the patients with cirrhosis Child-Pugh stage B and C, HCV genotype 1 and previous intolerance or non-response to interferon (IFN)-based therapy[3]. First generation DAAs (*i.e.*, telaprevir and boceprevir) improved significantly the sustained virological response (SVR) but their common interactions with the calcineurin inhibitors and the poor tolerance prevented their wide implementation in LT recipients. Various IFN-free combinations, including potent second generation DAAs with non-overlapping resistance profiles, have provided rapid and potent suppression of viral replication. The current available reports[27,28] indicated that the undetectable HCV RNA peritransplant has led to successful prevention of recurrent CHC post LT. In this line, the new DAA combinations have ensured excellent safety with minimal CNI interactions on LT recipients with recurrent CHC after LT (with the good results to be extended to LT recipients with fibrosing cholestatic hepatitis as well). The recent combinations of sofosbuvir with simeprevir or daclatasvir or ledipasvir plus/minus ribavirin (RBV) have boosted the SVR response to rates approaching 100% within LT recipients with CHC recurrence[29-31]. Similarly, SVR and HCV recurrence prevention was reported in a second LT recipient with decompensated cirrhosis without pre-LT SVR commenced on an novel antiviral combination immediately post-LT[32].

The latest data derived from the Coral study[33] showed 97% SVR at four and 12 wk after four-drug administration: Paritaprevir (potent NS3/4A protease inhibitor), ombitasvir (potent NS5A inhibitor), dasabuvir (non-nucleoside NS5B polymerase inhibitor) and RBV. The Cosmos study group reported complete treatment in 27 LT recipients with CHC genotype 1 by using sofosbuvir plus simeprevir for 12 wk underlining minor side effects such as mild transient rash, indirect hyperbilirubinemia and cyclosporine withdrawal (due to simeprevir interaction)[34]. Based on all these striking results, the European Association for the Study of the Liver[35] recommends that: (1) all patients with CHC listed for LT should receive antiviral therapy in order to prevent graft infection after LT; and (2) HCV recurrence post LT should be treated with one of the above antiviral combinations, irrespectively of the severity of liver disease: Sofosbuvir and RBV for 12 wk in genotype 2; fixed dose sofosbuvir, ledipasvir and RBV for 12 wk in genotypes 1, 4, 5, 6; sofosbuvir, daclatasvir plus RBV for 12 wk in all genotypes. In addition, patients without cirrhosis or with Child-Pugh class A post-LT could be also treated with: (1) sofosbuvir and simeprevir plus RBV if there are genotype 1, 4; and (2) paritaprevir/ombitasvir/dasabuvir and RBV for 12 wk if they are genotype 1b and for 24 wk if they are genotype 1a with cirrhosis; if they are genotype 4: Paritaprevir/ombitasvir plus RBV for 12 wk in non-cirrhotics or 24 wk in cirrhotics.

Recurrent hepatitis C post kidney transplantation (Tables 1 and 2)

CHC has been related with poor patient and graft survival after KT that corresponded to the pre-existing HCV infection before KT (the level of HCV RNA and the liver complications)[36,37]. Consequently, substantial attention should be applied to treat HCV infection before KT. Very few studies reported the use of first generation DAAs (boceprevir and telaprevir) on the top of reduced dose IFN and RBV in kidney transplant candidates. The results are promising in regards to efficacy, but side effects such as anemia have been still of concern[38-40]. The new DAAs administered as IFN- and RBV-free combinations might be proved the treatment of choice against HCV infection in KT patients. However DAA-efficacy, -tolerability and effect on graft function still warrant thorough evaluation. So far, the use of new antivirals in KT recipients has been reported solely in few cases and is being tested in two ongoing trials[41-43]. Fibrosing cholestatic hepatitis was successfully treated either with sofosbuvir combined with pegylated-IFN and RBV[41] or with sofosbuvir and simeprevir without IFN or RBV in combined kidney-liver transplant recipients[42]. Moreover, sofosbuvir combined with low dose RBV was efficient, presented minimum adverse events such as pruritus and mylalgia and did not require tacrolimus dose adjustments in eight KT recipients with HCV genotype 1, creatinine clearance higher than 30 mL/min and hemoglobin higher than 10 g/dL[44]. The antivirals tested in the ongoing trial among KT recipients with HCV are sofosbuvir and ledipasvir and no initial data have been published yet[43]. Concerns emerge in regards to performance of sofosbuvir in recipients with kidney function deterioration, since sofobusvir is renally excreted and is not appropriate for creatinine clearance below 30 mL/min. Initiation of sofusbuvir - based regimens post KT once glomerular filtration rate > 30 mL/min as a prophylaxis could be

Table 2 Main characteristics of the approved direct acting antivirals that are currently used in interferon-free regimens for the treatment of chronic hepatitis C

Name	Category, antiviral activity	Doses	Adjustments
Simeprevir	Second-wave NS3/4A protease inhibitor, genotypes 1 and 4	150 mg daily, orally	No renal adjustment is needed Contraindicated in patients with Child-Pugh B/C Contraindicated cyclosporine co-administration
Sofosbuvir	NS5B RNA Polymerase nucleotide inhibitor, pangenotypic	400 mg daily, orally	Only in glomerular filtration rate > 30 mL/min No CNI adjustment is needed
Daclatasvir	NS5A inhibitor, genotypes 1, 3 and 4	60 mg daily, orally	No renal adjustment is needed No CNI adjustment is needed
Ledipasvir	NS5A inhibitor genotypes 1, 3 and 4	90 mg daily, orally (fixed dose with sofosbuvir)	No renal adjustment is needed[1] No CNI adjustment is needed
Dasabuvir	Non-NUC NS5B polymerase inhibitor genotype 1	250 mg every 12 h	No renal adjustment is needed
Paritaprevir/Ritonavir/Ombitasvir	Ritonavir boosted NS3/4A protease inhibitor/NS5A inhibitor, genotypes 1 and 4	75/50/12.5 mg x 2 once daily	No safety data in Child-Pugh B, contraindicated in Child-Pugh C Cyclosporine: 20% of pretreatment total daily dose; tacrolimus: 0.2 mg/72 h or 0.5 mg once weekly

[1]Ledipasvir in combination with sofosbuvir should not be given in patients with glomerular filtration rate < 30 mL/min. CNI: Calcineurin inhibitor.

one option. Nevertheless, recent studies have reported acceptable safety and tolerance profile of sofosbuvir in CHC patients with end stage renal disease (glomerular filtration rate < 30 mL/min) or under hemodialysis[45].

CONCLUSION

Substantial progress is acknowledged in the field of antiviral treatment of HBV and HCV positive LT recipients, even if the existing data are preliminary. The applications of novel antiviral combinations are viable in concept, but provisionally under way for HBV and HCV positive KT recipients. However, the high antiviral cost, the drug resistance and the nephrotoxicity will be barriers to optimal therapy access.

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EDITORIAL

Flatography: Detection of gastrointestinal diseases by faecal gas analysis

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Abstract

Patients presenting with gastro-intestinal symptoms might suffer from a range of possible underlying diseases. An unmet need exists for novel cost-effective, reproducible, easy-to-perform and non-invasive tests. Hippocrates used body odours to diagnose diseases circa 460 before Christ. The art of diagnostic smelling is making a promising high-tech come-back with portable "electronic diagnostic noses". Analysis of faecal volatile organic compounds is a novel field in metabolomics with considerable potential to improve the diagnosis, phenotyping and monitoring of gastro-intestinal disease. Challenges will be to mature over the coming years by development of a standardized methodology for stool sample collection, storage, handling and analysis. Furthermore, key volatiles need to be identified to improve test accuracy and sensitivity by development of sensors tailored toward the accurate identification of disease specific volatiles. If these challenges are adequately faced, analysis of faecal volatiles has realistic potential to considerably improve screening, diagnosis and disease monitoring for gastro-intestinal diseases.

Key words: Flatography; Electronic nose; Smell; Volatile organic compounds; Gastro-intestinal diseases; Volatile metabolomics

Core tip: Analysis of faecal volatile organic compounds is a novel field in metabolomics with considerable potential to improve the diagnosis, phenotyping and monitoring of gastro-intestinal disease. Challenges will be to mature over the coming years by development of a standardized methodology for stool sample collection, storage, handling and analysis. Key volatiles need to be identified to improve test accuracy and sensitivity by development of sensors tailored toward the accurate identification of disease specific volatiles. Analysis of faecal volatiles has realistic potential to considerably improve screening, diagnosis and disease monitoring for gastro-intestinal diseases.

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EDITORIAL

Patients presenting with gastro-intestinal symptoms might suffer from a range of possible underlying diseases. Diagnosis and monitoring of disease activity in gastro-intestinal disease (GID) are often time-consuming and carry a high burden on patients and the health care system. Laboratory tests have usually only limited specificity and sensitivity, and endoscopic evaluation of the gastro-intestinal tract is unpleasant and costly. Therefore, an unmet need exists in daily clinical practice for novel cost-effective, reproducible, easy-to-perform and non-invasive tests.

Recent advances in chemical analytical techniques may help to meet these goals. The "Father of Western medicine", the ancient Greek physician Hippocrates, used body odours to diagnose diseases already circa 460 before Christ. Centuries later, the art of diagnostic smelling is making a promising high-tech come-back since portable "electronic diagnostic noses" are currently increasingly studied in medical research setting.

The human body is a metabolic machinery and metabolites, both odorous and non-odorous, are discharged in various bodily excretions, like urine, faeces, blood and breath[1]. The metabolome is the aggregate of the small molecules (< 2000 Dalton) that form the raw materials of a wide array of metabolic reactions, of both physiologic and pathologic metabolic pathways, and its resulting end products[2]. Volatile organic compounds (VOCs) are the carbon based end products and in humans up to 1840 different VOCs have been described[1]. As any disease is characterized by changes in metabolism, these molecules are potential diagnostic biomarkers for a wide range of diseases. This may be especially useful when diseases have clinically similar manifestations, but require different therapeutic approaches.

Analysis of VOCs is a rapid emerging field of basal and clinical research and two basic approaches are used. Firstly chemical analytical techniques, such as gas chromatography linked to mass spectrometry, selected ion flow tube mass spectrometry and ion mobility spectroscopy, help to identify physiochemical properties of the target volatiles in a sample. This can generate valuable insights into the pathophysiology of underlying diseases and the origins of these volatiles. Unfortunately these techniques are relatively cumbersome and costly, thereby limiting their applicability in daily clinical practice, making them primarily suitable as a research tool.

Alternatively VOCs can be analysed by broadly cross-reactive gas-sensor arrays[3] that employ pattern recognition techniques to discriminate patterns of volatile biomarkers, so-called smellprints[4]. The attractiveness of this approach lies in the simultaneous assessment of a full VOC-profile by sensors that are generally low-cost, rapid and suitable as point of care tools. Since such an approach closely mimics mammalian olfaction this technique has been termed an electronic nose (eNose)[3,5]. This technique does not allow identification of separate compounds, which is generally not a clinical limitation, since determination of individual molecules is mostly not necessary in clinical practice.

Recently published data has shown that analysis of VOCs can discriminate between patients with various diseases, like Clostridium difficile, colorectal cancer and inflammatory bowel disease and controls with promising accuracy[6-11]. On theoretical grounds, VOCs in GID have several metabolic origins. Firstly exogenous, comprising dietary intake and the microbiome, supported by studies showing effect of diet on VOCs/microbiome[12] and links between VOC and microbiome[13]. Secondly, local from the primary affected disease site, due to inflammation of mucosa and necrosis, suggested by studies on colorectal cancer[8] and inflammatory bowel disease[11]. Thirdly the systemic (immunological) response, such as increased oxidative stress[14], supported by fact that GI diseases can be detected in breath[7] and urine[15].

The composition of the volatile metabolome is highly depended on the analysed substrate. For example, some VOCs found in faeces might be absent in breath[1]. At least theoretically, VOCs originating from the gastro-intestinal tract dissolve from the intestines into the bloodstream and subsequently being transported to the lungs and might consequently appear in breath. However, some VOCs are chemically unable to dissolve into the bloodstream and some VOCs will be converted by the liver or other organs. Thereby some VOCs will remain or drop below the detection level and in addition new VOCs might be produced or existing VOCs will increase[1].

Based on the origins of faecal VOCs, analysis of VOCs emanating from faeces, so called flatography, might be the best non-invasive way of diagnosing GID as this substrate offers the most direct and integral reflection of the diseased gastrointestinal tract.

Results of several studies underline that flatography can be used for discrimination of patients with GID[8,11]. It is an attractive technique from both the patient's and physician's perspective, as the samples can be collected non-invasively. Moreover, in common daily clinical practice, most patients already need to hand in a stool sample for culture, faecal calprotectin or other laboratory tests, therefore collection of an extra sample for faecal gas analysis will take only little effort.

Although published results are promising, this technique is currently still in development and also has some limitations. At this moment, no standard methodology is verified yet. There are no studies available on extern validation and also the influences of procedure of stool sampling, storage and handling needs to be sorted out. These development pathways probably require a combination of eNose and chemical analytical techniques

to help identify target VOCs helping to guide the development of primed sensors that are suited for use in clinical practice.

To conclude, analysis of faecal VOCs is a novel field in metabolomics with considerable potential to improve the diagnosis, phenotyping and monitoring of GID. Challenges will be to mature over the coming years by development of a standardized methodology for stool sample collection, storage, handling and analysis. Furthermore, key volatiles need to be identified to improve test accuracy and sensitivity by development of sensors tailored toward the accurate identification of disease specific volatiles. If these challenges are adequately faced analysis of faecal volatiles has realistic potential to considerably improve screening, diagnosis and disease monitoring for gastro-intestinal diseases.

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EDITORIAL

Direct antiviral agent treatment of decompensated hepatitis C virus-induced liver cirrhosis

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Abstract

Recently, direct antiviral agents (DAAs) have been increasingly used for the treatment of chronic hepatitis C virus (HCV) infections, replacing interferon-based regimens that have severe adverse effects and low tolerability. The constant supply of new DAAs makes shorter treatment periods with enhanced safety possible. The efficacy of DAAs for treatment of compensated liver cirrhosis (LC) is not less than that for treatment of non-cirrhotic conditions. These clinical advantages have been useful in pre- and post-liver transplantation (LT) settings. Moreover, DAAs can be used to treat decompensated HCV-induced LC in elderly patients or those with severe complications otherwise having poor prognosis. Although encouraging clinical data are beginning to appear, the actual efficacy of DAAs for suppressing disease progression, allowing delisting for LT and, most importantly, improving prognosis of patients with decompensated HCV-LC remains unknown. Case-control studies to examine the short- or long-term effects of DAAs for treatment of decompensated HCV-LC are urgently need.

Key words: Decompensated liver cirrhosis; Prognosis; Direct antiviral agent; Hepatitis C virus; Comorbidity; Nucleic acid analogue

Core tip: Decompensated liver cirrhosis (LC) due to hepatitis C virus (HCV) infection is a severe disease with poor prognosis. Because interferon-included regimens are contraindicated at this stage, liver transplantation has been the only way to cure the disease. However, recent development of direct antiviral agents (DAAs) is offering a hope for this difficult situation. Promising antiviral effects of DAAs for LC have been observed, suggesting

they might be useful for treatment of decompensated HCV-LC. To explore this possibility, large case-control studies are needed.

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INTRODUCTION

Hepatitis C virus (HCV) - induced decompensated liver cirrhosis (LC) is a life-threatening illness with an average 5-year survival rate of 50%[1,2]. Although liver transplantation (LT) has been the only curative therapy for these patients, the recently-developed direct antiviral agents (DAAs) are now helping patients cope with these difficult situations. Because DAAs have far fewer adverse effects and are better tolerated than interferon (IFN), they can be used to treat the elderly or patients with comorbidities, such as leuco - or thrombocytopenia. It is well-known that IFN-based therapies provide lower sustained viral response (SVR) rates as the disease progresses and fibrosis levels increase. However, this may not be the case for DAA treatments, because the presence of compensated cirrhosis reportedly has not hampered the therapeutic outcomes[3,4]. Because these patients have the higher risk of mortality and hepatocellular carcinoma (HCC), the benefits of eradicating the virus, may be greater than doing so in those having less-advanced disease.

There have been only a few reports of the effects of DAA therapy in patients with decompensated HCV-induced LC (HCV-LC). The effects and safety of DAAs for treatment of severe liver diseases in both pre- and post-(LT) patients have been demonstrated[5,6]. After LT, reinfection of the grafted liver frequently occurs if HCV is not eradicated, and subsequently one third of liver transplant recipients with HCV infection either died, experienced allograft loss, or developed cirrhosis by the fifth post-operative year[7]. Thus, the primary endpoint of DAA therapy in the setting of LT is to clear the virus before transplantation and prevent reinfection of grafted livers. Importantly, findings obtained from studying this situation can be extended to therapy for elderly patients and those having comorbidities-patients having no indications for LT in the real-world settings.

On the other hand, there have been numerous clinical experiences of use of oral nucleoside analogues (NAs) for HBV-induced compensated or decompensated LC, and clinical benefits for both in short- and long-term prognosis have been established[8]. In this review, we summarize the data on use of DAAs for treatment of advanced HCV-LC and comorbidities, referencing the past experience with use of NAs for these HBV patients.

DECOMPENSATED HCV-LC: NATURAL HISTORY AND TREATMENT

Chronic hepatitis C infection is one of the leading causes of chronic liver diseases and the most common indication for LT. Because of its indolent clinical course, patients occasionally suffer from advanced disease before diagnosis is made. In its natural history, cirrhosis develops in 4%-24% of patients during 20 years of chronic infection[9]. The annual occurrence rate of HCC is about 2%-4% of patients with cirrhosis, and it is reported to be around 7% in Japanese patients[9,10]. Signs of decompensation include ascites, jaundice, encephalopathy and variceal bleedings. The 5-year survival rate for decompensated HCV-LC is about 50%; the condition is a good indication for LT[1,2]. However, implementation rates of LT vary among the countries depending on the accessibility of donor livers. In a country like Japan, where chronic insufficiency of donor livers is the usual situation, most of patients with decompensated HCV-LC cannot benefit from LT. Above all, most of these patients are elderly and have several clinical complications, and are generally not considered suitable candidate for LT.

IFN-BASED TREATMENT FOR ADVANCED HCV-INDUCED LIVER DISEASES

Currently IFN-based regimen for the patients with chronic HCV having genotype 1 (GT1) mainly consists of pegylated-interferon (PEG-IFN), ribavirin (RBV) and a protease inhibitor (PI). Xu *et al*[11] reported that SVR rates of PEG-IFN plus RBV therapy for patients with decompensated HCV-LC were 19.7% for GT1 and 42.9% for GT2, resulting in significant suppression of disease progression, compared to control patients. Thus IFN-based regimens might improve the prognosis of the patients with advanced type C liver diseases when treatment is safe and compliance is good. However, in the era of DAAs which have both strong anti-viral effect and sufficient tolerability, IFN-based regimens may no longer be prioritized for patients with advanced type C liver diseases; and rather, may be relatively contraindicated because of severe complications and low probability of attaining SVR[12].

RECENT PROGRESS OF DAA TREATMENTS

NS3/4A protease inhibitors (PIs) such as telaprevir, simeprevir, vaniprevir have been approved in Japan for treatment of GT1b, in combination with PEG-IFN plus RBV. These regimens have greatly improved therapeutic efficacy; SVR rates have been reported to be more than 70% for telaprevir or simeprevir plus PEG-IFN and RBV regimens[13,14]. However, the recent replacement, which was approved in 2014, of the IFN-based regimens, which had severe adverse effects and poor tolerability, by the

combination of asunaprevir (PI) and daclatasvir (NS5A inhibitor) for GT1b patients, has become a central role in their treatment[15]. On the other hand, sofosbuvir (SOF), a potent inhibitor of the HCV NS5B polymerase, has been approved for the treatment of chronic hepatitis C genotypes 1-4 in the United States and other countries. SVR was obtained in more than 85% of patients with GT1 when combined with PEG-IFN and RBV[16]. Because of its high efficacy and safety, low incidence of adverse effects and a high genetic barrier, SOF is useful when combined with another DAA or RBV[17,18]. The combination of SOF and ledipasvir (LDV, another NS5A inhibitor) treatment was tested in large-scale clinical trials, resulting in SVR rates of more than 90% in patients with GT1 infection[19-21]. It is expected to be approved in Japan in 2015, based on the SVR rate of close to 100% in Japanese GT1 patients[22]. Moreover, remarkable SVR rates (99.5% with RBV and 99.0% without RBV for GT1b infection) were reported with ABT-450/r-ombitasvir and dasabuvir treatment[23]. The combination treatment of grazoprevir (MK-5172, an NS3/4A protease inhibitor) and elbasvir (MK-8742, an NS5A inhibitor) with or without RBV for 12 to 18 wk achieved high SVR rates, ranging from 90%-100%, encompassing the treatment arms[24]. Thus, the constant supply of new DAAs is improving the efficacy and tolerability of this class of drugs.

THE CURRENT STATUS OF DAA TREATMENT FOR COMPENSATED HCV-LC

Most IFN-free DAA trails up to now have enrolled only a modest population (10%-20%) of cirrhotics with well-compensated diseases[15,20,25,26]. SVR rates for cirrhotics in these studies were comparable to those of non-cirrhotic patients, in contrast to the result of IFN-based regimens, for which, as fibrosis of liver progresses, the rate of SVR decreases and the incidence of adverse effects increases. A recently-published phase Ⅲ trial (Turquoise-Ⅱ) with ABT-450/r-ombitasvir- and dasabuvir plus RBV-based regimens, was performed exclusively in patients with GT1 HCV-LC[3]. This study included 380 patients with cirrhosis in Child-Pugh class A5 to A6, randomly assigned to receive either 12 or 24 wk of treatments. Patients achieved SVR12 of 91.8% and 95.9% in the 12-wk and 24-wk treatment arm, respectively. In addition, differences in rates of SVR12 between patients with mild to moderate fibrosis (F0 to F2) *vs* F3 to F4 were not statistically significant[27]. These outstanding SVR rates accompanied by a high safety and tolerability profile in cirrhotics allow IFN-free DAA regimens to be much more available to even decompensated cirrhosis patients.

DAA TREATMENT FOR DECOMPENSATED HCV-LC

DAA treatments for patients with severe liver diseases before and after LT have been reported[7,28]. The primary endpoint of treatment in the liver-transplant setting is to eradicate the virus to prevent reinfection by HCV of the grafted liver tissue. SOF-based DAA regimens have been playing a central role in these settings. A total of 61 patients, mainly with compensated LC [≤ CTP (Child-Turcotte-Pugh) 7, or < MELD (Model of End-Stage Liver Disease) 22] and HCC, on the waiting list for LT, had received up to 48 wk of SOF plus RBV treatment, and 30 of 43 (70%) whose HCV RNA levels were less than 25 IU/mL at transplantation obtained post-transplantation virologic response at 12 wk. Safety profiles were excellent[28]. In addition, combination treatment of SOF + RBV (± Peg-IFN) were tested in 104 patients who had early severe recurrence (52), LC (52) after LT[7]. SVR 12 was obtained in 35 of 48 (73%) with early severe recurrence. Importantly, 59 of 103 (57%) reported clinical improvement. These studies showed that SOF-based, IFN-free regimens provided high rates of SVR with a good safety profile in difficult peri-LT settings.

Clinical and epidemiological features of HCV-infected patients vary among the countries. In Japan, candidates for DAA treatment are mainly the elderly or those with complications. Presently, published results in patients with decompensated cirrhosis in real-world settings consist mostly of preliminary conference reports. Afdhal *et al*[29] reported on 48 wk of SOF plus RBV treatment for a total of 50 patients with GT1-4 HCV-LC (60% of them were CPT7-10 and 20% ≥ MELD 14) and 89% of rapid viral response (RVR) 4 and 97% RVR 8 were obtained. SVR 4 was obtained in 16/18 (89%) GT1 and CTP Class B patients after 12 wk of SOF/LDV treatment[30]. A total of 108 patients with CTP Class B or C cirrhosis with GT1 and 4 were treated with SOF/LDV and RBV, and SVR was achieved in 87% of those given the 12-wk treatment and 89% in the 24-wk treatment[31]. It is remarkable that these SVR rates are comparable to those for compensated LC or even non-cirrhotic patients. Although the numbers were still small and the results were preliminary, these studies suggest that such treatments may extend the life expectancy of patients who would otherwise be considered end-stage without the use of DAA.

DAA TREATMENT FOR COMORBIDITIES

IFN-based regimens are contraindicated for depressed patients and cautions should be exercised in their use for those with psychiatric disorders. The presence of leucocytopenia and thrombocytopenia may severely impair compliance with IFN-based therapies[32]. DAA can be administered safely in these situations and also to elderly patients with cardiovascular complications and diabetes. However, caution should be used in patients with anemia, renal insufficiency and cardiopulmonary complications if the regimen contains RBV.

Because SOF also has renal toxicity, caution is necessary in those with renal dysfunction. However, the newly-developed DAAs grazoprevir and elbasvir

are metabolized by the liver and easily administered to patients with renal dysfunction without adjustment of dose[24]. In general, unlike IFN, DAAs can be administered to patients with autoimmune disorders unless RBV, which might affect the immune status, is included in the regimen[33]. Human Immunodeficiency Virus (HIV)/HCV co-infected patients have the same cure rates, of over 90%, with IFN-free DAA combinations. Therefore, guidelines no longer separate mono- and co-infected patients. The only special consideration in HIV/HCV co-infected subjects is the need to check for drug-drug interactions between anti-HIV and -HCV agents[34].

EXPECTATIONS AND CONCERNS OF DAA TREATMENT FOR DECOMPENSATED HCV-LC

Past clinical experiences from the results of NA treatment of patients with advanced HBV liver diseases may provide useful information when predicting the short- and long-term effects of DAA therapy for decompensated HCV-LC, a practice that is still in the beginning stages. Jang *et al*[35] reported the long-term effect of the NAs in patients with decompensated HBV-LC. They followed 284 untreated patients and 423 treated with NAs for more than 7 years and found that transplant-free survival was significantly improved in those treated with NAs (59.7% *vs* 46.0%). CTP and MELD scores improved significantly as a consequence of continuous suppression of HBV. They found that the degree of improvement was greater in those with higher CTP or MELD scores and early commencement of therapy was more important in the improvement of prognosis.

There are some similarities and differences between the following two situations: That is, NAs for HBV and DAAs for HCV. Anti-viral effects of DAAs against HCV might possibly surpass that of NAs against HBV, given that DAAs eliminate HCV RNA from serum in a very short period. Safety profiles may be comparable to NAs. Most importantly, DAAs may induce virus-free state if SVR is accomplished with only short duration of treatment, whereas the NAs for HBV cannot eliminate covalently closed circular DNA in liver and even long-term treatment may not eradicate the virus. Thus, DAA treatment for decompensated HCV-LC might elicit a similar clinical impact as NAs for decompensated HBV-LC with only a short-term treatment. Actually, preliminary report showed that DAAs for decompensated LC improved MELD scores in 60% to 79% patients only 4 wk after treatment finalization[31]. For these reasons, DAA treatments would be indicated positively for those with decompensated LC-HCV in real world-settings[36].

Despite of these encouraging situations and that a standardized mortality rate analysis reported a lower liver-related mortality among HCV-cirrhotics with SVR by IFN treatment[37], to our knowledge there are currently no data on disease progression, delisting from LT, and improvement of life expectancy after the achievement of SVR for decompensated LC-HCV. It is well-known that the level of liver fibrosis can be decreased with the eradication of virus[38]. However, it is uncertain whether the severe fibrosis observed in decompensated HCV-LC could be reversed to some extent. Although successful treatment outcomes in HCV induced cirrhotics resulted in the significant prevention of HCC[39], it is not yet clear whether DAA treatment for decompensated HCV-LC lowers the incidence of HCC. Actually, despite clinical improvement, the occurrence of HCC was not significantly suppressed by NA treatment for decompensated HBV-LC[35]. However, given the difference of pathogenesis between HBV and HCV, in that one virus integrates into the genome and the other does not, the two infections might not always respond in a similar fashion.

There are also several issues to be considered when commencing the treatment of DAA for decompensated HCV-LC. Early mortality due to aggravation of liver function during therapy might occur as was observed during treatment with lamivudine for decompensated HBV-LC[40]. Patients with advanced cirrhosis still have a high risk for hospitalization after the initiation of DAA treatment[36]. In addition, decompensated cirrhosis patients are more prone to develop drug-induced side-effects when compared to patients with compensated cirrhosis. For example, simeprevir is contraindicated to Child C LC, asunaprevir to Child B-C and ABT-450/r to Child B[6]. Especially patients on SOF regimens should also be monitored for renal dysfunction[41].

Currently it is possible to perform a large scale case-control study to clarify short- or long-term effects of DAA treatment for decompensated HCV-LC patients in real-world settings. Until these results are available, treatment of decompensated HCV-LC patients should be individualized on a case-by-case basis, giving due consideration to viral factors like genotype and clinical background factors including age, severity of liver diseases and presence of comorbidities.

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EDITORIAL

Role of peroxisome proliferator-activated receptors alpha and gamma in gastric ulcer: An overview of experimental evidences

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Abstract

Peroxisome proliferator-activated receptors (PPARs) are ligand-activated transcription factors belonging to the nuclear hormone receptor superfamily. Three subtypes, PPARα, PPARβ/δ, and PPARγ, have been identified so far. PPARα is expressed in the liver, kidney, small intestine, heart, and muscle, where it activates the fatty acid catabolism and control lipoprotein assembly in response to long-chain unsaturated fatty acids, eicosanoids, and hypolipidemic drugs (*e.g.*, fenofibrate). PPARβ/δ is more broadly expressed and is implicated in fatty acid oxidation, keratinocyte differentiation, wound healing, and macrophage response to very low density lipoprotein metabolism. This isoform has been implicated in transcriptional-repression functions and has been shown to repress the activity of PPARα or PPARγ target genes. PPARγ1 and γ2 are generated from a single-gene peroxisome proliferator-activated receptors gamma by differential promoter usage and alternative splicing. PPARγ1 is expressed in colon, immune system (*e.g.*, monocytes and macrophages), and other tissues where it participates in the modulation of inflammation, cell proliferation, and differentiation. PPARs regulate gene expression through distinct mechanisms: Ligand-dependent transactivation, ligand-independent repression, and ligand-dependent transrepression. Studies in animals have demonstrated the gastric antisecretory activity of PPARα agonists like ciprofibrate, bezafibrate and clofibrate. Study by Pathak *et al* also demonstrated the effect of PPARα agonist, bezafibrate, on gastric secretion and gastric cytoprotection in various gastric ulcer models in rats. The majority of the experimental studies is on pioglitazone and rosiglitazone, which are PPARγ activators. In all the studies, both the PPARγ activators showed protection against the gastric ulcer and also accelerate the ulcer healing in gastric ulcer model in rats. Therefore, PPARα and PPARγ may be a target for gastric ulcer therapy. Finally, more studies are also needed to confirm the involvement of PPARs α and γ in gastric ulcer.

Key words: Peroxisome proliferator-activated receptors; Gastric ulcer; Evidences

Core tip: Peroxisome proliferator-activated receptors (PPARs) are a nuclear hormone receptor family and act as transcription factors. PPARs are of three subtypes, *i.e.*, PPARα, PPARβ/δ, and PPARγ. The common sites where PPARα is expressed are muscle, heart, liver, small intestine and kidney. PPARγ is involved in modulation of various functions like inflammation, cell proliferation, and differentiation and it is commonly expressed in white blood cells (*e.g.*, macrophages and monocytes) which are involved in immune activity and in the colon. Studies in animals have demonstrated the gastric antisecretory activity of PPARα agonists like ciprofibrate, bezafibrate and clofibrate. The majority of the experimental studies regarding the role of PPARγ activators is on pioglitazone and rosiglitazone. In all the studies, both the PPARγ activators showed protection against the gastric ulcer and also accelerate the ulcer healing in gastric ulcer model in rats. Therefore, PPARα and PPARγ can be explored as a target of gastric ulcer treatment. The aim of the present paper is to discuss the experimental evidences of the role of PPARs in gastric ulcer.

Saha L. Role of peroxisome proliferator-activated receptors alpha and gamma in gastric ulcer: An overview of experimental evidences. *World J Gastrointest Pharmacol Ther* 2015; 6(4): 120-126 Available from: URL: http://www.wjgnet.com/2150-5349/full/v6/i4/120.htm DOI: http://dx.doi.org/10.4292/wjgpt.v6.i4.120

INTRODUCTION

Peroxisome proliferator-activated receptors (PPARs) are a nuclear hormone receptor family and act as transcription factors. PPARs are of three subtypes, *i.e.,* PPARα, PPARβ/δ, and PPARγ. PPARα is involved in fatty acid catabolism and also in controlling of lipoprotein assembly. Factors which activate PPARα are hypolipidemic drugs (*e.g.*, Fenofibrate), long-chain unsaturated fatty acids and eicosanoids. PPARα is distributed in many tissues like heart, kidney, muscle, liver, and small intestine[1,2]. PPARβ/δ isoform of PPARs has been shown to involve in transcriptional-repression functions and inhibits PPARα or PPARγ target genes[2-7]. This isoform is abundantly distributed in the body and take part in various activities like keratinocyte differentiation, fatty acid oxidation, healing and very low density lipoprotein metabolism. PPARγ1 and γ2 are two subtype of PPARγ and derived from a single gene peroxisome proliferator-activated receptors gamma[8-12]. PPARγ1 is involved in modulation of various physiological functions like inflammation, cell proliferation, and differentiation and is found in tissues like white blood cells (*e.g.*, monocytes and macrophages) of the immune system, colon, and other tissues. Whereas PPARγ2 found in adipose tissue and plays an important role in the differentiation of adipocyte, storage of lipid, and energy dissipation[12]. Not only that, PPARγ is also taking part in the metabolism of glucose and improving the insulin sensitivity. Thiazolidinediones which is a PPARγ agonist, are commonly used for the treatment of type 2 diabetes as insulin-sensitizing drugs[2,4,5].

Molecular structure of PPARs

PPARs are consist of following function domains: (A/B) N-terminal region, (C) DBD (DNA-binding domain), (D) flexible hinge region, (E) LBD (ligand binding domain) and (F) C-terminal region[13,14]. The DBD contains two zinc finger motifs, which bind to specific sequences of DNA known as hormone response elements when the receptor is activated. The LBD has an extensive secondary structure consisting of 13 alpha helices and a beta sheet. Natural and synthetic ligands bind to the LBD, either activating or repressing the receptor. The transcriptional activating function (AF-1) motif is present in the N-terminus and it is not activated by ligands. On the other hand, E/F domain or LBD also contains a transcriptional activating function (AF-2) motif at the C-terminus helix 12, which is activated by ligands[13]. A large numbers of synthetic and natural ligands like eicosanoids, fatty acids, linoleic acid derivatives, oxidized and nitrated fatty acids, bind to the large binding pocket present on the E/F (LBD) domain. The dimerization of PPARs with the 9-cis retinoic acid receptor (RXR) requires both the D and E/F domains. Then this dimerized PPARs and RXR bound to their respective peroxisome proliferator-activated receptor response elements (PPREs) present on the DNA molecule.

Genes which take part in various body functions like the metabolism of lipid, homeostasis of energy, proliferation, differentiation, and survival of cells has their functional PPREs in their regulatory regions[1,2,13,15].

The expression of various genes which involved in various physiological functions are regulated by PPARs through three mechanisms: Transactivation (ligand-dependent), repression (ligand-independent) and trans-repression (ligand-dependent)[16,17] (Figure 1).

The classical mode of action of PPARs is the ligand-dependent transactivation. In this mechanism when a ligand binds to the PPARs, there is folding back of the helix 12 of the LBD which leads to the exposure of the AF-2 motif. The AF-2 motif is crucial for the recruitment of transcriptional coactivators. All these changes help in fitting together of all the transcriptional machinery at PPRE-containing promoters[16,17]. When there is no ligand, transcription of target genes is inhibited by PPARs by recruitment of co repressor complexes like nuclear receptor corepressor and silencing mediator for retinoid and thyroid receptors (Figure 1). "Transrepression" has recently been discovered as an additional nongenomic, ligand-dependent gene repression mechanism of PPARs which involves protein-protein interactions of NFκB, AP1, Smads, signal transducers and activators of transcription, and nuclear factor of activated T cells[17-19]. In both the genomic mechanisms of regulation of various genes by PPARs, *i.e.,* repression and transactivation required the

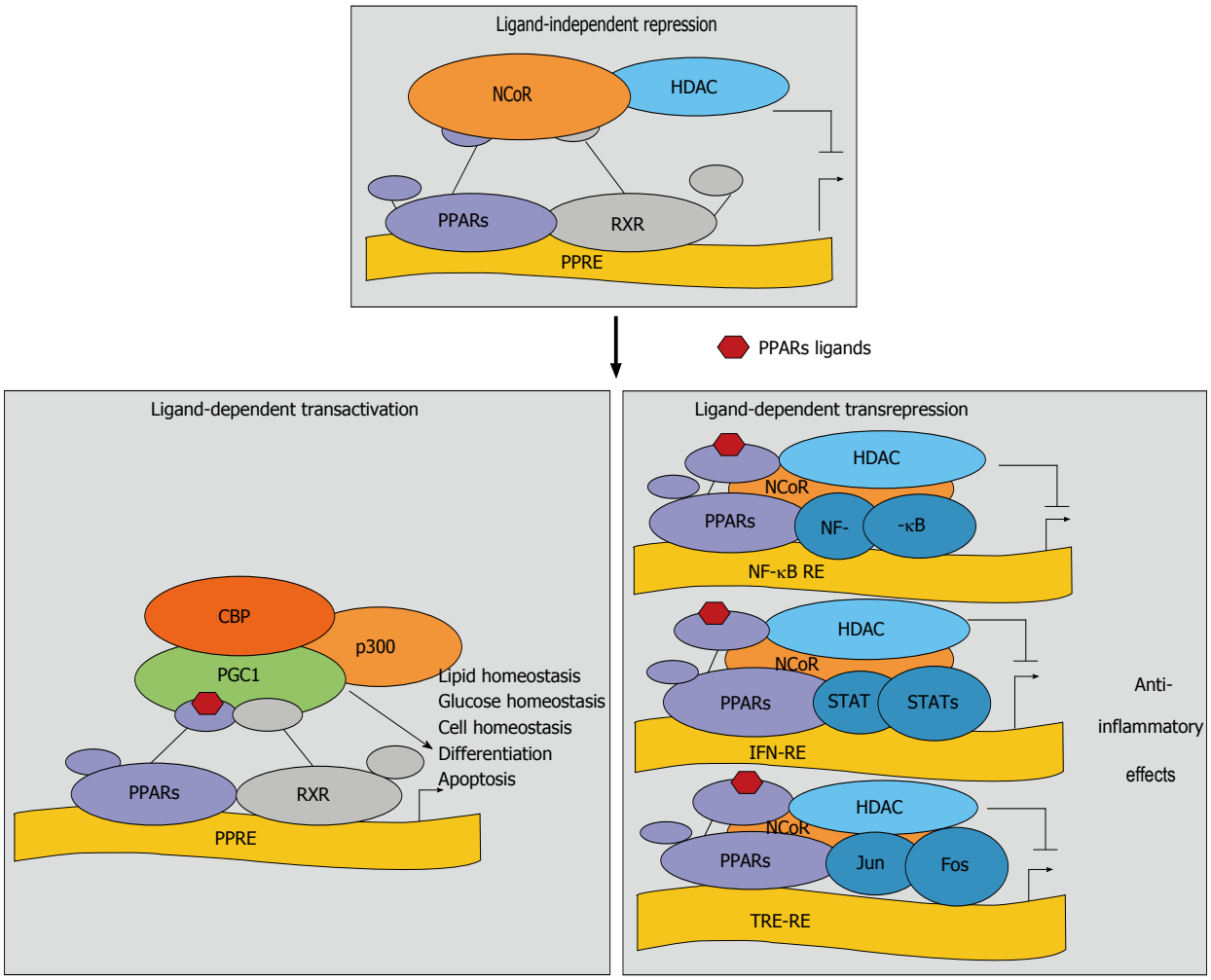

Figure 1 Peroxisome proliferator-activated receptors - mediated mechanisms of transcriptional regulation. In the absence of ligands, peroxisome proliferator-activated receptors (PPARs) bind the promoters of their target genes and repress transcription by recruiting the corepressor complex. In the presence of ligands, PPARs can induce either ligand-dependent transactivation or transrepression. Transactivation involves PPARs heterodimerization with the retinoid X receptors (RXRs) followed by recognition of specific PPAR response elements (PPREs) and interaction with coactivators. Transrepression involves interference with other signal transduction pathways, including NF-κB, STAT, and AP1. NCoR: Nuclear receptor corepressor; NF-κB-RE: NF-κB response element; IFN-RE: Interferon-stimulated gene factor responsive element; TRE-RE: O-tetradecanoylphorbol 13-acetate-responsive element; HDAC: Histone deacetylase; CBP: CREB binding protein; PGC1: Peroxisome proliferator activated receptor gamma coactivator 1; STATs: Signal transducers and activators of transcription.

binding of PPARs to PPREs, but transrepression, which is a nongenomic mechanism, does not require the binding of PPARs to PPREs. The anti-inflammatory properties of PPARs might be explained by the transrepression mechanism, where there is recruitment and stabilization of the corepressor complexes on the promoters of pro-inflammatory genes[17-20].

PPARα and gastric ulcer

PPARα is widely distributed in the small and large intestinal mucosa where dietary fatty acids delivered[21,22]. The genes which involved in functions like inflammation, cell cycle progression, angiogenesis and lipid metabolism are regulated by PPARα[23-27]. The role of PPARα in the processes like angiogenesis and cell cycle progression has been suggested its contribution toward the formation and progression tumor. To the best of my knowledge till date, no data have been available which indicates its role in gastric and esophageal cancer. However, its role in colorectal cancer has been investigated both *in vivo* and *in vitro* studies[24-30].

PPARα agonist enhanced the release of gastrin following the stimulation of PPARα[31]. The PPARα agonist induced hypergastrinemia is associated with less number of granules per cell as well as a relative increase in the number of electron-dense granules. All these changes with PPARα agonist are similar to those effects induced by proton pump inhibitor, pantoprazole, which indicates the signs of activation of the gastric cells in general[32]. Gastrin is a peptide hormone. It has two principal biological effects: Stimulation of acid secretion from gastric parietal cells and stimulation of mucosal growth in the acid-secreting part of the stomach (gastric cytoprotective effect)[33]. Nitric oxide is one of the well know gastro-cytoprotective agent and there is increased in the release of nitric oxide following PPARα stimulation[34]. Reports in the published literature have indirectly shown the antigastric ulcer effect of PPARα.

The gastric antisecretory activity of PPARα agonists like ciprofibrate, bezafibrate and clofibrate have been demonstrated in animal studies[35,36]. A short study by Eason *et al*[36] in the mid-1970s first time demonstrated the antisecretory activity of clofibrate in rats. In the same study other phenoxyisobutyrate derivatives like ciprofibrate and bezafibrate have also demonstrated a significant reduction in gastric acid secretion in rats like clofibrate, but the duration of action of ciprofibrate was longer as compared to ciprofibrate and bezafibrate[36].

Another study by Pathak *et al*[35] also studied the effect of bezafibrate, a PPARα agonist, on acid secretion and gastric cytoprotection in various rat models of gastric ulcer. Various gastric ulcer models were used in this study like acetic acid-induced chronic gastric ulcers, pylorus ligation, ethanol-induced, indomethacin-induced and ischemia-reperfusion-induced gastric ulcers. Bezafibrate (10 mg/kg and 100 mg/kg body weight) were used intraperitonealy. Significant antiulcer effects were seen with both the doses of bezafibrate in all the gastric ulcer models except acetic acid-induced chronic gastric ulcers and ischemia-reperfusion-induced gastric ulcer models. The healing of gastric ulcer was improved with bezafibrate in acetic acid-induced chronic gastric ulcer model. Bezafibrate (10 and 100 mg/kg) was also able to inhibit gastric ulcer formation induced by ischemia-reperfusion. So, this study not only demonstrated the antigastric ulcer property of the PPARα agonists, but also demonstrated its ulcer healing property in gastric ulcer models in rats[35].

We have also demonstrated the antigastric ulcer activity of bezafibrate in our laboratory (unpublished data). The present study was undertaken to validate antiulcer activity and the mechanism of action of bezafibrate in gastric ulcer. The aspirin induced gastric ulcer model was used. Bezafibrate was administered in graded doses (10-200 mg/kg) to detect the best effective anti-ulcer dose of bezafibrate. Keeping in view the diversity of defensive mechanisms, the present study was limited to exploring the involvement of the mucosal oxidant system, apoptotic pathway and nitric oxide pathway in the mechanism of the antigastric ulcer effect of bezafibrate. To explore the nitric oxide mechanism, a nitric oxide synthase inhibitor, N^{ω}-nitro-l-arginine was used. The following parameters were measured: Ulcer Index, Histopathological scoring of gastric ulcer, gastric juice analysis, Gastric mucosal lipid peroxidation parameters, Estimation of nitric oxide metabolite in blood, mRNA expression of inducible nitric oxide synthase (iNOS) and constitutive nitric oxide synthase (cNOS) enzyme in gastric mucosa, Gastric mucosal DNA fragmentation study. Bezafibrate demonstrated dose-dependent antiulcer activity, showed antisecretory and gastro protective action, reduced lipid peroxidation, inhibit iNOS expression, preserve cNOS expression and qualitatively inhibited DNA fragmentation and improved upon the Histopathological score of gastric mucosa.

The histopathological findings of the gastric mucosa: Aspirin administration showed superficial erosion and ulceration on the mucosa and infiltration of inflammatory cells. Co administration of bezafibrate with aspirin showed gastric mucosa with reepithilization, formation of pits and decreased infiltration of inflammatory cells. From this finding we can say that bezafibrate (a PPARα agonist) might have an anti inflammatory activity in gastric ulcer. This is the new finding seen with Bezafibrate and if proven clinically can be used in combination with aspirin (unpublished data).

PPARγ AND GASTRIC ULCER

PPARγ is a subtype type of PPARs which is a nuclear hormone receptor super family. The transcription of numerous cellular processes and various cytokines is controlled by activation of PPARγ[37]. The function and differentiation of immune cells and synthesis of many inflammatory cytokines like tumor necrosis factor alpha (TNF-α) might be controlled by stimulation of PPARγ[38]. Cytoprotective and antioxidant activities of PPARγ activation have been demonstrated in several experimental studies[39]. The role of PPARγ has been implicated in various disease conditions like atherosclerosis, inflammation, cancer and infertility. In adipose tissue, PPARγ is highly expressed and it plays an important role in the differentiation of adipocyte and maintenance of insulin responses.

PPARγ is widely distributed in the colon and the major sources might be the macrophages and epithelial cells. The other tissues which expressed PPARγ are small intestine, liver, pancreas and stomach. Low concentrations of PPARγ are seen in tissues like kidneys, glial cells, cartilage, airway epithelial cells and skin. Whereas gene of PPARγ is almost undetectable in muscles[40]. Inspite the fact that colonic epithelial cells highly expressed PPARγ, investigators have also demonstrated the effect of the PPARγ/RXR in gastric disorders. Most gastric cancer cell lines expressed PPARγ and RXRα both at mRNA and protein level[41]. Studies have not been fully explored the distribution of PPARγ in human stomach tissue. In the published literatures, reports are there about the constitutive and the ubiquitous expression of PPARγ mainly by epithelial cells in normal human gastric mucosa[42]. During gastritis, there noticeably increased in the expression of PPARγ in gastric mucosa[41]. This finding may substantiate the preventive regulatory role of PPARγ in inflammatory cascade and could be a target for the prevention of gastric inflammation. This hypothesis has been supported by many studies in which PPARγ agonists demonstrated the protective role in gastric inflammation in chemical-induced gastritis models in rats[43,44]. A study by Hamaguchi *et al*[43] demonstrated the ulcer healing property of 15d-prostaglandin J2, a natural PPARγ agonist, in gastric ulcer model without the effect on gastric-acid secretion. A study by Takagi *et al*[44] also supported this finding of Hamaguchi and colleagues by demonstrating the beneficial effects of pioglitazone, a specific ligand of PPARγ on aspirin-induced

gastric mucosal injury, which could be attributable to the inhibition of production of gastric TNF-α. Another study by Naito *et al*[45] further substantiated the anti-inflammatory properties of pioglitazone in experimentally induced gastric mucosal injury in rat which could be attributable to its. Naito *et al*[45] also explores the antioxidative property of pioglitazone in their study. The ulcer healing property of pioglitazone was also demonstrated by Konturek *et al*[46] in rat. They determined the effect of pioglitazone on the healing of gastric ulcers in rats. Konturek *et al*[46] explored the following parameters in their study to demonstrate the ulcer healing property of pioglitazone: Measuring the gastric mucosal blood flow, measuring the expression of various cytokines like interleukin-1α, TNF-α and measuring the expression of various protective proteins like heat shock protein 70 (HSP70), cNOS, iNOS, cyclooxygenase-1 and cyclooxygenase-2 in gastric mucosa. There was significant increased in gastric ulcer healing by pioglitazone pre-treatment as ther was significant increased in blood flow at the ulcer margin and there was reduction in the area of gastric ulcers. There was significant increased in the expression of PPARγ mRNA in the gastric mucosa (ulcerated). Therefore the study by Konturek *et al*[46] demonstrated the ulcer healing property of pioglitazone and also supports the anti-inflammatory action of pioglitazone.

The gastroprotective effect of another PPARγ agonist, rosiglitazone, has been documented by Villegas *et al*[47] against ischemia - reperfusion induced injury in the rat stomach. The potent gastro protective and ulcer healing properties of pioglitazone was also determined in another study by Brzozowski *et al*[37]. In this study, the authors were also able to demonstrate the role of endogenous PG and NO in gastroprotective and hyperaemic actions of pioglitazone. There were significantly decreased in the release and expression of various proinflammatory cytokines like TNF-α and IL-1β by pioglitazone. The acceleration of ulcer healing by the PPARγ ligand may be attributed to the increase in angiogenesis at the ulcer margin by a significant increase in the expression of PECAM-1 protein, a marker of angiogenesis[37].

The latest study by El-Moselhy *et al*[48] further documented the involvement of PPARγ activation in nitric oxide mediated gastric ulcer healing in rats. Stress - induced ulcer model was used in this study. The nitric oxide pathway was explored. To demonstrate the gastroprotective effect of rosiglitazone, the authors used the following groups of animals: Rosiglitazone alone treated group, rosiglitazone + PPARγ antagonist (BADGE) group, rosiglitazone + nitric oxide synthase inhibitor (L-NAME) group. The authors concluded that the gastroprotective effect of rosiglitazone might be because of its antisecretory, antioxidant and anti-inflammatory properties[48].

CONCLUSION

The findings of the experimental studies suggest the involvement of PPARα and γ in gastric ulcer. Regarding the involvement of PPARα, there are two studies published in the literature which showed gastric antisecretory effects of phenoxyisobutyrate (ciprofibrate, bezafibrate and clofibrate) and gastric cytoprotective effects of bezafibrate in the rat gastric ulcer model. The majority of the experimental studies is on pioglitazone and rosiglitazone, which are PPARγ activators. In all the studies, both the PPARγ activators showed protection against the gastric ulcer and also accelerate the ulcer healing in gastric ulcer model in rats. The mechanisms involved in the protection of gastric ulcer explored in these studies are antioxidant activity, anti-inflammatory activity, angiogenic activity and over expression of HSP70. Therefore, PPARα and PPARγ may be a target for gastric ulcer therapy. Finally, more studies are also needed to confirm the involvement of PPARα and γ in gastric ulcer.

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TOPIC HIGHLIGHT

2015 Advances in *Helicobacter Pylori*

Helicobacter pylori: Effect of coexisting diseases and update on treatment regimens

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Abstract

The presence of concomitant diseases is an independent predictive factor for non-*Helicobacter pylori* (*H. pylori*) peptic ulcers. Patients contracting concomitant diseases have an increased risk of developing ulcer disease through pathogenic mechanisms distinct from those of *H. pylori* infections. Factors other than *H. pylori* seem critical in peptic ulcer recurrence in end stage renal disease (ESRD) and cirrhotic patients. However, early *H. pylori* eradication is associated with a reduced risk of recurrent complicated peptic ulcers in patients with ESRD and liver cirrhosis. Resistances to triple therapy are currently detected using culture-based and molecular methods. Culture susceptibility testing before first- or second-line therapy is unadvisable. Using highly effective empiric first-line and rescue regimens can yield acceptable results. Sequential therapy has been included in a recent consensus report as a valid first-line option for eradicating *H. pylori* in geographic regions with high clarithromycin resistance. Two novel eradication regimens, namely concomitant and hybrid therapy, have proven more effective in patients with dual- (clarithromycin- and metronidazole-) resistant *H. pylori* strains. We aim to review the prevalence of and eradication therapy for *H. pylori* infection in patients with ESRD and cirrhosis. Moreover, we summarized the updated *H. pylori* eradication regimens.

Key words: Concomitant diseases; *Helicobacter pylori*; Culture susceptibility; Concomitant therapy; Hybrid therapy

Core tip: The authors outline that patients contracting concomitant diseases have an increased risk of developing ulcer disease through pathogenic mechanisms distinct from those of *Helicobacter pylori* (*H. pylori*) infections. Early *H. pylori* eradication is associated with a reduced risk of recurrent complicated peptic ulcers in patients with end stage renal disease and liver cirrhosis. Two novel eradication regimens, namely concomitant

and hybrid therapy, have proven more effective in patients with dual resistant *H. pylori* strains. High-dose amoxicillin therapy is promising and superior to standard regimens. Finally, culture and susceptibility testing should be performed before third-line treatment.

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INTRODUCTION

Helicobacter pylori (*H. pylori*), a widespread human pathogen affecting more than 50% of the human population[1], has been implicated in the development of peptic ulcer disease (PUD), gastric adenocarcinoma, and mucosa-associated lymphoid tissue lymphoma[2-4]. Hopkins *et al*[5] reported that the recurrence of peptic ulcers can markedly decrease from 70% to 10% or lower following *H. pylori* eradication.

Interactions among hosts, pathogens, and environmental factors are crucial to *H. pylori* colonization[6,7]. The presence of concomitant diseases is an independent predictive factor for non-*H. pylori* peptic ulcers[8]. Patients contracting concomitant diseases have an increased risk of developing ulcer disease through pathogenic mechanisms distinct from those of *H. pylori* infections[9,10]. Another explanation for the reported association between concomitant diseases and *H. pylori*-negative peptic ulcers is that many patients with comorbid conditions require nonsteroidal antiinflammatory drugs (NSAIDs). Whether *H. pylori* eradication can protect all patients with concomitant diseases, such as end-stage renal disease (ESRD) and liver cirrhosis, from ulcer recurrence requires further exploration.

Triple therapy is one of the oldest methods for *H. pylori* eradication. Resistances to triple therapy are currently detected using culture-based and molecular methods. However, such methods are difficult to apply in clinical practice because of the long period necessary before obtaining results as well as the high costs of routine performance. No therapy regimen can cure *H. pylori* infections in all treated patients, and some patients remain infected despite several consecutive standard therapies[11]. Concomitant therapy is a combination of antibiotics, including amoxicillin, metronidazole, clarithromycin, and a proton pump inhibitor (PPI), for a period of 5-7 d. This is a novel regimen, regarding which only a few evaluation studies have been published. Thus, the optimal therapy duration and success rates in populations with high dual resistance remain undefined.

END-STAGE RENAL DISEASE

H. pylori prevalence among patients with end-stage renal disease

Studies on *H. pylori* infections in uremic patients have reported rates varying from 27% to 73.0%[12-20]. This variation may have been caused by small sample sizes, nonuniform duration of dialysis, and varying methodologies and enrollment criteria. The *H. pylori* infection rate is lower in chronic kidney disease (CKD) (58.52%) and ESRD (56.25%) patients with PUD than in PUD patients without CKD, according to a Taiwanese population-based study[21]. Sugimoto *et al*[20] reported an *H. pylori* infection rate of 38.3% in patients with ESRD receiving dialysis for 4 years, suggesting that longer durations of dialysis reduce the risk of *H. pylori* infection. Factors other than *H. pylori* seem critical to peptic ulcer recurrence in patients with ESRD patients. These results imply that the diverse gastric environment of ESRD patients. Factors such as reductions in mucosa prostaglandin[12], hypergastrinemia[22], drugs such as NSAIDs[23], and systemic and local circulatory failure[20] influence the onset of recurrent PUD in patients with ESRD.

H. pylori tests for patients with end-stage renal disease

Invasive and noninvasive methods are available to detect *H. pylori* infections. However, dialyzed patients are often reluctant to undergo invasive procedures such as endoscopies[24]. Because patients with ESRD receive antisecretory drugs and require multiple antibiotic treatments for septic complications, inadvertent eradication of *H. pylori* infections is possible. Therefore, many *H. pylori*-negative patients with positive serologies were previously infected with *H. pylori* and inadvertently cured. The 2007 Maastricht Consensus Report on the diagnosis and treatment of *H. pylori* infections does not recommend the serological determination of *H. pylori* infections in routine clinical practice[25]. Huang *et al*[26] proposed that the diagnostic accuracy of *H. pylori* detection in patients with ESRD can be improved by performing the ^{13}C-urea breath test (UBT) after hemodialysis therapy and assessing the UBT with a cutoff excess 13CO2/12CO2 ratio value exceeding 5. However, the diagnostic efficacy of the UBT for patients with ESRD remains less accurate than that for dyspeptic patients without renal impairment.

The UBT seems to be the most reliable diagnostic method for *H. pylori* infections in patients with chronic renal failure (CRF), and stool antigen tests show heterogeneous results, with substantial differences among manufacturers[24]. However, Wang *et al*[27] proposed that stool antigen is a noninvasive and reliable tool for screening *H. pylori* infections before therapy and assessing the success of eradication therapy in patients with ESRD. Establishing the reliability of diagnostic methods for *H. pylori* is crucial for managing infection[28]. Patients contracting CRF often have reduced gastric acid secretion and increased levels of urea in the blood and gastric juices[29-31], which may affect the density and distribution of *H. pylori* infections and, consequently, the reliability of diagnostic tests. Because no diagnostic tests for *H. pylori* infections are 100% reliable, the European *Helicobacter pylori* Study Group guidelines[32] recommend determining a gold standard from at

least two techniques. The approach of combining all techniques has been used in previous studies evaluating the methods of diagnosing *H. pylori* infections[24,33,34]. However, this approach is too costly for use in clinical practice.

H. pylori therapy in patients with end-stage renal disease

The optimal therapeutic regimen for *H. pylori* infection remains undefined in patients with ESRD. Few studies concerning triple therapy in uremic patients have been reported[27,35-39]. A 1-wk course of PPI-based triple therapy (omeprazole, 20 mg twice daily; amoxicillin, 1 g twice daily; and clarithromycin, 500 mg twice daily) achieved a high eradication rate of *H. pylori* infection in patients with CRF and a creatinine clearance (CrCl) of less than 30 mL/min per 1.73 m^2, similar to that of controls with normal renal function[35]. The regimen was tolerated favorably. Another study showed that 7-d triple therapy with a low-dose OAC (omeprazole, 40 mg daily; amoxicillin, 500 mg daily; and clarithromycin, 500 mg daily) regimen was effective and safe for eradicating *H. pylori* infections in hemodialysis patients[40].

Amoxicillin[41] and clarithromycin[42], which are primarily eliminated renally, necessitate dosage reduction in patients with renal impairment according to CrCl. Wang *et al*[27] used 7-d triple therapy with omeprazole (20 mg twice daily), amoxicillin (1 g twice daily), and clarithromycin (500 mg twice daily) and reported an eradication rate of 86.8% among 40 hemodialysis patients. Tsukada *et al*[38] reported an eradication rate of 82.0% among 39 hemodialysis patients using 7-d triple therapy with omeprazole (30 mg twice daily), amoxicillin (500 mg twice daily), and clarithromycin (400 mg twice daily). Itatsu *et al*[39] used 7-d triple therapy with lansoprazole (30 mg twice daily), amoxicillin (375 mg twice daily), and clarithromycin (200 mg twice daily) in 11 hemodialysis patients and achieved an eradication rate of 72.7%.

Ideal treatment regimens should be effective, simple, and safe with fewer adverse effects and high patient compliance. One of the main reasons for treatment failure is irregular therapy with low compliance[43]. The prevalence of resistance to antibiotics, particularly clarithromycin and nitroimidazoles varies with gender, ethnic group and country[44]. Resistance to amoxicillin does not appear to be a critical problem in *H. pylori*-infected ESRD and non-ESRD patients in Turkey. By contrast, rates of resistance to clarithromycin are high, particularly in the ESRD population[45]. Prospective studies on the optimal therapy protocol, including antibiotic combination, dosage, and duration, in ESRD patients are warranted.

H. pylori eradication and recurrent peptic ulcers in patients with end-stage renal disease

Patients with ESRD receiving hemodialysis often have various gastrointestinal (GI) problems such as nausea, dyspepsia, appetite loss, epigastric discomfort, and heartburn. Patients with ESRD exhibit a higher incidence of PUD than do patients without renal disease[12]. *H. pylori* is critical in the development of peptic ulcers[46]. Therefore, physicians must consider these two factors when treating ESRD in patients with upper GI disease.

ESRD is associated with a substantial health care burden in hospitalized patients with peptic ulcer bleeding (PUB). The presence of ESRD contributes to a higher mortality rate, longer hospital stay, and increased need for surgery among such patients[47]. A population-based study conducted in Taiwan[48] over a 10-year period revealed that PUD incidence was 10-12 times higher in patients with CKD than in those without CKD. Luo *et al*[49] reported that patients with ESRD receiving hemodialysis exhibited a high risk of PUB. Another study showed that patients with ESRD had a higher long-term risk of peptic ulcer rebleeding[50]. A crucial question is whether *H. pylori* eradication therapy is necessary for *H. pylori*-infected dialysis patients. Although *H. pylori* eradication is unequivocally effective in preventing peptic ulcer recurrence in the general population[3], such effectiveness has not been established in patients with ESRD. Tseng *et al*[12] conducted a prospective study in a single hospital and reported that *H. pylori* eradication in patients with ESRD reduces recurrent PUD. In addition, a higher recurrent peptic ulcer rate was noted after successful *H. pylori* eradication in *H. pylori*-infected ESRD patients. Sugimoto *et al*[20,51] reported that initiating hemodialysis treatment triggers a decrease in the prevalence of *H. pylori* infection. Moreover, receiving a maximum of 4 years of dialysis treatment has naturally cured *H. pylori* infection, thus supporting the practice of administering eradication therapy to *H. pylori*-infected dialysis patients, particularly those receiving dialysis for 5 years or more. However, a population-based study in Taiwan[52] revealed that early *H. pylori* eradication is associated with a reduced risk of recurrent complicated peptic ulcers in patients with ESRD; the authors recommended administering *H. pylori* eradication within 120 d after peptic ulcer diagnosis in *H. pylori*-infected ESRD patients who have developed peptic ulcers.

LIVER CIRRHOSIS

H. pylori prevalence in patients with liver cirrhosis

Cirrhotic patients with PUB have a 5-fold higher risk of complications or death[53]. *H. pylori* infection promotes the production of ammonia, which is an etiological factor involved in gastric mucosa disorders[54]. However, decompensated liver cirrhosis also involves hepatic encephalopathy because of higher serum ammonia levels. Thus, decompensated cirrhotic patients with *H. pylori* infections seem to have a higher risk of gastric mucosa lesions and GI complications.

Kim *et al*[55] revealed that *H. pylori* infection in cirrhotic patients may be inversely related to the severity of liver cirrhosis (Child-Pugh class A group: 51.5%, class B group: 30.5%, and class C group: 20%). Jung *et al*[56] also showed that the *H. pylori* infection rate

is approximately 71.1% (162 of 228 patients) in all cirrhotic patients with peptic ulcers; however, the rate is only 50.0% (17 of 34 patients) in the Child-Pugh class C group. By contrast, Siringo *et al*[57] used serum anti-*H. pylori* IgG antibody levels to confirm a higher *H. pylori* infection rate of 76.5% in cirrhotic patients and 95.1% in cirrhotic patients with peptic ulcers. Such variability is likely explained by differences in *H. pylori* prevalence inherent in different populations and methodological differences among studies, including the means of testing used to define the presence of infection.

Bhargava *et al*[58] showed that gastric mucosa from patients with portal hypertension exhibited typical vascular dilatation and congestion, whereas the *H. pylori* infection rate was significantly lower in patients with portal hypertension (51.5% *H. pylori* infection rate) with particularly marked vascular dilatation (only 18.8% *H. pylori* infection rate) compared with the controls (75.5% *H. pylori* infection rate). Factors other than *H. pylori* seem critical in peptic ulcer recurrence in cirrhotic patients. In addition, *H. pylori* is not the predominant etiology for liver cirrhosis, particularly the decompensated type, with PUD or recurrent ulcer disease, according to a population-based study conducted in Taiwan[59]. This is consistent with previous studies[55,60,61], and a 35.6% to 51.92% *H. pylori* infection rate in cirrhotic patients with peptic ulcers.

H. pylori eradication and recurrent peptic ulcers in patients with liver cirrhosis

The prevalence of *H. pylori* in patients with cirrhosis and PUD is generally less than 60%[60,62-65], suggesting that the pathogenesis of ulcer disease in a substantial proportion of cirrhotic patients may not be related to *H. pylori* infection[66-69]. Host environments are crucial to *H. pylori* colonization[7,70]. *H. pylori* adapts to humans, colonizing in children and remaining persistent throughout life[71]. The pathogenesis of the high peptic ulcer rate in cirrhotic patients is multifactorial. Cirrhotic patients have a higher risk of gastric mucosa damage because of reduced mucosal prostaglandin, which is crucial in the cytoprotection of gastric mucosa, which causes PUD. Other factors including increased serum gastrin concentration[72], impaired mucus secretion[73], and portal hypertensive gastropathy[74,75] may contribute to peptic ulcers and peptic ulcer recurrence in cirrhotic patients.

Compared with the general population, patients with cirrhosis have greater bleeding complications, delayed healing, and higher ulcer recurrence rates[68]. Luo *et al*[76] proposed that the risk of developing PUB in liver cirrhosis patients is 4-fold. A group of researchers from Taiwan found that 21 (58%) patients in whom *H. pylori* infections were eradicated developed recurrent duodenal ulcers within a year[60]. These results suggest that the pathogenesis of ulcer disease in a substantial proportion of patients with cirrhosis may not be related to *H. pylori* infection, a possibility proposed by researchers[65,67]. However, early *H. pylori* eradication is associated with a lower risk of recurrent peptic ulcers in cirrhotic patients, according to a Taiwan population-based study[77]. *H. pylori* eradication therapy is the primary method of treating cirrhotic patients with peptic ulcers.

H. pylori eradication and hepatic encephalopathy in patients with liver cirrhosis

A meta-analysis showed that *H. pylori* infections are associated with elevated blood ammonia levels in cirrhotic patients. In addition, the association seems more prominent in Asian patients than in Caucasian patients[78]. Addressing whether *H. pylori* eradication may benefit the long-term management of hepatic encephalopathy. Some studies have shown the benefit of eradication therapy[79-81]; however, other studies have reported negative results[82-84].

An ideal study protocol should also address a homogenous study population according to the severity of liver cirrhosis, assessment of psychometric tests, and measurements of ammonia levels in the blood. Currently, the demonstrated beneficial effect of eradication therapy in *H. pylori*-positive cirrhotic patients is insufficient for recommending the use of this therapy in clinical practice. An appropriately randomized study designed to assess the influence of *H. pylori* eradication on hepatic encephalopathy in cirrhotic patients is warranted.

ENTEROHEPATIC *HELICOBACTERS* AND LIVER DISEASE

Bile-tolerant *helicobacter* species such as *H. hepaticus* and *H. bilis* have frequently been reported to cause hepatitis in mice and other rodents[85]. The most comprehensively studied member of this group of enterohepatic *Helicobacter* species is *H. hepaticus*[86]. *H. bilis* has been found in species of inbred mice and reported to induce chronic hepatitis and hepatocellular carcinomas[87]. *H. hepaticus* was initially detected by immunofluorescence, electron microscopy, and culture in the livers of certain inbred mice and has been shown to cause multifocal necrotizing hepatitis, hepatic adenomas, and hepatocellular carcinomas[88,89]. The reason for further study of the possible role of these new helicobacter species in human liver disease has been to develop noninvasive serological assays for determining the seroprevalence of hepatic helicobacter infections[85].

Fox *et al*[90] stated that *H. hepaticus* infection increased the risk of liver cancer in hepatitis C virus and/or Hepatitis B virus infection. Fukuda *et al*[91] revealed that serum anti-*H. hepaticus* and/or *anti-H. bilis* antibody levels were significantly higher in patients with liver disease that in patients with other autoimmune hepatitis. Liver cirrhosis patients exhibited a substantially higher anti-*H. hepaticus* serum antibody level than other liver disease and health donors[92]. Although *Helicobacter* spp. have been identified in the liver of humans, their pathogenic role in human liver diseases remains largely unclear[93]. Kleine *et al*[94] established an *in vitro* model

for demonstrating the pathogenic effect of a *Helicobacter* spp. on human liver cells, resulting in an inflammatory response with increased synthesis of inflammatory mediators and consecutive monocyte activation. The higher prevalence of *Helicobacter* spp. associated with more advanced stages of liver disease supports the possibility of their role in the progression of chronic hepatitis toward cirrhosis and hepatocellular carcinoma. An interventional study aimed at eradicating *Helicobacter* spp. from the liver would elucidate this association between *Helicobacter* spp. and liver cirrhosis and is thus warranted[93].

UPDATED *H. PYLORI* THERAPY

Successful eradication of *H. pylori* is a major component in treating these conditions[95]. However, the rate of *H. pylori* eradication after triple therapy is decreasing, with standard amoxicillin plus clarithromycin-based triple therapy achieving eradication rates of only approximately 75% in many series[96,97]. Apart from the conventional first-line regimen (triple therapy), other therapies have been proposed (sequential, concomitant, quadruple, and miscellaneous) to face the growing problem of antibiotic resistance. Although unsuccessful eradication can be caused by increasing antimicrobial resistance, an additional critical contributor is the treatment-related side effects and resultant incomplete therapy. New treatment regimens for *H. pylori* infections should be optimized to achieve an eradication rate of ≥ 95%[98]. Patient allergies and the local availability of drugs should be considered when selecting a treatment. *H. pylori* eradication should be confirmed after treatment, and, if second-line therapy is required, clarithromycin or a fluoroquinolone should not be reused[99].

Sequential therapy

First developed in Italy in the 1990s, sequential therapy (5-d PPI and amoxicillin, followed by 5-d PPI, clarithromycin, and metronidazole), is a regimen that has proven more effective in eradicating *H. pylori* than has triple therapy in many studies[100-104]. Recent multicenter randomized trials conducted in Taiwan have shown the superiority of sequential therapy over standard triple therapy. These findings also support that the most effective eradication regimen should be chosen on the basis of the prevalence of antibiotic-resistant *H. pylori* in a region[105]. The ability of sequential therapy to eradicate clarithromycin-resistant bacteria has been demonstrated, and sequential therapy has been included in a recent consensus report as a valid first-line option for eradicating *H. pylori* in geographic regions with high clarithromycin resistance[106].

Hybrid and concomitant therapy

Two novel eradication regimens, namely concomitant and hybrid therapy, have proven more effective than triple and sequential therapy in the last few years, particularly in patients with dual- (clarithromycin- and metronidazole-) resistant *H. pylori* strains[107-111]. Meta-analyses have shown that the outcome of concomitant therapy is duration-dependent[112]. Molina-Infante *et al*[113] proposed that optimized nonbismuth quadruple hybrid (consisting of omeprazole 40 mg twice daily and amoxicillin 1 g twice daily for 14 d, plus clarithromycin 500 mg twice daily and nitroimidazole 500 mg twice daily for the final 7 d as a quadruple therapy) and concomitant (consisting of omeprazole 40 mg twice daily, amoxicillin 1 g twice daily, clarithromycin 500 mg twice daily, and nitroimidazole 500 mg twice daily, all concurrently for 14 d) therapies cured more than 90% of patients with *H. pylori* infections in areas of high clarithromycin and metronidazole resistance. In concomitant therapy, all four drugs are administered for the entire therapy duration, indicating that this regimen is less complex than sequential therapy because it does not involve changing the number or type of drugs halfway through the therapy. A recently proposed hybrid therapy has proven as effective as a 14-d concomitant regimen in a pilot study[106].

High-dose amoxicillin therapy

The efficacy of treatment for *H. pylori* infection has decreased steadily because of increasing resistance to clarithromycin, metronidazole, and levofloxacin. Resistance to amoxicillin is generally low, and high intragastric pH increases the efficacy of amoxicillin. Therefore, Yang *et al*[114] proposed that a combination of a high-dose PPI and amoxicillin (dual therapy), consisting of rabeprazole (20 mg, four times daily) and amoxicillin (750 mg, four times daily) for 14 d, is superior to standard regimens as empirical first-line or rescue therapies for *H. pylori* infection and has similar safety profiles and tolerability.

Bismuth-based quadruple therapy

During the initial development of bismuth quadruple therapy, the dose and duration of bismuth therapy were shown to be crucial variables, particularly in regions where metronidazole resistance was common[115]. However, many studies on these regimens have used suboptimal doses and durations of bismuth therapy, producing relatively poor results[116]. Bismuth-containing quadruple therapy is an alternative to standard triple therapy in areas with low clarithromycin resistance and the main first-line therapeutic option in areas with high prevalence of clarithromycin resistance. Using this regimen at full doses for 14 d produces a 95% or greater treatment success rate, irrespective of the level of metronidazole resistance[117].

Salvage therapy

Tailored antimicrobial therapy for an infectious disease is typically selected on the basis of susceptibility testing[99,118]. The treatment of *H. pylori* infection differs from that of most common infectious diseases because culture and susceptibility testing is generally not offered for *H. pylori* infections by hospital laboratories. In

addition, many *H. pylori* infections can be identified using noninvasive testing (such as UBT or stool antigen testing), indicating that endoscopies are neither necessary nor acceptable to patients, and clinicians must choose treatment empirically. Therefore, culture and susceptibility testing should be performed before third-line treatment[106]. Salvage therapy (after multiple treatment failures) should be chosen on the basis of susceptibility testing whenever possible[101].

Probiotic therapy

Song *et al*[119] proposed that supplementation with *Saccharomyces boulardii* could be effective for improving *H. pylori* eradication rates by reducing side effects, thus facilitating completion of eradication therapy. Probiotics are mostly administered by modulating the balance of its microflora[120]. Probiotic adjunct therapy is particularly useful in patients with a history of GI intolerance to antibiotic treatment[121]. These effects have been demonstrated for several species of probiotics, including *Lactobacillus acidophilus*, *Lactobacillus salivarius*, *Lactobacillus rhamnosus*, and *Bifidobacterium bifidum*, among others[122-124]. Proposed mechanisms include modulating the colonization of the gastric mucosa and the direct killing of *H. pylori* through secreted metabolites with antimicrobial properties[95]. The lack of protocol standardization is problematic, and interpreting the various results is difficult.

CONCLUSION

Although other factors influence the onset of peptic ulcer and recurrent peptic ulcer in ESRD and cirrhotic patients, *H. pylori* eradication therapy is the primary method of treating ESRD and cirrhotic patients with peptic ulcers. Culture susceptibility testing before first- or second-line therapy is unadvisable. The known obstacles to culture susceptibility testing include the need for endoscopic examination and the fact that culture is time-consuming, costly, and not 100% sensitive. Using highly effective empiric first-line and rescue regimens can yield acceptable results[100,107,125]. In the near future, *in vivo* and *in vitro* studies may possibly be grouped according to geographic area to identify the most effective therapy for eradicating *H. pylori*, which relates to the local habitat.

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TOPIC HIGHLIGHT

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Mesalazine preparations for the treatment of ulcerative colitis: Are all created equal?

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Abstract

Oral mesalazine (also known as mesalamine) is a 5-aminosalicylic acid compound used in the treatment of mild to moderate ulcerative colitis, with high rates of efficacy in induction and maintenance of remission. The therapeutic effect of mesalazine occurs topically at the site of diseased colonic mucosa. A myriad of oral mesalazine preparations have been formulated with various drug delivery methods to minimize systemic absorption and maximise drug availability at the inflamed colonic epithelium. It remains unclear whether different oral mesalazine formulations are bioequivalent. This review aims to evaluate the differences between mesalazine formulations based on the currently available literature and explore factors which may influence the selection of one agent above another.

Key words: Colitis; Ulcerative; Drug delivery systems; Mesalamine; Sulfasalazine; Therapeutic equivalency

Core tip: Various formulations of oral mesalazine are available for management of mild to moderate ulcerative colitis. Selection of the most appropriate formulation requires tailoring of the therapy to the individual and must incorporate factors such as disease distribution, efficacy, side effect profile, pill burden, patient preference and health economics.

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INTRODUCTION

Ulcerative colitis is a chronic idiopathic inflammatory bowel disease (IBD) characterised by intestinal inflammation confined to the superficial mucosal layer. It may involve the rectum only, the distal colon

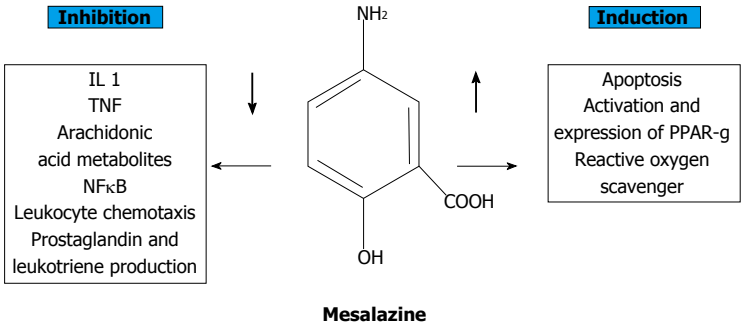

Figure 1 Proposed mechanism of action of mesalazine at the colonic mucosa. IL: Interleukin; NFκB: Nuclear factor κB; PPAR: Peroxisome proliferative activated receptor; TNF: Tumour necrosis factor.

or the entire colon, typically in a contiguous fashion. Classical symptoms of ulcerative colitis include bloody diarrhoea, urgency and tenesmus. Mesalazine, a 5-aminosalicylic acid compound (5-aminosalicylate, or 5-ASA), is most often used as the first line therapy for mild to moderate ulcerative colitis[1]. However, the exact mechanism of action of mesalazine remains poorly elucidated. It is believed to exert a negative effect on the cyclooxygenase and lipoxygenase pathways, thereby reducing the formation of pro-inflammatory prostaglandins and leukotrienes[2,3]. The peroxisome proliferator activated receptor-g is also implicated in colonic inflammation and has been identified as a target of 5-ASA action[4]. Furthermore, mesalazine may have antioxidant properties that reduce tissue injury and play a part in inhibition of T cell activation and proliferation[5,6] (Figure 1).

Oral mesalazine compounds have proven efficacy for inducing and maintaining remission in patients with ulcerative colitis[7,8]. Mesalazine exerts therapeutic effect through local topical activity at the inflamed mucosa[9]. Oral mesalazine in unaltered form is almost entirely absorbed by the small intestines, with very little intact drug reaching the colon[10,11]. Hence, the main goal of the various formulations currently available on the market is to optimise drug delivery to the affected colon and minimise systemic absorption. This promotes maximal therapeutic efficacy at the lowest possible dose, which in turn reduces side effects.

It remains unclear whether individual mesalazine formulations have differential effects in certain IBD patient subgroups. Anecdotally in the clinical setting, the choice of mesalazine appears at best to be rather experimental or idiosyncratic, and at worst, based on ambit claims by pharmaceutical representatives and/or advertisement, rather than evidence-based. In the absence of quality head to head comparative trials in appropriately selected patients, claims that one formulation is superior to another may be spurious. Nevertheless, physicians are often tasked with selecting a suitable mesalazine compound for their patients. These decisions require tailoring of the therapy to the individual and must incorporate factors such as disease distribution, efficacy, side effect profile, pill burden, patient preference and health economics. Hence, this review aims to evaluate the current literature relating to potential therapeutic differences between mesalazine formulations and thus inform an evidence-based approach to optimal mesalazine use in patients with ulcerative colitis.

DELIVERY MECHANISMS

Azo-bonded prodrugs

In these formulations, mesalazine is synthesized as a prodrug, binding *via* an azo bond to either a transporter molecule or another mesalazine molecule. This prevents absorption of the drug in the upper gastrointestinal tract. The azo bond is subsequently cleaved by bacteria containing azoreductase in the colon, releasing the active mesalazine component (Table 1).

Sulfasalazine (Azulfidine®, Salazopyrin®, Pyralin®, Pfizer Inc, New York, NY) was one of the first aminosalicylates shown to be effective in the induction and maintenance of remission in ulcerative colitis[12,13]. It consists of a mesalazine and a sulfapyridine molecule bound by an azo bond, which is cleaved upon exposure to colonic bacteria. Mesalazine is the active moiety and sulfapyridine acts as an inactive carrier molecule[14,15]. Systemic absorption of sulfapyridine is responsible for many of the adverse effects associated with sulfasalazine[16]. Approximately 20% of patients are intolerant[17].

Other azo-bonded prodrugs have been formulated with alternative carrier molecules, in an attempt to reduce side effects. Olsalazine sodium (Dipentum®; UCB Pharma, Slough, United Kingdom) is comprised of two mesalazine molecules also connected by an azo-bond. Balsalazide disodium (Colazide®, Fresenius Kabi AG, Hamburg, Germany; Colazal®, Salix Pharmaceuticals Inc, Morrisville, NC) consists of mesalazine bound to 4-aminobenzoyl-β-alanine. Both agents have been shown to be effective in treatment of patients with ulcerative colitis[18,19].

pH dependent formulations

Other mesalazine preparations encapsulate the active drug in an enteric coat in order to control the site of drug release. The enteric coating consists of a resin film designed to release mesalazine only at a designated pH, thereby preventing premature disintegration in the

Table 1 Summary of drug delivery mechanisms

Formulations	Generic name	Proprietary names	Mode of delivery	Site of drug release
Azo-bonded prodrugs	Sulfasalazine	Azulfidine®; Salazopyrin®; Pyralin®	Mesalazine bound to sulfapyridine	Colon
	Olsalazine	Dipentum®	Two mesalazine molecules bound together	Colon
	Balsalazide	Colazide®; Colazal®	Mesalazine bound to 4-aminobenzoyl-β-alanine	Colon
pH dependent	Mesalazine	Asacol®; Mesren®	Eudragit-S coating (dissolves at pH ≥ 7)	Terminal ileum, colon
		Salofalk®; Mesasal®; Claversal®	Eudragit-L coating (dissolves at pH ≥ 6)	Mid ileum to colon
		Salofalk Granules®	Eudragit-L coating and matrix core	Mid ileum to colon
Time dependent	Mesalazine	Pentasa®, Pentasa® granules	Microspheres encapsulated within an ethycellulose semi-permeable membrane	Duodenum to colon
MMX	MMX mesalazine	Lialda®; Mezavant XL®; Mezavant®	Enteric coating (dissolves at pH ≥ 7). MMX of lipophilic and hydrophilic excipients	Terminal ileum and entire colon

MMX: Multi-matrix system.

acidic environment of the stomach and proximal small bowel. Asacol® (Tillotts Pharma AG, Ziefen, Switzerland) and Mesren® (Ivax Pharmaceuticals Limited, Runcorn, Cheshire, United Kingdom) are manufactured with a methacrylate copolymer coating, Eudragit-S. This coating dissolves at pH ≥ 7, releasing the active drug in the terminal ileum and colon. Salofalk® (Dr Falk GmBH, Freiburg, Germany), Mesasal® (Aspen Pharmacare, NSW, Australia) and Claversal® (Merckle GmbH, Ulm, Germany), comprise mesalazine enclosed within an Eudragit-L coating which disintegrates at pH ≥ 6, thus preferentially releasing the drug throughout mid to distal ileum and colon[20]. A potential issue with this mode of delivery is that colonic pH, although highly variable, is overall reduced in patients with inflammatory bowel disease[21]. It has been postulated that the lowered colonic pH may impede the release of 5-ASA from the pH dependent enteric coating and reduce its efficacy. Certainly, it is recommended that pH dependent formulations should not be co-administered with lactulose or other medications which lower colonic pH.

Time dependent formulations

Pentasa® (Ferring Pharmaceuticals, Copenhagen, Denmark) adopts an alternative method of drug delivery consisting of microspheres of mesalazine encapsulated within an ethylcellulose semi-permeable membrane. This structure allows time and moisture dependent release of the active drug, independent of the luminal pH. Mesalazine is theoretically distributed gradually throughout the gastrointestinal tract from the duodenum to the rectum[22]. This in turn may be of therapeutic value in patients with small bowel Crohn's disease[23]. In ulcerative colitis, the efficacy of Pentasa® has been demonstrated in multiple studies, including one randomised control trial where 64% of patients maintained remission after 12 mo of Pentasa® 4 g/d compared with 38% of patient who received placebo ($P = 0.0004$)[24].

Granule formulations

There is data to suggest that improved efficacy in patients with moderate ulcerative colitis may be achieved with a higher daily dose of mesalazine[25]. In order to reduce pill burden and encourage adherence, both Pentasa® and Salofalk® (Dr Falk GmBH, Freiburg, Germany) are available as loose micro granules, packaged into sachets. This allows a higher drug dose to be administered without increasing pill burden and thus attempts to enhance patient tolerability. Furthermore, this formulation may be especially advantageous in patients who have difficulty ingesting large quantities of tablets.

Multi-matrix system

Mezavant® (Lialda®, United States), Mezavant XL® (United Kingdom and Ireland), Shire Pharmaceuticals Inc, Wayne, PA) is a once daily formulation of mesalazine which adopts a multi-matrix system (MMX). Mesalazine is incorporated into a lipophilic matrix which is in turn dispersed within a hydrophilic matrix. The tablet is enterically coated and dissolves at pH ≥ 7, in the terminal ileum. The hydrophilic matrix is then exposed to intestinal fluid and swells to form a viscous gel mass. This viscous gel potentiates slow diffusion of the active drug from the tablet core and thereby enabling slow controlled release of mesalazine throughout the entire length of the colon[26]. Kamm *et al*[27] evaluated the efficacy of MMX mesalazine in patients with active ulcerative colitis and found it to be significantly superior to placebo in inducing remission.

COMPARISON OF MESALAZINE FORMULATIONS

Pharmacokinetics

The ideal mesalazine formulation would minimise systemic absorption in the upper gastrointestinal tract and maximise delivery of the active drug to the colonic mucosa. Ingested 5-ASA is acetylated by the N-acetyltransferase 1 (NAT 1) enzyme in intestinal epithelial cells to form the inactive metabolite N-Ac-5ASA. This metabolite is then either absorbed systemically and excreted in the urine or secreted back into the colonic lumen and excreted in the faeces. Some 5-ASA is also absorbed directly into the bloodstream and undergoes metabolism by the NAT 1 enzyme in liver cells, followed

by elimination in the urine[11,28].

The assorted delivery technologies used by mesalazine formulations have a direct bearing on their pharmacokinetics. The drug release profile of MMX mesalazine has been compared with pH-dependent formulation Asacol® using radioactive labelling. MMX mesalazine tablets began to disintegrate earlier than Asacol®, at an average of 4.8 h compared to 6.2 h respectively. Complete disintegration occurred at 17.4 h for MMX mesalazine compared with 7.3 h for Asacol®, implying a more prolonged release of 5-ASA with MMX mesalazine. This allows slow and controlled distribution throughout the entire colon. In contrast, Asacol® released the active drug more rapidly, predominantly in the right colon. Consequently, disease distribution may be an important factor to consider in selection of mesalazine agents, with MMX mesalazine potentially more appropriate for patients with distal colitis.

The rate of intestinal transit may also impact the pharmacokinetics of different oral mesalazine preparations, and hence their efficacy. Faecal excretion of 5-ASA was evaluated in healthy volunteers after administration of laxatives to induce diarrhoea and accelerate intestinal transit. Diarrhoea resulted in a marked increase in faecal loss of the pro-drugs, sulfasalazine and olsalazine, indicating insufficient time for activation of the pro-drug by colonic bacteria[29]. In comparison, pH and time dependent formulations (Pentasa® and Salofalk®) appeared to maintain adequate release of 5-ASA despite accelerated intestinal transit[29,30]. Similarly, Das *et al*[31] evaluated this theory in the clinical setting by administering sulfasalazine to patients with active and inactive ulcerative colitis. The serum levels of sulfapyridine, a byproduct of sulfasalazine metabolism, were then measured as a marker of drug activation. Patients with active disease had lower systemic levels of sulfapyridine compared with patients with inactive disease, suggesting less sulfasalazine had been activated to release the 5-ASA molecule. As such, pro-drug formulations like sulfasalazine may potentially be less effective in the setting of active ulcerative colitis due to diarrhoea and accelerated intestinal transit, given their reliance on exposure to colonic bacteria for activation.

Efficacy

Comparing the efficacy of various oral mesalazine formulations is problematic as patient populations in each study differ in terms of disease severity, disease distribution and primary end points. Direct comparative studies have only identified minor yet inconsistent differences in efficacy between agents. In a randomised double-blind study of patients with active ulcerative colitis, balsalazide was found to be significantly more efficacious in inducing remission and better tolerated than the pH dependent formulation (Asacol®)[32]. Two subsequent studies, however, were not able to reproduce these results[33,34].

The influence of enteric coating on efficacy has also been evaluated. Gibson *et al*[35] demonstrated in a randomised double-blind trial that Eudragit-L (pH-dependent) and ethylcellulose-coated (time-dependent) mesalazine tablets achieved comparable rates of clinical remission after 8 wk of therapy. In contrast, another study by Ito *et al*[36] found that pH-dependent formulations were significantly more effective than time-dependent formulations in patients with proctitis-predominant ulcerative colitis.

As discussed, MMX mesalazine utilises multi matrix technology in an attempt to release 5-ASA in a controlled manner. Pharmacokinetic studies also suggest a more prolonged duration of drug release, theoretically enabling active drug delivery to more distal regions of the colon. Prantera *et al*[37] compared MMX mesalazine 2.4 g/d to Asacol® 2.4 g/d as maintenance therapy in 331 patients with left sided ulcerative colitis. After 12 mo, the two formulations were comparable in maintaining clinical and endoscopic remission based on clinician assessment, 60.9% and 61.7% respectively. However, based on patient diary records of symptoms, including stool frequency and rectal bleeding, 62.2% of patients treated with MMX mesalazine maintained remission compared with 51.5% treated with Asacol ($P = 0.053$)[37]. Although not statistically significant, there is a trend to suggest that MMX mesalazine may be more efficacious in patients with left sided ulcerative colitis. The disparity between clinician assessment and patient records may be a reflection of under reporting of symptoms during clinical consultations.

It is apparent that studies have to date delivered incongruent results regarding the efficacy of different oral mesalazine agents. A Cochrane review by Feagan and Macdonald[8] in 2012 aimed to accrue currently available data and compare the efficacy and safety of oral mesalazine formulations in ulcerative colitis. The meta-analysis did not show any statistically significant difference in efficacy between the various preparations of mesalazine in induction of remission. Interestingly, in maintenance of remission, sulfasalazine was significantly superior to other oral mesalazine agents, with 43% of sulfasalazine patients relapsing compared with 48% of patients treated with other oral mesalazine preparations (12 studies, 1655 patients; RR = 1.14, 95%CI: 1.03-1.27)[7]. However, it must be highlighted that comparative reviews should be interpreted with caution, as they may not account for patient population and study design variability between different trials. Given the paucity of direct comparative trials with adequate power, the relative efficacy of different oral mesalazine formulations cannot be definitively concluded. Patient characteristics, such as disease distribution nevertheless, do anecdotally influence clinicians towards the selection of a particular agent.

Safety

Mesalazine is generally well tolerated, with similar side effect profiles between different formulations. The rate of adverse events is estimated to be in the range of

20%-30%[38]. The most common side effects include arthralgia, myalgia, flatulence, abdominal pain, nausea, diarrhoea and headache. Rare but serious side effects include interstitial nephritis and pancreatitis.

Of the mesalazine formulations, olsalazine more commonly causes diarrhoea, with up to 29% of patient experiencing this side effect[39,40]. This has been attributed, at least in part, to the presence of the azo bond, which has prosecretory effects on rabbit mucosa *in vitro*[41].

As expected, sulfasalazine is poorly tolerated compared with other mesalazine formulations. A meta-analysis found 28% of patients treated with sulfasalazine experienced adverse events compared with 15% of other mesalazine agents (RR = 0.48, 95%CI: 0.37-0.63)[8]. In addition, it is also associated with agranulocytosis, a rare but potentially fatal haematological condition[42]. As a result, sulfasalazine is increasingly superseded by the newer generation oral mesalazine formulations. Patients who do not tolerate sulfasalazine may benefit from switching to an alternate mesalazine agent that does not contain the sulfapyridine moiety, which is believed to cause the majority of side effects.

Adherence

The natural history of ulcerative colitis entails a remitting and relapsing clinical course. Maintenance therapy is important in prevention of disease recurrence. Non-adherence, defined as taking less than 80% of prescribed medications, ranges between 40% to 72% in patients with ulcerative colitis[43,44]. This is particularly problematic in patients with quiescent disease, as the benefit of therapy is less obvious. Patients who are non-adherent have a five-fold greater risk of disease recurrence than adherent patients[45].

Determinants of adherence are varied and patient-specific. Risk factors for non-adherence include male sex, single status, full-time employment, and thrice daily dosing[44]. Dosing regimen is one facet of this multifactorial issue. A meta-analysis by Claxton *et al*[46] suggested that less frequent dosing is associated with higher adherence. Multi-dose regimens and large pill burdens have been identified as major barriers to adherence in ulcerative colitis[47]. Formulations such as MMX mesalazine with once daily (OD) dosing or granule-based preparations with lower pill burden should in theory assist adherence.

OD dosing was compared with conventional dosing in a meta-analysis by Ford *et al*[48] in 2011. Rates of adherence were not significantly different between the two groups. Similarly, in the meta-analysis by Feagan and Macdonald[7], OD dosing did not result in improved adherence compared with conventional dosing. The most plausible explanation for this finding is that medication adherence in most clinical trials is artificially higher due to the intensive clinical supervision and reinforcement, thus not necessarily a true reflection of real-world clinical practice. OD dosing of mesalazine is still promulgated as the preferred option for reducing pill burden and promoting adherence.

Cost effectiveness

Ulcerative colitis is a chronic disease which requires prolonged therapy to maintain remission. This can place a substantial financial burden on the patient or the healthcare provider. On a per tablet basis, novel formulations of oral mesalazine are often presumed to be more expensive. Yet, Prenzler *et al*[49] analysed the cost effectiveness of Mezavant® compared with Asacol® and showed a 76% probability for cost savings and a gain of 0.011 quality adjusted life-years (QALYs) with Mezavant®. A similar United Kingdom analysis of Mezavant® and Asacol® found a 62% chance of cost savings and a gain of 0.011 QALYs with Mezavant®[50]. Both these models suggest that Mezavant® may be a cost effective option amongst oral mesalazine formulations.

CHANGING MESALAZINE FORMULATIONS

Although mesalazine is overall an effective therapy in ulcerative colitis, not all formulations are appropriate for each individual patient. The clinical decision to change from one preparation to another is often influenced by factors including clinical response, tolerability, pill burden, compliance, cost and patient preference. (See Figure 2) An important clinical dilemma is whether patients who have failed one formulation of mesalazine should be switched to an alternate preparation, or should the lack of response to one formulation be considered a class effect.

In a small study, 9 ulcerative colitis patients with endoscopic evidence of active disease despite treatment with Asacol® 2.4 g/d were changed to Pentasa® 4.0 g/d. Following twelve weeks of treatment, there was a significant reduction in the endoscopic severity of disease[51]. It is important to highlight, however, that the dosages of the two mesalazine formulations were not equimolar. In another study, sub-analysis of two MMX mesalazine trials identified a pooled population of patients with active mild to moderate ulcerative colitis, who were switched from an existing oral 5-ASA (≤ 2.0 g/d) to 2.4 g/d or 4.8 g/d of MMX mesalazine. After 8 wk, significantly more patients treated with 4.8 g/d (37.5%, $P < 0.05$) and numerically more patients treated with 2.4 g/d (31.8%) achieved endoscopic remission compared to placebo (20.9%)[52]. Similarly, two small pilot studies also evaluated 87 patients who were inadequately maintained on mesalazine and switched to OD dosing Salofalk® granules. After 6 mo of therapy, 70% of patients demonstrated improved ulcerative colitis severity scores (Walmsley Index). There was also a 60% reduction in hospital visits due to flare of disease, 45% reduction in family doctor visits and 50% reduction in steroid usage[53]. In addition, Motoya *et al*[54] reported a retrospective analysis of 46 patients with active ulcerative colitis, who were switched from a

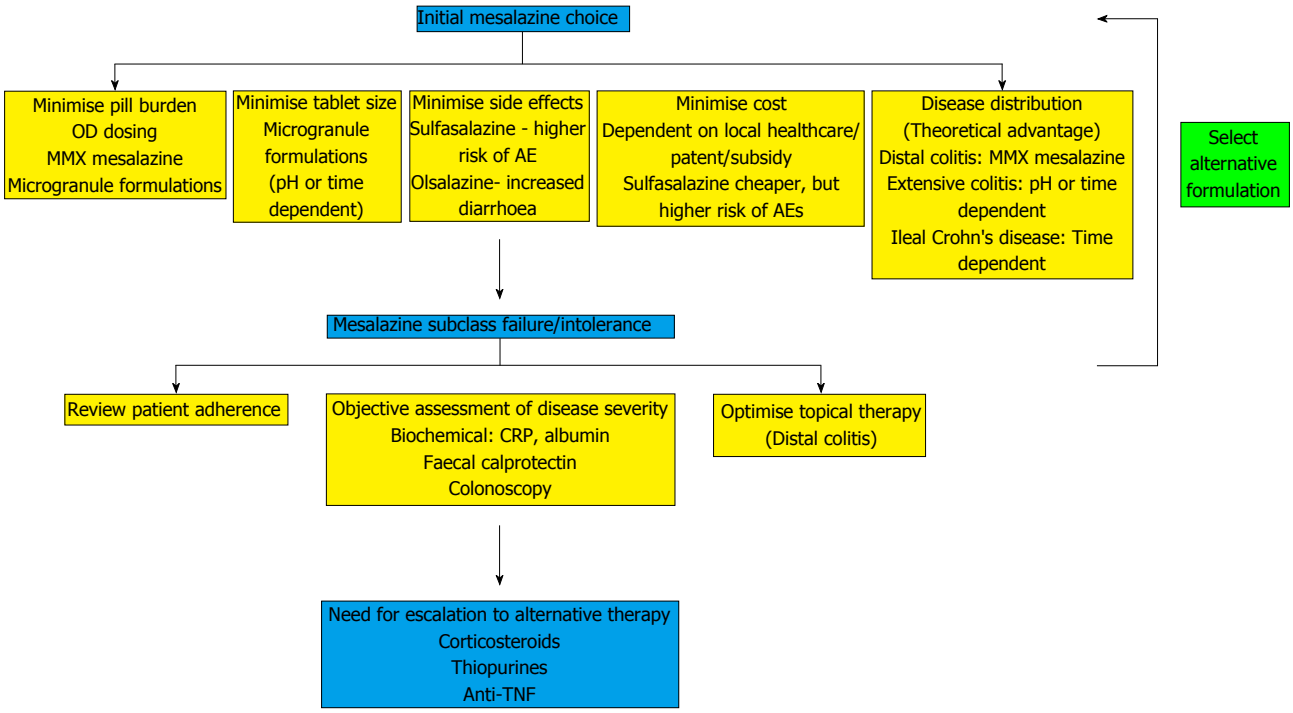

Figure 2 Algorithm for selection of mesalazine formulations. Anti-TNF: Anti-tumour necrosis factor; AEs: Adverse events; MMX: Multi-matrix system; OD: Once daily; CRP: C-reactive protein.

time-dependent mesalazine formulation (4.0 g/d) to a pH dependent formulation (3.6 g/d) due to inadequate clinical response. At 8 wk, 50% of patients achieved clinical remission, with a significant reduction in the Lichtiger clinical activity index. These studies suggest that patients with poor response to one formulation of oral mesalazine may benefit from switching to an alternate preparation, although the data remains sparse and warrants further investigation.

On the other hand, patients who have stable disease on a particular mesalazine formulation should not change preparations as it may destabilise disease control. Robinson *et al*[55] found in a retrospective study that stable patients who switched mesalazine formulations had a 3.5 fold greater risk of relapse compared to those who did not switch. This indicates that the mesalazine formulations are not bioequivalent and disruptions to maintenance mesalazine should be avoided.

CONCLUSION

In summary, oral mesalazine remains the cornerstone of management of mild to moderate ulcerative colitis. Various formulations have been developed in an attempt to optimise drug delivery to the region of active disease. Each differ in terms of enteric coating, site of drug release and mode of drug delivery, and thus are not interchangeable. Failure of one formulation, should not negate future use of the entire drug class. Although there is a lack of consistent comparative data to confidently state the superiority of one formulation over another, there are theoretical advantages of each formulation to provide some limited guidance. Ultimately, the choice of mesalazine formulation should be tailored to each individual patient, taking into consideration disease distribution, tolerability, adherence and cost effectiveness.

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REVIEW

Pharmacotherapy for the management of achalasia: Current status, challenges and future directions

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Abstract

This article reviews currently available pharmacological options available for the treatment of achalasia, with a special focus on the role of botulinum toxin (BT) injection due to its superior therapeutic effect and side effect profile. The discussion on BT includes the role of different BT serotypes, better pharmacological formulations, improved BT injection techniques, the use of sprouting inhibitors, designer recombinant BT formulations and alternative substances used in endoscopic injections. The large body of ongoing research into achalasia and BT may provide a stronger role for BT injection as a form of minimally invasive, cost effective and efficacious form of therapy for patients with achalasia. The article also explores current issues and future research avenues that may prove beneficial in improving the efficacy of pharmacological treatment approaches in patients with achalasia.

Key words: Botulinum toxin; Pharmacotherapy; Botox; Achalasia; Sprouting inhibitors

Core tip: Botulinum toxin (BT) injection is the most common and effective pharmacological therapy used in the treatment of achalasia, and is commonly used in the elderly, those with multiple comorbidities, patients at high risk for surgery and as a salvage therapy. This article discusses new advances related to the pharmacological management of achalasia that may help to optimize minimally invasive treatment approaches in patients with achalasia, and discusses improvements in endoscopic injection techniques, the use of sclerosants, new BT formulations, alternate serotypes, sprouting inhibitors and designer recombinant BT formulations.

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INTRODUCTION

Esophageal achalasia is an idiopathic motility disorder characterized by an incomplete relaxation of the lower esophageal sphincter (LES) in response to swallowing as well as aperistalsis in the esophagus resulting in impaired food bolus transport[1]. First described by Sir Thomas Willis in 1674, it was not until 1928 with the work of Hurst[2] and Rake[3] that the pathophysiology was realized to be a failure in LES relaxation.

Clinically, dysphagia is the most common presenting symptom in patients with achalasia. Other symptoms may include regurgitation, chest pain, heartburn, weight loss, postprandial aspiration and nocturnal coughing[4]. The incidence of achalasia in studies ranges between 0.5-1.2/100000 per year[5] and the estimated prevalence is around 10/100000[6]. There does not seem to be a distinct pattern of incidence as it occurs equally in both sexes, all races and at any age[5].

Although the cause of idiopathic achalasia is largely unknown, the general pathophysiology has been studied extensively. There is a hallmark loss of esophageal nitric oxide-inhibitory postganglionic neurons in the myenteric plexus of the lower esophagus[7]. The excitatory neurons remain unaffected, leading to an imbalance between excitatory and inhibitory neurons and resultant increase in LES pressure[8]. However, it is as of yet still unclear as to why there is a loss of these enteric neurons in patients who develop idiopathic achalasia. There is evidence to support a combination of autoimmune, infectious and genetic factors. It is now accepted that a viral or unknown environmental trigger causes an inflammatory cell infiltrate of the myenteric plexus, which in genetically predisposed individuals triggers an autoimmune response causing destruction of the inhibitory myenteric ganglion[8,9].

Diagnostic evaluation includes endoscopy, radiological imaging and esophageal manometry. Upper endoscopy may reveal resistance while traversing the LES with the endoscope, described as a "pop" sensation in the literature. Barium esophagogram shows a classic "bird's beak" appearance in the region of the LES which is highly suggestive of achalasia. However, esophageal manometry revealing incomplete LES relaxation and aperistalsis in the esophageal body is considered the gold standard investigation for diagnosis[1].

There is no curative treatment for achalasia. The most effective form of treatment is a myotomy which can be performed endoscopically or surgically. Endoscopic myotomy can be performed by pneumatic balloon dilation or *via* a new procedure called peroral endoscopic myotomy (POEM). Surgical options include the laparascopic Heller myotomy which is routinely accompanied by fundoplication to decrease the risk of severe symptomatic gastroesophageal reflux disease (GERD), as well as older open techniques which are now rarely used[10]. Medical forms of treatment primarily include the injection of botulinum toxin (BT) into the LES, nitrates and calcium channel blockers. BT is the most effective and commonly used pharmacological agent and will be discussed in detail in the subsequent sections.

BT FOR ACHALASIA

History

Since its use was first described in 1977 in children with strabismus, BT has been increasingly used in various fields and diseases, from the treatment of focal dystonias, spasticity and urinary incontinence to becoming the most widely used injection in cosmetic procedures worldwide[11,12].

The use of BT for the treatment of achalasia was first described by Pasricha *et al*[13] in 1994 in a pilot study, which was followed by a double blinded trial[14] in which patients with achalasia were randomized to treatment either with BT injection or placebo (saline) injection into the LES. At one week, 90% of the BT injection group showed significant symptom reduction and a significant decrease in mean LES pressure. At 6 mo approximately two thirds of the patients were still in remission. Since the publication of this seminal study many studies have investigated the role of BT in the management of achalasia[15].

Pharmacology and mechanism of action

Every BT serotype is initially synthesized as a 150 kDa neurotoxin polypeptide chain with low intrinsic activity along with a set of neuro-toxin associated proteins (NAP), which are believed to protect the neurotoxin from proteases in the gastrointestinal tract[16]. The BT precursor is cleaved *in vivo* into a 100 kDa heavy chain (HC) and 50 kDa light chain (LC) linked by a disulfide bridge as well as a poorly structured protein segment called the belt. The HC can functionally be split into the heavy chain carboxy terminus (H-C) and heavy chain amino terminus (H-N) (Figure 1). The H-C, which can further be split into two subdomains, is responsible for neuronal receptor recognition and binding whilst the H-N is responsible for facilitating translocation of the LC into the cytosol[17]. The HC binds to transiently expressed specific cell receptors as well as to a polysialoganglioside, the disulfide bond is reduced and the light chain is internalized by exploiting synaptic vesicle recycling and diffusing into the cytosol. BT has a high affinity and specificity for target cells and requires two different co-receptors found on the neuronal surface, although different serotypes have different receptors[12].

Once inside the cell, the light chain proceeds to cleave one or more of the soluble NSF-attachment protein

Table 1 Properties of commercially available botulinum toxin drugs

	Botox®	Dysport®	Xeomin®	NeuroBloc® Myobloc®
Manufacturer	Allergan Inc. Irvine, CA, United States	Ipsen Pharma Boulogne-Billancourt, France	Merz Pharmaceuticals Frankfurt/M, Germany	United States WorldMeds Louisville, KY, United States
Pharmaceutical preparation	Powder	Powder	Powder	Ready-to-use solution 5000 MU-E/mL
Storage conditions	Below 8 ℃	Below 8 ℃	Below 25 ℃	Below 8 ℃
Shelf life	36 mo	24 mo	36 mo	24 mo
Botulinum toxin type	A	A	A	B
Clostridium botulinum strain	Hall A	Ipsen strain	Hall A	Bean B
SNARE target	SNAP25	SNAP25	SNAP25	VAMP
Purification process	Precipitation and chromatography	Precipitation and chromatography	Precipitation and chromatography	Precipitation and chromatography
pH-value of the reconstituted preparation	7.4	7.4	7.4	5.6
Stabilisation	Vacuum drying	Freeze-drying (lyophilisate)	Vacuum drying	pH-reduction
Excipients	Human serum albumin 500 μg/100 MU-vial; NaCl 900 μg/100 MU-vial buffer system	Human serum albumin 125 μg/500 MU-vial; Lactose 2500 μg/100 MU-vial buffer system	Human serum albumin 1000 μg/100 MU-vial; Sucrose 4.7 mg/100 MU-vial buffer system	Human serum albumin 500 μg/mL; Disodium succinate 0.01 mol/L; Sodium chloride 0.1 mol/L; H2O; Hydrochloric acid
Biological activity	50/100 MU-A/vial	500 MU-I/vial	50/100 MU-M/vial	1.0/2.5/10.0 kMU-E/vial
Biological activity in relation to Botox®	1	1:2-1:3	1	1:40
Specific biological activity	60 MU-EV/ngBNT	100 MU-EV/ngBNT	167 MU-EV/ngBNT	5 MU-EV/ngBNT

SNARE: Soluble NSF-attachment protein receptor; NSF: N-ethylmaleimide-sensitive fusion protein; SNAP-25: Synaptosomal-associated protein of 25 kD; BNT: Botulinum neurotoxin; MU-A: Mouse unit in the Allergan mouse lethality assay; MU-E: Mouse unit in the Solstice mouse lethality assay; MU-I: Mouse unit in the Ipsen mouse lethality assay; MU-M: Mouse unit in the Merz mouse lethality assay; MU-EV: Equivalence mouse unit. 1 MU-EV = 1 MU-A = 1 MU-M = 3 MU-I = 40 MU-E. Adapted with permission from Dressler[11].

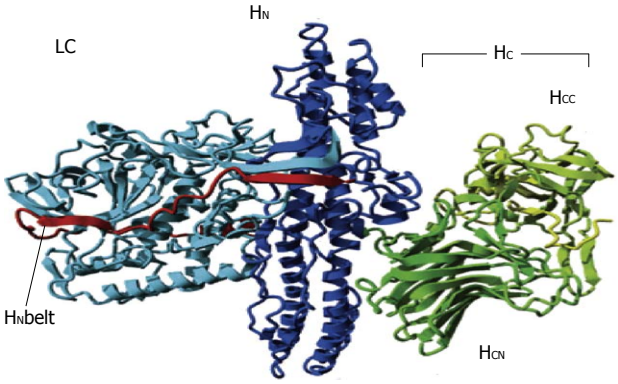

Figure 1 Structure of botulinum toxin A. The Cα backbone is represented as ribbons with the LC in cyan, the HN in dark blue and the HC in a green to yellow gradient highlighting the HCN and HCC subdomains. The HN belt is in red. H_N + H_C: 100 kDa heavy chain; H_N: 50 kDa amino terminus; H_C: 50 kDa carboxy terminal of the heavy chain; H_{CC}: β-beta tree foil fold heavy chain subdomain; H_{CN}: β-sheet jelly roll fold heavy chain subdomain; LC: Light chain. Adapted with permission from Montal[17].

receptor (SNARE) complex proteins, which are required for synaptic vesicle fusion with the active zone of the neuronal synapse (Figure 2). Cleavage by BT causes impairment of vesicle fusion and inhibition of synaptic activity[16].

Although the effects of BT on the nerve terminal are long lasting, they are however reversible and do not lead to neurodegeneration[18]. After inhibition of synaptic vesicle fusion by BT, neuronal sprouts begin to develop from motor nerve terminals that establish synaptic activity. Ultimately, synaptic activity at the motor neuron endplate is restored, and the neuronal sprouts retract completely[18,19].

Formulations (Table 1)

BT is commercially produced by the anaerobic fermentation of *Clostridium botulinum* although in nature it is also produced by other related species such as *C. barati and C. butyricum*[16]. There are eight immunologically distinct serotypes of Botulinum identified, with type H being only recently discovered[20].

As of now, the Food and Drug Administration (FDA) has approved two serotypes, type A and type B for use in humans for various clinical indications. There are seven subtypes of BT (A) (A1-A7) that have been described, but all three formulations of BT (A) available for clinical use in patients with achalasia are of the A1 subtype. They include Abobotulinum (ABO; Dysport®/Azzalure®), Incobotulinum (INCO; Xeomin®/Bocouture®) and Onabotulinum (ONA; Botox®/Vistabel®). BT (A) is the most widely used and best studied formulations of BT[21]. Although Rimaotulinumtoxin B (Neurobloc®/Myobloc®) is available commercially, it has not been widely studied in patients with achalasia.

The biological activity of BT is measured in a mouse lethality assay (LD_{50}), *i.e.*, the dose of toxin capable of killing 50% of a group of mice, and units are given in mouse units (MU)[11]. Concern for mouse welfare has prompted the investigation of more humane cell-based assays, with several being proposed such as the recently published compound muscle action potential assay[22]. The different formulations of BT (A) have varying

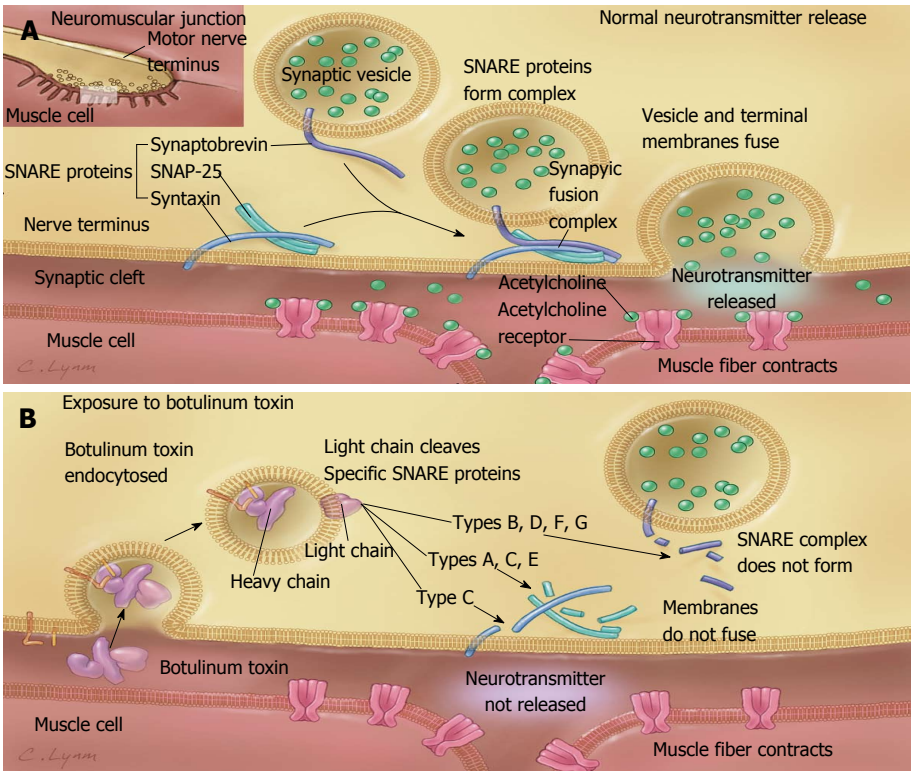

Figure 2 Mechanism of action of botulinum neurotoxin. A: Release of acetylcholine at the neuromuscular junction is mediated by the assembly of a synaptic fusion complex that allows the membrane of the synaptic vesicle containing acetylcholine to fuse with the neuronal cell membrane. The synaptic fusion complex is a set of SNARE proteins, which include synaptobrevin, SNAP-25, and syntaxin. After membrane fusion, acetylcholine is released into the synaptic cleft and then bound by receptors on the muscle cell; B: Botulinum toxin binds to the neuronal cell membrane at the nerve terminus and enters the neuron by endocytosis. The light chain of botulinum toxin cleaves specific sites on the SNARE proteins, preventing complete assembly of the synaptic fusion complex and thereby blocking acetylcholine release. Botulinum toxins types B, D, F, and G cleave synaptobrevin; types A, C, and E cleave SNAP-25; and type C cleaves syntaxin. Without acetylcholine release, the muscle is unable to contract. SNARE: Soluble NSF-attachment protein receptor; NSF: N-ethylmaleimide-sensitive fusion protein; SNAP-25: Synaptosomal-associated protein of 25 kD. Reproduced with permission from Arnon *et al*[91].

potencies which have been compared in several studies. One MU of ONA has been shown to be equivalent to 1 MU of INCO[23-25], whilst a conversion rate of 1 MU: 2-3 MU between ONA and ABO has been proposed in various studies[26,27], as well as 1:2.5 for aesthetic indications[25].

The potency of BT (A) and BT (B) is difficulty to compare directly. BT (B) has relatively weaker motor side effects and stronger autonomic effects than BT (A), even when used at standard dose. In one study for example, patients who received BT (B) reported higher incidences of constipation and lower saliva production[28].

All of the currently available BT (A) drugs are sold in powdered form and have to be reconstituted, whereas BT (B) (Neurobloc®/Myobloc®) is available as a ready to use solution. In addition, only INCO can be stored at room temperatures while the other formulations need special storage temperatures[11].

In the available commercial formulations, BT is stored with excipients such as NaCl/lactose/sucrose as well as albumin to decrease the risk of inactivation during preparation and storage. Out of the three formulations of BT (A), ABO has the least amount of albumin, which may partially explain the lower amount of available toxin per injected unit, as well as the shorter shelf life and decreased duration of stability after reconstitution compared to ONA and INCO[12].

Dosages and techniques

The technique used to inject BT into the LES is still largely followed as described in the pilot study by Pasricha *et al*[13]. The LES is visually identified during upper endoscopy and aliquots containing 20-25 U of BT (A) are injected in quadrants for a total of 80-100 U.

Several studies have used slightly different techniques in their studies, although to date there are no randomized controlled trials comparing these different methodologies. In one study, patients received two injections spaced 1 cm apart in each of four quadrants for a total of eight injections equaling 100 U of BT (A). The response rate was 89.65% at 30 d and 55.17% at one year but fell to 13.79% at 2 years[29]. In another study involving seven patients, 100 U of BT (A) was injected in eight aliquots, with four injections each at the LES and approximately 4 cm above the LES, respectively[30]. At follow up, only 28.6% of patients were in remission.

Other investigators have attempted to find the optimal dose of BT. In one study[31], 118 achalasia patients were randomized into three treatment arms to receive 50 U, 100 U or 200 U of BT (A). At 30 d, 82%

of patients were considered responders, and based on symptom scores the proportion of responders was slightly higher in the higher dose group, albeit not statistically significant. Similarly, the change in LES pressure was comparable in all three groups. To determine whether the timing of administration had any effect on duration of action, responders to 100 U of BT (A) were injected with a similar dose of 100 U 30 d later. At the end of follow up, patients that received 2 doses of 100 U 30 d apart were found to be more likely in remission (19%, $P < 0.04$) compared to 47% and 43% in the 50 U group and single dose 200 U group respectively. This study demonstrated a statistically insignificant therapeutic effect with dose escalation of BT, but clearly showed decrease in relapse rates with repeat BT injection.

Safety and side effects

BT (A) injection into the LES is extremely well tolerated, with the most common side effect usually being transient chest pain[32]. On the other hand, BT-B, by virtue of its stronger anticholinergic effects, has been noted to cause autonomic side effects like dry mouth and jitteriness even in small doses[11].

Efficacy in achalasia

BT injection is considered effective in the short term, but has a high rate of relapse requiring a need for reinjection. For example, one meta-analysis[33] evaluated nine studies with a total of 315 patients, and found that the rate of symptomatic improvement at one month to be 78.7%, but gradually decreased to 70% at 3 mo, 53.3% at 6 mo and 40.6% at 1 year. Furthermore, at least a second treatment was required in 46.6% of patients. Generally speaking, there is almost universal symptom relapse by two years[34], although some studies have shown continued efficacy in up to 34% of patients at two years[35]. The efficacy of BT with repeat injections decreases and is thought to be secondary to antibody formation.

Current role in treatment

Overall, pneumatic dilation and myotomy have superior long term efficacy than BT injection in treating patients with achalasia[10].

In a Cochrane review comparing BT with pneumatic dilation, there was no significant difference in rates of remission at 4 wk after intervention. However, BT was significantly less effective in maintaining symptom remission at six months and one year[33].

Similarly, one meta-analysis analyzed studies comparing pneumatic dilation with BT injection and found a remission rate of 65.8% at one year for pneumatic dilation compared to 36% for BT injection (RR = 2.20, 95%CI: 1.51-3.20, $P < 0.0001$)[36].

In a prospective randomized study evaluating BT injection with Heller myotomy, the authors found that results at 6 mo were comparable; however, the efficacy of BT injection decreased thereafter and the probability of being symptom free at 2 years was 87.5% after Heller myotomy and only 34% after BT injection ($P < 0.05$)[35].

POEM is a novel endoscopic treatment for achalasia which is a minimally invasive alternative to conventional Heller myotomy. Although to our knowledge no prospective trials have compared BT injection to POEM, various studies have compared Heller myotomy with POEM and found favorable results. One meta-analysis evaluating a total of four studies found that POEM had comparable complications [odds ratio (OR) = 1.17, 95%CI: 0.53-2.56, $P = 0.70$] and symptom recurrence (OR = 0.24, 95%CI: 0.04-1.55, $P = 0.13$) as Heller Myotomy on short term follow up[37].

The response to BT seems to be unaffected by prior therapy such as prior pneumatic dilation or myotomy, which highlights an important role for BT injection in patients who have failed prior surgical or endoscopic therapy. In one study[38], the response to BT injection in achalasia was compared in patients without prior therapy, with prior dilation and with prior myotomy. Neither LES pressures nor symptom scores differed between groups. At 6 mo the remission rate was 71.4% in those who received prior dilation, 71.4% in prior myotomy and 73.9% in prior BT injection.

Pneumatic dilation comes with recognized risks, including esophageal perforation (approximately 1.6%) as well as symptomatic heartburn in up to 45% of patients[33]. Likewise, myotomy may be associated with risks, most noticeably esophageal perforation and postoperative GERD[39], and due to the nature of the procedure may not be suitable for patients with multiple comorbidities or at high risk.

For this reason, several expert guidelines have suggested that BT injection may play a role in the elderly, in patients with extensive co-morbidities or who are poor surgical candidates and as a salvage therapy in patients who have failed other therapeutic modalities[1,39-43].

FUTURE DIRECTIONS

Inhibition of sprouting

As mentioned previously, the major cause of BT's limited long term efficacy is the sprouting phenomenon, in which the presynaptic nerve terminals begin to produce sprouting nerve collaterals with a corresponding increase in acetylcholine receptors and ultimately restoration of conduction at the neuromuscular junction (Figure 3).

Several animal model studies investigating nerve sprouting demonstrated a complex array of neural factors and neuroreceptors upregulated and involved in the process of neuronal regeneration and sprout formation, including nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), glial cell line derived neurotrophic factor (GDNF) and insulin-like growth factor 1 (IGF-1)[44-48].

A limited number of studies have investigated the effect of neutralizing substances against these factors

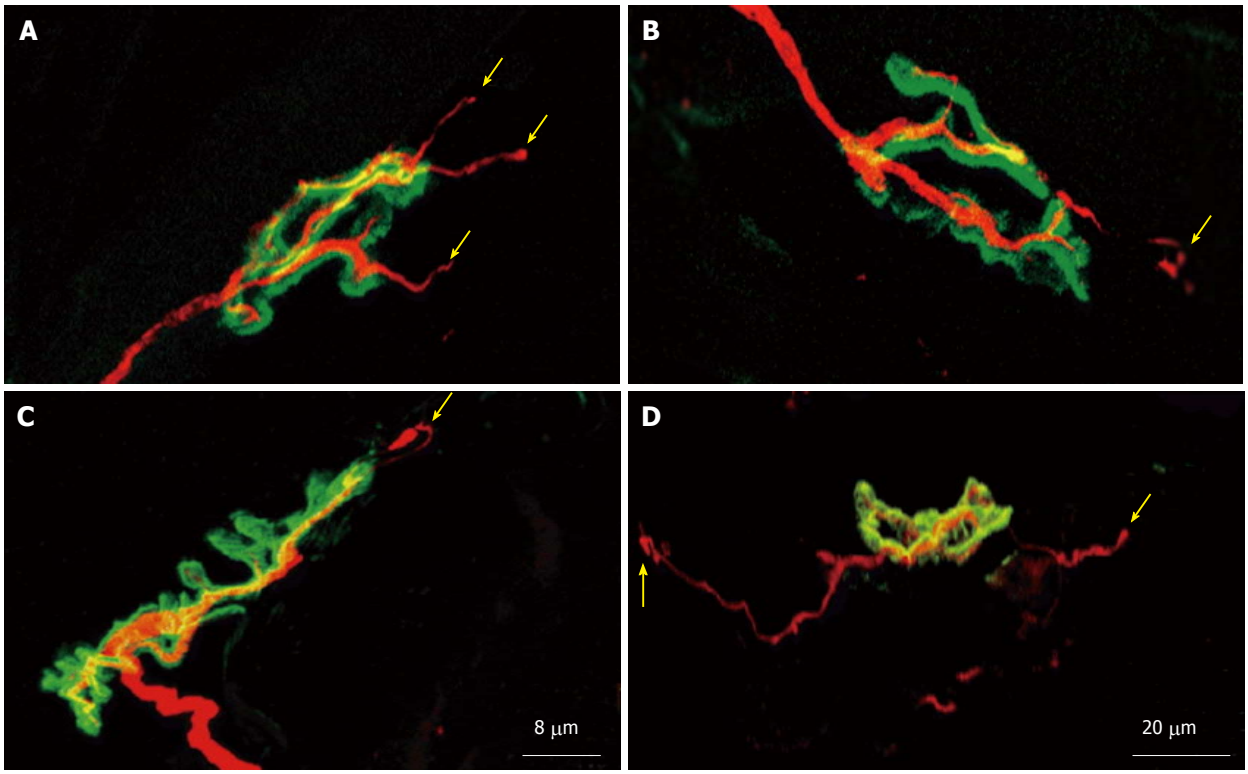

Figure 3 Neuronal sprouting and remodeling of the neuromuscular junction. Remodeling of the neuromuscular junction in extensor digitorum longus muscle at 10 (A-C) and 21 d (D) after a single injection of botulinum neurotoxin type C. Axons and nerve terminals were immunolabelled (red). To localize the junctions, nicotinic acetylcholine receptors were stained (green). Note the sprouts that emerge from the original motor endplate and project along muscle fibers (yellow arrows). Reproduced with permission from Morbiato *et al*[82].

on the process of sprout formation, as well as the effects of co-injection of alternate substances with BT in hopes of decreasing the rate of neuronal sprouting and thereby increasing the duration of action and efficacy of BT injection. In a murine model Streppel *et al*[49] demonstrated that neutralizing antibodies to NGF, BDNF, and IGF1 significantly reduced nerve sprouting. In an animal model, Harrison *et al*[50] demonstrated that injection of anti-IGF1 or corticotropin-releasing factor after BT injection reduced axonal sprouting, prevented the up regulation of neuromuscular junctions and increased duration of BT efficacy.

Ricin-mAb35 is an immunotoxin composed of ricin, a toxin that inhibits protein synthesis, conjugated to a monoclonal antibody to the nicotinic acetylcholine receptor. Christiansen *et al*[51] found that injection of BT followed by Ricin-mAB35 into animal optic muscles resulted in decreased twitch and tetanic force at 6 mo compared to BT alone. Most recently, Jiang *et al*[52] investigated the adjunct use of acrylamide injection to botulinum toxin injection. Acrylamide is a cumulative dose related neurotoxin that causes retrograde necrotizing neuropathy. Acrylamide injection after BT injection in the gastrocnemius muscles of rats was found to inhibit nerve sprouting as measured by electromyography and pathological observation of nerve fibers per unit. In this study, after the injection of BT (A) plus acrylamide, the increase in the number of nerve fibers was significantly reduced, the peak time of sprouting was delayed to 8-10 wk, and the peak nerve fiber counts were significantly lower than that in the BT-A group ($10.65 \pm 0.32 \times 10^8/m^2$ *vs* $14.33 \pm 0.45 \times 10^8/m^2$, $P < 0.05$).

No study has yet investigated use of BT injection with sprouting inhibitors in achalasia models. Hence, it remains to be seen if this can prolong the therapeutic efficacy of BT injection in patients with achalasia.

Use of specific subtypes of BT (A)

As mentioned above, as of now studies have primarily utilized BT (A) for achalasia. Of the three formulations of BT (A) that are available on the market (ABO, ONA INCO), INCO (Xeomin®, Boucouture®) differs from ABO and ONA in that only the 150 kDa neurotoxin itself is included and is devoid of the NAPs that are included in the ABO and ONA formulations. NAPs are naturally produced with the neurotoxin and are thought to protect the toxin from proteolytic and acidic degradation whilst in the gastrointestinal tract[12]. However, they are not known to play a role in the neurotoxin induced blockade of cholinergic transmission. Building on previous animal studies that demonstrated increased immune response and presence of neutralizing antibodies in BT (A) complex compared to pure BT (A), Wang *et al*[53] recently studied the inflammatory cytokine release between human neuroblastoma cells treated with pure BT (A), BT (A) + NAPs complex or NAPs alone. They found that exposure to BT (A) + NPA complex significantly increased release of IL-6, MCP-1, IL-8, TNF-α and RANTES compared to controls.

In addition, it appears that while pure BT binds solely to neuronal cells, NAPs bind to neuronal as well as to non-neuronal cells, demonstrating that they may not simply be as passive as previously thought. It is likely that the hemagglutinins in the BT (A) complex bind to dendritic cells which act as antigen presenting cells in the early stages of the immune response. This is likely to result in increased immunogenicity and the formation of an immune response against the BT agent, although the level of antibody titers necessary to effect BT's clinical efficacy is unknown due to heterogeneity of available studies, and their presence may or may not affect patient's responsiveness to BT[12].

Factors that decrease the efficacy of BT over time are poorly understood. Suboptimal reconstitution of BT by physicians may decrease the efficacy of the BT preparation as shown in a recent study[54]. Similarly, it has been proposed that establishing practices to reduce the risk of neutralizing antibodies such as lower dosing frequency, longer treatment intervals, and lower number of injections may decrease the likelihood of their development[12,55].

Testing and availability of serotypes other than (A)/(B)

As of yet, only serotypes A and B have been FDA approved for use in human subjects[11]. However, almost all studies investigating the use of BT in achalasia patients were done using serotype A[15]. Hence, it is unknown if the other serotypes may prove more effective by decreasing the response of neutralizing antibodies or whether their use of different neuronal surface type receptors or cleavage of different SNARE proteins may prove less likely to elicit neuronal sprouting which is what primarily contributes towards the high relapse rate seen in achalasia patients treated with BT.

Recombinant BT

Part of BT's unique attraction is its particularly long persistence in the neuron and prolonged mode of action. Interestingly, different BT serotypes have been found to have varying duration of action. For example, in one study BT (E) was found to exert its activity for only about 2-3 wk in murine models compared to around 10 mo with BT (A)[56]. However, BT (E) cleaves more C-terminal residues from synaptosomal-associated protein of 25 kD (SNAP-25) than BT (A) causing a greater disruption of exocytosis resulting in quicker and greater neurotransmission blockade[57]. Studies have shown that the differential persistence of BT (A) *vs* BT (E) may be secondary to different susceptibility of the LC to various ubiquitin-dependent proteolysis pathways in the neuron through its interaction with RING finger protein TRAF2[58].

A few studies have investigated the translational applications of genetically modified BT. One study genetically fused BT (E) LC to an enzymatically inactive BT (A) mutant, resulting in an end product that had BT (A)'s stability and persistence, as well as BT (E)'s potent cleavage of SNAP-25 and resultant neuromuscular paralysis[57]. Given BT ubiquitous use in many different fields, ongoing research to produce BT mutant strains that have stronger potency and persistence may prove beneficial in diseases such as achalasia.

Improvisations in BT injection techniques

As of yet, the endoscopic injection procedure for achalasia has largely remained unchanged since first introduced by Pasricha *et al*[14]. BT injection into the LES is routinely performed during endoscopy, which may mean that precise injection into the LES may not be possible due to lack of visualization. This may contribute to the lower rates of efficacy of BT injection or early relapse in some patients.

The utility of endoscopic ultra sound (EUS) in achalasia patients was first reported by Devière *et al*[59] in 1989 using an early 7.5 MHz ultrasound endoscope to measure the thickness of the LES in achalasia patients. A subsequent case series demonstrated technical feasibility, safety and decrease in dysphagia scores in a series of four patients. However, the study included a very small sample size and there was no comparison between standard endoscopic injection and EUS-guided BT injection[60]. Recently, a study compared EUS-guided BT injection in achalasia patients compared to conventional endoscopic BT injection[61]. Patients that received EUS-guided injections benefited from higher rate of relief and significantly lower rate of symptomatic relapse.

In summary, there is no gold standard for injection technique or dosage of BT injected, with different authors utilizing different dosages and techniques. Randomized trials are needed to determine optimum dosage, formulation and technique of injection.

Injection of sclerosing agents

Alternative agents for the treatment of achalasia have been investigated. Ethanolamine Oleate (EO) is a sclerosant which is FDA approved for the treatment of bleeding esophageal varices, and is also used for lower extremity varicosities and vascular lesions. EO is a synthetic salt comprising of ethanolamine and oleic acid. When injected, EO produces an inflammatory response resulting in necrosis and fibrosis in the epithelium and submucosal tissue. First investigated for the use in the treatment of achalasia by Moretó *et al*[62] over 20 years ago, a long term study has since been published evaluating the long term efficacy of EO injection[63]. The authors included 103 patients in the study, with a mean follow up of 84.5 mo for patients who received EO. The cumulative expectancy of being free from recurrence was 90% at 50 mo.

Niknam *et al*[64] investigated the long term efficacy of EO in patients with achalasia who were resistant or poor candidate for pneumatic dilation or surgery. Of 220 patients who were evaluated for inclusion, thirty one patients met the inclusion criteria. EO was injected into the LES three times at two week intervals, and followed for a duration of 30.16 ± 11.3 mo with primary endpoints

being symptomatic improvement on the achalasia symptom scale (ASS) and results of timed barium esophagram. At 1.5 mo post injection, the mean ASS, volume of barium and LES were significantly decreased compared to pretreatment ($P = 0.0001$). Symptom score and volume of barium remained significantly improved at 6 mo and one year intervals. Recently, Mikaeli *et al*[65] prospectively compared BT to EO in a cohort of patients who were poor candidates for pneumatic dilation or surgery. Out of the 189 patients evaluated, 10 were included in EO group *vs* 11 in the BT group, with a mean duration of 27.38 ± 16.49 mo of follow up. No statistically significant differences were found between either treatment groups.

NITRATES

The effects of short acting nitrates such as amyl or acetyl nitrate and sublingual nitroglycerin on achalasia patients was first evaluated by investigators in the 1940's and 1950's[66-69], but their use was abandoned due to the short duration of action (less than 30 min) and significant side effects such as hypotension and headaches.

With the subsequent introduction and widespread use of isosorbide dinitrate, Gelfond *et al*[70] evaluated its use in patients with achalasia and found significant clinical improvement in dysphagia and decrease in LES pressures on manometry. However, the use of nitrates in achalasia has been severely limited by its transient or poor efficacy in a significant subset of patients, high rate of tachyphylaxis, and high incidence of side effects such as headache and hypotension. Hence, nitrates have been poorly evaluated in high quality randomized controlled trials as was shown in a 2004 Cochrane review[71].

The use of nitrates has largely been relegated for use in those patients unable or unwilling to undergo any other treatment modality, although even in this respect it has largely been replaced by calcium channel blockers, which have been shown to be better tolerated and have a larger body of data investigating their use.

CALCIUM CHANNEL BLOCKERS

The efficacy of calcium channel blockers on achalasia was first investigated in the late 1970's and 1980's[72-76]. Calcium channel blockers inhibit cellular uptake of calcium, thereby impeding contraction and promoting relaxation. Nifedipine has been shown to decrease LES pressures and provide symptomatic relief, although with variable efficacy demonstrating benefit in between 50% and 90% of cases[77]. In one small prospective study where nifedipine was titrated to a dose of 10-30 mg before meals, the overall symptom scores decreased compared with placebo, but symptoms such as dysphagia, chest pain or regurgitation were still present on most days[78].

Up to 30% of patients may experience significant side effects from calcium channel blockers such as peripheral edema and headaches which may limit their clinical utility. In addition, tachyphylaxis is also an issue with calcium channel blockers and the use of these drugs often induces tolerance[79]. Only a handful of studies have looked at calcium channel blockers prospectively, and the studies are limited by short follow up and very small sample sizes[77]. Studies comparing nitrates and calcium channel blockers have suggested that nitrates work faster and may be more effective compared with nifedipine[76,80], but have limited clinical use due to more significant side effects. In another study, nifedipine was prospectively compared with verapamil, and while verapamil also decreased mean LES pressures, it did not provide effective symptomatic relief compared with nifedipine[81].

OTHERS PHARMACOLOGICAL AGENTS

Other early studies have investigated the effect of different pharmacological agents on the LES in patients with achalasia. Administration of phosphodiesterase inhibitors such as sildenafil[82,83] was found to have a reduction in LES pressure in achalasia patients, as was carbuterol, a beta 2 adrenergic agent[84], morphine[85], loperamide[86], anticholinergics like cimetropium bromide[87] and butylscopolamine[88], vasoactive intestinal peptide[89], terbutaline[90] and aminophylline[90]. However, the clinical utility of these agents is not clear as they have only been studied in very small sample sizes demonstrating proof of concept results and short duration of action.

CONCLUSION

BT injection into the LES is the most commonly used initial therapy in patients with achalasia. Although lauded for its remarkably safety profile and short time efficacy, issues with need for repeat injection and decreased efficacy over time have relegated it to use in patients unable to undergo more long lasting procedures such as myotomy and as a form of salvage therapy. However the large body of ongoing research into BT may provide a stronger role for BT injection as a form of minimally invasive, cost effective and efficacious form of therapy for patients with achalasia. Further research in achalasia models are needed to investigate the role of different BT serotypes, better pharmacological formulations, better BT injection techniques, the use of sprouting inhibitors, designer recombinant BT formulations and alternative substances used in endoscopic injections. In addition, as more research demonstrates an autoimmune component in the pathophysiology of achalasia, the need for studies investigating the use of immune therapy in achalasia models becomes ever important. Various medications like calcium channel blocker and nitrates have been shown to have a role in treatment, but their widespread use is limited largely by the high incidence of side effects. Newer oral pharmacological agents with high potency and fewer side effects may prove beneficial

as an alternative treatment modality in patients with achalasia.

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REVIEW

Pregnancy and inflammatory bowel diseases: Current perspectives, risks and patient management

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Abstract

Inflammatory bowel diseases (IBD) are chronic idiopathic inflammatory conditions characterized by relapsing and remitting episodes of inflammation which can affect several different regions of the gastrointestinal tract, but also shows extra-intestinal manifestations. IBD is most frequently diagnosed during peak female reproductive years, with 25% of women with IBD conceiving after their diagnosis. While IBD therapy has improved dramatically with enhanced surveillance and more abundant and powerful treatment options, IBD disease can have important effects on pregnancy and presents several challenges for maintaining optimal outcomes for mothers with IBD and the developing fetus/neonate. Women with IBD, the medical team treating them (both gastroenterologists and obstetricians/gynecologists) must often make highly complicated choices regarding conception, pregnancy, and post-natal care (particularly breastfeeding) related to their choice of treatment options at different phases of pregnancy as well as post-partum. This current review discusses current concerns and recommendations for pregnancy during

IBD and is intended for gastroenterologists, general practitioners and IBD patients intending to become, (or already) pregnant, and their families. We have addressed patterns of IBD inheritance, effects of IBD on fertility and conception (in both men and women), the effects of IBD disease activity on maintenance of pregnancy and outcomes, risks of diagnostic procedures during pregnancy and potential risks and complications associated with different classes of IBD therapeutics. We also have evaluated the clinical experience using "top-down" care with biologics, which is currently the standard care at our institution. Post-partum care and breastfeeding recommendations are also addressed.

Key words: Inflammatory bowel diseases; Pregnancy; Biologics; Breast-feeding; Immunomodulatorsi

Core tip: Inflammatory bowel diseases (IBD) are chronic inflammatory conditions characterized by relapsing and remitting episodes of intestinal inflammation. IBD is most frequently diagnosed during peak female reproductive years, with 25% of women with IBD conceiving after their diagnosis. While therapies have improved dramatically, IBDs have important effects on pregnancy and present challenges for maintaining optimal outcomes for mothers and their developing fetus/neonate. Women with IBD and physicians must often make challenging decisions on conception, pregnancy, and breastfeeding. This review discusses concerns and recommendations for pregnancy during IBD and is intended for gastroenterologists, general practitioners and IBD patients and their families.

Hosseini-Carroll P, Mutyala M, Seth A, Nageeb S, Soliman D, Boktor M, Sheth A, Chapman J, Morris J, Jordan P, Manas K, Becker F, Alexander JS. Pregnancy and inflammatory bowel diseases: Current perspectives, risks and patient management. *World J Gastrointest Pharmacol Ther* 2015; 6(4): 156-171 Available from: URL: http://www.wjgnet.com/2150-5349/full/v6/i4/156.htm DOI: http://dx.doi.org/10.4292/wjgpt.v6.i4.156

INTRODUCTION

Inflammatory bowel diseases (IBD) are chronic idiopathic inflammatory conditions characterized by relapsing and remitting episodes of inflammation affecting several regions of the gastrointestinal (GI) tract[1,2]. In the United States, upwards of 1.4 million people have IBD[2], and there is a trend for increasing IBD incidence over the last decades[3]. The global incidence of Crohn's disease (CD) varies between 0.1-16/100000 and that of ulcerative colitis (UC) varies between 0.5-24.5/100000, with an overall prevalence IBD of 396/100000[4]. IBD is more common in women than in men[5], occurs more frequently in adolescents and young adults[6], and is most frequently diagnosed during peak reproductive years in women.

IBD includes at least 3 different subtypes: CD, UC and indeterminate colitis[1]. UC and CD are distinguished by their affected locations and the histopathology of the disease at each affected site[6]. While UC primarily affects the colon and the rectum, with involvement of the submucosa and mucosa, CD can affect any region in the GI tract (often sparing the rectum) and is characterized by transmural inflammation[6]. When there is difficulty in discriminating between CD and UC, either based on colonoscopic evidence or excised colectomy specimens, the term "indeterminate" colitis is used[7].

Clinically, IBD symptoms reflect inflammatory changes within the GI tract. Hallmark GI symptoms of IBD include diarrhea, constipation, bloody stools, increased bowel movements, abdominal cramping, nausea, and vomiting[8]. In addition to GI symptomology, fever, weight loss, arthralgias, and malaise are other frequent systemic symptoms seen in IBD. Fistulizing disease, fat and vitamin malabsorption are long-standing complications that are associated with CD[6] but are less common in UC. These complications have serious consequences even in normal patients and can be devastating for pregnant women with IBD and their developing fetuses.

IBD therefore creates a unique and challenging set of conditions to effectively manage and control. Gastroenterologists can now provide specific and targeted treatment plans which can often be managed according to each patient's individual needs. Women with IBD, the physicians that care for them and their families must often face complex decisions on issues of conception, pregnancy and breastfeeding. As previously stated, at least 50% of patients are diagnosed by age 35[9], more frequently[10], affecting women during their peak reproductive years. Importantly, 25% of women with IBD will conceive after their diagnosis of IBD has been established[9]. This review will examine some of the important considerations for women with IBD and their families including heritability, fertility, risks unique to IBD and IBD therapy in the setting of pregnancy and lactation.

FERTILITY

Women with active IBD experience reduced fertility for several reasons compared to the general population, with an overall "fertility rate" (lifetime births per woman) of 2.45 for healthy women, but only 2.06 for IBD patients (in the United States)[10]. Population studies show infertility rates in CD to be somewhere between 5% to 14%[11]. By comparison, UC has less of an effect on fertility, unless patients had undergone any IBD related surgery[12,13]. Several other factors associated with active IBD can also contribute to the overall lower rate of conception in IBD including dyspareunia, low libido, and depression[14-16]. Dyspareunia (painful sexual congress) often occurs secondary to pelvic surgery, from IBD-associated inflammation, or psychological stress

associated with IBD. Interestingly, the main cause of decreased rates of fertility in CD patients with history of previous surgeries was found to be a conscious and concerted decision against conception[17].

For women with inactive IBD and without history of pelvic surgery, fertility is however comparable to their respective age-matched peers[18]. Pelvic surgery in IBD thus remains a major factor negatively impacting fertility, which varies with the extent and type of surgery[19]. Post-surgical adhesions also appear to play a key role in tubal infertility[20].

Proctocolectomy (PCL) and ileal-pouch anal anastomosis (IPAA) surgeries are associated with reduced fertility. Two studies showed approximately 50% of the women experienced fallopian tube obstruction (either unilaterally or bilaterally) following these procedures[21,22]. A meta-analysis evaluating IPAA in UC patients suggested that the risk of infertility increased 3-fold post-IPAA[23]. PCL[24] with IPAA has a more pronounced effect on fertility compared to the laparoscopic approach, which produces fewer adhesions[18,19,23-26]. Studies involving laparoscopic IPAA indicate that women undergoing these procedures have significantly higher pregnancy rates as compared to open field IPAA[27]. Therefore, laparoscopic procedures are always preferable particularly when conception is a goal.

INHERITANCE

Questions on inheritance patterns in IBD remain concerns for patients and their families. If one parent has any form of IBD, their child will have between a 2 to 13 fold increased lifetime risk of developing IBD[28] and is empirically estimated as an approximately 5% heritable risk in CD and 1.6% in UC[29]. However when both parents have a form of IBD, this risk increases to approximately 33%-36% for their offspring to inherit a form of IBD[30,31]. Genomic studies have shown that at least 100 heritable loci may influence IBD onset and penetrance[32]. Genomic studies have identified a vast heterogeneous distribution of genes linked with IBD, possibly suggesting different populations clusters that exhibit these conditions. Consequently, while in a population, the risk of IBD may be elevated by the presence of any particular gene variant, this does not necessarily hold true for any individual IBD patient bearing such alleles. The large number of genes creates several diverse patterns of IBD activity and inheritance involving different levels of disease activity and thereby necessitate individualized therapy. Both UC and CD have been associated with excessive interleukin-23 (IL-23) pathway activation with the dysregulation of several transcription factors, including SMAD3, STAT3, c-REL, zinc-finger-MIZ-type containing 1 and NK2[32]. Several genes specifically associated with UC include cytokines *e.g.,* IL-26, IL-22, structural proteins LAMB1 (encodes laminin β1), and hepatocyte nuclear factor 4α. With respect to CD, its pathogenesis has been linked with disturbances in nucleotide binding oligomerization domain protein 2 and genes that control autophagy (*e.g.,* ATG)[32], as well as disturbances in IL-10, tumor necrosis factor superfamily (TNFSF) 8, TNFSF-15, ZMIZ-1, NK2 transcription factor (NKX2-3), SMAD-3, caspase recruitment domain family, member 9 (CARD-9), and CARD-15[32].

DISEASE ACTIVITY DURING PREGNANCY

The severity of IBD disease activity during pregnancy also significantly influences pregnancy outcomes. While pregnancy has not been shown to specifically increase the risks of IBD "flares"[19], approximately 30%-40% of women with IBD active at the time of conception will develop more intense disease or endure disease flares during pregnancy[33,34]. Some studies show that disease outcomes and flares in IBD outside of pregnancy are linked to environmental factors and lifestyle including hormone use, diet, mental health status, cigarette smoking, and vitamin D levels[35]. IBD activity at the time of conception apparently determines the clinical course IBD patients will experience during pregnancy. That is to say, 2/3 of women with IBD in remission at the time of conception are likely continue to remain in remission throughout their pregnancy[36-39]. Remarkably[37-40], because only 1/3 of those patients with active disease at time of conception will relapse during their pregnancy, Editor, the gravid state may suppress some disease processes in IBD[40]. Effective IBD control in prenatal planning is therefore essential for favorable pregnancy outcomes, (birth weight > 5.5 lbs, no spontaneous abortion, congenital malformations or antepartum hemorrhage)[41]. Women with inactive IBD at the time of conception have only similar risks of adverse pregnancy outcomes as the general female population[12]. Women with active IBD however have increased risks of preterm deliveries, intrauterine growth restriction, and low birth weight (LBW) babies (defined as live born infants < 2500 g regardless of total gestational age)[37,42-44]. This suggests that the IBD process itself produces greater fetal risks during pregnancy. These complications are also more often seen in CD patients as compared to patients with UC.

The Crohn's Disease Activity Index (CDAI) is used in CD patients to evaluate baseline disease severity quantify cumulative symptoms and assess the changes of the disease in response to therapies in afflicted individuals[45]. There are eight factors involved in determining CDAI, which are assessed daily for 7 d, including: (1) frequency of watery stools; (2) well-being; (3) abdominal pain; (4) presence of any complications; (5) presence of abdominal mass; (6) usage of opioids; (7) low hematocrit < 0.47 and < 0.42 in men and women respectively, and lastly; and (8) standard weight percentage deviation[45]. A CDAI below 150 is defined as "in remission", while a CDAI > 450 is termed severe disease[46]. Pregnant women with IBD should seek early prenatal care similar to other pregnant women but need additional education

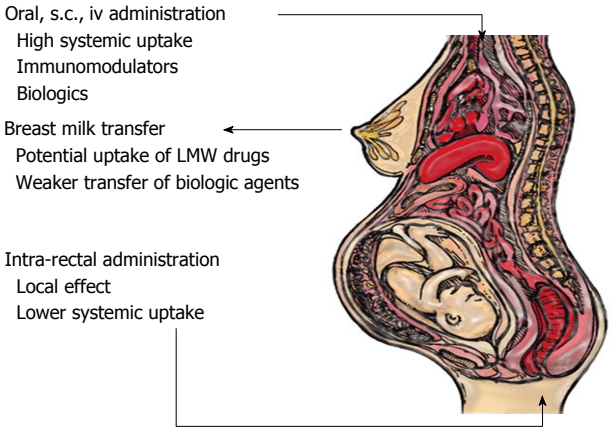

Figure 1 Inflammatory bowel disease drug metabolism considerations in pregnancy. LMW: Low molecular weight.

regarding effects of drug usage, vaccinations and vitamin regimens. Ideally, gastroenterologists should confer with obstetricians in the care of females with IBD.

COMPLICATIONS OF IBD DURING PREGNANCY

Expectant mothers with IBD are at a greater risk for several complications including malnutrition, venous thromboembolism (VTE), antepartum hemorrhage, and cesarean delivery[19,47,48]. VTE is increased in women with UC, while antepartum hemorrhage risk is more prevalent in women with CD[47]. There is nearly a 4-fold increase in the risk of VTE in women with UC; while CD affects women have a risk of VTE that was comparable with the general population[47]. The antepartum hemorrhage risk is shown to be doubled in women with CD[47]. Approximately 2% of women with CD and UC were seen to develop placental abruption in a study on obstetric hospitalizations[49]. The risk of cesarean delivery is also increased in the setting of either UC or CD[48]. According to Ng *et al*[19], women with perianal disease should opt for cesarean section, while those without perianal involvement can safely opt for a normal vaginal delivery.

As stated earlier, pregnant women with active IBD at conception suffer more frequent complications compared to those with quiescent disease (at conception), with those with low levels of disease activity for IBD having outcomes similar to the healthy pregnant population[40,50,51]. Complications associated with IBD activity at the time of conception include: Abortion, low birth weight (LBW), and premature births[50,52-55]. These complications again, are usually seen more often in CD patients than UC patients. A 2007 study, which evaluated birth outcomes in CD, showed an increased risk of preterm births but did not report any other adverse birth outcomes[56]. Khosla *et al*[36] showed that individuals with active CD at the time of conception had a 35% higher rate of miscarriage than women with CD in remission. Moser *et al*[57] demonstrated ileal disease was a particularly reliable index predicting LBW. Relapse of UC in pregnant women was also associated with LBW and preterm births[58]. Fortunately, increased risks for congenital abnormalities have not been demonstrated in neonates whose mothers had IBD compared to the general population[57].

Women also suffer from diverse forms of inadequate nutrition during active IBD due to decreased appetite and/or history of multiple small bowel surgeries, both of which can negatively affect absorption of specific nutrients[59,60]; protein losing enteropathies can also exacerbate these nutritional deficits.

Overall, IBD disease activity at the time of conception will play some role in the outcome of the pregnancy. It is therefore advisable to optimally control disease prior to conceiving to diminish the likelihood of adverse outcomes from disease flares, the need to suppress symptoms and the need to medicate all of which can be harmful to both the mother and fetus. Monitoring maternal nutrition and providing proper prenatal care giving heparin prophylactically to the gravid IBD patient, may help in prevention of VTE and malnutrition respectively.

IBD DIAGNOSIS DURING PREGNANCY

Imaging modalities used during evaluation of IBD during pregnancy can present risks to both mother and fetus and should be limited to ultrasound and MRI. Ultrasound avoids radiation exposure to the fetus and is always the preferred imaging compared to CT scan[19]. When more detailed imaging study is required, MRI without gadolinium contrast can be used to avoid teratogenicity, especially in the first trimester[19]. X-rays should be avoided throughout the pregnancy. Colonoscopy should be considered during pregnancy when life-threatening lower GI bleeds are observed or when surgical interventions are the only available option[61]. However, flexible sigmoidoscopy is considered safe during pregnancy and is the endoscopic procedure of choice[61,62]. Recommendations by the American Society for Gastrointestinal Endoscopy, state that procedure associated sedation is also safe during the 2nd trimester but is not recommended during the 1st and 3rd trimesters, except in emergent situations[63].

MANAGEMENT

Serious discussions and consideration need to be made regarding treatment modalities at different phases of pregnancy (Figure 1). IBD therapy is still evolving and the focus of IBD management has now moved away from short-term control of symptoms to more long-term suppression of disease mechanisms which alter the course and complications of IBD. Older drug classes such as 5-aminosalicylic acid (5-ASA) compounds (sulfasalazine, mesalamine, balsalazide), steroids, antibiotics and other immunomodulators [*i.e.*, 6-mercaptopurine (6-MP), azathioprine (AZA),

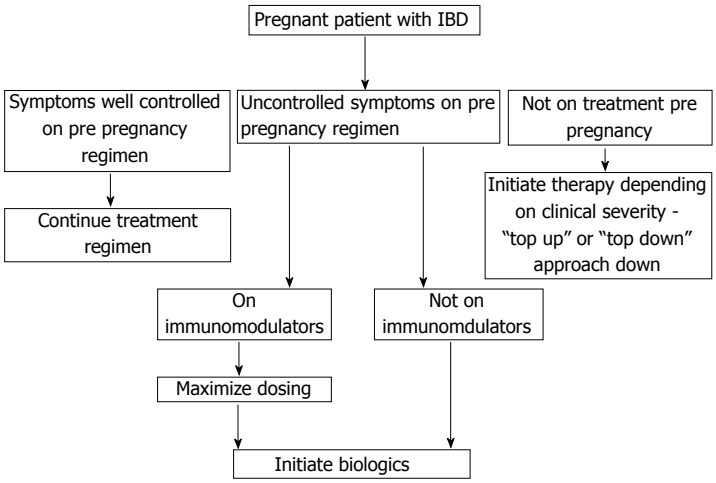

Figure 2 Treatment approach strategy. IBD: Inflammatory bowel disease.

cyclosporine and methotrexate] have given way to newer "biologic" agents. The biologics currently used for treatment of IBD are most often humanized monoclonal antibodies directed toward inflammatory cytokines such as tumor necrosis factor-alpha (TNF-α) or adhesive determinants on leukocytes (*e.g.,* integrin $\alpha4\beta7$), which bind counter-ligands expressed on inflammation-activated intestinal endothelial cells [like mucosal addressin cell adhesion molecule-1 (MAdCAM-1)].

Traditional IBD therapy has been to "step up" or incrementally increase treatment in a stepwise fashion finally introducing more powerful medications for IBD. It involves gradual addition of relatively benign drugs early in the course of IBD like aminosalicylates and steroids. When these drugs eventually fail, they are substituted by more aggressive therapies like immunomodulators and lastly biologics. The final step for escalation in the "step up therapy" approach was the introduction of biologic agents.

Over the last few years, clinical studies have suggested that aggressive medical therapies initiated earlier in the disease course helps to arrest the progressive nature of IBD (especially CD) leading to a "disease modifying effect"[64]. "Disease modifying" describes the slowing or stabilization of IBD progression, which leads to a more benign clinical picture, often eliminating the need for multiple or complex surgeries and importantly, reducing the overall lifetime risk for colorectal malignancies. The most convincing IBD therapy data now seems to favor the use of biologics; either alone or in combination with immunomodulator therapy. The more intensive combination therapy has gained widespread clinical acceptance of switching from the "step-up therapy" to the "top-down" approach (Figure 2). The same principle of this treatment approach also appears applicable during pregnancy, albeit with several safety considerations.

Currently, "top-down" therapy is our standard approach to IBD at Louisiana State University Health Sciences Center-Shreveport (LSUHSC-S), which provides state-sponsored free care to all patients despite the high annual cost of biologic drugs. LSUHSC-S has approximately 500 patient population with IBD, and we have used the "top-down" approach for over 15 years since biologics were first introduced (1998), starting with infliximab (INF)[65]. We commonly initiate therapy with biologics and immunomodulators in new IBD patients to obtain control of the disease early on and achieve remission, rather than allowing the disease opportunities to develop an aggressive course before taking action. Other institutions in the surrounding region have followed suit and now also use "top-down" approach. "Top-down" therapy, while highly effective may be altered during pregnancy based on safety considerations and disease severity.

The Food and Drug Administration (FDA) has established five distinguishing categories to designate the potential for a drug to cause birth defects if used during pregnancy[66] and reflect both documentation reliability and relative risk to benefit ratio considerations (Table 1).

AMINOSALICYLATES

There are several different formulations of aminosalicylates with differing levels of risk in the setting of pregnancy. Sulfasalazine (SSZ), the first aminosalicylate used in treating IBD, is an FDA category "B" drug. Since SSZ crosses the placenta, there are several concerns in pregnancy[67]. SSZ is known to inhibit folate synthesis[68] and impairs folate absorption potentially leading to fetal neural tube defects. This has raised some concerns about its safe use during pregnancy, but these appear to have been alleviated by several studies[36,56,68]. For example, Mogadam *et al*[69] performed a study on 181 pregnant women with IBD who were treated with SSZ. When matched with the overall population, these patients showed a net lower incidence of having adverse outcomes. Nørgård *et al*[56] also conducted a regional retrospective cohort study, which also showed no adverse outcomes in 17 CD patients who were treated with SSZ. Therefore, SSZ may be used in pregnancy with the requirement of patients taking folate supplements[68]. In fact, pregnant women taking SSZ are advised to increase

Table 1 Food and Drug Administration pregnancy category definitions[66]

Category	Definition
A	AWC studies in humans have failed to demonstrate a risk to the fetus in the all trimesters of pregnancy
B	Studies in animals have failed to demonstrate a risk to the fetus and there are no AWC studies in humans and the drug usage benefits outweigh its potential risks, or, there were no studies performed either in animals nor in humans
C	There are no AWC studies in humans but studies in animals have shown a side effect on the fetus, and the drug usage benefits outweigh its potential risks, or, there were no studies performed either in animals nor in humans
D	Investigational or marketing experience or studies in humans reported positive evidence of human fetus risk, but it can still be used in spite of its potential risks if there are extreme measures as in a life-threatening situation or serious disease in which safer drugs are ineffective or contraindicated
X	Studies in animals or humans have demonstrated fetal abnormalities, or, there investigational or marketing experience, or both reported positive evidence of fetal risk, and, the potential risks of drug usage clearly outweigh any possible benefit (for example, other forms of therapy are available)

AWC: Adequate and well-controlled.

their daily dose of folate to 5 mg[70], compared to women not taking SSZ who only require between 0.4 mg and 1 mg of folate daily[70].

Men on SSZ therapy for IBD should switch to an alternate drug treatment 3-4 mo prior to conceiving given SSZ's known suppressive effect on sperm[19]. One study compared the fertility of 10 men with IBD being treated with SSZ over 5 years to those of 19 control subjects. It showed that while SSZ treatment did reduce semen quality, this effect was reversed upon drug discontinuation[71].

Over the last two decades, preparations based on 5-ASA have remained a standard of IBD therapy, avoiding the use of SSZ, which has been associated with several serious adverse effects[72,73]. Both topical 5-ASAs and non-enteric coated formulations of 5-ASA are accepted to be safe in pregnancy. Bell *et al*[74] in a study of 19 pregnant patients with distal colitis on maintenance with topical 5-ASA therapies at the time of conception found that 5-ASA was safe and effective for managing distal colitis during pregnancy. Marteau *et al*[75] also conducted a study in which 123 pregnant IBD patients were monitored while taking between 1-4 g/d of mesalamine microgranules and found no serious complications during the course of pregnancy, nor did they find any adverse fetal outcomes.

Asacol (mesalamine) (Procter and Gamble Pharmaceuticals, OH, United States of America) and Asacol HD (mesalamine delayed-release tablet) (Procter and Gamble) have been moved from pregnancy category "B" to "C" due to the use of dibutyl phthalate (DBP), which is used in the coating of these medications. DBP has adverse effects on the male reproductive system[76] and has been linked to precocious puberty[77]. However, it is important to note that the doses used in these animal models which have linked to skeletal deformities and reproductive system disturbances were approximately 190 times greater than the maximum doses used in humans. Precocious puberty was also caused by DBP doses that were 10 × the maximum recommended levels[77]. There have been no studies to date showing increased birth defects in patients taking mesalamine. All other formulations of mesalamine, as well as other aminosalicylates are classified as category "B" drugs. No statistically significant increases in congenital abnormalities, stillbirths, spontaneous abortions, preterm deliveries, or LBW have been reported in association with Asacol use[78].

Mesalamine is available as an enema or suppository in the United States. Since enemas can reach the left colon, rectal therapies are typically considered for patients with disease activity found in locations from the rectum to the left colon. UC often starts in the rectum with 1/3 of patients with UC having disease restricted to the rectum, with another 1/3 having disease extending into the left colon, both of which can be reached by administration of drugs by enema[79]. CD affecting the rectum and sigmoid colon is also very commonly treated by enema[80]. Enemas are the treatment of choice for pregnant women suffering from distal UC because its therapeutic effect on the lining of the bowel is maximized while the systemic side effects are compartmentalized.

Mesalamine can penetrate the placenta and also enters into breast milk. SSZ itself may be present in breast milk at 30% of the maternal plasma concentration, and sulfapyridine (an SSZ metabolite metabolite) is found at 50% of the concentration in the maternal circulation[81]. Therefore, SSZ should be avoided, if possible, for mothers of premature infants or those less than 1 mo of age. There is also a concern for kernicterus, a bilirubin-induced brain dysfunction, as sulfonamides can displace bilirubin from albumin, though there are no reported cases in the literature[82,83]. There is however one case report of an infant with severe but reversible diarrhea after being breastfed by a mother using rectal 5-ASA[84]. Infant stool patterns should therefore be monitored if the mother is using mesalamine for IBD therapy and is breastfeeding[19]. There is a general preference for clinicians in limiting limiting the maximum dose to 2 g mesalamine daily during pregnancy based on an association with neonatal renal insufficiency in a 1994 report[85]. This dose is low compared to the normal non-pregnant patient maximum dose of 2.4 to 4.8 g daily depending on the preparation being used.

Using 5-ASA for treating CD is controversial. Ford *et al*[86] performed a meta-analysis to determine the effectiveness of 5-ASA in inducing and maintaining CD

remission. That study suggested that 5-ASA based drugs were superior to placebo at inducing remission in patients with active CD, with a reported "number needed to treat" of 11. Approximately 68% of CD patients treated with 5-ASA failed to achieve remission *vs* 75% of patients who were receiving placebo. 5-ASA had no benefit in maintaining remission. A relapse rate of 56% was found in patients treated with 5-ASA compared with 57% for patients on placebo treatments. In a 2011 study, mesalamine and budesonide were found to be equally efficacious in inducing remission in patients who had mild to moderate activity CD[87]. Remission rates were similar between those who were receiving budesonide and mesalamine (70% *vs* 62%). Therefore, 5-ASA, based on studies, is not as effective in the treatment of CD and its use in treating pregnant women is unclear.

5-ASA is however, an optimal drug for inducing and maintaining remission in mild to moderate UC[88]. Trallori *et al*[72] conducted a safety study on 5-ASA use for treating UC during pregnancy. All patients in the study were in clinical remission from UC at the beginning of pregnancy and were receiving regular maintenance therapy with 1.2 g/d of 5-ASA. It was noted that 5-ASA usages during pregnancy did not affect the course or outcome of pregnancy, but it could prevent disease relapse of UC. Therefore, in general, aminosalicylates like 5-ASA can be used in pregnant women with IBD, but caution should be still advised regarding dosing.

ANTIBIOTIC USE FOR IBD DURING PREGNANCY

Metronidazole is an antimicrobial drug and a pregnancy category "B" drug that works to limit proliferation of anaerobic bacteria and is used for treating active colonic and perianal CD[89]. There have also been some benefits seen with the combined use of metronidazole and ciprofloxacin in treatment of pouchitis (inflammation of the ileal pouch), which is a long-term complication of IPAA surgery for UC[90,91]. Metronidazole should however be avoided in the 1st trimester as it has been linked to an increased rate of cleft lip/palate in a 1998 study[92]. Metronidazole teratogenicity has also been demonstrated in animal models (when used in the same developmental stage equivalent of the 1st trimester) but there is apparently less risk of teratogenicity in the 2nd and 3rd trimesters. A study of metronidazole in rats also demonstrated a depression of plasma gonadotropins (luteinizing hormone and follicle stimulating hormone), testosterone, testes weight, and spermatogenesis[93].

Metronidazole is also incompatible with breastfeeding as breastfed infants of mothers taking metronidazole have exhibited diarrhea, secondary lactose intolerance, and Candidiasis[94,95]. Women receiving a single dose of metronidazole may resume breastfeeding after 12-24 h[96].

Ciprofloxacin, another antibiotic used to control flares in IBD, carries a pregnancy category "C" rating. There is increased uptake of ciprofloxacin in bone tissue which can cause arthropathy in children and therefore, its use is discouraged during pregnancy[97]. Limited data exists on the safety of ciprofloxacin use during breastfeeding. It is recommended that women receiving a single dose of ciprofloxacin can resume breastfeeding after 48 h[98]. Conversely, there have been studies that considered whether short-term ciprofloxacin use is acceptable during breastfeeding as it decreases in breast milk over time. One study involving 10 lactating women who were given ciprofloxacin in 3 doses every 12 h estimated that an infant fed only by breast would receive a maximum of 0.57 mg/kg daily dose of the drug. The dosage that an infant would receive is low compared to the levels used to treating newborn infants (10 to 40 mg/kg)[99-102]. Another study showed that an infant nursing from a woman being treated with ciprofloxacin for 10 d had no measurable ciprofloxacin in the infant's serum (< 30 μg/L) 2.7 h after breastfeeding[103]. Therefore, modest or acute use of ciprofloxacin appears relatively safe for use in most pregnant women and even nursing mothers. In men, ciprofloxacin does not seem to affect sperm quality, however, the function of the accessory glands (including the seminal vesicles, prostate gland, and bulbourethral glands) can in some cases be modified[104].

Rifaximin, a broad-spectrum antimicrobial, has shown to be useful in treating pouchitis and small bowel bacterial overgrowth in IBD. Rifaximin is used in IPAA, stricturing small bowel disease, and in patients with a history of multiple bowel surgeries, which can contribute to intestinal stasis. Rifaximin is relatively new to both the clinic and market place and is listed as a category class "C" drug during pregnancy since its fetal effects and transfer in breastfeeding is still unclear. Studies have shown rifaximin-induced birth defects in animals, including abnormalities in bone maturation and cleft palate[105], however another study failed to demonstrate as strong evidence for birth defects in rats[106]. The fertility of male rats was not affected the consumption of rifaximin[107]. Based on the limited data for rifaximin in pregnancy, this drug cannot yet be safely recommended to pregnant women with IBD. Amoxicillin/clavulanic acid, are pregnancy class "B" drugs, which are a safe alternative option for use in treating pouchitis. Unlike rifaximin, amoxicillin/clavulanic acid failed to show birth defects in both a prospective controlled study[93] and a population-based case-control study[108].

IMMUNOMODULATORS

6-MP/AZA

AZA is a prodrug that is metabolized to 6-MP, which is then later metabolized into several metabolites including the active metabolite 6-thioguanine (6-TG) and the inactive metabolite 6-methylmercaptopurine (6-MMP). Therapeutic efficacy in IBD is related to 6-TG levels, while high 6-MMP levels are correlated with liver and bone marrow toxicity. Several strategies have been utilized to try to optimize 6-TG levels while minimizing 6-MMP levels when administering thiopurines to patients that

would not otherwise tolerate these drugs. 6-TG levels between 230 and 400 pmol/8 × 10^8 red cells correlate with response and remission of IBD but levels which exceed 400 pmol/8 × 10^8 red cells correlate with bone marrow suppression. 6-MMP levels over 5700 pmol/8 × 10^8 red blood cells have been linked with hepatotoxicity (measured by release of liver enzymes)[109,110].

AZA and 6-MP, both purine analogs, are pregnancy category "D" drugs, since gestational animal studies show a defined association with birth defects. Because of their cytotoxicity, and potential risk of birth defects, they should be used with great care during pregnancy[111]. Immunomodulators alter the activity of the immune system in order to decrease the body's inflammatory response and cause an overall immunosuppressive effect. AZA and 6-MP both target the expansion of T lymphocytes, and suppress lymphocyte survival to depress inflammatory responses in IBD. These drugs effectively establish and maintain remission of IBD and are especially helpful in patients who have do not respond strongly to milder therapies (such as aminosalicylates), or are steroid-dependent[112]. There are variable and conflicting data on side effects of immunomodulators in humans. In a 2006 study, Cleary *et al*[113] studied 476 women, the majority of which had IBD. This study found a 3 × increase in the frequency of cardiac defects in children of women who took these immunomodulators early in their pregnancy. It was also found that there was a risk for increased preterm deliveries, LBW, and "small for gestational age" babies in AZA treatment associated pregnancies. A meta-analysis performed in 2012 showed that men fathering children who were exposed to thiopurine around the time of conception did not increase rates of congenital birth defects and so did not recommend discontinuation of treatment in men[112]. However, if there is a medical history of unexplained infertility or miscarriages, men should discontinue taking thiopurines at least four months before conception to improve fertility[114].

Conversely, Goldstein *et al*[111] studied 189 women who took AZA for different indications and later contacted a birth defects registry following delivery. That study failed to find a statistically significant increase in the rate of malformations (compared to 230 women who contacted the same service that were not on any potentially teratogenic treatment). However, the Goldstein study did confirm a statistically significant difference in premature birth and LBW associated with AZA. Akbari *et al*[112] 2012 performed a meta-analysis and found that exposure to thiopurines during conception was not clearly associated with birth abnormalities and concluded that maternal use of thiopurine was not associated with low LBW, but confirmed an increase in the risk of preterm births associated with thiopurine exposure. In fact, preterm birth had an increased odds of 70% and was the only outcome found to be significantly affected by thiopurine use. Again, whether this is directly related to thiopurine use, or simply the result of the more more severe disease state in which thiopurines are more often required is unclear[112]. For example, other studies have demonstrated a more severe disease course in IBD was also significantly associated with preterm births not related to drug loading[50,112]. Furthermore, data from the Pregnancy in Inflammatory Bowel Disease and Neonatal Outcomes (PIANO) registry in 2012 found no evidence for an increased frequency of gestational or fetal complications in Group A (6-MP/AZA) as compared to other groups. Data presented at Digestive Disease Week 2014 revealed improved milestone achievement in babies of mothers in Group A[115]. These milestones were statistically significant for social interaction at 24 mo (50.75 *vs* 47.34, $P = 0.04$), problem solving 36 mo (mean 52.04 *vs* 48.66, $P = 0.05$), and problem solving 48 mo (mean 59.92 *vs* 57.66, $P = 0.02$).

Cancers Et Surrisque Associé aux Maladies inflammatoires intestinales En France (CESAME), a prospective cohort population-based study included 11006 women that followed patients between 2004-2007 in France, had a primary goal to determine the risk of malignancies in patients on thiopurines[116]. Coelho *et al*[117] ran a sub study that was added to the CESAME in 2005, which included 86 thiopurine-treated pregnancies compared to 129 IBD controlled pregnant patients. One of the main findings of the study was that there were no increases in congenital abnormalities in thiopurine-treated pregnancies[117].

Therefore we believe that benefits from maintenance on these immunomodulators during pregnancy may in some cases outweigh potential fetal risks. AZA and 6-MP are also believed to be generally compatible with breastfeeding. So far studies have demonstrated only very low levels of the drugs transferred in breast milk and thus clinically insignificant concentrations accumulated in healthy breastfeeding infants. However, Mahadevan *et al*[118] suggests caution in infants with weaker than normal immune systems. We agree with this comment since these drugs may intensify an already immunodeficient state due to their mechanism of action, which could become more serious in the setting of perinatal pathogen exposures.

In conclusion, several different outcomes are possible with the use of thiopurines with the worst being a potential increase in congenital malformations. Consequently some, caution is warranted with the use of these drugs. Despite limitations of these studies *e.g.*, relatively small sample sizes, or failure to consider the disease activity of IBD, these data are consistent with very moderate thiopurine use as potentially safe during pregnancy[56,57,117].

Methotrexate

The action of Methotrexate (MTX) in IBD involves several mechanisms. MTX interferes with DNA synthesis producing a suppression of T-cell expansion and also diminished immune cell persistence. MTX also inhibits both lymphocyte and endothelial cell expression of intercellular adhesion molecule-1 (ICAM-1) to

lower leukocyte extravasation and its accompanying inflammation. MTX is a pregnancy category X drug, and acts as a folic acid antagonist that has been previously linked with several forms of birth defects affecting fetal organ development. Although MTX has beneficial anti-inflammatory actions in IBD, this particular drug is considered to be so dangerous in the setting of pregnancy that women should be advised to wait at least 6 mo after discontinuing MTX before resuming any attempts to conceive. MTX is also not to be used during breastfeeding as it is passed into breast milk. For men on methotrexate, one study which considered 42 pregnancy outcomes involving paternal exposure to MTX around the time of or up to 3 mo prior to conception concluded that this treatment did not enhance the risk of birth defects[119]. However, given the limited data available to date, some health providers still recommend that men also wait at least 3 mo after discontinuing MTX before attempting to conceive based on the depressive effect of MTX on spermatogenesis leading to oligospermia[120].

IMMUNOSUPPRESSANTS

Cyclosporine A/tacrolimus

Cyclosporine A (CsA) and tacrolimus are immune suppressing drugs, which are listed as pregnancy category "C" drugs. The majority of CsA recommendations have been derived from transplant experiences. CsA blocks IL-2 formation by helper T-cells. Binding of CsA to cyclophilin, inhibits calcineurin, a cytoplasmic phosphatase which participates in the control of T-cell activation. CsA also indirectly inhibits the function of B-cells by suppression of T-helper cells. CsA has a more rapid onset of clinical action than either 6-MP or AZA, which can require 3-6 mo before showing detectable disease suppressing activity[121]. Patients with CD who respond to CsA show rapid improvements within 2-3 wk[121]. Clinical improvements have also been seen within 1-2 wk following the initiation of therapy with CsA in severe UC[121].

Tacrolimus, a macrolide antibiotic, has immunomodulator properties like CsA but two orders of magnitude more potent than CsA. One advantage of tacrolimus is that it doesn't require bile or mucosal integrity for its absorption. As a result, tacrolimus can be used in patients with small bowel involvement, including both CD and UC[122]. So far these drugs have not yet been linked to increased rates of congenital abnormalities. However, increased rates of maternal and perinatal complications have been reported in kidney transplant recipients on different regimens of immunosuppressant medications, including those using either cyclosporine and tacrolimus[123]. These drugs are further restricted during breastfeeding as they develop high concentrations in breast milk, with the potential for perinatal immune suppression. However, according to Nielsen *et al*[97], tacrolimus is excreted into breast milk at only 0.05% of the maternal dose suggesting that it does not need to be discontinued while breastfeeding. Cyclosporine is also weakly transferred/passed into in breast milk and is possibly safe while breastfeeding, although caution should be recommended and exercised based on its potential for immunosuppression[97]. At LSUHSC-S we routinely discuss the such risks with our patients before initiating therapy with either CsA or tacrolimus.

Steroids

As mentioned earlier, biologic agents and immunomodulators remain key therapies in achieving remission of IBD. However, during acute flares many practitioners often fall back upon the use of corticosteroids to provide patients with temporary relief from their symptoms. Due to side effects associated with their long term use, corticosteroids (prednisone and methylprednisolone) are used only sporadically and are not used for maintaining remission. Corticosteroids, specifically prednisone, are considered a pregnancy category "C" drug. Prednisone use in pregnancy has been associated with development of when used within the first month after conception or during the first trimester[98]. However, no evidence currently links glucocorticoid use with major malformations[63,124,125]. Corticosteroids have also further been linked to premature rupture of placental membranes and adrenal suppression (so far seen only in mothers observed in transplant studies)[19]. Corticosteroids are however usually thought to be compatible with breastfeeding since only very low levels of steroids are transferred into the breast milk and the risks to the neonate appear to be considered to be very low clinically[126,127]. Consequently, no absolute guidelines/recommendations have yet been developed regarding timing of breastfeeding around administration of the corticosteroids[19].

Biologics

Biologics are now widely used for the treatment of IBD. However, since they function by targeting inflammatory cytokines or adhesive determinants, they may not be highly effective for treating acute flare-ups because they frequently can often weeks to months to become effective. Biologics are broadly divided into TNF-α inhibitors and non TNF-α inhibitors. TNF-α inhibitors are often humanized recombinant IgG1 monoclonal antibodies that neutralize TNF-α with high affinity. INF (Remicade, Janssen), adalimumab (ADA) (Humira, Abbvie) and certolizumab-pegol (CZP) (Cimzia, UCB) are currently the most commonly used drugs of this type in our practice, and are considered pregnancy category "B" drugs.

The second class includes biologics like Tysabri (natalizumab, Biogen), which is a class IgG4 monoclonal antibody IgG4 which has been "humanized" to more closely resemble human IgG that inhibits leukocyte binding mediated by the integrin α4 adhesion molecule. IgG4 antibodies are not as efficiently transported across the placenta as IgG1, however fetal levels of IgG4 still exceed those in the maternal circulation. The risk

of congenital malformations has not been seen to be increased in a study of pregnant patients with CD or multiple sclerosis who were treated with Tysabri during their first trimester[128]. Vedolizumab (Entyvio, Takeda) is the latest biologic (approved by FDA on May 2014) used for treating IBD. Vedolizumab is a humanized monoclonal antibody (IgG1) that binds to the human $\alpha4\beta7$ integrin (expressed on the surface of T cells), thereby inhibiting T cell adhesion presumably to MAdCAM-1[129]. However vedolizumab and natalizumab are not equivalent. Although vedolizumab is listed as a category B agent, natalizumab is listed as a class C agent and therefore carries an unknown level of and is not recommended for use during pregnancy. Newer biologic therapies under development are currently in different phases of clinical trials and target other cytokines. For example, ABT-874 (Briakinumab, Abbott) and CNTO 1275 (Ustekinumab, Centocor) are both anti-IL-12/-23 antibodies and tocilizumab is anti-IL-6 antibody[130]. Briakinumab has no preganancy category assigment yet, while Ustekinumab is listed a category B agent. Tocilizumab is listed as a category C drug based on abortifacient potential of this agent; its use should be terminated prior to and during pregnancy.

Biologics like INF and ADA cross the placenta and do so at the greatest extent during the third trimester. Mahadevan *et al*[131] evaluated 31 pregnant women with IBD being treated with INF, ADA or CZP and compared concentrations of these biologics in infant and cord blood with concentrations in the mothers circulation. The levels of INF and ADA were elevated in infant and cord blood compared to their respective maternal levels with the median level of INF in cord blood being 60% higher than that of the mother. Similarly the median concentration of ADA found in cord blood was found to be 53% higher than that in the maternal circulation. The level of CZP was lower in neonatal circulation and in cord blood than in the mothers blood (median level of CZP in cord was by comparison only 3.9% of that within the mother). This may reflect the fact that CZP is, not actively transported across the placenta, because it lacks an Fc domain to bind to the FcR and is confined to the maternal compartment[131]. In an independent clinical study on 10 pregnant women with IBD, CZP levels were measured in maternal, fetal, and cord blood *via* ELISA on the day of birth. CZP concentrations in fetal and cord blood were seen to be low, reduced in concentration by 75% as compared to levels in maternal blood thereby indicating low placental transfer[132].

While highly effective, there have been reported cases of infections following live vaccines in newborns following INF. For instance, there has been one case report of a fatal BCG infection in an infant who received the Rotavirus vaccine at 3 mo whose mother had been on INF as therapy for CD[133]. As such, great caution is recommended with the use of any live vaccines (particularly rotavirus vaccine) given during the first 6 neonatal months for any infants potentially exposed in utero to maternal biologics, since some biologics can cross the placenta. According to Nielsen *et al*[97], the vaccine schedule should be initiated 2-3 mo post-natally, as this should provide enough time for biologics to become sufficiently cleared to accomodate immunization. In our practice, we typically wait until the 6th month post-natally to give any live vaccines to infants potentially exposed to biologics in utero.

The PIANO study registry, a prospective analysis of 1315 currently enrolled pregnant women as of (March 2014) at 31 IBD centers around the country, is intended to determine whether complication rates are significantly higher among women with IBD and their offspring who may be exposed to AZA, 6-MP or anti-TNF agents biologics during pregnancy, compared to women with IBD who do not take these medications. Pregnant women with IBD were registered for the study prospectively and evaluated at each trimester, at delivery and during for the first 4 years of the child's life. Patients have been divided into groups based on their patterns of exposure from conception through delivery. The groups were either "unexposed" receiving neither thiopurines nor anti-TNF agents, those receiving 6MP/AZA, those receiving INF, ADA or CZP and the last group receiving combination therapy with thiopurines plus anti-TNF. Newborn complications during the first year of life, alterations in developmental milestones, maternal medications, disease activity and complications encountered during pregnancy are all being recorded. Of the patients studied so far, those on biologics alone had a slightly increased rate of spontaneous abortions and C-section deliveries. These observations may however be confounded by the fact that patients with more severe disease were given biologic therapies and may already have clinical stress from advanced IBD. Of the patients studied, those on combination therapy (biologics and immunomodulators) had slightly elevated rates of preterm birth and infections at 12 mo. However, updated data from the registry later showed (as of April 2013) that relative risk at 1 year, adjusted for premature birth was 0.9 for biologics alone, and 1.0 for women using combination therapy[134]. The final results of the PIANO registry are pending. Thus far the data are reassuring for the application of immunomodulators and biologics in pregnant IBD patients.

The PIANO registry as of 2012 studied 291 patients exposed to biologic therapy alone and 75 patients exposed to biologics and immunomodulators[117], and found no increase in congenital abnormalities, infections, or developmental delays which could be clearly attributed to these drugs. Interestingly in the combination group, when CZP was left out of the analysis and only INF and ADA were analyzed individually, there was an increase in infections in the combination therapy group[117]. This suggested that the presence of placentally transferred IgG1 antibodies in INF and ADA treatment groups might have contributed to an increased infection risk. These antibodies can persist in the neonate for up to 6 mo. However, most of the infections occurred between months 9 and 12, a time when drug levels should have

been undetectable in the infants, and further research will be needed to determine if these infections chronic immune system development in these children[19]. CZP does not, however, actively cross the placenta and infection risk was not noted in CZP patients[132]. Data presented at the 2013 American Gastroenterological Association (AGA) Spring Postgraduate Course (Orlando, FL, United States of America) has suggested that among the women studied, there has been no report of increased risk of serious infections seen in the newborns of mothers with IBD who had been treated with TNF inhibitors.

Based on the information discussed, many clinical experts in this field would agree that continuing biologic therapy during pregnancy is likely to be safe with a favorable benefit/risk ratio. Despite a slightly higher infection risk in children of mothers treated with INF and ADA, if possible biologics should not be switched during pregnancy as the switch could precipitate disease flares with worse overall disease outcomes. Based on our knowledge of placental transfer, timing of biologic drug dosage can be manipulated to avoid transfer to the child while controlling disease in the mother. Ng *et al*[19] therefore suggest administering the final dose of INF at 32 wk and to continue CZP per usual dosing schedule. Since ADA requires biweekly dosing, the last dose would therefore be given at 36 wk. According to Yiu *et al*[135] paternal exposure to anti-TNF-α therapy has not been shown to be teratogenic. Interestingly, anti-TNF-α therapy has actually however been shown to improve male fertility by increasing sperm count and motility.

More studies are also needed regarding breastfeeding while using biologic agents. The available data suggests that transfer of biologic agents into breast milk may be low. Few data are available regarding the fetal absorption of biologics transferred into breast milk and more studies are needed to draw meaningful clinical conclusions. At present, it is thought that biologics, being very large proteins, would weakly transfer into breast milk. The small amount that does pass into breast milk is unlikely to substantively penetrate the baby's circulatory system as orally consumed biologics may ultimately be poorly absorbed by the gut. However, premature infants may absorb more drugs through breast milk due to potentially having digestive tracts that are more permeant to large molecules like biologics[136]. Thus, the decision to breastfeed during biologic use in IBD is still unclear and should be made with some consideration for the health of the infant and the preferences of the mother.

OUR EXPERIENCE WITH IBD THERAPY AND THE EVOLUTION OF BIOLOGICS AT LSU HEALTH

In 1998, "step up therapy" for IBD was more a norm than a choice made by physicians treating IBD patients. At that time, Remicade had just been approved for use in IBD patients, but was not yet popular for this indication[65]. Physicians at LSUHSC-S were among the first in our state to use Remicade for the treatment of CD patients. The rationale behind the decision to use biologics in our patient class involved several stages. The prime objective was to rapidly decrease the inflammatory process and prevent ongoing accumulating damage to the bowel. This led to the concept of classifying our patients as "early" or "late" CD based on severity of disease, rather than simple and complex disease. We started by using biologics in our "late" CD patients and the results were extremely encouraging. However, there were instances where patients who appeared to be in remission clinically had a contrasting picture of disease activity based on endoscopic visualization. This led us to include endoscopic evidence of remission in addition to symptomatic clinical improvement, to objectively describe success of biologic therapy in IBD patients.

The positive results noted with use of biologics in "late-phase" CD patients encouraged us to incorporate biologic use in the early or recently diagnosed CD patients as well. The rationale for this approach was to halt the inflammatory process early enough in disease course so as to decrease or arrest disease progression into the severe morbidity and complications seen in late-phase disease.

Similarly, our goal for choosing appropriate treatment regimens for our pregnant IBD patients was disease control, as long as we were assured that medication regimens used to achieve therapeutic control would cause no harm to the fetus. With no evidence to suggest any adverse fetal outcomes and with biologics available and promising outcomes with their use in non-pregnant IBD patients, we decided to use biologics for uncontrolled disease in pregnant patients. We explained the potential risks and benefits to our patients who chose to be treated with these medications. Fortunately, we have not experienced any adverse outcomes to date in pregnant patients nor their fetuses as a result of treatment. Disease control with use of biologics has been excellent, and most patients have remained in remission throughout pregnancy. Therefore, we have continued to use the "top down" approach for initiation of therapy in our pregnant patients.

At LSUHSC-S, we encounter IBD patients in different phases of disease severity who may wish to conceive or already be pregnant. Our approach for management of pregnant IBD patients varies with respect to them being treatment naïve or already on some form of treatment for their disease.

As stated earlier, patients who have not been on IBD treatment are classified as per their disease activity. For patients well controlled on their regimens, we continue them on the same drug treatments. For patients with uncontrolled pathology we use "step up" approach, *i.e.,* maximizing their immunomodulator regimen if already on one. If this fails we prefer initiation of biologics. In an event of no response or suboptimal response to one biologic agent, we switch the patient to a different biologic, preferably within same class (anti-TNF or anti-

integrin). For instance, patients can be switched between INF, ADA and CZP. Similarly, for patients on a biologic prior to their pregnancy found to be with uncontrolled disease are switched from one biologic to another.

As of October 1, 2012 all CD patients at our institution were administratively directed to receive CZP for their biologic therapy needs. Another study in progress at our institution done by Motlis *et al*[137], is currently evaluating both short and long-term outcomes of CD patients diagnosed with moderate to severe CD treated with INF or ADA as they undergo transition to CZP treatment. So far most patients exhibited a good clinical response to CZP and had stable disease at 1 year. This in addition to the relative safety of CZP with no placental barrier transmission has made the use of CZP popular for our pregnant patients as well. Full results of this study will be submitted later this year.

The decision to initiate immunomodulator and/or biologic therapy should always be preceded by a thorough clinical workup in addition to extensive family and patient counseling regarding the risks and benefits of these medications these conversations should address each patient's disease severity and underlying co-morbidities. IBD patients definitely need to be pre-screened for Hepatitis B and latent tuberculosis according to standard guidelines for all IBD immunomodulators. No guidelines exist for HCV screening in these patient populations. However, we also routinely screen for hepatitis B, C and latent tuberculosis as a part of workup before initiation of therapy with these agents. A tuberculin skin test or interferon gamma release assay (Quantiferon® gold assay) is used to detect latent TB as there is a much higher incidence of reactivation of latent infection upon or following initiation of biologic therapy and subsequent immune suppression. Consideration of use of immunomodulators like thiopurines also requires evaluation for thiopurine methyl transferrase (TPMT) activity. Phenotypic TPMT enzyme activity is measured in red blood cells and classified as - low, intermediate and normal reflecting 0.3% (homozygous for mutations of TPMT), 11% (heterozygous for mutations of TPMT), and 88.7% (wild type TPMT) in the population respectively. Patients found to have either low to intermediate activity are at risk of decreased clearance of the drug and therefore are more prone to its adverse effects. TPMT testing helps gastroenterologists to make more judicious decisions regarding the use of these medications in specific TPMT phenotype patient groups.

CONCLUSION

IBD remains characterized as a group of chronic and idiopathic inflammatory conditions of the gut exhibiting relapsing and remitting episodes. At present it is been estimated that as many as 1.4 million people in the United States have been diagnosed with a form of IBD[2]. IBD in pregnancy presents several important challenges for gastroenterologists, women with IBD, the unborn fetus, and family members. Physicians particularly gastroenterologists, must often assist in making complicated and personal decisions on conception, pregnancy, and breastfeeding-postnatal considerations, which need to be weighed to optimize the course of pregnancy and long-term postnatal risk. At the same time, controlling disease and minimizing flares in IBD reduces disease severity and helps to maintain pregnancy but still carries risks to both mother and fetus. Future therapies that are more mechanism-specific (*e.g.,* biologics) may improve clinical outcomes with overall lower to both the mother and fetus and may replace several currently used agents which have significant off-target effects.

Here at LSUHSC-S our approach for management of pregnant IBD patients depends on their treatment status (naïve *vs* being treated) and their response to the treatment (uncontrolled disease activity *vs* remission). Patients who have not been on treatment are classified based on their disease activity. For patients well controlled on their regimens, we generally try to maintain them on the same course of therapy. For patients with uncontrolled pathology we use a "top down" approach. By working closely with the patients, assessing benefits and risks of various treatment options, patient and physicians can together make prudent decisions in the management of IBD in pregnancy.

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REVIEW

Diagnosis and therapy of non-variceal upper gastrointestinal bleeding

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Abstract

Non-variceal upper gastrointestinal bleeding (UGIB) is defined as bleeding proximal to the ligament of Treitz in the absence of oesophageal, gastric or duodenal varices. The clinical presentation varies according to the intensity of bleeding from occult bleeding to melena or haematemesis and haemorrhagic shock. Causes of UGIB are peptic ulcers, Mallory-Weiss lesions, erosive gastritis, reflux oesophagitis, Dieulafoy lesions or angiodysplasia. After admission to the hospital a structured approach to the patient with acute UGIB that includes haemodynamic resuscitation and stabilization as well as pre-endoscopic risk stratification has to be done. Endoscopy offers not only the localisation of the bleeding site but also a variety of therapeutic measures like injection therapy, thermocoagulation or endoclips. Endoscopic therapy is facilitated by acid suppression with proton pump inhibitor (PPI) therapy. These drugs are highly effective but the best route of application (oral *vs* intravenous) and the adequate dosage are still subjects of discussion. Patients with ulcer disease are tested for *Helicobacter pylori* and eradication therapy should be given if it is present. Non-steroidal anti-inflammatory drugs have to be discontinued if possible. If discontinuation is not possible, cyclooxygenase-2 inhibitors in combination with PPI have the lowest bleeding risk but the incidence of cardiovascular events is increased.

Key words: Gastrointestinal bleeding; Gastric ulcer; Duodenal ulcer; Endoscopy; Endoscopic therapy

Core tip: Non-variceal upper gastrointestinal bleeding (UGIB) is still accompanied by a significant mortality rate in older patients. Causes of UGIB are ulcers, Mallory-Weiss lesions, erosions, esophagitis or angiodysplasia. Endoscopy offers the localisation of the bleeding site as well as a variety of therapeutic measures. Patients with peptic lesions are effectively treated with proton pump inhibitors. *Helicobacter pylori* is a risk factor for the genesis of peptic ulcers and eradication therapy should be given if it is present.

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INTRODUCTION

Upper gastrointestinal bleeding (UGIB) is defined as bleeding proximal to the band of Treitz. Approximately 10% to 20% of bleeding episodes are from esophageal, gastric or duodenal varices or from portal hypertensive gastropathy related to portal hypertension. This article will deal only with non-variceal UGIB. Table 1 gives an overview of possible causes of UGIB.

The reported annual incidence of UGIB ranges from 48 to 160 cases per 100000 adults[1-6], with a mortality from 10% to 14%[4,7]. Besides the advances in endoscopy and intensive care medicine, these mortality rates have not changed very much during the last decades[3,4,7]. Most likely, this is caused by the fact that patients with UGIB are nowadays older and more likely to have relevant co-morbidity than in the past. Accordingly, the mortality rate in patients under the age of 60 years and no relevant co-morbidity is almost zero[8].

Clinical signs of UGIB are vomiting of blood (haematemesis) and/or passage of black, tarry stools (melena). In some cases, melena might be caused by bleeding from the small intestine downwards the duodenum. Tarry stools are usually seen if more than 50 mL to 100 mL of blood is lost per day. The passage of bright red blood per rectum (haematochezia) could be caused by severe, brisk bleeding. Non-specific signs like fatigue, prostration or shortness of breath could be caused by occult bleeding. Typical laboratory findings are anaemia, low MCV, low ferritin and an increase in the reticulocyte count. Patients are haemodynamically affected (hypotension, tachycardia) if more than 10% to 20% of the total intravascular blood volume is lost. Several clinical signs provide clues to the localisation of the bleeding: Melena and/or haematemesis indicate UGIB. Haematochezia indicates lower gastrointestinal bleeding or massive bleeding in the upper GI-tract, typically distal of the pylorus. Ascites and/or jaundice make the diagnosis of liver cirrhosis very likely and point at variceal bleeding. Special attention should be paid to the medical history of the patient: Non-steroidal anti-inflammatory drugs (NSAID) or acetyl-salicylic acid (ASA) make bleeding from ulcers or severe erosive gastritis likely. The presence of an aortic prosthesis increases the risk of an aorto-intestinal fistula.

Patients who present with signs and symptoms of UGIB should first be stratified into low or high risk[8,9] to guide further treatment. The stratification is done on the basis of clinical, endoscopic and laboratory criteria using prognostic scales. The most used scores in clinical practice are the Blatchford *et al*[10] and Rockall *et al*[11] scores. Tables 2 and 3 give a concise overview of the two scores. Both scores allow the identification of patients with low risk, meaning that these patients do not require emergence endoscopy and could safely be managed as outpatients. Clinical criteria include pulse, blood pressure, melena, cardiac failure, syncope and evidence of liver disease. Placement of a naso-gastric tube and aspiration of blood make the diagnosis of an acute bleeding very likely. Haemoglobin and blood urea levels are laboratory criteria.

Every patient who is haemodynamically instable should first be stabilized in an intensive care-unit before endoscopic diagnostic or therapy is initiated.

The risk of re-bleeding is based on the Forrest classification[12] (Table 4) and endoscopic findings like the localisation of the bleeding and type of bleeding (ulcer, cancer or variceal bleeding).

BLOOD TRANSFUSION

In contrast to immediate and sufficient volume resuscitation, the timing and amount of blood transfusions in patients with UGIB is a subject of intense discussion. It is widely accepted that patients with a haemoglobin level of 7 g/dL or less should receive a transfusion, whereas it is rarely indicated in patients with a haemoglobin level of 10 g/dL or more. The threshold for each patient has to be individually defined and depends on factors like age, haemodynamic status, markers of tissue hypoxia and presence of coronary artery disease. A meta-analysis of studies in a heterogeneous group of critically ill patients (trauma, surgery, intensive care)[13] showed that transfusion was associated with an increase in mortality, multi-organ failure as well as an increase in nosocomial infection and acute respiratory distress syndrome. Yet, confounding factors of this meta-analysis by the need for transfusion itself could not be excluded.

Most national[8] and international guidelines[14,15] on UGIB recommend a target level for blood transfusions in patients without signs of tissue hypoxia and/or coronary artery disease in the range of 7 g/dL to 9 g/dL. This was confirmed by a trial in critical care patients that demonstrated a lower mortality in patients with a haemoglobin level of 7 g/dL to 9 g/dL compared to patients with a haemoglobin level of 10 g/dL to 12 g/dL[16]. However, in the context of UGIB the study has to be interpreted with caution since patients with UGIB were excluded in this study. A lower mortality in patients with UGIB and a restrictive transfusion regimen (haemoglobin below 7 g/dL *vs* haemoglobin below 9 g/dL) was shown in a recent trial including 921 patients[17]. In patients with massive bleeding the haemoglobin level is of limited use only, since there is no time for haemodilution and a drop in haemoglobin concentration to develop. Therefore, patients with massive bleeding should be managed with transfusion of blood, platelets, clotting factors and volume resuscitation according to local protocols for managing massive bleeding.

Table 1 Causes of upper gastrointestinal bleeding

Peptic ulcer	
Oesophagitis	
Drug-induced mucosal damage (NSAID)	Ulcer
	Erosion
Traumatic or postoperative lesions	Mallory-Weiss lesion
	Arterio-intestinal fistula
Malignant tumor	
Sequelae of portal hypertension	Oesophageal varices
	Varices of the gastric fundus
	Portal hypertensive gastropathy
Vascular anomalies	Dieulafoy lesion
	Gastric antral vascular ectasia (GAVE syndrome)
	Angiodysplasia
	Rendu-Osler-Weber syndrome (hereditary hemorrhagic telangiectasia)
Bleeding from the hepato-pancreatico-biliary system	
Bleeding from a duodenal diverticulum	

NSAID: Non-steroidal anti-inflammatory drugs.

Table 2 Glasgow-Blatchford Score[10]

Admission risk marker	Score component value
Blood urea (mmol/L)	
6.5-8.0	2
8.0-10.0	3
10.0-25	4
> 25	6
Haemoglobin (g/dL) for men	
12.0-12.9	1
10.0-11.9	3
< 10.0	6
Haemoglobin (g/dL) for women	
10.0-11.9	1
< 10.0	6
Systolic blood pressure (mmHg)	
100-109	1
90-99	2
< 90	3
Other markers	
Pulse ≥ 100 (per minute)	1
Presentation with melaena	1
Presentation with syncope	2
Liver disease	2
Cardiac failure	2

The Blatchford score has to be used before endoscopy. The score component values are added up for each component. A score of 0 is the cut-off with any patient scoring > 0 at risk of requiring an intervention.

ANTICOAGULATION

A reasonable amount of patients with UGIB is on a medication with anticoagulants, but data from clinical trials that investigated correction of an underlying coagulopathy is sparse. A retrospective study was not able to show that patients with a baseline international normalized ratio (INR) greater than 1.3 had a higher risk of re-bleeding, surgery or mortality[18]. These findings were substantiated by another study[19], in which neither platelet count nor INR predicted re-bleeding. In contrast to these findings, one study, published in abstract form only, showed that an INR of 1.5 or greater at presentation is a predictor of mortality[20]. Correction of coagulopathy to an INR of less than 1.8 led to a lower mortality compared to a historical control group[21] without differences in time to endoscopy and units of transfused blood. Another study that compared cohorts of patients that underwent endoscopic treatment was not able to show differences in mortality, re-bleeding or need for surgery between patients on warfarin whose INR was corrected using fresh frozen plasma compared to patients without correction of coagulopathy[22]. The recommendation for clinical practice is that coagulopathy should not delay early endoscopic treatment and that coagulopathy should be corrected to an INR of 1.5 or less to facilitate endoscopic treatment. Correction of coagulopathy is best done by the application of prothrombin complex[23]. In patients on warfarin therapy, iv Vitamin K should be administered. The situation is even more complicated by the fact that an increasing amount of patients is on a therapy with target-specific oral anticoagulants like rivaroxaban or epixaban. Antidotes or specific reversal agents for these drugs are lacking. The INR is of no value in target-specific oral anticoagulants and correction of coagulopathy using prothrombin complex on the basis of the clinical needs and judgement is necessary[24].

Platelet transfusion is not necessary in patients who are haemodynamically stable and have no signs of active bleeding. In contrast, patients with active bleeding and a platelet count of less than 50 G/L should receive platelets[8]. A substantial gap in evidence still remains in the case of massive bleeding. At the moment, there are no high-quality trials on the effect of component therapies and the ratio of red blood transfusion to component therapies or therapy with recombinant factor VIIa[25].

TIMING OF ENDOSCOPY

Endoscopy is able to identify the bleeding site in more than 80% of patients. It is the principle diagnostic tool in UGIB and haemostatic therapy could be applied. While diagnostic endoscopy in clinically stable patients without relevant co-morbidity is safe, complications may arise in actively bleeding patients with co-morbidities. Therefore, patients should be sufficiently stabilized before endoscopy is performed[8,15].

Several studies investigated the best time point for endoscopy in patients with suspected UGIB. Endoscopy within the first 24 h (early endoscopy) improves outcomes of high-risk patients and allows for early discharge of low-risk patients[9,26]. Only in a minority of high-risk patients endoscopy should be delayed due to reasons that make endoscopy an additional risk factor (*e.g.*, perforation, acute coronary syndrome). Endoscopy within 24 h after presentation was performed in the majority (> 75%) of patients in a US-study[27], whereas

Table 3 Clinical (pre endoscopy) and full (post endoscopy) Rockall score[11]

Variable	Score 0	Score 1	Score 2	Score 3
Age	< 60	60-79	≥ 80	
Shock	No shock	Pulse ≥ 100 Systolic blood pressure ≥ 100	Systolic blood pressure < 100	
Co-morbidity	Non major		Chronic heart failure, ischemic heart disease, major comorbidity	Renal failure, liver failure, metastatic cancer
Diagnosis	Mallory-Weiss lesion	All other diagnoses	GI malignancy	
Evidence of bleeding	None		Blood, adherent clot, visible or spurting vessel	

The first three rows make up the clinical score. After endoscopy the scores from the last two rows are added to create the full score. Scores are additive. A score of 0 for the clinical and scores from 0-2 for the full score are the clinical cut-offs to indicate patients at low risk of re-bleeding or death. GI: Gastrointestinal.

Table 4 Forrest classification[12] and the risk of re-bleeding within 24 h after exclusively medical therapy

	Re-bleeding risk (%)
Acute bleeding	
Forrest Ⅰ a (spurting bleeding)	90
Forrest Ⅰ b (oozing bleeding)	50
Signs of recent bleeding	
Forrest Ⅱ a (visible vessel)	25-30
Forrest Ⅱ b (adherent clot)	10-20
Forrest Ⅱ c (flat pigmented haematin on ulcer base)	7-10
Lesions without active bleeding	
Forrest Ⅲ (lesions without signs of recent bleeding or fibrin-covered clean ulcer base)	3-5

in a study from the United Kingdom[28] only half of the patients received endoscopy within the first 24 h. Early endoscopy is considered safe and effective in the vast majority of patients and is associated with a reduction in the length of hospital stay in patients of all risk groups[29-35]. A cohort analysis[28] showed a relevant trend (however not statistically significant) that the availability of after-hour endoscopy decreased mortality. These results are substantiated by findings that patients with UGIB who were admitted on weekends had higher in-hospital mortality[29]. However, a more recent study from the United Kingdom was not able to show a higher mortality in patients who were admitted on weekends[36]. Whereas these findings are in favour of early endoscopy within 24 h after presentation, a meta-analysis found no difference in mortality, reduction in re-bleeding or surgery comparing very early endoscopy (< 12 h) over early endoscopy (> 24 h)[15]. One study analysed the need for transfusions and length of hospital stay in patients with blood in the gastric tube aspirate and time to endoscopy < 12 h or > 12 h[33]. They found less need for blood transfusions and shorter hospital stay in the patients who underwent endoscopy in the first 12 h after presentation. Most likely the conflicting results in the available studies are due to the heterogeneity of the included patients. A study identified independent predictors for the need of endoscopy within 12 h after presentation[37]: Fresh blood in the gastric tube aspirate, hemodynamic instability, haemoglobin below 8 g/dL and a leukocyte count of more than 12 G/L. The recommendation from the available data is that patients with suspected UGIB should undergo endoscopy within 24 h after presentation. Patients who are hemodynamically instable and/or blood in the naso-gastric tube aspirate should undergo endoscopy immediately after resuscitation, at least within 12 h after presentation.

MEDICAL TREATMENT

The rationale for an acid suppressing therapy is to increase intra-gastric pH and to achieve stabilization of the blood clot that plugs the vessel defect and to promote ulcer healing. Whereas proton pump inhibitors (PPI) therapy is well tolerated and side effects in the acute, short-term use are rare, it is questionable if all patients that present with haematemesis or melena actually need PPI-therapy, since approximately 80% of ulcers stop bleeding without any form of intervention and re-bleeding is rare.

While the debate whether pre-endoscopic PPI-treatment is cost-effective or not[38-40] is ongoing, it is advisable in situations where endoscopic treatment is delayed or endoscopic expertise is not sufficient.

The effect of pre-endoscopic treatment with PPI was investigated in several trials and summarized in a Cochrane analysis[41] that was later updated by additional studies[42]. Of the included studies, one used an oral PPI regimen whereas the remaining five studies investigated iv PPI treatment. The meta-analysis was not able to show differences in re-bleeding, surgical intervention or mortality between the patients on PPI-treatment and patients in the control group. Nevertheless, the patients in the treatment group had less high-risk stigmata and need for endoscopic treatment.

The use of PPI therapy in patients with UGIB was investigated in numerous studies. A Cochrane analysis from 2006[43] as well as an update of this meta-analysis[44] comprising 24 and 31 randomized controlled trials (RCTs), respectively, studied PPI-treatment. Therapy with PPI - alone or in combination with endoscopic treatment - compared to placebo or histamine receptor antagonists reduced re-bleeding and need for surgery

but did not reduce mortality[40]. Subgroup analysis of the data revealed a lower mortality for patients with active bleeding and endoscopic haemostasis who were treated with an 80 mg PPI bolus followed by continuous infusion of 8 mg/h. In contrast, lower doses of PPI reduced re-bleeding but had no effect on mortality. These findings were substantiated by another meta-analysis from the year 2009[45] that found lower re-bleeding rates, need for surgery and mortality in patients with high-dose intravenous PPI therapy. One meta-analysis compared continuous intravenous PPI therapy with bolus intravenous therapy and found bolus therapy as effective as continuous therapy[46]. Lower PPI doses were also associated with less re-bleeding but had no effect on surgery and mortality. Even though there is strong evidence that high-dose PPI therapy combined with endoscopic therapy is highly effective, it is still a subject of intense discussion whether oral PPI therapy is as effective as intravenous therapy. A recent Cochrane analysis was not able to draw a final conclusion since the available studies are not sufficient[47]. A more recent meta-analysis came to the conclusion that oral and intravenous PPI therapies are comparable[48] but also criticized the low quality of the available studies. One recent single-center Asian study that compared high-dose oral PPI therapy with intravenous high-dose PPI therapy in patients with Forrest Ⅰa/Ⅰb or Ⅱa/Ⅱb peptic ulcer found no difference in the risk of re-bleeding between the two groups[49].

Cost effective analyses revealed a clear advantage for high-dose intravenous PPI therapy for three days following successful endoscopic haemostasis[50-52] compared to placebo-as mentioned above, adequate RCTs comparing high-dose intravenous PPI with standard dose intravenous PPI or high dose oral PPI therapy are not yet available.

Two trials showed that PPI therapy in hospitalized patients might be associated with Clostridium difficile infection[53,54]. These findings were substantiated by a recent retrospective cohort study[55]. However, the benefits of PPI treatment in UGIB clearly outweigh this risk.

Post-endoscopic PPI therapy depends on the underlying aetiology of UGIB. In most RCTs, oral PPI therapy was initiated three days after the acute bleeding episode and a dose once daily is thought to be appropriate[56-60]. One trial that investigated the role of PPI therapy in the non-acute setting demonstrated effective ulcer healing with a once daily dose[61]. The duration of therapy is not clearly defined. Patients with Helicobacter negative ulcers who require long-term NSAID therapy might need concomitant continuing PPI therapy.

ENDOSCOPIC MANAGEMENT

Several endoscopic techniques to achieve haemostasis are available. Epinephrine injection is easy to perform and effective in the acute setting but re-bleeding occurs in almost all patients. Therefore, it should be used in combination with another method. The application of clips, thermocoagulation, injection with a sclerosing agent or fibrin or thrombin glue could be performed alone or in combination with epinephrine injection. A new method for the treatment of refractory bleeding is the over the scope clip, that allows the treatment of large defects[62].

First of all, the ulcer bed should be cleaned from blood and blood clots by vigorous irrigation to visualize the underlying lesion. By irrigation alone, the underlying stigmata are exposed in 26% to 43% of cases[63,64]. It is a subject of discussion[45,65] whether adherent clots should be removed by using more vigorous methods like cold guillotining with a snare. There is good evidence that the risk for re-bleeding with clots that remain adherent after washing without endoscopic therapy (only therapy with a proton-pump inhibitor) is as low as 0% to 8%[63,66]. One Asian study that compared endoscopic therapy plus high-dose iv PPI therapy with high-dose iv PPI therapy alone[66] found no re-bleeding in the patients in whom the adherent clots could not be removed by irrigation. Since it is known that the PPI metabolism in Asian people differs from the metabolism in patients with Caucasian background, it is not clear whether these results could be extrapolated to an European or North American population. Furthermore, other studies revealed a re-bleeding risk of 25% to 35%[64,67-69] in high-risk patients. This subject was further evaluated in two meta-analyses: One meta-analysis from 2009[45] comprising 5 RCTs of patients with adherent clots found no advantage of endoscopic *vs* medical therapy alone. These data was substantiated by another meta-analysis comprising 6 RCTs[70] that was also not able to show a reduction in the re-bleeding risk in patients with endoscopic therapy compared to patients with medical therapy alone. On the other hand, a systematic review[71] did not show that endoscopic therapy increased the risk for complications. As a recommendation for clinical practice, patients who are at high risk for re-bleeding and an adherent clot to the ulcer base that is resistant to irrigation, endoscopic therapy after cold guillotining may be beneficial. In patients with a low risk of re-bleeding and those who are Helicobacter positive, high-dose PPI therapy alone might be sufficient.

Numerous studies and meta-analyses studied the efficacy of the available endoscopic techniques in patients with high-risk lesions[45,71-77]. Injection with epinephrine as a monotherapy has been shown to be superior to medical therapy alone but it is clearly inferior to other monotherapies like clip application, thermocoagulation or injection with alcohol, fibrin or thrombin glue[45,71-76,78]. The combination of epinephrine injection with one of the above mentioned therapies for the treatment of high-risk stigmata significantly reduces re-bleeding, need for surgery and mortality[75,78]. The combination of clip application with epinephrine injection is superior to epinephrine injection alone but not to clips alone[72,74]. This is also true for the combination therapy of injection with epinephrine and a second injectate or

thermocoagulation[71]. Complication rates with mono- or combination therapy do not vary significantly[71,79,80].

There is an ongoing discussion whether a routine endoscopic control after the initial endoscopy is necessary or not. The advantages of a programmed second look endoscopy like the identification of residual stigmata that need re-treatment has to be outweighed against potential risks like an increase in ulcer perforation. Five studies[81-85] as well as two meta-analyses of these trials[86,87] investigated the benefit of a second-look endoscopy. The results from these trials were inconclusive due to methodological flaws. A more recent meta-analysis[88] found that routine second-look endoscopy and endoscopic treatment with thermocoagulation as appropriate reduced the risk of re-bleeding. In contrast to the use of a heater probe, second-look endoscopy with injection therapy did not reveal any advantages. Another meta-analysis[89] demonstrated that second look endoscopy decreased re-bleeding and need for surgery but not mortality. The impact of this meta-analysis is decreased by the fact that only one study with concomitant high-dose PPI therapy was included. All of the above mentioned studies had several methodological shortcomings: the included patients were heterogeneous; intervention and control treatments were not standardized. When looking at high risk patients who presented with haemorrhagic shock and/or active bleeding[81] or patients with a very high risk for re-bleeding based on the Forrest criteria[84] second look endoscopy led to a decrease in the re-bleeding rate. A trial, which included a control group that received high-dose iv PPI therapy-as it is standard now-found no benefit for second look therapy[81]. These findings suggest that second look endoscopy is not necessary in patients with high dose PPI therapy. Similar results were obtained from a cost-effectiveness study[90] that compared second-look endoscopy in selected high-risk patients only to second-look endoscopy in all patients and found endoscopy in selected patients to be more effective and less expensive. From the available data, routine second-look endoscopy is not recommended. However, patients at high-risk of re-bleeding might benefit from a programmed second-look endoscopy.

The highest risk for re-bleeding in patients treated with a combination of endoscopic and PPI therapy is within the first 72 h after the initial bleeding episode. Sixty to 76% of re-bleeding occurred in the first three days[56,57,59]. Thus, patients with bleeding from high-risk lesions should be treated as in-patients for at least three days. Patients at high-risk for re-bleeding should be monitored more intensely on an intensive or intermediate care unit for at least 24 h. Nevertheless, selected patients with ulcers not more than 15 mm in size, no relevant co-morbidity, appropriate family support and absence of haemorrhagic shock at presentation could be safely managed as outpatients[91].

If haemostasis could not be achieved or repeated re-bleeding occurs, it is associated with a high mortality. Patients rarely die because of exsanguination but because of problems that arouse from associated co-morbidity like cardiac events, acute kidney failure, infection or stroke. Accordingly, patients in whom endoscopic therapy failed should be admitted to surgery without delay. In patients who are high-risk candidates for surgery, percutaneous or transcatheter arterial embolization might be an alternative[92-99]. Data from uncontrolled trials revealed technically success rates from 52% to 98% with a reported re-bleeding rate of 10% to 20%[92-99]. The reported periprocedural mortality is as high as 25% to 30%. This is most likely due to the negative selection of patients with advanced age and co-morbidity to unstable to undergo surgery[92,93,95,97]. Possible complications of the procedure are mainly bowel ischemia or infarction of the stomach, liver or spleen[94,95,98-101].

Helicobacter pylori

Patients with UGIB from ulcers or haemorrhagic gastritis should be tested for *Helicobacter pylori* (*H. pylori*) infection and should undergo eradication therapy if *H. pylori* is present. The effectiveness in prevention of re-bleeding in peptic ulcer disease was demonstrated in a meta-analysis[102]. It is well known that *H. pylori* testing might reveal false negative results in the setting of an acute bleeding episode[103]. The reason is not fully understood but is most likely due to the alkaline setting that results in pH buffering from blood in the stomach[103] as well as from PPI therapy, which is dose-dependent. Therefore, an initially negative testing for *H. pylori* should be repeated during follow-up.

NSAID AND ASA USE

The use of NSAID and ASA is associated with a markedly increased risk of ulcer disease. Several studies addressed this issue and investigated whether the combination of NSAID and PPI decreased the risk for recurrent bleeding and also compared traditional NSAID with cyclooxygenase-2 (COX-2) inhibitors. Two small trials with a relatively low patient number showed that the combination of NSAID with PPI therapy as well as COX-2 inhibitor therapy alone lowered the risk for recurrent bleeding compared to historical controls on a therapy with NSAID alone[104-106]. These findings are substantiated by population-based studies that also found a reduction in UGIB by adding PPI to traditional NSAID or by therapy with a COX-2 inhibitor alone[3,107]. The combination of a COX-2 inhibitor with PPI further decreased the bleeding risk compared to a COX-2 inhibitor alone[108]. These finding were in-line with the results of a meta-analysis of three RCTs[109] and two studies[108,110] that also revealed a lower bleeding risk in patients who were on a combination of COX-2 inhibitors and PPI compared to patients on a COX-2 inhibitor alone.

Although COX-2 inhibitors, especially in combination with PPI therapy, lower the risk for UGIB, it was

demonstrated that the use of a COX-2 inhibitor is associated with an increased risk of cardiovascular events[111,112].

In clinical practice, NSAID therapy should be discontinued if possible. In patients without an increased risk for cardiovascular events and the need for NSAID therapy, patients should receive the combination of a COX-2 inhibitor and PPI. However, possible long-term side effects of PPI therapy should be kept in mind.

Things are more complicated in patients who receive cardioprotective ASA therapy. Prolonged discontinuation of ASA therapy (*e.g.,* to complete ulcer healing) is associated with an increase in adverse cardiovascular events[113,114]. In most cases, thrombotic events occur between 7 and 10 d after discontinuation of ASA therapy[113,115,116]. This is well explained by the fact that ASA therapy inhibits irreversibly platelet function and the half-life of platelets of around 7 d. In patients at high risk of cardiovascular events, the early reintroduction of ASA therapy outweighs the risk of re-bleeding[117]. Discontinuation of ASA therapy in patients with acute ulcer bleeding was shown to increase the eight-week mortality rate, whereas the early reintroduction of ASA therapy in combination with PPI revealed an insignificant trend to a higher re-bleeding rate only. The findings of another RCT[117] were even more convincing with no reported re-bleeding in patients on ASA therapy and ulcer bleeding in whom therapy with ASA or clopidogrel in combination with PPI was initiated one day after endoscopy. In summary, therapy with ASA or clopidogrel in patients with cardiovascular risk factors should be restarted as soon as the risk for cardiovascular events outweighs the risk for re-bleeding.

Compared to ASA, the risk of ulcer bleeding associated with clopidogrel mono therapy is lower, but is still as high as 14%[118,119]. Clopidogrel therapy alone has a higher re-bleeding risk than ASA therapy combined with PPI therapy[118,119]. Clopidogrel requires cytochrome P450 isoenzyme CYP2C19 to be converted to its active metabolite[120]. Since PPI and clopidogrel compete for the same cytochrome P450 isoenzyme, PPI may decrease the effect of clopidogrel. An increase in cardiovascular events in patients who received clopidogrel and PPI therapy in combination has been shown by some observational studies[121-125], but other studies did not reveal an increase in cardiovascular events[125,126]. Since reliable RCT addressing this issue are lacking, the interval between the intake of PPI and clopidogrel should be as long as possible (*e.g.,* PPI in the morning and clopidogrel in the evening).

CONCLUSION

Non-variceal UGIB could be a life-threatening event, especially in older patients with co-morbidities. With a combination of endoscopic and PPI therapy haemostasis could be achieved in the majority of patients. When endoscopic measures fail, patients should undergo surgery or interventional radiology without delay. In peptic ulcer disease, testing for *H. pylori* is mandatory and eradication reduces the re-bleeding risk. Caution is necessary in patients that need a long-term therapy with NSAID. In patients at risk, NSAID have to be combined with PPI therapy.

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REVIEW

Antibiotic treatment for *Helicobacter pylori*: Is the end coming?

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Abstract

Infection with the Gram-negative pathogen *Helicobacter pylori* (*H. pylori*) has been associated with gastroduodenal disease and the importance of *H. pylori* eradication is underscored by its designation as a group I carcinogen. The standard triple therapy consists of a proton pump inhibitor, amoxicillin and clarithromycin, although many other regimens are used, including quadruple, sequential and concomitant therapy regimens supplemented with metronidazole, clarithromycin and levofloxacin. Despite these efforts, current therapeutic regimens lack efficacy in eradication due to antibiotic resistance, drug compliance and antibiotic degradation by the acidic stomach environment. Antibiotic resistance to clarithromycin and metronidazole is particularly problematic and several approaches have been proposed to overcome this issue, such as complementary probiotic therapy with *Lactobacillus*. Other studies have identified novel molecules with an anti-*H. pylori* effect, as well as tailored therapy and nanotechnology as viable alternative eradication strategies. This review discusses current antibiotic therapy for *H. pylori* infections, limitations of this type of therapy and predicts the availability of newly developed therapies for *H. pylori* eradication.

Key words: *Helicobacter pylori*; Treatment; Antibiotic resistance; Therapeutic regimens; Novel agents

Core tip: This article reviews the recent literature describing antibiotic resistance and trends in *Helicobacter pylori* (*H. pylori*) treatment. As there is no effective conventional therapy, new treatments are being developed and bismuth quadruple, sequential, concomitant therapies are recommended as a first-line regimen in regions with high levels of clarithromycin resistance. Quinolones have also been used for *H. pylori* treatment, although the cure rate has gradually reduced with this approach. New therapeutic directions include probiotic supplementation, tailored therapy, novel agents, and nanotechnology.

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INTRODUCTION

Helicobacter pylori (*H. pylori*) is a Gram-negative, flagellated, spiral shaped microaerophilic bacterium first identified by Marshall and Warren[1-3]. These bacteria have morphological characteristics penetrate the mucosa and colonize the stomach and duodenum[4]. *H. pylori* are responsible for the pathogenesis that leads to gastritis, peptic ulcer disease (PUD), gastric adenocarcinoma and gastric mucosa-associated lymphoid tissue (MALT) lymphoma[3,5,6]. The World Health Organization has classified *H. pylori* as a group I carcinogen with a risk of stomach cancer[7,8]. *H. pylori*-related stomach cancer represents 5.5% of all cancers worldwide and 25% of all infection-associated malignancies. Socioeconomically, *H. pylori* infection increases the risk of malignancy and the expense of *H. pylori*-associated morbidity[9]. *H. pylori* infection has also been related to non-digestive conditions such as ischemic heart disease, stroke, Alzheimer's disease, Parkinson's disease, and iron deficient anemia[4,10]. In other report, some patients with gastritis resolved *H. pylori* infection without using antibiotic treatment[11]. Although the prevalence of *H. pylori* infection has been reduced in developed countries, it has remained prevalent in developing countries[12,13] with rates of infection varying according to nation, patient age, and socioeconomic states[14]. Eradication of *H. pylori* is an effective treatment for PUD, gastric MALT lymphoma, and preventing the recurrence of stomach cancer after endoscopic treatment[15-17].

A standard triple therapy (STT), consisting of a proton pump inhibitor (PPI), clarithromycin and amoxicillin, was established in clinical practice for the eradication of *H. pylori* infection[18,19]. However, in recent years, the efficacy of STT has been critically altered in many regions of the world as eradication rates have diminished to inadequately low levels[18,20]. The causes for this decline may involve patient compliance, bacterial factors, obesity, smoking, reinfection, and genetic polymorphisms in CYP2C19. However, antibiotic resistance may be the primary reason for reduced eradication of *H. pylori* infection worldwide[20-22]. In addition, the eradication rates differ by region, even in the same country. In South Korea, one study reported that the eradication rates of first-line therapy decreased from 81.3% to 77.5% from 2001-2007[23], while another showed that no definite evidence of a significant change in the eradication rate during 2000-2010[24]. This may be due to geographical differences in antibiotic resistance and the methods used to confirm eradication. In a region with high rates of clarithromycin resistance, sequential or concomitant therapy is recommended as the first-line *H. pylori* eradication treatment[25]. The primary reason for the growth in antibiotic resistance is the emergence of point mutations in the *H. pylori* genome[26]. Thus, the development of novel treatment methods to increase eradication rates and reduce antibiotic resistance is needed. The focus of this review will be on current *H. pylori* therapies and limitations, as well as alternative anti-*H. pylori* regimens.

CURRENT ANTIBIOTIC RESISTANCE IN WORLDWIDE

The most important antibiotics in *H. pylori* treatment are clarithromycin, metronidazole, and amoxicillin. Figure 1 illustrates recently reported clarithromycin and metronidazole resistance rates worldwide. Resistance to these antibiotics is thought to be the main cause of eradication failure[27-29]. Antibiotic resistance is discovered by bacterial culture-based techniques (*E*-test, modified disk diffusion, agar dilution method, and breakpoint susceptibility test) and molecular methods [polymerase chain reaction (PCR), real-time PCR, allele-specific PCR, sequencing, and fluorescent *in situ* hybridization][30]. Although these methods are useful for examining antibiotic resistance, their implementation at the early stages of *H. pylori* remains impractical due to the time required to obtain results and the high cost of the tests.

Clarithromycin is a macrolide antibiotic that inhibits protein synthesis by binding to and slowing the actions of the bacterial ribosome[30]. Clarithromycin resistance is due to three point mutations at A2142C, A2142G, and A2143G in the *23s rRNA* gene[31]. In particular, the A2143G mutation has been related to a very low eradication rate[32]. In contrast, the A2143G mutation occurs in only 23% of resistant strains in Eastern countries[31]. This suggests that clarithromycin point mutations may be geographically distinct between Eastern and Western countries and new point mutations have appeared in South America[33]. Clarithromycin resistance is also different depending on the area. In Brazil, stomach biopsy specimens positive for *H. pylori* were analyzed by PCR to detect the point mutation associated with clarithromycin resistance[34]. The results uncovered primary clarithromycin resistance in 16.5% patients. Recently, the clarithromycin resistance rate in South Korea was reported to range from 17.2% to 23.7%[35]. In a study published in Japan, the clarithromycin resistance rate in 2002 was 18.9%; however, the clarithromycin resistance rate in 2006 increased to 27.2%[36]. Even with third-line eradication therapy, clarithromycin resistance rates in Japan were reported as 86.4%[37]. Several studies in China have reported increased resistance rates Shanghai[38], 21.5% resistance in the southeast coastal region[39], and a relatively high rate of 33% in Vietnam, which is near Southeast China[40]. In Western Asia, resistance to clarithromycin has been reported to be > 10% in Iran and > 20% in Turkey[13]. In one study, clarithromycin resistance was reported in 47.5% of patients with dyspepsia in Turkey[41]. In sharp contrast to other Asian countries, no resistance to clarithromycin has been reported in Malaysia[42] and the prevalence of resistance to clarithromycin in Gambia and Senegal also remains

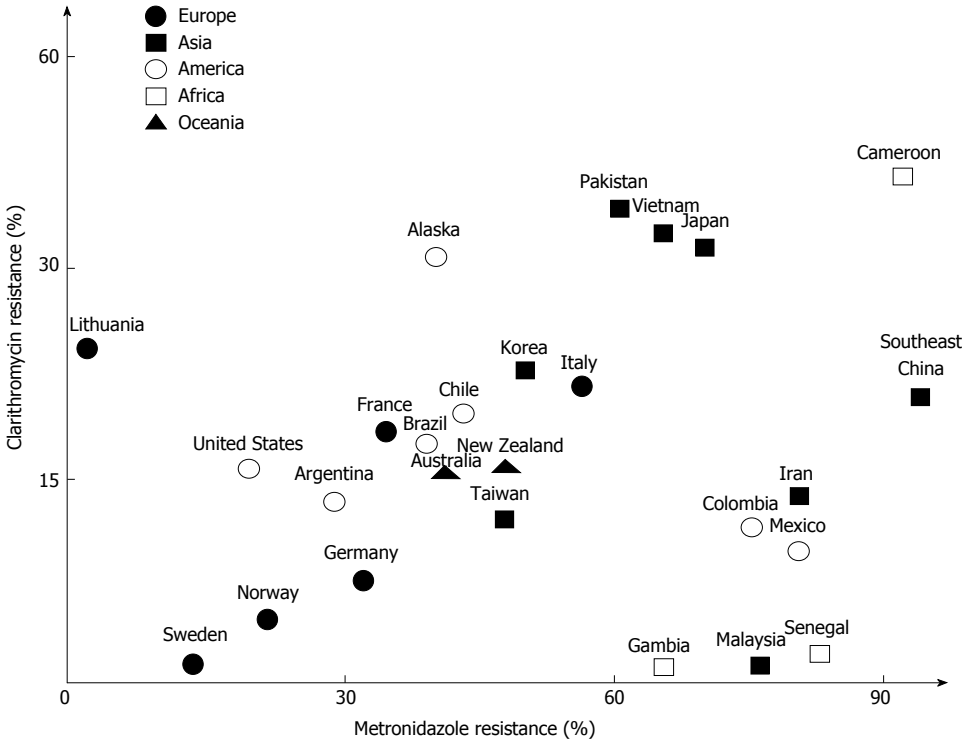

Figure 1 Worldwide rates of resistance to clarithromycin and metronidazole.

very low[43,44]. Resistance to clarithromycin has also risen by > 20% in Southern Europe, although in Northern Europe the resistance rate is less than 10%[45] compared to 1.5% in a random adult Swedish population[46] and 7.5% in central Germany[47]. During the last 15 years, a twofold increase in clarithromycin resistance was reported in Italy[48] and in Spain, where the mean clarithromycin resistance rate was 18.3% in 1709 patients[49], and 34.7% in Portuguese children[50]. In contrast to the general trend, the rate of *H. pylori* strains resistant to clarithromycin decreased from 34% to 22% during 6 years in Southern Poland[51]. Despite these variations, the overall frequency of clarithromycin resistance has risen from 10.2% to 21.3% worldwide, and A2143G is the most frequently reported point mutation. Present European guidelines recommend 7 d of STT in regions in which the rate of clarithromycin resistance is < 20%, and 14 d in regions with clarithromycin resistance rates of > 20%[25,45].

The mechanism mediating resistance to metronidazole is complex. Modifications in the *rdxA* gene, assumed to be point mutations, are considered a primary cause[30]. Metronidazole resistance may also influence the treatment outcome, although it is generally considered less clinically important than clarithromycin resistance[52,53]. Overall, the Eastern Asian region has higher metronidazole resistance rates with 95.4% in the southeast coastal region of China[39] and 71.3% in Japan[37]. In Vietnam, the resistance rate was 69.9% among 103 strains[40]. Unlike the clarithromycin resistance rate, there was a high prevalence of resistance to metronidazole (75.5%) in Malaysia[42], a 76.8% rate in Iran[54] and a high resistance rate in Africa[43,44]. Another study showed that 80% of strains in Mexico were resistant to metronidazole[55]. Overall, metronidazole resistance is > 50% in much of the world but there are reports that metronidazole resistance has declined in Northern Europe[9,30], while in the United States and Europe, the metronidazole resistance rate was reported to be < 40%[30,56], and 22.5% in 102 isolates from Norway[57]. However, in Central and Southern Europe, resistance rates remain markedly higher-34.9% in France and 32.7% in Germany[47,58].

Amoxicillin is a beta-lactam antibiotic that was first used for *H. pylori* therapy[25]. Unlike clarithromycin and metronidazole, amoxicillin resistance rates are low worldwide[30]: 0% or < 1% in Europe[30]. However, other studies revealed high amoxicillin resistance rates in Iran, Japan, and Cameroon[37,45,54].

Fluoroquinolones are the sole class of antibiotics for treatment of *H. pylori* that directly inhibit bacterial DNA synthesis. Resistance to fluoroquinolones occurs primarily by mutation in the genes for topoisomerase Ⅳ and gyrase[59]. Levofloxacin is currently recommended as a second-line *H. pylori* treatment when first-line therapy containing clarithromycin has failed, although levofloxacin resistance has been predicted to increase in the near future[25]. Levofloxacin resistance rates in Asia differ from region to region with rates of 20.6% in the southeast coastal region of China and 18.4% in Vietnam[39,40]. Fluoroquinolone resistance was noted as 62.3% in Pakistan[60], while Japan and Malaysia had low resistance rates of 8.2% and 0%, respectively[37,42]. Primary *H. pylori* resistance to ciprofloxacin occurred at a high frequency (15.7%) in South Korea[61]. A study by Mégraud[62] of more than 2000 patients with *H.*

Table 1 Decline in rates of *Helicobacter pylori* eradication following first-line standard triple therapy

Country	Ref.	Publication	Treatment duration	Patients	Therapy regimen	Eradication rate (ITT)	Eradication rate (PP)
South Korea	Na *et al*[177]	2007	7 d	3267	Standard PPI Cla 500 mg bid Amo 1 g bid	NA	84.3%
	Chung *et al*[178]	2012	10 d	80	Lan 30 mg bid Cla 500 mg bid Amo 1 g bid	58.7%	67.6%
Japan	Asaka *et al*[179]	2001	7 d	96	Lan 30 mg bid Cla 200 mg bid Amo 750 mg bid	NA	90.7%
	Fujioka *et al*[180]	2012	7 d	3162	Rab 10 mg bid Amo 750 mg bid Cla 200 mg bid	80.7%	NA
	Nishizawa *et al*[27]	2012	7 d	55	Lan 30 mg bid Cla 400 mg bid Amo 750 mg bid	74.5%	80.4%
	Nishida *et al*[181]	2014	7 d	134/134	Eso 20 mg bid Cla 400 mg bid Amo 750 mg bid Lan 30 mg bid Cla 400 mg bid Amo 750 mg bid	69.4%/73.9%	76.9%/79.8%
Taiwan	Sheu *et al*[182]	2000	7 d or 2 wk	286	Ome 20 mg bid Amo 1 g bid Cla or Met bid	NA	87.8%
	Chen *et al*[117]	2014	7 d	73	Rab 20 mg bid Cla 500 mg bid Amo 1 g bid	57.5%	61.8%
Turkey	Ozçay *et al*[183]	2004	4 wk: PPI 2 wk:Cla, Amo	102	Ome or Lan Cla 7.5 mg/kg bid Amo 20 mg/kg bid	NA	75.7%
	Kutluk *et al*[184]	2014	10 d	74	Lan 1 mg/kg per day Cla 20 mg/kg per day Amo 50 mg/kg per day	52.7%	55.7%
Italy	Catalano *et al*[185]	1999	10 d	84	Ome 20 mg bid Cla 500 mg bid Amo 1 g bid	NA	94.0%
	Paoluzi *et al*[186]	2010	7 d	90	Eso 20 mg bid Cla 500 mg bid Amo 1 g bid	66.0%	75.0%
Latin America	Greenberg *et al*[106]	2011	14 d	488	Lan 30 mg bid Cla 500 mg bid Amo 1 g bid	82.2%	87.1%

ITT: Intention to treat; PP: Per protocol; PPI: Proton pump inhibitor; NA: Not available; Cla: Clarithromycin; Amo: Amoxicillin; Lan: Lansoprazole; Rab: Rabeprazole; Eso: Esomeprazole; Ome: Omeprazole; Met: Metronidazole.

pylori infection showed resistance rates of 14.1% for levofloxacin, with significantly higher fluoroquinolone resistance in Western/Southern Europe than in Northern Europe[62]. O'Connor *et al*[63] reported that 11.7% of patients had strains resistant to levofloxacin in Ireland and there was a 29.1% resistance rate in 2011 in Germany[64], 15% in Senegal[44] and 23% in Brazil[65].

THE EFFICACY OF STT AND BISMUTH QUADRUPLE THERAPY ARE DECREASING

The first-line regimen for the eradication of *H. pylori* infection consists of STT using a PPI, amoxicillin and clarithromycin and was first introduced by Dr. Bazzoli. In studies conducted during the 1990s, STT yielded > 80% treatment success with reports of > 90% possible[66,67]. However, the increased prevalence of clarithromycin resistance has accounted for the diminished efficacy of STT. Table 1 shows eradication rates from recent studies using STT. Generally, STT is not recommended as a first-line regimen when the clarithromycin resistance rate is > 15%-20%, and other therapies such as quadruple therapy or sequential therapy are suggested[25]. Thus, a steady increase in *H. pylori* resistance to amoxicillin and metronidazole has also resulted in reduced treatment success of STT[27,68,69]. The ideal outcome of *H. pylori* eradication is > 80% by intention to treat (ITT) analysis and > 90% by per protocol (PP) analysis. According to a recent study, the eradication rate was unacceptably low for treatment success, with only 18% exceeding 85% and approximately 60% failing to attain 80% eradication by ITT analysis[20]. Over the past 20 years, the efficacy

of STT has decreased, with eradication rates < 80% by ITT analysis[41]. According to the present formula by Dr. Graham[70], if clarithromycin resistance rate of 20%, the outcome of clarithromycin containing triple therapy is reduced to 77.2% by PP analysis. Already in some countries the eradication rates have been reported to be < 50% and if this trend continues for another 20 years, the efficacy of STT will be negligible.

Various methods have been considered to circumvent the STT eradication rate decrease. The first method suggested that increasing the STT duration would improve treatment efficacy. In an early meta-analysis, a 14-d STT regimen raised the eradication rate compared to a 7-d regimen[71]. Another meta-analysis supported this result by showing that extending STT over 7 d improved the eradication rate[72]. However, other reports determined that extending STT was not cost-effective and increased adverse events and decreased compliance, resulting in no significant difference between the eradication rate and extended treatment duration[73]. Another means of addressing the decrease in STT eradication rate is to increase the dose of PPI, which has a positive effect on treatment success. PPIs delay gastric emptying and increase gastric pH, which improves the effect of antibiotics by preventing acid-related degradation[74]. A meta-analysis reported increased eradication rates from STT involving PPI administration twice per day compared with once per day[75]. Another systematic review reported that utilizing a high dose of PPI increased the *H. pylori* treatment rate[76] and the use of high-dose PPI increased the effectiveness of STT compared with a single does PPI[77]. In spite of these positive outcomes, STT is now regarded as an outdated therapy.

Bismuth quadruple therapy (bismuth subcitrate potassium, metronidazole, tetracycline, PPI) has been suggested as a first-line treatment option for regions with a high (> 20%) incidence of clarithromycin resistance[53]. In a meta-analysis of nine randomized controlled trials (RCTs), bismuth quadruple therapy and STT resulted in similar compliance rates, side effects, and eradication rates as a primary therapy for *H. pylori* infection[78]. For example, the ITT eradication rate with modified bismuth quadruple therapy was 92.7% in a recent randomized study in Chinese patients[79]. A pilot study in United States Hispanics showed that 14-d bismuth quadruple anti-*H. pylori* therapy achieved a > 95% eradication rate[80]. However, in some studies the eradication rate of bismuth quadruple therapy was < 80%[81-83]. A decrease in the bismuth quadruple therapy eradication rate was highly associated with metronidazole resistance[20].

ARE THERE SUITABLE SEQUENTIAL AND CONCOMITANT THERAPY ALTERNATIVES?

Sequential therapy was introduced by Zullo *et al*[84] in Italy in 2000. This regimen includes a PPI and amoxicillin for 5 d, followed by a PPI, clarithromycin, and tinidazole triple therapy for another 5 d. Several studies have indicated that the eradication rate of sequential therapy was significantly higher than that of STT[85-87]. The reason that sequential therapy has a higher eradication rate than STT is that amoxicillin and PPI administered during the first 5 d decreases *H. pylori* density in the stomach, which increases clarithromycin and metronidazole efficacy[32,88-90]. In addition, amoxicillin damages the bacterial cell wall and limits production of an efflux channel underlying drug resistance. However, it is uncertain whether improvement in the eradication rate is due to sequential therapy or additional use of antibiotics such as tinidazole. Recent data from South Korea, showed a lower *H. pylori* eradication rate with sequential therapy with eradication rates by ITT analysis of 79.0% and by PP analysis of 84.9%[91]. Another study showed that the eradication rates by ITT were 72.1% and 80.2% in 10-d and 15-d sequential groups, respectively[92]. Although the 15-d sequential therapy group cure rate was higher than that of the 10-d sequential therapy group, the eradication rate remains low. In the study by Zhou *et al*[93], there was no significant difference between the eradication rates achieved with STT (66.4%) and sequential therapy (72.1%) by ITT analysis. Moreover, the sequential therapy group with dual clarithromycin resistance and metronidazole resistance had a lower eradication rate (43.9%) compared to the rate seen with only clarithromycin resistance (88.9%)[93]. In a 2015 study from India that compared sequential therapy to ciprofloxacin-containing sequential therapy, the ITT cure rate in the sequential therapy group was 66% and only 73.5% in the ciprofloxacin group[94]. Thus, the sequential therapy efficacy in Asia was lower than reported by earlier European studies. Another meta-analysis showed that the overall eradication rate of sequential therapy was 84.3% (95%CI: 82.1%-86.4%), although this was not superior to 14-d STT[86]. However, sequential therapy was able to eradicate 72.8% of the *H. pylori* resistant to clarithromycin[86]. In addition to the problem of sequential therapy eradication rate reduction, treatment compliance can be reduced due to medication changes during treatment. Furthermore, if eradication fails, no second-line treatment regimen has been established[95].

Concomitant therapy, also known as non-bismuth quadruple therapy, consists of PPI and all three antibiotics (clarithromycin, amoxicillin, metronidazole) administered concomitantly to provide a simpler treatment regimen compared to sequential therapy[96]. Recently, several studies have compared concomitant therapy to STT and sequential therapy. In one study, 10-d concomitant therapy resulted in a better eradication rate in settings with antibiotic-resistant *H. pylori* strains[97]. Eradication rates for concomitant and sequential therapies were 100% *vs* 75% for clarithromycin-resistant strains and 75% *vs* 60% for clarithromycin-resistant/metronidazole-resistant strains[97]. A meta-analysis of 15 studies showed a mean *H. pylori* eradication rate of 90% by ITT analysis

for concomitant therapy and reported that longer treatment improved the outcomes compared to STT[98]. Another meta-analysis showed that concomitant therapy was superior to STT[99]. In studies published in South Korea, the eradication rate for concomitant therapy was considerably higher than that for sequential therapy[100,101]. However, several other studies have reported no difference in eradication rates between sequential and the concomitant therapy[102-104]. In a randomized open-label study, ITT eradication rates were 75.6% (95%CI: 66.3%-84.9%) in the sequential therapy group and 80.8% (95%CI: 71.8%-88.5%) in the concomitant therapy group[104]. In both groups, there was no difference in eradication rates and the treatment rate was lower than expected[104]. Furthermore, in some studies, concomitant therapy had a lower eradication rate than other regimens[83,105,106]. A total of 200 patients were randomized and the ITT eradication rates were 79% (95%CI: 71.0%-87.0%) in the bismuth group and 74% (95%CI: 68%-81%) in the concomitant group, although this was not statistically significant[83]. Another study compared the eradication rate between 10-d sequential therapy, 5-d concomitant therapy, 14-d concomitant therapy and 14-d hybrid therapy[105]. In ITT analysis, sequential therapy showed the highest eradication rate, which was higher than even 5-d concomitant therapy[105]. This is supported by an RCT of 1463 patients in seven Latin American sites (Chile, Colombia, Costa Rica, Honduras, Nicaragua, Mexico) that reported the eradication rate with 14-d standard therapy was 82.2%, compared to 73.6% with 5-d concomitant therapy and 76.5% with 10-d sequential therapy[106]. Currently, concomitant therapy has several limitations. First, side effects were reported to occur more frequently than with sequential therapy[107]. Second, there are few data describing the effect of metronidazole resistance in concomitant therapy. Moreover if dual-resistance to clarithromycin and metronidazole was > 15%, the eradication rate decreased[108,109]. Finally, as with sequential therapy, when first-line treatment fails no second-line treatment for concomitant therapy has been established. Tables 2 and 3 indicate that current trends of *H. pylori* eradication for sequential and concomitant therapy.

ADDING LEVOFLOXACIN AND OTHER QUINOLONES TO EXISTING TREATMENT

Levofloxacin has a large spectrum of activity against diverse Gram-positive and -negative bacteria[110] though inhibition of bacterial topoisomerase Ⅱ[111]. There have been several studies of levofloxacin use as a first-line treatment[112]. To overcome increasing clarithromycin resistance, levofloxacin has been used as a alternative to clarithromycin in either STT or sequential therapy[53]. Table 4 shows *H. pylori* eradication rates following levofloxacin-containing therapy. According to a meta-analysis, 10-d of levofloxacin triple therapy is more efficacious than 7-d bismuth-based quadruple therapy (RR = 1.41, 95%CI: 1.25-1.59) in the eradication of *H. pylori* infection[113]. In another study, levofloxacin-based triple therapy (ITT, 80.8%; 95%CI: 73%-88%) was more effective than STT (ITT: 64%, 95%CI: 55%-73%) and there were no differences in compliance or side effects[114]. However, other studies reported that levofloxacin-containing regimens did not have superior eradication rates compared to other treatments. Meta-analyses and a recent study in 2014 have shown that the outcome of levofloxacin-based first-line therapy was similar to STT[115,116] with an overall crude eradication rate of 79.1% in the levofloxacin group compared to 81.4% in the STT group[116]. A recent RCT in Taiwan with over 153 patients determined there was an advantage to levofloxacin-amoxicillin/clavulanate-PPI therapy over STT, although there was a low eradication rate (ITT analysis: 78.1% *vs* 57.5%)[117]. Unsatisfactory results were reported in an Asian meta-analysis, which showed that 7-d STT was more effective than 7-d levofloxacin-based therapy[118]. However, in European countries, levofloxacin-based therapy was more effective than STT[118]. Regional differences in *H. pylori* resistance to antibiotics might account for these results. Although levofloxacin has been suggested as a replacement for clarithromycin in *H. pylori* treatment, increasing quinolone resistance is a larger problem. According to Graham *et al*[119], in the presence of fluoroquinolone resistance treatment success with quinolone-containing therapy decreases and these results can be predicted using a formula. A report published in United States in 2015 determined that the prevalence of levofloxacin resistance was 31.3% (95%CI: 23.1%-39.4%)[120] and another study showed a high rate of quinolone resistance (50%) in Congo[121]. We calculated the effect of quinolone resistance on treatment success using the proposed formula, which indicated success rates of 87.6% and 73.5%, respectively[119,122]. The levofloxacin resistance rate is also relatively high in East Asia, where there is also a higher prevalence of stomach cancer compared to other regions[122,123]. Sitafloxacin, which has lower minimum inhibitory concentration for *H. pylori*, and levofloxacin triple therapy combined with bismuth quadruple therapy have been suggested as solutions to this problem, although further evidence is required to establish this approach[122,124].

PROBIOTICS AS ANOTHER APPROACH TO IMPROVE ERADICATION RATES

Many studies have demonstrated that probiotics have an inhibitory effect on *H. pylori*. Although some studies have reported that probiotics alone have limited efficacy[125,126], they can be useful when used as a supplemental drug. In a study by Lv *et al*[127], the cure rates in the probiotic supplementation group were superior to those in the group that did not receive probiotics (RR, 1.12; 95%CI: 1.06-1.19), and probiotics reduced the risk of *H. pylori* therapy related side effects (RR, 0.60; 95%CI:

Table 2 ***Helicobacter pylori*** **eradication rates following first-line sequential therapy**

Country	Ref.	Publication	Treatment duration	Patients	Therapy regimen	Eradication rate (ITT)	Eradication rate (PP)
South Korea	Lee *et al*[92]	2014	10 d	111	1st 5 d: Eso + Amo 2nd 5 d: Eso + Cla + Met	72.1%	78.4%
	Lee *et al*[91]	2015	10 d	100	1st 5 d: Rab + Amo 2nd 5 d: Rab + Cla + Met	79.0%	84.9%
China	Zhou *et al*[93]	2014	10 d	140	1st 5 d: Eso + Amo 2nd 5 d: Eso + Cla + Tin	72.1%	76.5%
Qatar	Ben Chaabane *et al*[94]	2015	14 d	106	1st 7 d: Rab + Amo 2nd 7 d: Rab + Cla + Met	66.0%	76.0%
Italy	Pontone *et al*[187]	2010	10 d	84	1st 5 d: Lan + Amo 2nd 5 d: Lan + Cla + Met	83.3%	90.9%
Spain	Molina-Infante *et al*[114]	2010	10 d	115	1st 5 d: Ome + Amo 2nd 5 d: Ome + Cla + Met	76.5%	80.8%

ITT: Intention to treat; PP: Per protocol; Lan: Lansoprazole; Amo: Amoxicillin; Cla: Clarithromycin; Met: Metronidazole; Ome: Omeprazole; Eso: Esomeprazole; Tin: Tinidazole; Rab: Rabeprazole.

Table 3 ***Helicobacter pylori*** **eradication rates following first-line concomitant therapy**

Country	Ref.	Publication	Treatment duration	Patients	Therapy regimen	Eradication rate (ITT)	Eradication rate (PP)
South Korea	Lim *et al*[104]	2013	14 d	78	Rab 20 mg bid Amo 1 g bid Cla 500 mg bid Met 500 mg bid	80.8%	81.3%
	Lee *et al*[100]	2015	7 d	170	Rab 20 mg bid Amo 1 g bid Cla 500 mg bid Met 500 mg tid	79.4%	94.4%
Thailand	Kongchayanun *et al*[188]	2012	5 d/10 d	55/55	Rab 20 mg bid Amo 1 g bid Met 400 mg tid Cla 1 g qd	89.1%/96.4%	NA
Singapore	Ang *et al*[102]	2015	10 d	153	PPI standard does Amo 1 g bid Cla 500 mg bid Met 400 mg bid	81.7%	95.4%
Spain	Molina-Infante *et al*[97]	2012	10 d	209	PPI standard does Amo 1 g bid Cla 500 mg bid Met 500 mg bid	87.0%	89.0%
	McNicholl *et al*[103]	2014	10 d	168	Ome 20 mg bid Amo 1 g bid Cla 500 mg bid Met 500 mg bid	87.0%	91.0%
Latin America	Greenberg *et al*[106]	2011	5 d	489	Lan 30 mg bid Amo 1 g bid Cla 500 mg bid Met 500 mg bid	73.6%	NA

ITT: Intention to treat; PP: Per protocol; NA: Not available; Lan: Lansoprazole; Amo: Amoxicillin; Cla: Clarithromycin; Met: Metronidazole; PPI: Proton pump inhibitor; Rab: Rabeprazole; Ome: Omeprazole.

0.40-0.91). In another meta-analysis, the pooled odd ratios (ORs) for the eradication rate were higher in the probiotic group than the control group (OR = 1.67; 95%CI: 1.38-2.02) by ITT, and adverse effects were lower in the probiotic group (OR = 0.49, 95%CI: 0.26-0.94)[128]. This study also showed racial differences in the sensitivity to probiotics, with greater differences in Asian populations compared to Caucasians. Adults, as well as children, have reported that probiotics positively affect eradication rates[129]. *Saccharomyces boulardii* is a type of probiotic gaining attention as a supplement for *H. pylori*. Recent reports have suggested that STT combined

Table 4 ***Helicobacter pylori*** **eradication rates following first-line levofloxacin-containing therapy**

Country	Ref.	Publication	Treatment duration	Patients	Therapy regimen	Eradication rate (ITT)	Eradication rate (PP)
South Korea	Choi *et al*[189]	2011	7 d	98	Ome 20 mg bid Lev 200 mg bid Amo 1 g bid	65.3%	73.6%
China	Liao *et al*[122]	2013	14 d	81	Lan 30 mg bid Lev 500 mg qd Amo 1 g bid	82.7%	85.9%
Taiwan	Liou *et al*[190]	2010	7 d	217	Lan 30 mg bid Lev 750 mg qd Amo 1 g bid	74.2%	80.1%
	Chen *et al*[117]	2014	7 d	73	Rab 20 mg bid Lev 500 mg bid Amo 1 g bid	78.1%	80.9%
Spain	Molina-Infante *et al*[114]	2010	10 d	115	Ome 20 mg bid Lev 500 mg bid Amo 1 g bid	80.8%	82.6%

ITT: Intention to treat; PP: Per protocol; Lan: Lansoprazole; Lev: Levofloxacin; Amo: Amoxicillin; Rab: Rabeprazole; Eso: Esomeprazole; Cla: Clarithromycin; Ome: Omeparzole.

with *S. boulardii* could be effective for enhancing *H. pylori* eradication rates[130]. Compared with no intervention, *S. boulardii*-including regimens significantly increased treatment success (RR, 1.13; 95%CI: 1.05-1.21) and reduced *H. pylori* therapy-related adverse effects (RR, 0.46; 95%CI: 0.3-0.7)[131]. Furthermore, *Lactobacillus* and *Bifidobacterium* species also have an anti-*H. pylori* effect. A meta-analysis of 10 studies on *Lactobacillus*-containing and *Bifidobacterium*-containing probiotics use as a supplementation to *H. pylori* eradication therapy found that the pooled ORs by ITT analysis and PP analysis were 2.066 (95%CI: 1.398-3.055) and 2.321 (95%CI: 1.715-3.142), respectively[132]. In addition to the above references, *Lactobacillus acidophilus* and *Bifidobacterium bifidum* supplementation to STT is effective for *H. pylori* eradication and dynamic changes in intestinal flora[133]. In recent RCT study, *Lactobacillus reuteri* was identified as a new probiotic proposed for the treatment of *H. pylori* infection. A combination that includes *L. reuteri* was able to reduce antibiotic-associated adverse events and to increase the *H. pylori* eradication rate[134]. Although more research into these probiotics is needed, it is important to note that there are reduced drug complications and treatment is comparatively free from resistance. Therefore, probiotics will be considered important future therapeutics for *H. pylori* eradication.

IS TAILORED THERAPY ON THE HORIZON FOR *H. PYLORI* TREATMENT?

It is well known that clarithromycin sensitivity of the *H. pylori* infection contributes to the success of the STT eradication rate[52,135]. Generally in infectious disease treatment, bacterial culture is carried out prior to determine the antibiotic selection of the organism. However, *H. pylori* bacterial culture is difficult and time-consuming, with various protocols for evaluating resistance. Thus, *H. pylori* treatment has depended on empirical antibiotic treatment[109]. In their study, Gerrits *et al*[136] determined that the A2142G, A2143G mutations were highly related to resistance using PCR, which was partially used to identify resistance to clarithromycin in *H. pylori*[137]. There are several advantages to this method because it is relatively simple and efficient with a cost similar to a rapid urease test[135]. In a recent study of 1232 patients, the eradication rate by selective treatment in the tailored group was 91.2%, which was significantly higher than control groups (amoxicillin, rabeprazole, clarithromycin; 75.9% and amoxicillin, rabeprazole, metronidazole; 79.1%)[138].

Appropriate stomach acid suppression, as well as resistant strains of *H. pylori*, remains a problem for successful eradication. PPI plays an important role in *H. pylori* eradication and the main enzyme involved in PPI metabolism is CYP2C19[139] and CYP2C19 genotypes can influence PPI efficacy[140]. Homozygous extensive metabolizer (HomEM) results in the highest rates of PPI metabolism, heterozygous extensive metabolizer (HetEM) results in moderate rates of PPI metabolism, while poor metabolizers (PM) exhibit the lowest rates of PPI metabolism[139]. The frequency of CYP2C19 polymorphism differs depending on ethnicity. Asians have a higher proportion of PM compared with Western populations, particularly Caucasians and African-Americans[139,141]. In contrast, Caucasians have a higher prevalence rate of HomEM compared with Asians[142]. Accordingly, geographic differences should be considered in selecting doses or types of PPIs for *H. pylori* treatment since there is a significant difference between HetEM and HomEM (OR = 1.90; 95%CI: 1.38-2.60) in *H. pylori* eradication rate[143]. In additional subanalysis of individual PPIs revealed that omeprazole was influenced by the CYP2C19 genotype[143]. In another meta-analysis, successful eradication rates differed considerably between PM and HetEM (OR = 1.73, P = 0.002) and between PM and HomEM (OR = 2.79, $P < 0.0001$) and even between HetEM and HomEM (OR = 2.00, $P < 0.0001$)[144]. This study showed that

a regimen including rabeprazole was not affected by CYP2C19 genotype status[144]. According to a meta-analysis of a RCT in 2013, regardless of the PPI being taken, the eradication rates of PM were higher than HetEM and HomEM[145]. In addition, results of the sub-analysis of the PPI type, omeprazole and lansoprazole were affected by CYP2C19 genotype. Unlike above, esomeprazole and rabeprazole were not affected by CYP2C19 genotype[145]. In studies published in Japan, esomeprazole and rabeprazole are less influenced by CYP2C19 genotype compared with another PPIs[146,147]. The efficacy of tailored *H. pylori* eradication treatment was demonstrated by Sugimoto *et al*[148]. In a tailored regimen, *H. pylori* patients with clarithromycin-sensitivity were treated with clarithromycin, amoxicillin, rabeprazole, while clarithromycin-resistant patients were treated with metronidazole, amoxicillin, rabeprazole for 1 wk. As a result, the overall eradication rate was 96.7% (95%CI: 92.5%-98.9%) by ITT analysis and 97.4% (95%CI: 93.4%-99.3%) by PP analysis[148]. The method achieved high eradication rates of 94.3% in CYP2C19 rapid metabolizers[148]. Although CYP2C19 genotyping remains difficult clinically, tailored therapy may be useful in overcoming decreased eradication rates.

NEWER AGENTS AND NONTRADITIONAL THERAPIES FOR *H. PYLORI* ERADICATION: HOPE IS COMING?

In the last decade, many researchers have argued that new classes of antimicrobials with novel mechanisms of action are necessary to overcome increasing drug resistance. Some agents have shown an antibacterial effect against *H. pylori in vitro* regardless of drug resistance and are effective even at low pH. Among them, pyloricidin A, B, and C have a strong and selective anti-*H. pylori* effect which an MIC_{90} value of 0.013 mg/L[149]. Benzimidazole derivatives (MIC_{90} = 0.025), polycyclic compound (MIC_{90} = 0.2-0.39), arylthiazole derivative 44 (MIC_{90} = 0.0065) also were highly effective against *H. pylori*[149].

Cathelicidins and defensins are examples of human antimicrobial peptides (AMPs) native to the innate immune system of many eukaryotes that have activity against *H. pylori*[150]. LL-37 is a cathelicidin with an anti-*H. pylori* effect[151], and a recent study demonstrated that cathelicidin limited *H. pylori* colonization and related gastritis in mouse models[152]. Defensin peptides have also been indicated to impede *H. pylori*[153]. Human beta defensin 2 and 3 are differentially expressed in gastric mucosa during *H. pylori* infection[154]. Oligo-acyl-lysyl (OAK) peptides, which have a structure and function similar to those of natural AMPs, have broad-spectrum antibacterial activity and anti-*H. pylori* effect *in vivo*[155]. Unlike the natural AMPs, OAK peptides are without known proteolytic cleavage sites and thus, resistant to enzymatic cleavage.

SQ109 was developed as a tuberculosis treatment and known to be safe and tolerated in human trials[150]. In an *in vitro* study, SQ109 had anti-*H. pylori* activity and a low *H. pylori* resistance rate[156]. Pyridodiazepines are potent and selective molecules that target the *H. pylori* MurI inhibitor not effective against other bacteria[157]. Sulfonamides and sulfamates were potent anti-beta-carbonic anhydrase molecules[150]. *H. pylori* beta-carbonic anhydrase catalyzes the hydration of carbon dioxide to proton and bicarbonate to facilitate *H. pylori* metabolism of urea and bicarbonate and survive in low pH. Sulfonamides and sulfamates, inhibit the enzyme and are effective against *H. pylori*[158].

Phytotherapy is expected to be another promising therapy for *H. pylori* eradication. Ginger rhizome extract has been demonstrated to have defensive activity in the stomach, increase stomach mucin regeneration, reinforce antioxidant enzymes, and suppress *H. pylori* growth[159]. Capsaicin has an anti-inflammatory effect and inhibited *H. pylori*-induced interleukin (IL)-8 production by gastric epithelial cells[160]. Sulphoraphane has also been indicated to suppress colonization and inhibit gastritis in *H. pylori*-infected mice and humans[161]. Red ginseng extract has inhibitory 5-LOX enzyme activity and LOX-inhibiting action that suppresses inflammation of *H. pylori*-infected gastric epithelial cells[162]. Epigallocatechin gallate, one of the green tea catechins, showed significant cytoprotective effects against *H. pylori* associated gastric cytotoxicity[163]. Red wine and resveratrol have also been shown to inhibit the growth of *H. pylori cagA*+ strains *in vitro*[164]. In an open-label RCT, adding vitamin C and E to antibiotic regimens showed excellent *H. pylori* eradication rates. Compared to the group that did not contain vitamin, the group that combined vitamin C and E to lansoprazole, amoxicillin, clarithromycin, and bismuth citrate treatment had significantly higher eradication rates of 91.3% by ITT analysis and 93.5% by PP analysis[165]. Thus, vitamin supplementation may be a future treatment option for *H. pylori*-related disease.

MICRO- AND NANO-TECHNOLOGY: IS THE ROAD TO *H. PYLORI* ERADICATION IN THE FUTURE?

Recently, several studies have determined the antibacterial activity of micro- and nano-technology against *H. pylori*. Liposomes are spherical vesicles that contain amphiphilic lipids in a bi- or multi-layer with an aqueous core used to encapsulate several compounds[4]. This material contains biocompatible and biodegradable constituents without significant toxicity[4]. According to Obonyo *et al*[166], a liposomal nanoformulation of linolenic acid is a favorable nanotherapeutic with bactericidal activity against resistant strains of *H. pylori*. Another study suggested that an epitope-based therapeutic *H. pylori* vaccine may be beneficial in eradicating *H. pylori*[167] and a double liposome-based dual drug system may be helpful for treatment of *H. pylori* infection[168].

Polymeric particles have a number of advantages for use as an antibiotic delivery factor with an anti-*H. pylori* effect. It is possible to manipulate their shape to affect biodistribution to increase interactions with the target cell. They also have mucoadhesive properties and protects drugs from proteolytic enzyme. Importantly polymeric particles possess several mechanisms to overpower microbes[4]. Encapsulation of clarithromycin and omeprazole using gliadin nanoparticles as a mucoadhesive component has been reported for the treatment of *H. pylori*[169]. Another study also showed that positively charged gelatin microspheres could be a feasible applicant delivery system for eradication of *H. pylori*[170]. The amoxicillin-loaded chitosan mucoadhesive microspheres could increase gastrointestinal residence time and enhance amoxicillin stability to contribute to *H. pylori* treatment[171]. In addition, chitosan nanoparticles improved the anti-*H. pylori* effect of chitosan[172]. Genipin-cross-linked fucose-chitosan/heparin nanoparticles diminished drug release in stomach acid and then released amoxicillin in an *H. pylori* survival situation to inhibit *H. pylori* proliferation. In addition, amoxicillin-loaded nanoparticles increased *H. pylori* eradication and decreased *H. pylori*-associated gastric inflammation in an animal model[173]. The metronidazole-loaded porous microparticles that exhibit sustained release of metronidazole could assist *H. pylori* eradication and healing from mucosal damage[174]. Silver nanoparticles may also be safer bactericidal agents for the treatment of *H. pylori*-induced gastritis[175]. Berberine-loaded targeted nanoparticles stimulated *H. pylori* clearance and suppressed stomach inflammation in *H. pylori* infection[176].

CONCLUSION

Many studies have determined that novel agents and treatment regimens can improve eradication of *H. pylori*. With STT, high doses of PPI and prolonged therapy duration can increase eradication rates; indeed, in Europe and some regions of Asia these results are improved further with concomitant therapy. Concomitant therapy is less affected by antibiotic resistance, which adds value as an alternative treatment. Nevertheless, the eradication rates following concomitant therapy will gradually decrease due to the rapidly emerging antibiotic resistance of *H. pylori* worldwide.

In this review, we highlighted new and promising directions in *H. pylori* eradication. Although there are some practical limitations in applying probiotics and tailored therapy, they could of assistance in fighting *H. pylori*. Newer agents, nontraditional therapy, and microtechnology are also expected to play a major role in *H. pylori* eradication. However, several issues need to be solved to apply these treatments to the clinic. First, novel agents must be devoid of known proteolytic cleavage sites and thus, resistant to human digestive enzymatic cleavage. Second, these agents must be effective in an acidic environment. Third, these novel agents should be free from antibiotic resistance such as OAK. OAK have multiple nonspecific actions, so it would be hard to occur antibiotic resistance of *H. pylori*. Fourth, further studies are necessary to assess micro- and nano-toxicity, *in vitro* as well as *in vivo*. The safety and pharmacokinetic properties of novel treatments for *H. pylori* in humans also need to be evaluated. Finally, although novel treatments have many advantages, clinical studies are required to determine whether these findings can be applied to humans. In order to improve the eradication rate for *H. pylori* infection, further studies must be required.

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MINIREVIEWS

Autoimmune pancreatitis and cholangitis

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Abstract

Autoimmune pancreatitis (AIP) is part of a systemic fibrosclerotic process characterized by lymphoplasmacytic infiltrate with immunoglobulin G subtype-4 (IgG4) positive cells. It characteristically presents with biliary obstruction due to mass-like swelling of the pancreas. Frequently AIP is accompanied by extra-pancreatic manifestations including retroperitoneal fibrosis, thyroid disease, and salivary gland involvement. Auto-antibodies, hypergammaglobulemia, and prompt resolution of pancreatic and extrapancreatic findings with steroids signify its autoimmune nature. Refractory cases are responsive to immunomodulators and rituximab. Involvement of the biliary tree, termed IgG4 associated cholangiopathy, mimics primary sclerosing cholangitis and is challenging to manage. High IgG4 levels and swelling of the pancreas with a diminutive pancreatic duct are suggestive of autoimmune pancreatitis. Given similarities in presentation but radical differences in management and outcome, differentiation from pancreatic malignancy is of paramount importance. There is controversy regarding the optimal diagnostic criterion and steroid trials to make the diagnosis. Additionally, the retroperitoneal location of the pancreas and requirement for histologic sampling, makes tissue acquisition challenging. Recently, a second type of autoimmune pancreatitis has been recognized with similar clinical presentation and steroid response though different histology, serologic, and extrapancreatic findings.

Key words: Immunoglobulin G; Pancreatitis, chronic; Pancreatitis; Pancreatitis, sclerosing cholangitis, and sicca complex; Sclerosing cholangitis; Retroperitoneal fibrosis

Core tip: Autoimmune pancreatitis is a component of a systemic immunoglobulin G subtype-4 mediated disease which also impacts the bile duct, salivary glands, kidney, and numerous other sites. It presents with jaundice and pancreas mass but it responds promptly to steroids and immunomodulators. A careful diagnostic approach is mandatory as autoimmune pancreatitis and its biliary manifestations closely resemble pancreas cancer and primary sclerosing cholangitis, diseases which have a more ominous course.

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BACKGROUND AND CLINICAL PRESENTATION

While autoimmune pancreatitis (AIP) has been the subject of intense recent interest, its description has actually been unfolding over the past half century. In 1961, Sarles *et al*[1] reported a series of patients with chronic inflammatory pancreas sclerosis. In the 1970's and 1980's astute clinicians correlated its association with salivary gland and bile duct dysfunction and it was theorized to represent a systemic immunologic dry gland syndrome[2-5]. It came to be known by a number of different monikers including sclerosing pancreatitis, primary sclerosing cholangitis with pancreatitis, and duct narrowing chronic pancreatis. Kawaguchi *et al*[6] used gross resection specimens to report the characteristic histology of autoimmune pancreatitis. They described a dense lymphocytic infiltrate rich in antibody producing plasma cells (lymphoplasmacytic infiltrate) which surrounded and compressed the pancreatic ducts. Additionally, they observed storiform fibrosis and obliterative phlebitis (but sparring of arterioles) due to the lymphocytic infiltrate[6,7].

Yoshida *et al*[8] coined the term autoimmune pancreatitis in 1995 when he noticed its clinicopathologic similarities to autoimmune hepatitis. He treated a patient who had undergone exploratory laparotomy and diagnosed (though not confirmed with tissue) with unresectable pancreatic cancer. The patient had waxing and waning jaundice and pancreatic swelling on computed tomography imaging. Yoshida observed markedly elevated immunoglobulin G, antinuclear, anti-thyroglobulin, and anti-microsomal antibodies. He treated the patient with corticosteroids and the swelling, biochemical, and serologic abnormalities all remitted.

Multiple series have further characterized the clinical presentation. Typically, it mimics chronic pancreatitis or pancreatic cancer though acute pancreatitis occurs in 7%-25% of cases[9-11]. Hypergammaglobulinemia and positive serum autoantibodies are seen in three quarter of patients with antinuclear antibody being most frequently elevated. Extra-pancreatic manifestations occur in 50% of cases. Radiographic, serologic, and extrapancreatic manifestations help to further confirm the diagnosis.

IMAGING FINDINGS

In AIP the pancreas exhibits diffuse gland enlargement with loss of clefts and a peripheral rim of altered enhancement (the halo sign) on cross sectional imaging[12,13]. In 20%-30% of patients, there is mass-like enlargement of the head with tail atrophy. Peripancreatic lymphadenopathy is seen in 25%. Endoscopic and magnetic resonance cholangiopancreatography reveals an irregular, diminutive pancreatic duct[14,15]. Endoscopic ultrasound demonstrates a diffuse altered (hypoechoic echotexture) gland enlargement (Figure 1). It typically lacks the hyperechoic foci and strands seen in chronic pancreatitis. Cross sectional imaging is also useful in detecting extrapancreatic manifestations (see below) and assess response to therapy[14].

IMMUNOGLOBULIN G SUBTYPE-4

Immunoglobulin G subtype-4 (IgG4) is elevated in number of autoimmune diseases including pemphigus vulgaris and membranous nephropathy. In a breakthrough paper in 2001 Hamano *et al*[16] demonstrated that it was markedly elevated in 20 patients with classic findings of autoimmune pancreatitis but not in normal patients. While it was modestly elevated in a number of pancreatico-biliary processes such as chronic pancreatitis and primary sclerosing cholangitis (PSC), IG4 was much more specific for AIP.

However, subsequent series have revealed that when > 140 mg/dL is defined as abnormal, the specificity of IgG4 is only 93%[9]. As AIP has a low prevalence, even in referral centers, most abnormal levels in patients with nonspecific gastrointestinal symptoms are false positives. Additionally, approximately 10% of pancreas cancer patients and 9% of patients with PSC have elevated levels of IgG4[9,17]. Increasing the cutoff to 280 mg/dL decreases sensitivity but improves specificity to 99%. Similarly, IgG4+ plasma cells may be seen in the periductal and stromal tissue. A small number of IgG4+ cells may be seen in chronic pancreatitis and pancreas cancer whereas dense infiltration with IgG4+ cells is specific for AIP[18]. Additionally, IgG4 staining in cancer does not exhibit the periductal pattern seen in autoimmune pancreatitis.

EXTRAPANCREATIC MANIFESTATIONS

Concomitant salivary gland and biliary involvement were reported even in the initial reports of autoimmune pancreatitis. Shortly after the acceptance of AIP as a true autoimmune entity a number of authors demonstrated that it was associated with a form of retroperitoneal fibrosis which promptly resolved with steroid therapy[19,20]. AIP patients also may present with concomitant thyroiditis, fibrotic pseudotumor of the orbit, and nodules of the renal cortex and collecting system, all of which demonstrate an IgG4 positive lymphoplasmacytic infiltrate on histology and respond to steroids[21-24]. Lung fibrosis, cervical and mediastinal lymphadenopathy are also associated with AIP[25].

Asymptomatic lymphoplasmacytic infiltration of the gallbladder, duodenum, and stomach with IG4 positive cells has been demonstrated on surgical specimen (cases misinterpreted as pancreas cancer) further suggesting the systemic nature of this disease[21]. Hypothyroidism

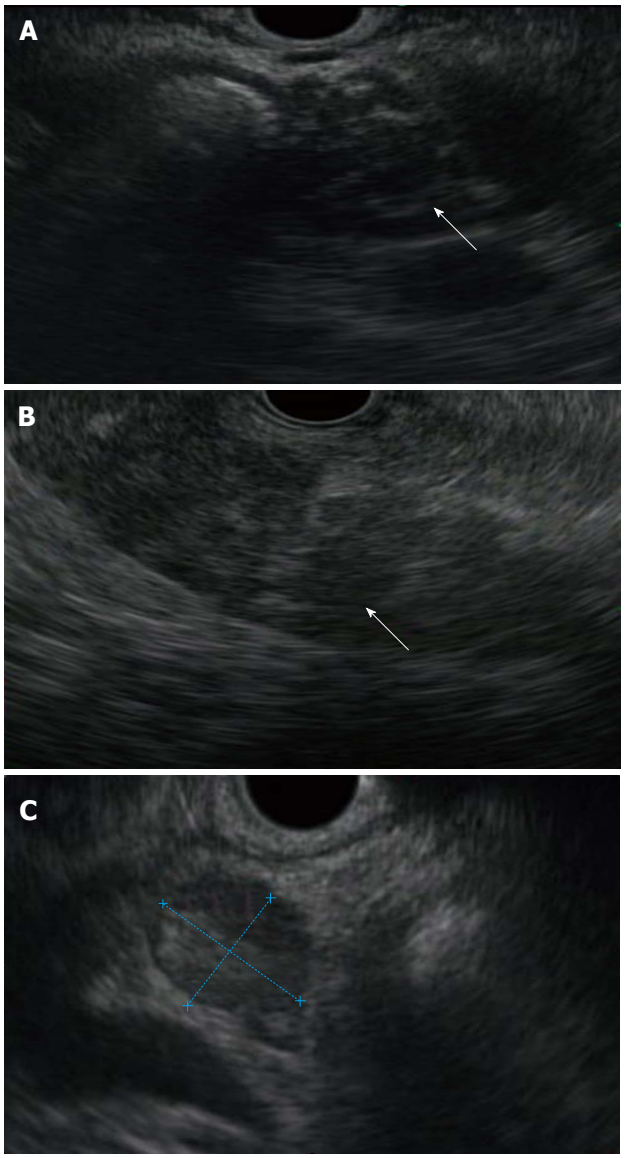

Figure 1 Endoscopic ultrasound features of autoimmune pancreatitis include diffuse hypoechoic changes in the head (A) and body (B) of the pancreas (note the metallic stent placed when the mass was assumed to represent carcinoma), and hypoechoic peripancreatic lymph nodes (C) of a similar echogenicity are frequently seen.

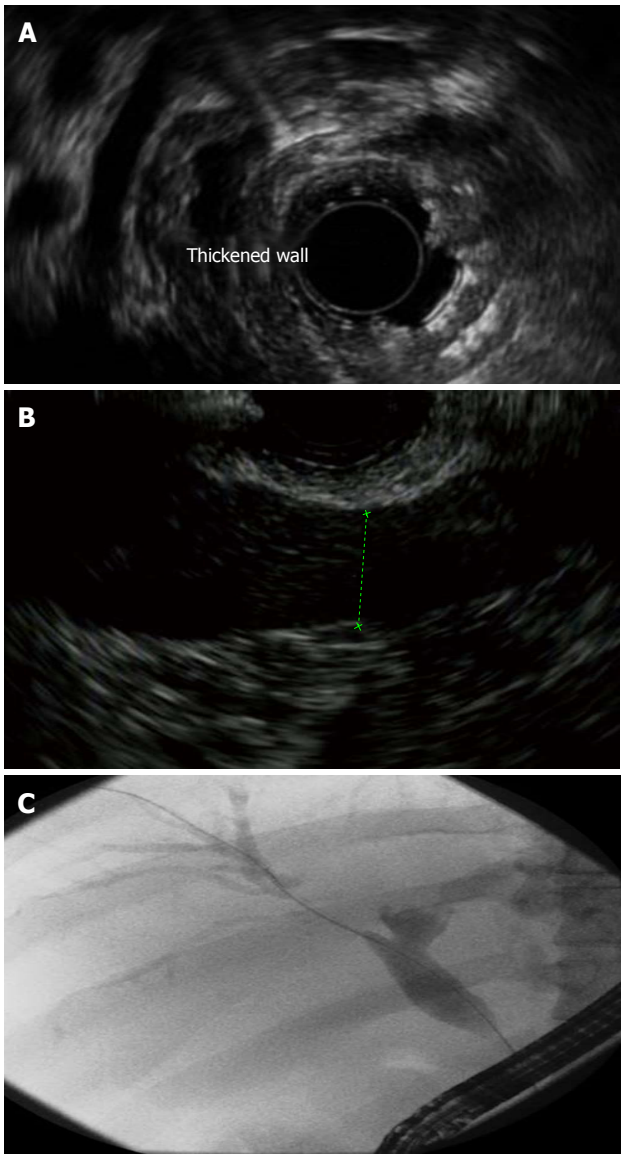

Figure 2 Endoscopic ultrasound reveals marked bile duct thickening (A) in a patient with longstanding autoimmune pancreatitis as evidenced by diffuse hypoechoic changes in the pancreas (B), and endoscopic retrograde cholangiopancreatography demonstrated segmental narrowing with prestenotic dilation (C).

is seen in 26.8% of patient with autoimmune pancreatis and antithyroglobulin Ab in 7.3%. compared to 0% and 7.3% of those with chronic pancreatitis[26]. In Western populations, there is a correlation of AIP with inflammatory bowel disease[25]. Thus, it is increasingly recognized that IgG4 mediated disease is a systemic, multifocal fibrosclerotic process[22,27].

IGG4 ASSOCIATED CHOLANGIOPATHY

Biliary involvement may be present at the time of AIP diagnosis but more frequently follows diagnosis[28]. Histologically, it is characterized by a peri-bile duct lymphoplasmacytic infiltrate similar to AIP and imaging reveals bile duct thickening and enhancement (Figure 2)[28]. Intraductal ultrasound may demonstrate inflammation before it is clinically significant. IgG4 Associated Cholangiopathy (IAC) often simultaneously involve both the intrahepatic and extrahepatic biliary tree.

It is challenging to differentiate IAC from PSC. Cholangiographic features of segmental narrowing with prestenotic bile duct dilation suggests IgG4 IAC compared to beading, pruning, and diverticuli which are more consistent with PSC[29]. Additionally IAC is more likely to involve the distal bile duct than PSC[17,30]. Clinically, IAC manifests with the sudden onset of jaundice while a slow rise in cholestatic enzymes is seen in PSC. At comparable bilirubin levels, IAC tends to demonstrate much less advanced histology than PSC and there is a predominance of lymphocytes[30].

Cholangiocarcinoma complicates PSC but typically

not IAC though an inflammatory pseudotumor due to a dense IG4-lymphoplasmytic infiltrate may mimic an intrahepatic bile duct tumor[31]. High levels of IgG4 (4 times upper limit of normal) positive cells in brushings helps to distinguish IAC from cholangiocarcinoma[32]. Retrospective assessment of IgG4 in patients who underwent transplant for PSC revealed that those who were positive had much higher bilirubin levels, Mayo risk scores, and progression to transplant. It is plausible that they might have actually had IAC. These findings emphasize the importance of making this diagnosis as IAC is responsive to steroids[17].

TREATMENT OF AIP

Steroid response was one of the initial features which suggested that AIP was an autoimmune process and corticosteroids remain the primary treatment modality. A large multicenter trial demonstrated that corticosteroid treatment induces remission in 98%[33]. The recommended starting dose of prednisone is 0.6 mg/kg per day (30-40 mg) followed by a slow taper over 3-6 mo[33-35]. Maintenance treatment with prednisone 2.5-10 mg for an additional 3-6 mo decreased relapse rates to 24%, which is significantly lower than those who stop taking steroids early, 34%, or are not treated 42%[33].

IgG4 levels may help determine disease activity especially following treatment with steroids and absence of improvement in the first 2-4 wk may suggest underlying malignancy[36,37]. As long term steroid therapy in this patient population has been associated with major complications including avascular necrosis, steroid cessation or transition to an immunomodulator is recommended within 1 year[33].

There are several predictors of relapse including extrapancreatic manifestations, diffuse pancreas swelling, and concomitant IAC[38,39]. While steroids are effective in 95% of cases of relapse, immunomodulators including azathioprine, 6-mercaptopurine, and mycophenolate mofetil should be considered for patients with multiple relapses or steroid refractory disease. Failure or inability to tolerate immunomodulators occurs in a significant percentage of those with AIP[40]. In these refractory cases the monoclonal antibody rituximab (anti-CD20), which depletes B cells, is successful in most (> 80%) of cases[40,41]. Rituximab's efficacy may lie in its ability to modulate the Th2 process implicated in AIP[42].

SPECIAL TREATMENT CONSIDERATIONS FOR IAC

The clinical and pathologic manifestations of AIP with IAC are also responsive to corticosteroid therapy[28]. However, these patients have a 30% higher rate of relapse than those without biliary involvement[40]. Relapse is particularly common in proximal (65%) *vs* distal (23%) biliary disease[33,39]. Immunomodulators are required in relapsing and refractory IAC[39]. Rituximab is an important option for refractory IAC and its use in biliary IgG4 mediated disease preceded its use in AIP without IAC[41]. In addition, a biliary stent is temporarily required in > 70% of cases to alleviate jaundice but typically may be removed following medical therapy[40].

DIAGNOSTIC CRITERION

The diagnosis of autoimmune pancreatitis must be made with caution as pancreatic cancer has a similar presentation but is much more prevalent. In an early series, Kamisawa *et al*[21] reported that > 50% of patients with AIP may have findings which are highly suggestive of pancreas cancer including elevated CEA, CA 19-9, portal vein narrowing, and bile duct stenosis. Additionally 10% of pancreas cancer patients will have significantly elevated levels of IgG4[9]. The Japanese Pancreas Society proposed the first diagnostic criterion for AIP which required diffuse enlargement of the pancreas with irregular pancreatic duct narrowing and histologic anomalies or elevated IgG4 levels[43,44].

Chari *et al*[45] found that the Japanese criterion would diagnose < 30% of patients in a Western population with histologic confirmed autoimmune pancreatitis. Additionally, routine diagnostic endoscopic pancreatography is not feasible in the litigious culture of the United States. Chari *et al*[45] proposed 3 potential methods to diagnose AIP: (1) classic histology; (2) typical imaging and elevated serum IgG4; and (3) pancreatic disease or extrapancreatic disease which responds to steroids[46]. These histology imaging serology, other organ involvement, response to treatment were adopted in most Western centers to diagnose autoimmune pancreatitis[45].

However, following application of these criteria, core biopsy, surgery, or steroid trials are required in 30% to differentiate AIP from cancer[46]. Experts from Asia and Western Countries recently proposed the International Consensus Diagnostic Criteria, which include 3 out of 4 characteristic histologic criterion: (1) lymphoplasmacytic infiltrate; (2) obliterative phlebitis; (3) storiform fibrosis; and (4) > 10 IgG4 positive cells/high power field or a combination of characteristic imaging and either 2/4 pathologic criterion, elevated IgG4, or extrapancreatic manifestations[47]. The consensus guidelines discourage the use of diagnostic steroid trials to diagnose AIP as pancreatitis and IgG4 elevations related to pancreas cancer may improve and give a false diagnosis of autoimmune pancreatitis.

In difficult cases, surgical evaluation is a reasonable approach given the difficulty of differentiating AIP from malignancy. A combined series from Johns Hopkins Medical Center and the Mayo Clinic included 37 patients with autoimmune pancreatitis among 1648 who had undergone pancreaticoduodenectomy[48]. While blood loss in AIP was higher than for other indications for Whipple resection, the quality of life in 68% was improved and none had recurrent jaundice at a median follow up of 33 mo.

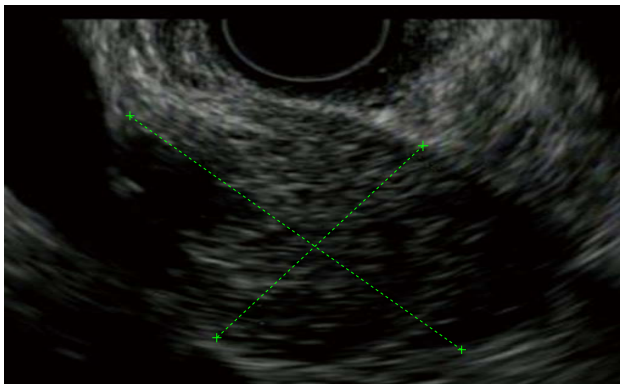

Figure 3 Endoscopic ultrasound reveals a hypoechoic pancreas mass in a jaundiced sixteen-year-old patient with crohn's disease confirmed by histologic sampling to have type Ⅱ autoimmune pancreatitis.

TISSUE ACQUISITION

Histology, which enables architectural assessment is needed to confirm the diagnosis in challenging cases. While endoscopic ultrasound guided aspiration methods offer the most direct access to the pancreas, this technique is more amenable to providing cytology specimens, which are less useful in AIP. A core endoscopic ultrasound (EUS) needle equipped with a spring loaded cutting sheath was used in a small series of patients with autoimmune pancreatitis[49]. However, the torque on the device required to access the pancreas head and uncinate from the duodenum prevented it from firing[50]. A new reverse bevel core needle has shown promise in the acquisition of tissue for histology and is the topic of an ongoing randomized trial for AIP tissue acquisition. Larger 19 gauge standard EUS needles have been used with meticulous specimen preparation techniques to yield histologic samples in lymphoma[51]. However, the results of this approach were disappointing in autoimmune pancreatitis as histologic confirmation was only achieved in 43% of patients[52]. Even with acquisition of histology, the patchy involvement of autoimmune pancreatitis limits EUS sampling and tissue acquisition remains a challenge. Surgery may be required for definite diagnosis.

Surgical specimens from AIP resection (cases thought to be cancer) reveal IgG4 infiltration of the stomach, duodenum, and other surrounding structures which provides another avenue for diagnosis. Endoscopically, AIP demonstrate papillary swelling in 41% of those with AIP and biopsies in cases of papillary swelling may reveal dense IgG4 cell (> 10/high power field) infiltration[53,54]. Pale thickened regions of mucosa of the stomach, duodenum and colon on biopsy also reveals focal infiltrates of IG4 (> 10/high powerfield) positive plasma cells[53,55]. Mucosal biopsies represent a minimally invasive adjunct to other sampling methods[53,56].

LONG TERM OUTCOMES

In some cases, AIP disease is progressive. Relapse is associated with chronic pancreatic injury. Between 33%-55% of those with recurrence develop pancreatic duct stones, though 4% without recurrence also develop evidence of chronic disease[35,40,57]. While steroids improve both the exocrine and endocrine abnormalities seen in autoimmune pancreatitis, abnormalities are only partially reversible with some patients developing chronic diabetes mellitus and steatorrhea[58]. In addition to the pancreatic dysfunction, chronic salivary gland dysfunction has also been shown to occur[58]. In some cases IAC results in progression to cirrhosis and portal hypertension[39]. AIP is also associated with increased risk of malignancy; the most common cancers were gastric, lung and prostate[59].

TYPE Ⅱ AIP

Recently a distinct variant of autoimmune pancreatitis, type Ⅱ, has been described.

Similar to Type Ⅰ AIP it presents with jaundice and mass-like changes in the gland (Figure 3). However, Type Ⅱ AIP presents with a completely different histological pattern, an idiopathic duct-centric chronic pancreatitis with increased intraluminal and epithelial neutrophils[60,61]. It has primarily been described in western nations and is not associated with extrapancreatic manifestations with the exception of inflammatory bowel disease[60]. In addition serologies and IgG4 are typically negative[40,61]. Given the absence of potential corroborative markers, definitive diagnosis requires histology[62]. Type Ⅱ is found predominantly in much younger patients than those with type Ⅰ AIP[61]. The pediatric entity of idiopathic fibrosing pancreatitis in which children (often with concomitant IBD) present with jaundice and pancreas mass is likely the same disease as type Ⅱ AIP[63]. Type Ⅱ AIP responds to steroids in 92% and is much less likely to relapse than Type Ⅰ AIP[40,61]. However, given that it is a recently described entity, studies of long-term sequelae and clinical course are lacking.

CONCLUSION

In summary, autoimmune pancreatitis presents with jaundice and pancreas mass mimicking pancreatic cancer. However, prompt resolution with steroids, autoantibodies, and hypergammaglobulinemia led to its recognition as an autoimmune process. It is frequently accompanied by diverse steroid responsive, IgG4 mediated abnormalities including cholangiopathy, retroperitoneal fibrosis, and sialadenitis. Challenges include the appropriate use of IgG4 and various criterion for its diagnosis, better methods to obtain tissue, and treatment of steroid refractory cases. Type Ⅱ AIP has a similar clinical presentation and treatment, but distinct histology, serology, and extrapancreatic manifestations.

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MINIREVIEWS

Age-related differences in celiac disease: Specific characteristics of adult presentation

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Abstract

Celiac disease may appear both in early childhood and in elderly subjects. Current knowledge of the disease has revealed some differences associated to the age of presentation. Furthermore, monitoring and prognosis of celiac subjects can vary depending on the pediatric or adult stage. The main objective of this review is to provide guidance for the adult diagnostic and follow-up processes, which must be tailored specifically for adults and be different from pediatric patients.

Key words: Celiac disease; Diagnosis; Complications; Gluten intolerance; Duodenal biopsy

Core tip: Current knowledge of celiac disease (CD) has revealed differences linked to the age of onset. These differences are related to the epidemiology, pathogenicity, clinical signs and prognosis of the disease. Here we present a comprehensive review of CD focusing on the age-specific management of patients. The knowledge of particular aspects linked to either adults or children would improve both the diagnosis and follow-up of this disease. This review can be helpful to the clinician involved in the management of adult and pediatric patients.

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INTRODUCTION

Celiac disease (CD) is an autoimmune inflammatory enteropathy triggered by gluten intake in genetically susceptible persons. It was initially described in children and, for many years, has been considered an

almost exclusively pediatric entity. The main clinical presentations in the pediatric population include, malnutrition and growth delay along with persistent diarrhea and high mortality[1]. Although the long standing assumption is that CD develops in childhood, the disease can occur at any age. The epidemiology and symptomatology of CD have both considerably changed in recent years, with a significant increase in adult prevalence that was not previously suspected[2]. The clinical presentation of CD has also changed, from typical malnutrition to oligosymptomatic cases such as anemia, osteoporosis and even asymptomatic cases diagnosed by screening high-risk groups[3,4].

The aim of this review is to describe the main differences in CD according to age and highlight the most characteristic aspects of its adult forms.

EPIDEMIOLOGY

CD is a disorder more frequent than previously described. The current prevalence of CD ranges between 1/100-1/500 subjects depending to the different populations surveyed and the method employed, with a higher positive diagnosis in females[2]. The accuracy of CD prevalence has been substantially improved by the high sensitivity and specificity of serological tests detecting anti-endomysial, anti-tissue transglutaminase or anti-deamidated gliadin antibodies. These tests have allowed screening of large cohorts, which resulted in estimating CD prevalence as high as 1% of the general population, both in Europe and North America[5]. However, most of these studies are focused on children. A recent epidemiological study in Catalonia that used serology to analyze prevalence, showed that CD prevalence in children (1/71) was five times higher than in adults (1/357). The authors proposed a tendency towards latency in adults, which would explain the lower prevalence of CD during adulthood[6]. Similar studies in Brazil and India also found twice the CD frequency in children[7,8].

However, these data are in contrast with the observed prevalence in adult populations from European countries such as the United Kingdom (1.2%)[9] and, more surprisingly, Finland, where a 2.4% prevalence of CD was demonstrated by biopsy in a population of adults over fifty years of age[10]. The pediatric population was not analyzed in these studies, but prevalence of over 1%, even in older populations, is similar to that observed in pediatric studies. A recent systematic review on world CD frequency in recent years, mainly based on European studies, found variable ratios of prevalence and incidence, which was similar, in many cases, between children and adults[2].

Although a hypothesis based on the evolution of pediatric CD towards latency would explain a lower frequency in adults; more studies on the natural evolution of this disease are needed in order to confirm it. It is clear that adult forms are becoming more frequent, sometimes reaching similar or even higher numbers than those of the pediatric population. The screening methods and the type of population selected may have an influence, since in high-risk groups of adults as well as in first-degree relatives, affected individuals may exceed 15%[3]. It must also be taken into account that, as we discuss below, studies based on serological screening may not really demonstrate adult prevalence, where antibody titers are low or even negative, while histological damage and symptoms are compatible with CD[11].

SYMPTOMATOLOGY

The clearest difference between children and adults is the clinical expressiveness of the disease at the time of diagnosis. Different studies have shown that classic clinical malabsorption pattern is frequently observed in children during diagnosis, while classical symptoms occur in less than 25% of adult cases[12]. Table 1 contains an overview of different clinical manifestations in CD according to the presentation age.

A tendency towards lower clinical manifestations can be observed as age increases[4]. In older children and adults only limited symptoms are prevalent, which often consist of an increase in stool volume, or intestinal gas generated by lactose malabsorption or bacterial overgrowth. In fact, constipation may be the only manifestation in a celiac adult.

Extraintestinal symptoms or manifestations are quite frequent in adult CD, and may appear associated with other digestive symptoms such as asthenia, oral sores, osteoporosis or skin lesions. In fact, some patients only present these extraintestinal symptoms at diagnosis. Although celiac children may exhibit non-digestive manifestations, these are less frequent and show a predominant digestive symptomatology[13].

There are numerous diseases associated with CD in children and adults. However, the presence of associated pathologies in adults seems to be more frequent and may have an autoimmune origin like CD. Autoimmune disorders such as autoimmune thyroiditis, type Ⅰ diabetes, Sjögren's syndrome or dermatitis herpetiformis have been linked to CD[14,15].

Even though malnutrition is a frequent manifestation in CD, mainly in adults, excessive weight or obesity may also be present at diagnosis. Recent studies have shown that more than half the adults diagnosed with CD have obesity while only 15% of them are below their normal weight[16,17]. These figures are from European and North American studies, where the prevalence of obesity among the adult population is high. Excess weight can also be observed in children at a lower frequency. Therefore, excess weight in an adult must not be a reason to lower the suspicion threshold for CD.

Functional dyspepsia and irritable bowel syndrome are two functional digestive pathologies, which are quite prevalent in the adult population, but are also seen during infancy. CD prevalence in adults with functional dyspepsia or irritable bowel syndrome can rise to over

Table 1 Age-related major clinical findings at celiac disease diagnosis

Children < 2 yr	Children > 2 yr	Adults
Diarrhea	Loose stools	Dyspepsia/irritable bowel syndrome
Malnutrition	Iron deficiency	Iron deficiency
Bloating	Abdominal pain	Constipation
Vomiting	Dyspepsia	Osteoporosis
Irritability	Growth delay	Arthritis
Muscular atrophy	Headache	Hypertransaminasemia
Anemia	Pubertal delay	Extraintestinal symptoms

Table 2 Diagnostic criteria for celiac disease proposed by Catassi and Fasano[22]

The presence of signs and symptoms compatible with celiac disease
Positivity of serum celiac disease IgA class autoantibodies at high titer
Presence of the predisposing genes *HLA-DQ2* and/or *-DQ8*
Celiac enteropathy at the small intestinal biopsy[1]
Resolution of the symptoms and normalization of serology test following the implementation of a gluten-free diet[2]

[1]Including Marsh-Oberhuber 3 lesions, Marsh-Oberhuber 1-2 lesions associated with positive celiac antibodies positive at low/high titer, or Marsh-Oberhuber 1-3 lesion associated with IgA subepithelial deposits; [2]Histological normalization in patients with sero-negative celiac disease or associated IgA deficiency. At least 4 of 5 (or 3 of 4 if the HLA genotype is not performed). HLA: Human leukocyte antigen.

10% of cases, as reported in some studies. Currently, before diagnosing both functional pathologies in adults, an underlying CD must always be ruled out, either by serology or endoscopy[18,19].

DIAGNOSIS

The diagnosis of CD is based on the presence of serum antibodies against deamidated antigliadin, antiendomysium or anti-tissue transglutaminase antibodies. In addition, the presence of intraepithelial lymphocytosis and/or villous atrophy and crypt hyperplasia of small-bowel mucosa, and clinical remission after withdrawal of gluten from the diet, are also used for diagnosis antitransglutaminase antibody (tTGA) titers and the degree of histological lesions inversely correlate with age[12]. Thus, as the age of diagnosis increases antibody titers decrease and histological damage is less marked. It is common to find adults without villous atrophy showing only an inflammatory pattern in duodenal mucosa biopsies: Lymphocytic enteritis (Marsh Ⅰ) or added crypt hyperplasia (Marsh Ⅱ), as seen in Figure 1.

This lower clinical, analytical and histological expressiveness in adult forms makes their diagnosis more complex than in pediatric forms. The European Pediatric Gastroenterology and Nutrition Society (ESPGHAN) criteria were edited in 2012 for CD diagnosis in children. These current pediatric criteria allow CD diagnosis upon finding high tTGA titers, without the need for a duodenal biopsy[20]. This is, again, based on the evidence of high antibody titers having a high predictive value for villous atrophy, and thus avoiding the need for biopsy. Thus, in pediatric patients, up to 75% of duodenal biopsies can be avoided. However, the presence of high antibody titers (> 10 times normal levels) that allow bypassing biopsies, appear in less than half of adult cases[21]. Besides, currently, when adult subjects present symptoms such as dyspepsia, an upper endoscopy with duodenal biopsy is routinely performed independently of CD serology[18].

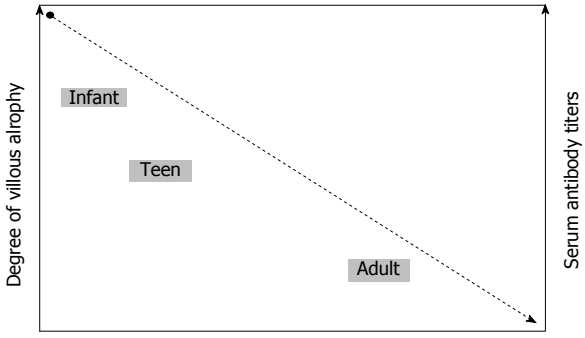

Figure 1 Relationship among age, antibodies and villous atrophy.

Therefore, correct diagnosis can only be achieved on a few occasions, where high tTGA titers are present in adult patients. For low titers, duodenal biopsies have to be performed to verify histological damage and begin a gluten-free diet. However, adult patients with symptoms and serology could have two types of enteropathies based on duodenal biopsy: (1) Duodenal mucosa with slight villous atrophy and increased intraepithelial lymphocytes (Marsh 3A). When a DQ2 or DQ8 human leukocyte antigen (HLA) genotype is found, the patient should be put on a gluten-free diet, and the clinical and histological response must be subsequently evaluated. Thus, 4 out of the 5 criteria for gluten-sensitive enteropathy diagnosis, proposed by Catassi and Fasano[22], would be fulfilled (Table 2); and (2) Mucosa with no villous atrophy, but with a marked increase in intraepithelial lymphocytes (> 25 LIE/100 enterocytes). Such cases present lymphocytic enteritis (Marsh Ⅰ) with diverse etiologies that are independent of CD: *Helicobacter pylori* infection, Non Steroid Anti-inflammatory Drugs intake, infections or Crohn's disease, among others. If none of these conditions are suspected, and the patient has a CD-compatible HLA genotype, the biopsy study can be extended by: Analyzing IgA antitransglutaminase subepithelial deposits[23] and flow cytometry or immunohistochemistry of the duodenal lymphocytic population, in order to evaluate the prevalence of gamma-delta receptor expression in T lymphocytes, which could point to CD[24].

Although, these tools could implicate CD, in order to fulfill the criteria proposed by Catassi, the lymphocytic enteritis response to the gluten-free diet has to be evaluated, along with a biopsy, once clinical improvement is seen. The evolution of lymphocytic enteritis associated with adult CD, and its relation to complications are not clearly understood. Therefore, once lymphocytic enteritis

is found in an adult subject with negative serology, CD diagnosis must be carefully made with a subsequent follow-up check.

POSSIBLE PATHOGENIC DIFFERENCES

A number of studies have evaluated intestinal microbiota in relation to CD[25]. The colonization process during the early stages of life, and the interaction between the microbiota and the immune system, could have an important role in the pathogenesis of CD.

The composition of the intestinal microbiota, both in duodenal biopsies and stool samples of celiac children and adults, exhibits alterations in relation to non-celiac controls[26]. Age-related differences in duodenal microbiota composition have also been found between celiac and non-celiac subjects[27,28]. In general terms, the richness in intestinal microbiota increases with age, as expected. Moreover, differences were also observed in the kind of bacterial community in children and adults. Although these are speculative data, it could be hypothesized that the interaction between microbiota and the immune system differs based on the age-varying microbial composition. This would originate a different response, which could be related to the clinical, analytical and histological responses, we see in adults.

Intraepithelial lymphocytes (IEL) have an important role in the immune response, which is part of the pathogenesis of CD. Within these lymphocytes, the most characteristic feature of CD, independent of the presentation age, is the increase in CD3+ lymphocytes, which express the γδ receptor. The function of γδ IELs is not known, but they could have a regulatory role in the immune response[29]. This would explain their relationship with the degree of villous atrophy both in children and in adults[30]. Another characteristic population is CD3-, which is inversely related to age. It appears at twice the frequency in children under three years of age than adults[31]. Changes in IEL population may be related to the presentation form according to age. Advances in the knowledge of these lymphocytes and CD pathogenesis will offer answers in the near future.

EVOLUTION AND PROGNOSIS

CD treatment consists of a strict life-long gluten-free diet (GFD). Once the diet has begun, a clinical response can be observed in most patients. However, in contrast to children, up to 30% adults may still have symptoms despite having withdrawn gluten from their diet. This fact demands investigation, in adult forms, of other possible causes associated to symptoms in celiac patients: Lactose intolerance, bacterial overgrowth, pancreatic insufficiency, microscopic colitis or refractory CD.

Not only do a high percentage of adults not respond to the GFD, there is also an observable lack of histological recovery during follow-up. Recent studies have shown that more than 50% of adults do not recover from villous atrophy, even after two years on a proper diet[32]. In the case of children, even though information is limited, there is duodenal mucosa recovery in the vast majority (95%) during the first two years after the diagnosis[33]. The main cause of this lack of mucosal recovery in adults could be the continuous and inadvertent ingestion of small amounts of gluten. This cause is likely to be more frequent in adults, since the daily diets of children are more closely monitored.

A strict adherence to the diet along with duodenal mucosa normalization should be the main objectives of adult follow-up. The lack of dietary compliance and persistence of histological damage, are two factors associated with the development of lymphoproliferative disease in adults, which is the most severe complication associated to CD[34]. A recent Swedish population study noted that celiac patients with persistent villous atrophy, during follow-up, had twice the risk of developing a malignant lymphoproliferative disease, mostly T lymphoma[35]. These findings make it necessary to recommend a control biopsy during the second year after diagnosis, in order to evaluate villous atrophy recovery. Thus, those individuals at higher risk of developing CD-associated complications could be identified and a closer follow-up performed in conjunction with monitoring dietary compliance.

CD patients have other complications that should be monitored. Decrease in bone mineral density is probably due to vitamin D deficiency. However, the risk of fracture in CD patients is unclear, and the predictive value of bone densitometry is not sufficient to identify individuals at high-risk of fracture. It seems reasonable to perform bone densitometry on adult CD patients in high-risk situations who include post-menopausal women, men > 55 years and those with known osteopenia before the diagnosis of CD[36]. Further studies are required to identify the efficacy and cost-effectiveness to perform bone densitometry on all adult CD patients at diagnosis, and to identify the follow-up frequency of performing such analysis[37]. Children may have reduced bone mass at diagnosis. However, they are more likely to have fully restored bone mass after 6-12 mo of a GFD than adults. Bone densitometry is not generally required in newly diagnosed pediatric patients with uncomplicated CD. Special attention to ensure normal growth and development is recommended in children[38].

Hyposplenism may affect more than one-third of adult CD patients, while it is not a complication in pediatric patients. The incidence of hyposplenism correlates with the duration of pre-exposure to gluten, and it is higher in those with concomitant autoimmune disorders or pre-malignant conditions[39]. Based on these associated factors, splenic function may be determined in a selected group of adult CD patients: Older patients at diagnosis, those with concomitant autoimmune or premalignant disorders, and patients with a previous history of major infections or thromboembolism. As a diagnostic tool, pitted red cell counting remains an accurate, quantitative and inexpensive method[40].

Table 3 Summary of key features associated with the presentation of celiac disease in adults

High prevalence, even in advanced age
Oligosymptomatic presentation
Serology may have a low diagnostic yield
Duodenal biopsy usually shows mild atrophy or lymphocytic enteritis
Lymphocytic enteritis is a common presentation in adult celiac
The study of duodenal biopsy by an expert pathologist and the use of advanced techniques like flow cytometry may be useful for the diagnosis
Monitoring of the strict dietary compliance and the recovery of villous atrophy
The presence of associated complications should be identified at an early stage

Protein-conjugate vaccines should be recommended in patients with major hyposplenism, defined by a pitted red cell value higher than 10% and/or an IgM memory B cell frequency lower than 10%.

Pediatric CD may be followed using the same scheme as adults. However, bone densitometry and follow-up biopsy should only be performed in select cases. Children with good adherence to GFD and normal antibody levels, should be followed annually instead of every two years. The main reason for this shorter interval is the need for an early recognition of conditions associated to pediatric CD, and especially to ensure normal growth and development. The appearance of malignant complications associated to CD, as well as the development of refractory CD, is both exclusively associated to adult forms. Even when both situations are infrequent, they dictate a different follow-up from that performed for pediatric patients.

CONCLUSION

CD has many differences between children and adults. In adults, the presentation of the disease is less marked than during childhood. Clinicians must be aware of the less marked clinical profile in adults than children, such as atypical or minor symptoms, lower antibody titers and mild mucosal lesions (Table 3). Pediatric diagnostic criteria (ESPGHAN), cannot always be applied to adulthood. In fact, duodenal biopsy is necessary in most adult CD diagnosis, and during follow-up, to assess mucosal recovery and to detect complications.

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ORIGINAL ARTICLE

Basic Study

Plecanatide and dolcanatide, novel guanylate cyclase-C agonists, ameliorate gastrointestinal inflammation in experimental models of murine colitis

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Author contributions: Shailubhai K, Arjunan KP, Saykhedkar S, Nefsky B, Foss JA and Comiskey S contributed to study concept, design, data acquisition, analysis and interpretation; Shailubhai K, Palejwala V, Jacob GS and Plevy SE contributed to manuscript preparation, critical revision and provided important intellectual content.

Institutional review board statement: Experiments reported in this manuscript did not involve human samples and hence institutional review is not applicable.

Institutional animal care and use committee statement: Studies employing BALB/c and TCR$\alpha^{-/-}$ mice were performed under the direct supervision of Doctor Scott Plevy at the University of Pittsburgh School of Medicine (Pittsburg, PA). Animals obtained from Jackson Laboratories (Bar Harbor, ME) were housed in accordance with guidelines from the American Association for Laboratory Animal Care and Research. Institutional Animal Care and Use Committee of the University of Pittsburgh approved all protocols. Epistem Ltd (Manchester, United Kingdom) conducted DSS and TNBS-induced colitis studies employing BDF1 mice. Animals obtained from Harlan Laboratories, United Kingdom, were housed individually in ventilated cages in a specific pathogen-free barrier unit in compliance with animal welfare regulations. All procedures were certified according to the United Kingdom Home Office (Animal Procedures) Act 1986.

Conflict-of-interest statement: Shailubhai K, Foss JA, Comiskey S, Palejwala V and Jacob GS are employees of Synergy Pharmaceuticals. Nefsky B, Arjunan KP, Saykhedkar S have no conflict of interest. Plevy SE received compensation as a consultant from Synergy Pharmaceuticals Inc.

Data sharing statement: Authors agree to share the raw data included in the manuscript with other researchers.

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Abstract

AIM: To evaluate the effect of orally administered

plecanatide or dolcanatide, analogs of uroguanylin, on amelioration of colitis in murine models.

METHODS: The cyclic guanosine monophosphate (cGMP) stimulatory potency of plecanatide and dolcanatide was measured using a human colon carcinoma T84 cell-based assay. For animal studies all test agents were formulated in phosphate buffered saline. Sulfasalazine or 5-amino salicylic acid (5-ASA) served as positive controls. Effect of oral treatment with test agents on amelioration of acute colitis induced either by dextran sulfate sodium (DSS) in drinking water or by rectal instillation of trinitrobenzene sulfonic (TNBS) acid, was examined in BALB/c and/or BDF1 mice. Additionally, the effect of orally administered plecanatide on the spontaneous colitis in T-cell receptor alpha knockout (TCR$\alpha^{-/-}$) mice was also examined. Amelioration of colitis was assessed by monitoring severity of colitis, disease activity index and by histopathology. Frozen colon tissues were used to measure myeloperoxidase activity.

RESULTS: Plecanatide and dolcanatide are structurally related analogs of uroguanylin, which is an endogenous ligand of guanylate cyclase-C (GC-C). As expected from the agonists of GC-C, both plecanatide and dolcanatide exhibited potent cGMP-stimulatory activity in T84 cells. Once-daily treatment by oral gavage with either of these analogs (0.05-0.5 mg/kg) ameliorated colitis in both DSS and TNBS-induced models of acute colitis, as assessed by body weight, reduction in colitis severity ($P < 0.05$) and disease activity index ($P < 0.05$). Amelioration of colitis by either of the drug candidates was comparable to that achieved by orally administered sulfasalazine or 5-ASA. Plecanatide also effectively ameliorated colitis in TCR$\alpha^{-/-}$ mice, a model of spontaneous colitis. As dolcanatide exhibited higher resistance to proteolysis in simulated gastric and intestinal juices, it was selected for further studies.

CONCLUSION: This is the first-ever study reporting the therapeutic utility of GC-C agonists as a new class of orally delivered and mucosally active drug candidates for the treatment of inflammatory bowel diseases.

Key words: Guanylate cyclase-C; Inflammatory bowel disease; Uroguanylin; Plecanatide; Dolcanatide

Core tip: Plecanatide (SP-304) and dolcanatide (SP-333) are structurally close analogs of the human endogenous natriuretic peptide uroguanylin, a ligand of guanylate cyclase-C (GC-C). Here we report that oral treatment with plecanatide or dolcanatide effectively ameliorates colitis in acute and chronic models of murine experimental colitis. The anti-inflammatory activity of plecanatide and dolcanatide was comparable to that achieved after treatment with sulfasalazine or 5-amino salicylic acid. This is the first-ever study reporting the therapeutic utility of GC-C agonists as a new class of orally delivered and mucosally active drug candidates for the treatment and management of inflammatory bowel diseases in humans.

Shailubhai K, Palejwala V, Arjunan KP, Saykhedkar S, Nefsky B, Foss JA, Comiskey S, Jacob GS, Plevy SE. Plecanatide and dolcanatide, novel guanylate cyclase-C agonists, ameliorate gastrointestinal inflammation in experimental models of murine colitis. *World J Gastrointest Pharmacol Ther* 2015; 6(4): 213-222 Available from: URL: http://www.wjgnet.com/2150-5349/full/v6/i4/213.htm DOI: http://dx.doi.org/10.4292/wjgpt.v6.i4.213

INTRODUCTION

Chronic inflammatory bowel diseases (IBD) such as Crohn's disease (CD) and ulcerative colitis (UC) are multifactorial gastrointestinal (GI) diseases with high incidences worldwide[1]. While the etiology of these diseases remains largely unknown, one of the contributing factors may be poorly regulated immune response against the enteric microbiota in genetically predisposed individuals[2]. Existing therapies for symptomatic relief of IBD include anti-inflammatory drugs, immunosuppressants, biologic agents, antibiotics, and aminosalicylic acid (5-ASA) based drugs. However, these treatments are not entirely satisfactory due to limited effectiveness and associated side effects[3-6]. Thus there is a need to identify drugs that provide safer, convenient and efficient means of therapeutic intervention for IBD. An ideal conceptual advance would be to develop oral drugs that act locally at the site of inflammation to maximize efficacy and to minimize systemic side effects.

Uroguanylin (UG) and guanylin (GN) activate guanylate cyclase-C (GC-C) receptors expressed on the epithelial cells lining the GI mucosa to stimulate production of cyclic GMP (cGMP), which in turn sequentially activates protein kinase G-Ⅱ and cystic fibrosis transmembrane conductance regulator to regulate ion and fluid transport, epithelial cell homeostasis and to maintain barrier function in the GI mucosa[7-10]. The expression of mRNAs encoding UG and GN are markedly suppressed in human colonic polyps, tumors and in inflamed tissues from UC and CD patients[10-13]. These findings raise the possibility that the deficiency of UG and GN might be associated with the pathogenesis of these diseases. Consistent with this notion, we have demonstrated that oral administration with UG not only inhibits polyp formation but also delays their progression to adenocarcinomas[10]. Similar findings have been reported by other researchers underscoring the evolving role of GC-C signaling in suppression of GI inflammation and prevention of colorectal tumorigenesis[13-16].

Maintenance of the intestinal epithelial cell barrier function is considered to be crucial for host immune defense. Thus, dysfunctional barrier function and

increased gut permeability might be crucial pathophysiological factors underlying the etiology of IBD. Recent studies with the GC-C$^{-/-}$ and UG$^{-/-}$ knockout mice further illustrate the involvement of GC-C signaling in the maintenance of homeostatic intestinal barrier function, permeability and intestinal epithelial cell proliferation[17,18]. Moreover, disruption in GC-C signaling is associated with a reduced number of colonic goblet cells, resulting in decreased production of mucin and intestinal trefoil factor[13], which are the principal components of the gut-coating mucus layer for maintenance of epithelial barrier protection and for post-injury restitution. Importantly, a recent study indicated that colitis in GC-C$^{-/-}$ and IL-10$^{-/-}$ double knockout mice was significantly more severe as compared to that in GC-C$^{+/+}$ and IL-10$^{-/-}$ mice, suggesting a role of GC-C as a suppressor of spontaneous T-cell-driven intestinal inflammation[19]. In this regard, it has been reported that cell-permeable analogs of cGMP exhibit anti-inflammatory activity, possibly *via* inhibition of NF-κB activation[20]. In addition, atrial natriuretic peptide, an agonist of natriuretic peptide receptor A (NPR-A), also exhibits anti-inflammatory activity in both *in vitro* and *in vivo* models through stimulation of cGMP production[21]. Consistent with these observations, disruption of the *NPR-A* gene leads to augmented production of pro-inflammatory cytokines and growth factors[22]. Collectively, these studies suggest that activation of GC-C/cGMP signaling may have therapeutic potential in the management of GI inflammatory diseases.

Based on results from thermal bond energy calculations, 3-D structure modeling, molecular simulation and structure-activity relationship studies, we discovered plecanatide and dolcanatide as novel analogs of UG that bind and activate GC-C receptors to stimulate production of cGMP in a pH-dependent manner. Thus, these agonists are expected to mimic UG function in the GI tract[23-25]. This is the first study demonstrating that oral treatment with plecanatide or dolcanatide ameliorates GI inflammation in acute as well as in chronic models of experimental colitis in mice.

MATERIALS AND METHODS

Materials

Human colonic carcinoma T84 cells obtained from Leonard Forte, University of Missouri, MO, were cultured as described earlier[10]. Plecanatide and dolcanatide were chemically synthesized by the procedures as described previously[24,26]. All other chemicals and other reagents were obtained from commercially available vendors.

Methods

Animal studies: Studies employing BALB/c and T-cell receptor alpha knockout (TCRα$^{-/-}$)mice were performed at the University of Pittsburgh School of Medicine (Pittsburg, PA). Animals obtained from Jackson Laboratories (Bar Harbor, ME) were housed in accordance with guidelines from the American Association for Laboratory Animal Care and Research Protocols as approved by the Institutional Animal Care and Use Committee. Epistem Ltd (Manchester, United Kingdom) conducted dextran sulfate sodium (DSS) and TNBS-induced colitis studies in BDF1 mice. Animals obtained from Harlan Laboratories, United Kingdom, were held in individually ventilated cages in a specific pathogen-free barrier unit in compliance with animal welfare regulations. The day-night cycle was constant, with light and dark phases of 12 h each. At the end of experimental protocols, mice were euthanized by CO_2 followed by cervical dislocation.

Cyclic GMP stimulation assay in T84 cells: Potencies of plecanatide and dolcanatide to stimulate cGMP synthesis in T84 cells was assayed as described previously[10]. Briefly, confluent monolayers of T84 cells were pre-incubated with 1 mmol/L isobutylmethylxanthine, a phosphodiesterase inhibitor, in DMEM for 10 min at 37 °C followed by incubation with test peptide for 30 min. The reaction was terminated by adding 3% perchloric acid. Following centrifugation and neutralization with 0.5 N NaOH, supernatants were used for measurements of intracellular cGMP using an ELISA kit (Cayman Chemical Co., Ann Arbor, MI). Results are expressed as pmol of cGMP/mg of protein in the cell extracts.

TNBS-induced colitis in BALB/c mice: BALB/c mice (n = 5-8/group) were used to evaluate the anti-inflammatory effects of plecanatide on TNBS-induced colitis by previously described procedures[27,28]. Briefly, colitis was induced in 2-4 mo old female BALB/c mice by administering 2.5 mg TNBS in 50% ethanol into the lumen of the colon (injection volume 100 μL). Plecanatide (0, 0.5 and 2.5 mg/kg) formulated in PBS was administered by oral gavage for 7 d, with the first dose given the same day as TNBS challenge. After 7 d of treatment animals were euthanized, GI tissues were collected for histopathological examination, and inflammation scoring was performed[29].

TNBS-induced colitis in BDF-1 mice: A TNBS-induced colitis study in 10-12 wk old BDF-1 mice (n = 10/group) was conducted at the Epistem Ltd., United Kingdom, using a procedure essentially similar to that described above except that mice were dosed with plecanatide (0.005-5 mg/kg) a day before TNBS treatment. Daily administration of plecanatide was continued until 7th day when the mice were euthanized. Colon tissues were removed and weighed. Distal sections were fixed, stained with H and E, and evaluated for histopathology and visual severity scores[27,28]. Scoring of the H and E-stained tissue sections employed the following criteria: Normal-appearing crypts (score 0); abnormal crypt pathology without ulceration (score 1); depleted crypts with some ulceration/ inflammation (score 2); 20%-70% depleted crypts and increased ulceration/inflammation (score 3); > 70% depleted crypts with substantial ulceration/

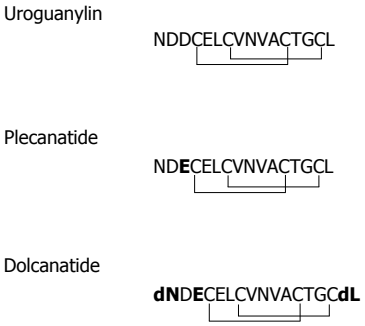

Figure 1 Primary structures of uroguanylin, plecanatide, and dolcanatide. Single-letter abbreviations for amino acids are depicted. Plecanatide is similar to UG except for the substitution of aspartic acid (D) at position 3 from the N-terminus of UG with glutamic acid (E). The structure of dolcanatide is similar to plecanatide except that the amino acids at both termini are replaced with their respective D-stereoisomers. Uroguanylin as well as its analogs have four cysteines (C) enabling the formation of two intramolecular disulfide bonds. Substituted amino acids in plecanatide and dolcanatide are shown in bold type. UG: Uroguanylin.

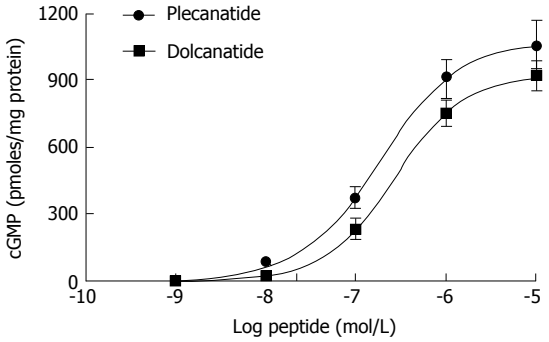

Figure 2 Plecanatide and dolcanatide mediated stimulation of cyclic guanosine monophosphate production in T84 cells. The potency of plecanatide and dolcanatide to stimulate cGMP production was evaluated using the T84 cell bioassay (see Materials and Methods). The data presented are an average of three determinations and are expressed as pmoles cGMP/mg protein ± SD. cGMP: Cyclic guanosine monophosphate.

inflammation (score 4); and totally ulcerated/inflamed colon with no crypts remaining (score 5). All slides were scored in a blinded manner. Scoring criteria were similar to that described below for DSS induced colitis studies.

DSS induced colitis in BDF-1 mice: This study was conducted by the Epistem Ltd., United Kingdom. BDF1 mice (*n* = 8-10; age 10-12 wk) were given 5% DSS in the drinking water on day 0 to induce colitis. Plecanatide or dolcanatide (0.005-5 mg/kg) in 0.1 M phosphate buffer (pH7) was administered daily by oral gavage starting a day prior to DSS administration (day-1) through 7th day. Oral gavage with sulfasalazine (80 mg/kg) or 5-ASA (100 mg/kg) served as a positive control. Mice were euthanized at the end of the treatment and tissues from large intestines were removed and weighed. The distal sections were fixed for histopathology evaluations. Colitis severity was assessed by histopathological evaluation of the H and E-stained tissue sections as described above. Disease activity index (DAI) was calculated by determining body weight, stool consistency, and the presence of overt blood in stools or around the anus, substantially similar to that reported earlier[30].

Spontaneous colitis in TCR$\alpha^{-/-}$ knockout mice: This study was conducted at the University of Pittsburgh School of Medicine, PA. The TCR$\alpha^{-/-}$ mice were matched for age and sex in all groups. Sixteen-week-old mice were administered plecanatide (0.5 or 2.5 mg/kg) or vehicle by oral gavage for 14 d (6 mice/group). Mice were euthanized 12 h after the final dose and GI tissues collected for histopathological evaluation of colitis severity. Scoring of colitis severity was as previously described[31].

Myeloperoxidase activity: Myeloperoxidase (MPO) activity in colonic tissue samples was measured according to methods described previously[32]. Briefly, the rate of change in absorbance at 450 nm was recorded when 20 μL of tissue extract was incubated with 150 μL of reaction buffer containing 0.26 mg/mL o-dianisidine and 0.6 μL/mL H_2O_2. Each reaction was performed in triplicate. The rate of reaction was determined (initial slope) and normalized to the protein concentration of the sample.

Statistical analysis

Statistical significance was performed by comparing mean values of the control with that of the treated samples, using 2-way unpaired Student *t*-tests, assuming unequal variance, in Microsoft Excel and PRISM. A *P* value < 0.05 was considered to be statistically significant.

RESULTS

Plecanatide and dolcanatide are analogs of human UG

Plecanatide is structurally similar to UG, differing only in the substitution of Asp^3 with Glu^3. Dolcanatide is similar to plecanatide in structure except that L-Asn^1 and L-Leu^{16} are replaced by D-Asn^1 and D-Leu^{16} at the N and C-termini, respectively (Figures 1 and 2). In T84 cells assay, both plecanatide and dolcanatide activate GC-C receptors to stimulate cGMP synthesis in a dose-dependent manner with EC_{50} values of 1.9×10^{-7} mol/L and 2.8×10^{-7} mol/L, respectively (Figure 2).

Plecanatide ameliorates spontaneous and chemically induced colitis

In a preliminary study, the ability of orally-administered plecanatide to ameliorate colitis was evaluated in TNBS-induced colitis in BALB/c mice (Figure 3A). Treatment with plecanatide at 0.5 and 2.5 mg/kg for 7 d effectively reduced colitis severity scores as compared to vehicle treatment. Oral treatment with plecanatide was also evaluated in TCR$\alpha^{-/-}$ mice that develop chronic colitis spontaneously. In this model, oral treatment with 0.5 and 2.5 mg/kg continuously for two weeks reduced colitis scores as compared to those in the vehicle-treated group (Figure 3B). Taken together, these preliminary

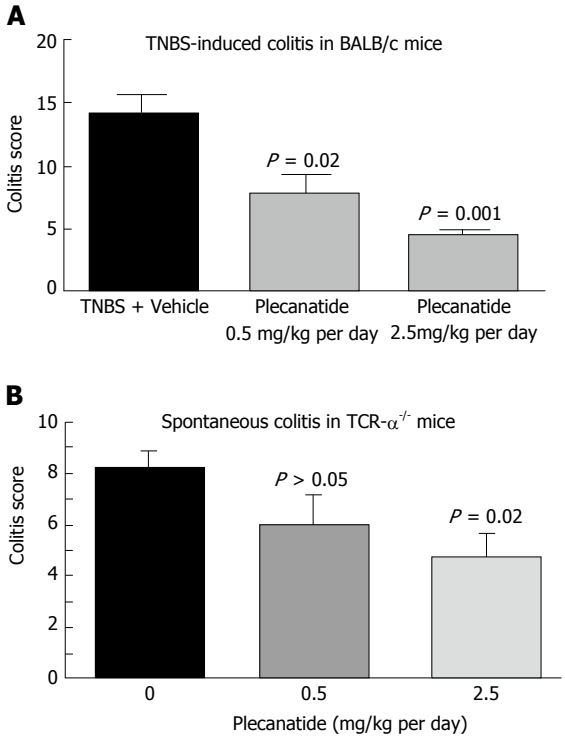

Figure 3 Oral treatment with plecanatide ameliorates colitis in acute and chronic mouse models. A: Acute colitis examined in BALB/c mice (*n* = 5-8/group) was induced by rectal instillation of TNBS. Mice were administered an oral gavage of vehicle or plecanatide (0.5 and 2.5 mg/kg per day) starting on 0 d; animals were euthanized on 7th day and colitis scores were determined; B: TCRα-/- mice, a model for chronic spontaneous colitis, were administered an oral gavage of plecanatide (0.5 and 2.5 mg/kg) or vehicle for 14 d. At the end of the study, colon tissues were harvested and used for histopathological analysis to assess colitis. Results are depicted as mean colitis score ± SD. TCRα: T-cell receptor alpha; TNBS: 2, 4, 6-trinitrobenzenesulfonic acid sol.

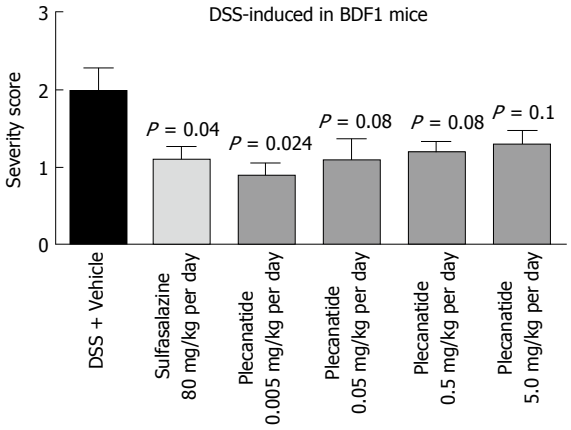

Figure 4 Oral treatment with plecanatide ameliorates gastrointestinal inflammation in the dextran sulfate sodium-induced colitis in BDF1 mice. BDF1 mice (*n* = 8/group) were administered 5% DSS in drinking water. An oral gavage was administered once daily containing 0.005 to 5.0 mg/kg of plecanatide beginning a day prior to initiating DSS regimen. Sulfasalazine (80 mg/kg) and vehicle (phosphate buffer) served as positive and negative controls, respectively. At the end of the study mice were euthanized; distal sections of the colon were fixed, embedded in paraffin, sectioned, stained with H and E and visualized to assign colitis severity scores. All slides were scored in a blinded manner. The mean histological severity of colitis score ± SEM was plotted for the indicated treatment groups. Statistical significance was calculated by comparing severity scores observed for the plecanatide or sulfasalazine treated group *vs* corresponding values in the vehicle treated group. DSS: Dextran sulfate sodium; SEM: Standard error of the mean.

results prompted a further evaluation of plecanatide and dolcanatide in DSS and TNBS-induced colitis with larger cohorts.

Oral treatment with either vehicle, sulfasalazine (80 mg/kg) or plecanatide (0.005, 0.05, 0.5 and 5.0 mg/kg), was evaluated in both DSS and TNBS-induced colitis in mice. In BDF1 mice, colitis was induced by including 5% DSS in drinking water, and mice were randomly divided into 6 groups. Colitis severity was determined by the histopathological evaluation of H and E-stained colonic tissue sections employing the criteria described under Materials and Methods. Consistent with the results from the preliminary studies described above, orally administered plecanatide even at a dose as low as 0.005 mg/kg was as effective as sulfasalazine (80 mg/kg) to ameliorate DSS induced colitis in BDF-1 mice (Figure 4). However, doses higher than 0.005 mg/kg per day did not produce an incremental effect on the amelioration of colitis, possibly due to the saturation of available GC-C receptors in the gut lumen.

The anti-inflammatory activity of plecanatide was further examined in the TNBS-induced colitis in BDF1 mice. Once-daily administration of plecanatide at 0.005 mg/kg was not effective, but all of the higher doses (0.05-5 mg/kg) produced statistically significant reduction in severity of colitis as compared to TNBS plus vehicle treated animals (Figure 5). Again, there was no further reduction in colitis severity with incremental doses above 0.05 mg/kg, suggesting that this dose might be at the saturation level of the available GC-C binding sites in the GI lumen. Notably, the efficacy of plecanatide at 0.05 mg/kg per day dose was comparable to that of sulfasalazine at the dose 80 mg/kg per day.

Dolcanatide ameliorates DSS induced colitis in BDF-1 mice

To further confirm the anti-inflammatory activity of this class of GC-C agonists, the effect of oral treatment with dolcanatide was evaluated in DSS induced colitis in BDF-1 mice. Except for the 0.5 mg/kg dose of dolcanatide, treatment with all other doses produced statistically significant reduction in colitis severity scores as compared to those in DSS + vehicle control. The reduction in colitis severity was comparable to that observed in 100 mg/kg dose of 5-ASA (Figure 6A). Similarly, treatment with dolcanatide also produced considerable reduction in the DAI score (Figure 6B), although the statistical significance (P = 0.04) was achieved only with a dose of 0.05 mg/kg. The effect of dolcanatide treatment on levels of MPO activity in colon tissues was measured as an indirect way to assess the severity of GI inflammation. As expected, the colon tissues from DSS plus vehicle-treated mice exhibited the highest levels of MPO (0.048 ± 0.004 units/min). A considerable reduction in MPO activity was observed following treatment either with 5-ASA (100 mg/kg) or with dolcanatide at all doses (Figure 6C). Even at the dose as low as (0.05 mg/kg

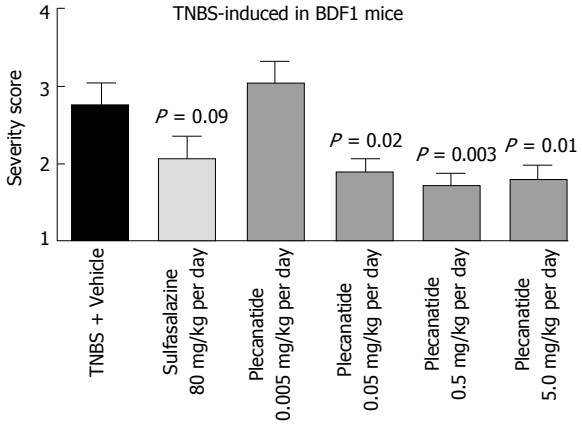

Figure 5 Oral treatment with plecanatide ameliorates gastrointestinal inflammation in 2, 4, 6-trinitrobenzenesulfonic acid-induced colitis in BDF1 mice. BDF1 mice (*n* = 10/group) were administered TNBS *via* rectal instillation on 0 d as described under Materials and Methods. An oral gavage was administered once daily containing 0.005 to 5.0 mg/kg plecanatide beginning a day prior to TNBS treatment. Sulfasalazine (80 mg/kg) and vehicle (phosphate buffer) served as positive and negative controls, respectively. At the end of the study mice were euthanized; distal sections of the colon were fixed, embedded in paraffin, sectioned, stained with H and E and visualized to assign colitis severity scores. All slides were scored in a blinded manner. The mean histological severity of the colitis score ± SEM was plotted for the indicated treatment groups. Statistical significance was calculated by comparing the severity score observed for the plecanatide or sulfasalazine-treated group *vs* the corresponding values in the vehicle-treated group. TNBS: 2, 4, 6-trinitrobenzenesulfonic acid sol; SEM: Standard error of the mean.

per day) of dolcanatide, showing approximately 50% reduction in total MPO activity, was comparable to that achieved with 5-ASA at 100 mg/kg dose.

Colon tissues from mice treated with dolcanatide at all doses or with 5-ASA showed a considerable reduction in histopathological scoring. Representative slides of H and E-stained sections of colon tissues used for histopathological evaluation are shown in Figure 7. Substantial loss of crypts, changes in crypt architecture, ulceration, and localized infiltration of inflammatory cells was observed in tissue slides from DSS plus-vehicle-treated mice (panel B; score = 3) as compared to that in the slides from naïve mice exhibiting normal mucosal architecture and evenly-spaced crypts of uniform length (panel A; score 0). Treatment with dolcanatide at 0.05 mg/kg (panel C; score 2) or with 5-ASA (panel D; score 2) exhibited minimal loss of crypts, changes in crypt architecture, ulceration, and localized infiltration of inflammatory cells, which is indicative of improvement in colitis severity.

DISCUSSION

This is the first-ever study reporting that oral treatment with either plecanatide or dolcanatide, analogs of UG, ameliorate colonic inflammation in both acute and chronic models of murine colitis. Oral treatment with plecanatide or dolcanatide at a dose range between 0.05-2.5 mg/kg per day was as effective as once-daily treatment with 5-ASA (100 mg/kg) or sulfasalazine (80 mg/kg). However, the amelioration of colitis by the treatment with plecanatide or dolcanatide was

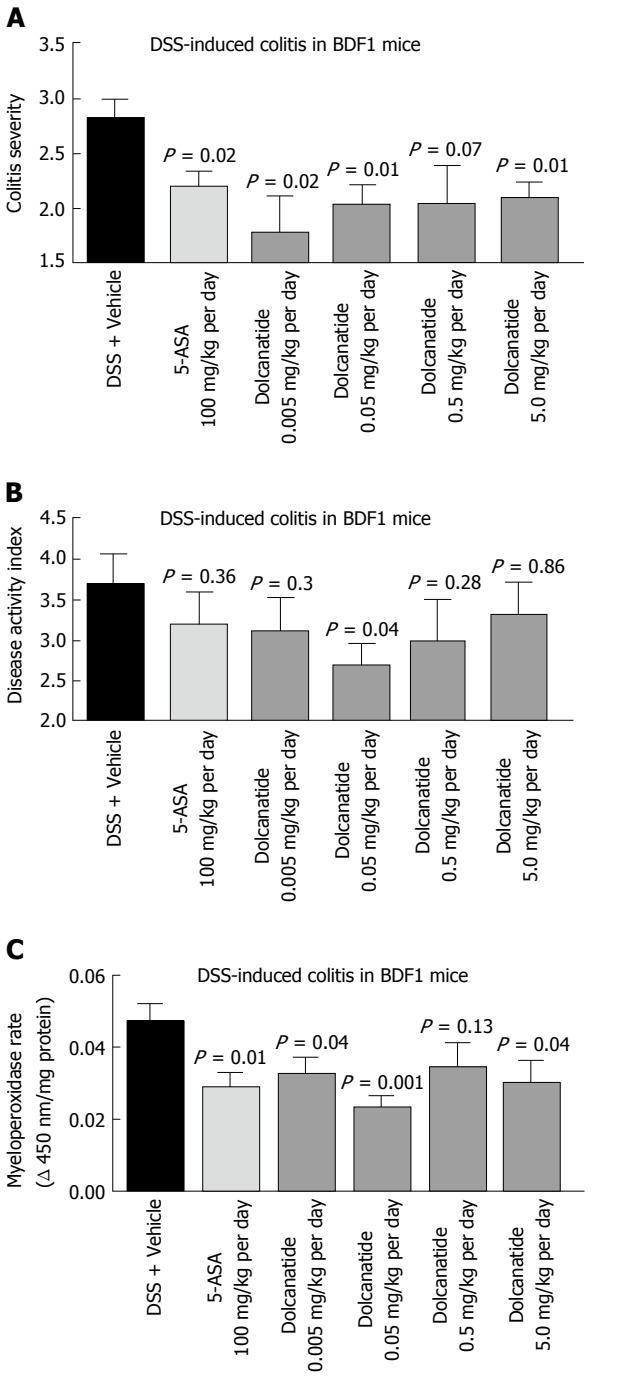

Figure 6 Oral treatment with dolcanatide ameliorates gastrointestinal inflammation in the dextran sulfate sodium induced colitis in BDF1 mice. BDF1 mice (*n* = 8-10/group) were administered 5% DSS in drinking water to induce colitis. Dolcanatide was administered *via* oral gavage beginning a day before initiating the DSS regimen. Oral gavage with 5-ASA (100 mg/kg) and vehicle (phosphate buffer) served as positive and negative controls, respectively. At the end of the study, mice were euthanized and colon tissues were removed for histopathological analyses (see Materials and Methods section). Additional groups of mice were treated under identical conditions for measurement of MPO activity in colon tissues. The Figure depicts results for colitis severity (A), disease activity index (B), and MPO activity (C). Data represent mean ± SEM for each group. Statistical significance was calculated by comparing the values observed for the dolcanatide or 5-ASA-treated group *vs* the corresponding scores for the vehicle-treated group. DSS: Dextran sulfate sodium; GI: Gastrointestinal; MPO: Myeloperoxidase; SEM: Standard error of the mean; 5-ASA: 5-amino salicylic acid.

not dose-dependent. This lack of dose response may

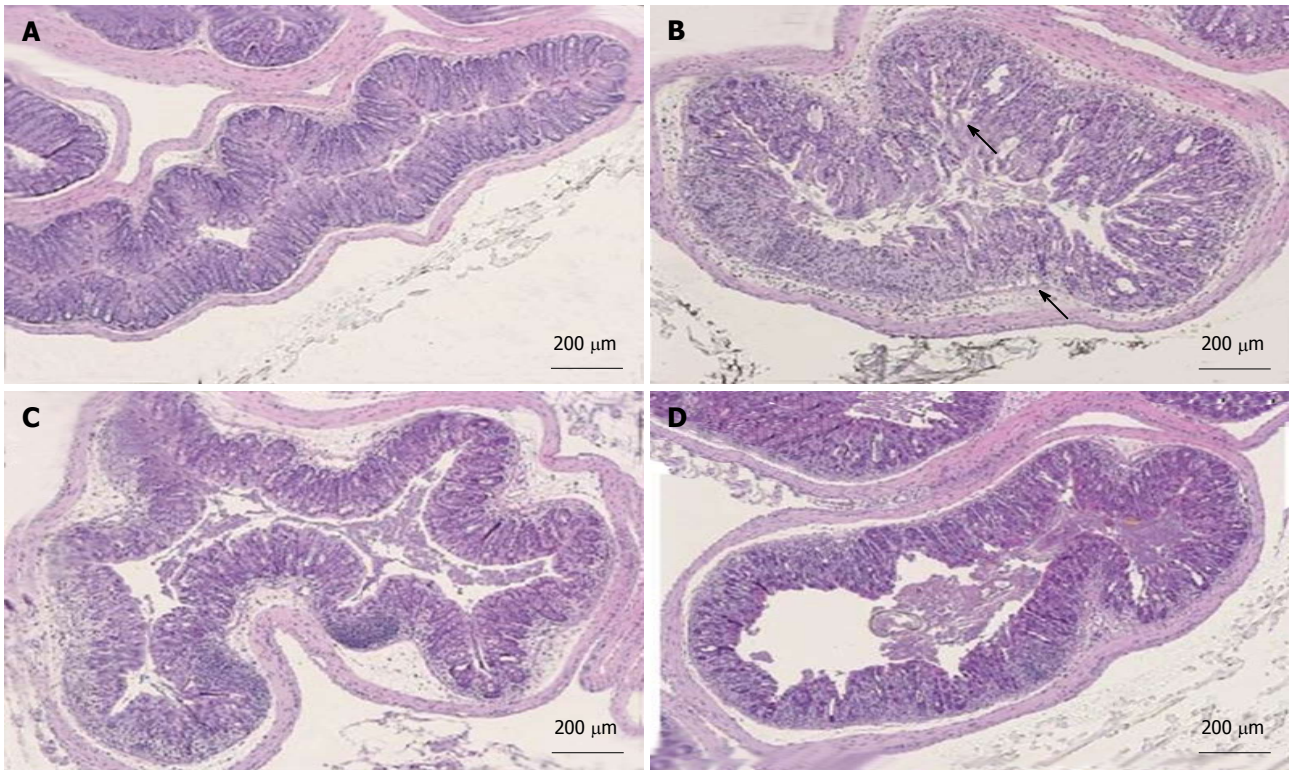

Figure 7 Histopathology analysis of colon tissues after treatment with dolcanatide. Representative histopathological images of the large bowel from the DSS-induced colitis study are depicted. Criteria for scoring colitis severity were as described under Materials and Methods. A: Untreated naïve mice, histopathology score = 0; B: DSS + vehicle treated, histopathology score = 3; C: DSS + dolcanatide (0.05 mg/kg), histopathology score = 2; D: DSS + 5-ASA (100 mg/kg), histopathology score = 2. Arrows in panel B indicate morphological deterioration in crypts and villi of GI mucosa. DSS: Dextran sulfate sodium; 5-ASA: 5-amino salicylic acid; GI: Gastrointestinal.

be attributable to the saturation of the available GC-C receptors on the epithelial cells lining the GI mucosa. Interestingly, the lowest effective dose of plecanatide varied from 0.005 to 2.5 mg/d in different models examined at the three different research facilities used. These apparent differences in the effective dose of plecanatide may be due to the differences in external factors, such as diet and animal husbandry conditions of contract research organizations in the United States and the United Kingdom. These external factors are known to impact the composition of gut microflora influencing the severity of colitis in different species of mice[33,34].

The mechanism of actions of the endogenous natriuretic peptides UG and GN are known to be through activation of GC-C expressed on the apical surface of epithelial cells lining the GI mucosa[8]. Thus, orally administered GC-C agonists enhance cGMP, which mediates their pharmacological actions resulting in increased fluid secretion to promote GI transit and bowel movement. Previously, we reported that orally administered plecanatide acts primarily in the lumen of the proximal intestine to facilitate bowel movement in mice and monkeys[35]. In addition, orally administered plecanatide not only ameliorated GI inflammation but also delayed its progression to colorectal carcinogenesis *via* enhancement of cGMP production through activation of GC-C signaling in $Apc^{+/min\text{-}FCCC}$ mice[36]. Importantly, recent clinical studies confirm that orally administered GC-C agonists are minimally absorbed into systemic circulation and they act locally in the gut lumen[25,37]. Taken together, these studies suggest that the pharmacological actions of orally administered plecanatide and dolcanatide are primarily through activation of GC-C receptors in the gut lumen.

During the renewal process, the intestinal epithelium goes through a cycle (initiated in the crypt) of proliferation, migration, differentiation, apoptosis and ultimately loss of epithelial cells into the lumen[38]. This process is crucial for maintaining the integrity of the intestinal mucosa. UG and GN are secreted in a gradient along this vertical axis. Maximal secretion of UG is in the villus region and minimum in the crypt[39]. GC-C receptor signaling is believed to be important in maintaining the balance between apoptosis and regeneration[14]. Decreased expression of UG and GN in colon polyps, tumors and in inflamed tissues from UC and CD patients[10-12], can potentially impair intestinal homeostasis, suggesting a pathophysiological significance of GC-C signaling in the etiology of these diseases. Consistent with this notion, studies with $GC\text{-}C^{-/-}$ and $UG^{-/-}$ knockout mice revealed a functional role of GC-C signaling in the maintenance of homeostatic intestinal barrier function, intestinal permeability, and epithelial cell proliferation[18,40]. Of relevance, intestinal permeability is higher in the GC-C and UG knockout mice than in the wild mice. Expression of the major

tight junction proteins is also reduced in the intestines of GC-C deficient mice. Luminal antigens permeate the defective barrier, promote inflammation, and damage the intestinal mucosal architecture[18,41]. These studies suggest a critical role of GC-C signaling in preserving the intestinal integrity. Histological analyses reported here lend support to this argument. Mucosal damage following DSS treatment results in loss of crypts, changes in crypt architecture, ulceration, and infiltration of inflammatory cells into colonic tissues. Treatment with dolcanatide resulted in minimal loss of crypts, low distortions in crypt morphology, and a significant reduction in myeloperoxidase activity. These results suggest less infiltration of inflammatory cells, possibly by strengthening the barrier function. Consistent with this notion, we recently reported that treatment with dolcanatide attenuated LPS-mediated enhancement in cellular permeability in T84 and Caco-2 cells[42].

Following the loss of barrier function, this regulatory balance is disrupted by the massive recruitment of leucocytes and macrophages, resulting in augmented levels of destructive inflammatory cytokines in the intestinal tissues[43]. In a preliminary study, we previously reported that treatment with plecanatide inhibited secretion of pro-inflammatory cytokines such as IL-12p40, IL-23, and TNF in explant cultures of colon tissues from TNBS-treated BALB/c mice. Plecanatide treatment similarly reduced production of RANTES, IL-17 and MIP-1α, with a concomitant increase in IL-10 in colon explants from TCR$\alpha^{-/-}$ mice[36]. Our results using T84 cells further suggest that treatment with dolcanatide inhibited LPS-mediated activation of NF-κB activation, presumably *via* a cGMP-mediated mechanism[23]. Oral treatment with plecanatide also reduced the formation of colon dysplasia in DSS-treated Apc$^{Min/+-FCCC}$, possibly through down-regulation of some pro-inflammatory cytokines and growth factors[44]. Taken together, these results suggest that orally-administered plecanatide or dolcanatide ameliorates colitis, possibly through suppression of pro-inflammatory cytokines production. However, this is a simplistic view on the possible mechanism of action for the anti-inflammatory activity of GC-C agonists. Additional studies are necessary to define the precise mechanism by which GC-C agonists promote intestinal barrier function, suppress production of cytokines, and exert their anti-inflammatory activity.

Oral treatment with analogs of the endogenous natriuretic peptide UG is thus an attractive approach with a unique mechanism of action, enabling restoration of homeostatic signaling responsible for maintenance of colonic mucosa integrity. This study may have the following significant implications for treatment and maintenance of IBD in humans: First, oral treatment with locally acting GC-C agonist may eliminate toxicity concerns associated with the existing systemic therapies of IBD; second, the evolving paradigm implies that the reduced production of UG and/or GN might be involved in the pathologies of UC and CD in humans. If so, oral therapy with UG analogs can be considered as a replacement therapy to overcome the deficiency underlying the etiology of IBD. Finally, it is now well established that patients suffering from chronic UC and CD are at higher risk of developing colon cancer[45]. Chronic treatment with orally-safe drug candidates such as plecanatide or dolcanatide could be useful as maintenance therapy to delay the onset of IBD to colon carcinogenesis. In this regard, we recently reported that oral treatment with plecanatide considerably reduced colonic dysplasia in DSS treated Apc$^{min/+FCCC}$ mice[44].

Although the potency of plecanatide to stimulate cGMP in T84 cells, and to ameliorate colitis in animal studies was comparable to that of dolcanatide, the latter drug candidate was advanced further for clinical development because of its enhanced stability against proteolysis in simulated intestinal fluid[23]. The non-clinical safety and toxicology studies conducted in rodents and monkeys suggest that dolcanatide is an orally-safe and minimally absorbed drug candidate. Synergy Pharmaceuticals Inc. has successfully completed Phase I single-ascending-dose and multiple ascending dose safety studies with dolcanatide in healthy volunteers. The drug candidate is well-tolerated and is currently being further evaluated as an orally safe and mucosally-active drug candidate for treatment in patients with ulcerative colitis.

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COMMENTS

Background

There is an unmet need to develop a safer and effective therapeutic intervention for inflammatory bowel diseases (IBD). Existing therapies are of limited effectiveness and often associated with side effects. Biologic injectables are costly and effective in less than 40% of patients, with a potential for systemic side effects. An important conceptual advance would be to develop drugs that act locally at the site of inflammation to maximize efficacy and minimize systemic side effects. Plecanatide and dolcanatide are analogs of the human endogenous peptide uroguanylin (UG), a guanylate cyclase-C (GC-C) agonist that regulates fluid/ion and epithelial cell homeostasis and maintains the barrier function within the gastrointestinal (GI) tract. Given its multitude of functions, GC-C agonists are potentially useful therapeutic candidates for treating IBD.

Research frontiers

Therapeutic intervention with locally acting, minimally absorbed analogs of UG, an endogenous natriuretic peptide, represents a novel and safe approach for treating IBD. This therapy could potentially be useful as a maintenance therapy to delay the onset of IBD into colon carcinogenesis.

Innovations and breakthroughs

This is the first report highlighting the therapeutic potential of orally administered and mucosally active GC-C agonists for treating inflammatory bowel diseases in humans.

Applications

UG is an endogenous peptide hormone that regulates fluid/ion homeostasis

and epithelial cell homeostasis and maintains the barrier function within the GI tract. Several studies have demonstrated that transcript levels of UG and related peptide guanylin are markedly reduced in inflamed colonic tissues from ulcerative colitis and Crohn's patients, as well as in human colonic polyps and tumors, implying that the pathogenesis of these diseases might be associated with the deficiency of UG and guanylin. Oral therapy with UG analogs, therefore, could be considered as a replacement therapy to overcome the deficiency underlying the etiology of IBD.

Peer-review

The manuscript presents some points of concern, and additional experiments should be performed, particularly regarding the evaluation of cyclic guanosine monophosphate production in intestinal mucosa. Nevertheless, the manuscript displays novel findings, which sustain the possible use of GC-C agonists for the treatment of IBDs.

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ORIGINAL ARTICLE

Basic Study

Orally administered extract from *Prunella vulgaris* attenuates spontaneous colitis in mdr1a$^{-/-}$ mice

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Author contributions: Haarberg KMK, Wymore Brand MJ, Ramer-Tait AE, and Wannemuehler MJ designed the research; Haarberg KMK, Wymore Brand MJ, and Overstreet AMC performed the research; Hauck CC and Murphy PA prepared the extract; Hostetter JM provided histopathological analysis, Haarberg KMK and Wymore Brand MJ analyzed the data, Haarberg KMK, Wymore Brand MJ, Ramer-Tait AE, and Wannemuehler MJ prepared the manuscript, figures, and data analysis.

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Abstract

AIM: To investigate the ability of a *Prunella vulgaris* (*P. vulgaris*) ethanolic extract to attenuate spontaneous typhlocolitis in mdr1a$^{-/-}$ mice.

METHODS: Vehicle (5% ethanol) or *P. vulgaris* ethanolic extract (2.4 mg/d) were administered daily by oral gavage to mdr1a$^{-/-}$ or wild type FVB^{WT} mice from 6 wk of age up to 20 wk of age. Clinical signs of disease were noted by monitoring weight loss. Mice experiencing

weight loss in excess of 15% were removed from the study. At the time mice were removed from the study, blood and colon tissue were collected for analyses that included histological evaluation of lesions, inflammatory cytokine levels, and myeloperoxidase activity.

RESULTS: Administration of *P. vulgaris* extracts to mdr1a$^{-/-}$ mice delayed onset of colitis and reduced severity of mucosal inflammation when compared to vehicle-treated mdr1a$^{-/-}$ mice. Oral administration of the *P. vulgaris* extract resulted in reduced ($P < 0.05$) serum levels of IL-10 (4.6 ± 2 *vs* 19.4 ± 4), CXCL9 (1319.0 ± 277 *vs* 3901.0 ± 858), and TNFα (9.9 ± 3 *vs* 14.8 ± 1) as well as reduced gene expression by more than two-fold for *Ccl2, Ccl20, Cxcl1, Cxcl9, IL-1α, Mmp10, VCAM-1, ICAM, IL-2,* and *TNFα* in the colonic mucosa of mdr1a$^{-/-}$ mice compared to vehicle-treated mdr1a$^{-/-}$ mice. Histologically, several microscopic parameters were reduced ($P < 0.05$) in *P. vulgaris*-treated mdr1a$^{-/-}$ mice, as was myeloperoxidase activity in the colon (2.49 ± 0.16 *vs* 3.36 ± 0.06, $P < 0.05$). The numbers of CD4^{+} T cells (2031.9 ± 412.1 *vs* 5054.5 ± 809.5) and germinal center B cells (2749.6 ± 473.7 *vs* 4934.0 ± 645.9) observed in the cecal tonsils of *P. vulgaris*-treated mdr1a$^{-/-}$ were significantly reduced ($P < 0.05$) from vehicle-treated mdr1a$^{-/-}$ mice. Vehicle-treated mdr1a$^{-/-}$ mice were found to produce serum antibodies to antigens derived from members of the intestinal microbiota, indicative of severe colitis and a loss of adaptive tolerance to the members of the microbiota. These serum antibodies were greatly reduced or absent in *P. vulgaris*-treated mdr1a$^{-/-}$ mice.

CONCLUSION: The anti-inflammatory activity of *P. vulgaris* ethanolic extract effectively attenuated the severity of intestinal inflammation in mdr1a$^{-/-}$ mice.

Key words: *Prunella vulgaris*; Spontaneous colitis; Inflammatory bowel disease; Mdr1a; Botanical extract; Mucosal inflammation; Nutraceutical

Core tip: Extracts of *Prunella vulgaris* (*P. vulgaris*) contain multiple anti-inflammatory phenolics and flavonoids and we report that oral administration of an ethanolic extract of *P. vulgaris* ameliorated the severity of spontaneous colitis in 20 wk old mdr1a$^{-/-}$ mice. Because these mice are genetically prone to develop colitis by 10 wk of age, daily oral treatments were initiated at 6 wk of age. This treatment regimen resulted in the inhibition of multiple parameters of inflammation that collectively contributed to ameliorate the severity of mucosal inflammation suggesting that botanical extracts may be used as effective complementary intervention strategies for the treatment of colitis.

Haarberg KMK, Wymore Brand MJ, Overstreet AMC, Hauck CC, Murphy PA, Hostetter JM, Ramer-Tait AE, Wannemuehler MJ. Orally administered extract from *Prunella vulgaris* attenuates spontaneous colitis in mdr1a$^{-/-}$ mice. *World J Gastrointest Pharmacol Ther* 2015; 6(4): 223-237 Available from: URL: http://www.wjgnet.com/2150-5349/full/v6/i4/223.htm DOI: http://dx.doi.org/10.4292/wjgpt.v6.i4.223

INTRODUCTION

The intestinal epithelium is the interface between the host and the lumen of the gastrointestinal tract and cooperates with other innate immune mechanisms to protect the host from microbial-induced inflammation as well as to hinder colonization and invasion by intestinal microorganisms. The ability to maintain low levels of mucosal inflammation in the gut is believed to be important for mucosal homeostasis. However, in the context of inflammatory bowel diseases (IBD), such as Crohn's disease (CD) and ulcerative colitis (UC), regulation of intestinal inflammation often fails resulting in mucosal damage and chronic disease[1]. While idiopathic in nature, current hypotheses regarding the etiology of IBD point to complex multifactorial causalities, which include disruption of the intestinal epithelial barrier, dysbiosis of the microbiota, genetic predispositions, chronically activated inflammatory immune cells, and failed adaptive immune regulatory responses[2,3].

Much IBD research has focused on aberrant adaptive immune responses to antigens derived from the microbiota. More emphasis is now being placed on elucidating the role innate immune cells (*e.g.*, neutrophils), cytokines, chemokines, and their related transcription factors play in the initiation and/or maintenance of epithelial damage as the initial step in the onset of IBD[4]. In the absence of effective epithelial barrier function, compartmentalization that is meant to separate immune cells in the lamina propria from the numerous bacterial and food antigens normally sequestered in the lumen is lost[5]. A loss of epithelial barrier integrity is characteristic of UC and CD, and the consequential loss of immunologic tolerance to the microbiota initiates a cascade of signaling pathways that activate both innate and adaptive immune mechanisms[6].

The most common therapies used for the treatment of IBD are immune suppressive and anti-inflammatory drugs and biologicals such as monoclonal antibodies (*e.g.*, anti-TNF)[7,8]. Metronidazole and ciprofloxacin have also been utilized in several clinical trials related to the treatment of UC, CD, and pouchitis with underwhelming results[9]. Immunosuppressive therapies include monoclonal antibodies against TNF-α, 5-aminosalicylates (5-ASA), and steroids[10-12]. For many of these treatments, there is the potential for adverse effects that may include increased susceptibility to bacterial and viral infections and increased risk of cancer. One study showed that IBD related hospitalizations at high volume IBD treatment centers around the United States increased 6-fold

from 1998 to 2004[13]. This data illustrates that despite advances in IBD research, current therapies have not decreased the frequency of IBD related hospitalizations and surgical interventions are still common for severe forms of IBD. These facts and the financial burdens associated with expensive therapeutic regimens have lead patients to explore unconventional means of coping with IBD.

A 1998 study showed that up to 51% of surveyed IBD patients had used alternative or complementary therapies and, in particular, 16% of patients used the alternative therapies specifically for their IBD[14]. For most complementary therapies (*e.g.*, nutraceuticals), many anecdotal claims of health benefits exist with very little scientific data to support or negate those claims.

Prunella vulgaris (*P. vulgaris*is) commonly used in traditional Chinese medicine for wound healing, indigestion, burns and anti-inflammatory therapy. *P. vulgaris* contains several bioactive phenolics, triterpenoids and flavonoids[15]. Dietary phenolics such as rosmarinic acid, ursolic acid, and caffeic acid are all found in extracts of *P. vulgaris*, and have been shown to possess antioxidant, anti-inflammatory and anti-cancer activities[16-21]. Caffeic acid has also been shown to ef)fectively attenuate chemically induced experimental colitis through upregulation of cytochrome P450 (CYP4B1)[22]. Flavonoids, like those found in *P. vulgaris*, have been implicated as potential therapeutics for IBD as well[23]. In contrast to its ability to attenuate DSS-induced colitis, the flavonoid luteolin was found to attenuate spontaneous colitis by inhibiting the activation of NF-κB. Despite this promising evidence, there are no published reports evaluating the use of *P. vulgaris* extracts as a treatment for IBD. In this context, we have designed this study to test the hypothesis that an ethanolic extract of *P. vulgaris* will decrease gastrointestinal mucosal inflammation and thereby ameliorate the severity of spontaneous colitis in mdr1a$^{-/-}$ mice.

MATERIALS AND METHODS

Prunella vulgaris extract preparation

Information about the specific provenance of *P. vulgaris* accession Ames 27664, obtained from Dr. Mark Wiederlichner at the USDA-ARS North Central Regional Plant Introduction Station (Ames, IA), is available on the Germplasm Resources Information Network database at http://www.ars-grin.gov/npgs/acc/acc_queries.html. Above ground portions of plants from *P. vulgaris* (Ames 27664), harvested in 2008 were prepared for storage by drying for 8 d at 38 ℃ in a forced-air dryer with constant humidity. The dried material was ground with a 40-mesh screen and stored at -20 ℃ under N_2 until extraction. Weighed plant material was extracted with 95 ethanol with Soxhlet extractors for 6 h. The extract was concentrated by rotary evaporation at < 30 ℃ and lyophilized. The residue weight was recorded and the residues stored at -20 ℃ until solubilized in a final working solution of 5% ethanol in sterile distilled water at a final plant extract concentration of 12 mg/mL. The working *P. vulgaris* extract was divided into 2 mL aliquots and stored at -20 ℃ until use. *P. vulgaris* extracts from North Central Regional Plant Introduction Station were screened for endotoxin by using the Limulus Amebocyte Lysate Test (BioWhittaker, Inc., Walkersville, MD) according to manufacturers' specifications, and there was no detectable endotoxin present in the extract (data not shown). Extracts were tested for antimicrobial activity *in vitro* with no activity demonstrated.

Animals

Prior to the initiation of any work being performed, all animal related experiments were approved by the Institutional Animal Care and Use Committee (IACUC) at Iowa State University. Incumbent with IACUC approval, methods and procedures were used tomimimize pain and/or distress of all animals used in this study. Four to five week old male mdr1a$^{-/-}$ FVB.129P2-Abcb1a tm1BorN7 and wild type (WT) FVB.129P2 mice were obtained from Taconic Farms, Inc. (Germantown, NY). Animals were housed and maintained in the Laboratory Animal Resource facility at the College of Veterinary Medicine, Iowa State University. Established specific pathogen-free husbandry practices were followed, and twelve-hour light/dark cycles were applied. Upon arrival and throughout the study, mice were fed a defined Harlan Teklad AIN93 (M) rodent chow (Madison, WI) to control the amount of phytochemicals in their diet.

Experimental design

Three treatment groups of mice where utilized: Mdr1a$^{-/-}$ mice that were orally gavaged with 2.4 mg/d *P. vulgaris* extract in a 200 μL volume (prepared as described above) and mdr1a$^{-/-}$ and FVB^{WT} mice were orally gavaged with 5% ethanol vehicle alone; there were 4 to 10 mice/group per experiment. Gavage was performed using a 20 gauge feeding needle once daily beginning at 6 wk of age until the mice reached 20 wk of age or were removed from the study because of severe clinical wasting and/or weight loss exceeding 15% of their peak body weight in order to minimize pain and discomfort. At necropsy, mice were euthanized by CO_2 asphyxiation. Following euthanasia, blood was collected by cardiac puncture and separate sections of each cecum and proximal colon were excised, washed, and stored for further histological, myeloperoxidase (MPO) enzymatic and real-time PCR analysis. Serum was analyzed by multiplex assay to measure cytokine and chemokine levels as well as western blot analysis for antibody reactivity to antigens derived from selected members of the microbiota. Cecal tonsils were also collected for flow cytometric analysis of T and B cell populations. All results are representative of two independent experiments.

Macroscopic typhlocolitis assessment

Following euthanization, the colon and cecum were excised, photographed, measured and scored for

severity of macroscopic lesions. Gross typhlocolitic lesions were scored using a 9-point additive scale: A score of zero being a healthy animal and a score of 9 being a maximally diseased animal. Score parameters evaluated included: (1) cecal atrophy; (2) enlarged cecal tonsil or other enlarged lymphoid aggregates; (3) emptying of cecal contents; (4) abnormally watery or mucoid intraluminal cecal and/or colonic contents; (5) bloody cecal contents; (6) bloody colonic contents; (7) visible thickening and rigidity of the cecum; (8) presence of visible thickening and rigidity of the colon; and (9) absence of formed fecal pellets in the colon. In accordance with approved IACUC protocol, mice that developed severe colitis prior to 20 wk of age were removed from study when they lost ≥ 15% of their maximal body weight. Mice were also removed from the study within 5 d of the onset of persistent clinical signs of disease as characterized by bloody stools, diarrhea, ruffled fur, and hunched gate.

Histopathological assessment

Sections of excised cecum and proximal colon were placed in 10% buffered formalin overnight, paraffin embedded, sectioned, and routinely stained with hematoxylin and eosin. Stained colonic and cecal sections were scored by a board-certified veterinary pathologist, Dr. Jesse Hostetter of Iowa State University (Ames, IA), blinded to the treatments as previously described[24,25]. Microscopic mucosal lesion scores were assessed by five parameters, with each parameter scored on a scale of 0-5 (5 = maximum severity). Score parameters include: (1) ulceration of the mucosa; extent of inflammatory cell infiltrate; (2) mucosal edema characterized by the extent of lymphatic and vascular distortion and expansion of the mucosa/submucosa by clear space; (3) stromal collapse and necrosis of the glands; and (4) glandular hyperplasia characterized by the crowding and immaturity of enterocytes along the gland and gland dilation. In addition to score, mucosal height was determined and recorded as a ratio of gland height to gland width, and the specific inflammatory cell populations, if present, were recorded. Score parameters were considered individually and as an additive histopathological score with mucosal height included in the additive score.

Myeloperoxidase assay

MPO activity was assessed as a measure of neutrophil/granulocyte accumulation in proximal colonic tissues. The MPO assay was performed as previously described with several modifications[22]. Proximal colon sections collected at necropsy were gently flushed with PBS to remove luminal contents and stored in 1 mL of freshly prepared PBS supplemented with the protease inhibitor phenylmethanesulfonyl fluoride (PMSF) at 0.1 mmol/L and 15% dimethylsulphoxide (DMSO) at -20 ℃ for no more than 7 d prior to assay. Samples used as positive controls for MPO activity were prepared fresh the day the assay from peripheral blood. One FVB^{WT} mouse, not on study, was euthanized by CO_2 asphyxiation, and 500 μL to 1 mL of blood was immediately collected by cardiac puncture with a heparinized needle (heparin at 5000 USP heparin units/0.5 mL is drawn into the needle and syringe and then expelled to coat the inside of the needle with heparin). The heparinized blood was centrifuged at 250 x g for 10 min, the supernatant discarded and the red blood cells (RBC) lysed. In brief, 1 mL of ACK lysis buffer (8042 mg/L ammonium chloride, 1001 mg/L potassium bicarbonate, 3.722 mg/L ethylene diamine tetraacetic acid disodium, pH 7.2) was added to the pellet, vortexed gently for 1 min, 1 mL of PBS was added, and the mixture was centrifuged for 10 min at 250 x g. The lysis was repeated until the pellet was white and the supernatant was clear. Following RBC lysis, the pellet was resuspended in 1 mL of PBS/PMSF (0.1 mmol/L), cell numbers were enumerated using a cell counter (average yield of 3 x 10^6 cells/mL) and the cells were sonicated at an amplitude of 5, pulse on for 4 s, pulse off for 1 s, for 20 s total. The sonicated tissue samples were then centrifuged at 250 x g for 15 min and the supernatant stored at 4 ℃ until the tissue samples were prepared. Frozen proximal colonic sections were thawed, blotted to remove as much excess fluid as possible, trimmed to roughly 35 mg and their weights recorded. Tissues were then homogenized for 1 min at maximum power in 1 mL PBS/PMSF (0.1 mmol/L) and the homogenizer probe was washed 5 times with PBS between tissue samples. Homogenate cell counts were recorded, and each sample was then sonicated as described above. The tissue sonicates were then centrifuged at 250 x g for 15 min, the supernatant collected and the pellet discarded. Each lysate prepared from tissue or peripheral blood monocytes (PBMC) was analyzed for total protein using a NanoDrop ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE). Individual lysates were pipetted into 96-well, flat bottom microtiter plates. The PBMC lysates (150 μL/well) were serially diluted (10, two-fold dilutions) and analyzed in triplicate wells. For each tissue lysate, 150 μL was pipetted into separate wells and analyzed in triplicate. To each well, 50 μL of 0.78 mg/mL 3,3,5,5-tetramethylbenzidine dihydrochloride hydrate was added, followed immediately by the addition of 50 μL hydrogen peroxide (H_2O_2) (5 mmol/L). The reaction was allowed to proceed for 2 min (wells turned bright blue), followed by the addition of 50 μL of sulfuric acid (1 mol/L) to stop the reaction. The optical density (OD) was measured at 405 nm spectrophotometerically (V-Max, Molecular Devices, United States) using SOFTmax PRO 4.0 software. The MPO content was determined by comparison to the standard curve and MPO activity was expressed as the relative units of enzyme activity per gram of wet weight of tissue.

Serum cytokine/chemokine quantification

Following euthanasia of mdr1a$^{-/-}$ and FVB^{WT} mice, blood was collected *via* cardiac puncture. The blood was allowed to clot for 24 h at 4 ℃ after which samples were

centrifuged at 10000 x g for 10 min. Serum was then removed and stored at -20 ℃ until use. The day of assay, serum samples were thawed to room temperature. Concentrations of cytokines and chemokines of interest were measured using the Millipore (Billerica, MA) mouse cytokine-chemokine multiplexed assay kit. Analytes screened include: Eotaxin, G-CSF, GM-CSF, IFN-γ, IL-10, IL-12 (p40), IL-12 (p70), IL-13, IL-15, IL-17, IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IP-10, KC, LIF, LIX, M-CSF, MCP-1, MIG, MIP-1α, MIP-1β, MIP-2, RANTES, TNF-α, and VEGF. The assay was performed according to the manufacturer's instructions. In brief, supplied analyte standards (range: 10000 to 3.2 pg/mL), quality control standard, and buffer only control samples were analyzed in duplicate wells of the supplied 96 well plate. Mouse serum samples were diluted 1:1 in supplied assay buffer plated for each mouse. Supplied serum matrix and supplied assay buffer were added to all wells. Supplied pre-conjugated multiplex analyte beads were added to each well and the samples were incubated at 4 ℃ overnight on a plate shaker (Barnstead International Titer Plate Shaker, setting 5, Model No. 4625). Supplied detection antibody was added to all wells and allowed to incubate at room temperature while shaking for 2 h. Supplied streptavidin-phycoerythrin was incubated for 30 min at room temperature while shaking. The mean fluorescence intensity (MFI) was measured using Luminex platform technology (The FlowMetric System, Luminex, Austin, TX). MFIs were subsequently converted to concentrations using a 5-parameter logistic or line curve-fitting method in MasterPlex QT Software (MiraiBio Group, San Francisco, CA).

Flow cytometric analysis of cecal tonsil cell populations

Cecal tonsils from mdr1a$^{-/-}$ and FVB^{WT} mice were excised, placed in complete cell culture medium (10 mL heat-inactivated FBS, 1 mL penicillin/streptomycin, 1 mL glutamine, 0.1 mL 50 mmol/L β-mercaptoethanol, 2.5 mL 1M Hepes buffer in 85.4 mL DMEM containing 4.5 g/L glucose and sodium pyruvate), and homogenized mechanically on ice. Stainless steel wire strainers (60 mesh) were used to prepare single cell suspensions and remove particulate matter. Cells (5 × 10^5 cells/tube) were washed in FACS buffer, centrifuged at 250 x g and incubated in FACS buffer containing 1:100 rat IgG and fluorochrome labeled reagents for 15 min on ice. Following labeling, cells were washed with FACS buffer, centrifuged and fixed in 200 μL of BD stabilizing fixative. Cellular preparations from individual mice were labeled with the following fluorochrome-labeled reagents: Germinal center B cells (PNA$^+$B220$^+$)[26] identified using FITC-conjugated PNA and Alexa 700-conjugated anti-B220 mAb, CD4$^+$ T cells were identified using PE-Cy7-conjugated anti-CD4 mAb and CD8$^+$ T cells were identified using APC-conjugated anti-CD8β mAb. The following isotype controls were utilized: Alexa 700-conjugated anti-rat IgG2aκ, PE-Cy7-conjugated anti-rat IgG2a$^+$, APC-conjugated anti-rat IgG2b$^+$ and PE-conjugated anti-rat IgG2a$^+$ (eBioscience, San Diego, CA). PNA has no isotype control. Analysis was performed using a BD FACSCanto flow cytometer (BD, San Jose, CA) made available through the Flow Cytometry Core Facility at Iowa State University (Ames, IA). Data analysis was performed using FlowJo software (TreeStar Inc., Ashland, OR).

Western blot analysis

Sera from mdr1a$^{-/-}$ and FVB^{WT} mice were used to evaluate the presence of serum antibody against select members of the intestinal microbiota. Whole cell sonicates (WCS) of three members of the clostridial cluster group XIVa (ASF356, ASF500, and ASF502) were cultivated anaerobically, cells were harvested by centrifugation, washed in PBS, lyophilized, and stored at -20 ℃ until use[27,28]. Cells were then weighed and suspended in PBS to 2 mg/mL. The resulting suspension was sonicated on ice for 3 min at the following settings: Amplitude of 50 for 2, 30 s pulses with 5 s between each pulse; amplitude 75 for 2, 30 s pulses with 5 s between each pulse; amplitude 100 for 2, 30 s pulses with 5 s between each pulse. The sonicate was sterilized by UV light (six-minute exposure) and sterility was confirmed bacteriologically. For each preparation, protein content was determined by bicinchoninic acid (BCA) analysis (Pierce Laboratories, New Haven, Connecticut, United States), aliquoted and stored at -20 ℃. Whole cell sonicates of ASF356, ASF500, and ASF502 (8 μg of total protein content) were subjected to SDS-PAGE using 12% tris-glycine gels (BioRad, Hercules, CA) and transferred to PVDF membranes. Each individual antigen was analyzed using pooled anti-sera (1:250) from separate treatment groups as described above The membranes were then reacted with alkaline phosphatase (AP) conjugated anti-mouse IgG (H+L) (1:1000, Southern Biotech, Birmingham, AL) in a solution containing tris buffered saline (pH 7.6), 1% Tween 20 (TBST) and 2.5% non-fat, skim milk. Immunoreactive proteins were visualized using Sigma fast red tablets (Sigma, St. Louis, MO) according to manufacturers' instructions.

Pathway finder R2 profiler PCR array analysis

To evaluate the activation of signal transduction pathways modulated by treatment with the *P. vulgaris* extract, cecal gene expression was analyzed using the RT^2 profiler signal transduction pathway finder PCR array from QIAGEN (Germantown, MD) as per the manufacturer's instructions. In brief, total RNA was isolated from cecal tissue collected that had been stored at -20 ℃ in RNAlater using the TRIzol method[29]. RNA was further purified using the RT^2 qPCR-grade RNA isolation kit from QIAGEN (Germantown, MD) according to manufacturer's instructions. RNA quality (8.2 to 9.4) was assessed using an Agilent 2100 BioAnalyzer (Agilent Technologies, Palo Alto, CA). Prior to preparation of cDNA, RNA samples were tested by PCR using oligonucleotide primers for GAPDH to

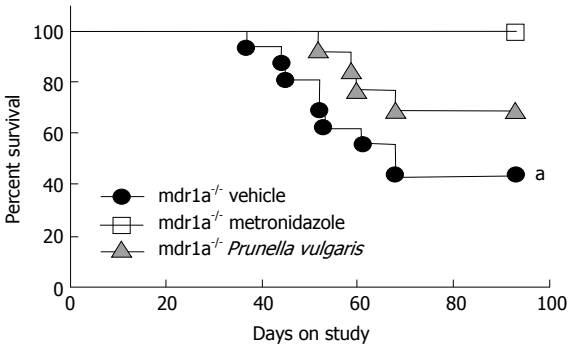

Figure 1 Effect of treatment with a *Prunella vulgaris* ethanolic extract on the onset of colitis in mdr1a$^{-/-}$ mice. Mdr1a$^{-/-}$ mice were removed from the study as they developed severe clinical disease (*e.g.*, > 15% weight loss) before the termination of the experiment as described in Materials and Methods. $^{a}P < 0.05$, as compared to FVB^{WT} control mice. Vehicle-treated mdr1a$^{-/-}$ mice $n = 16$, metronidazole-treated mdr1a$^{-/-}$ mice $n = 10$, *Prunella vulgaris*-treated mdr1a$^{-/-}$ mice $n = 13$. This survival (*i.e.*, mice remaining on study) curve is representative of two independent experiments.

confirm the absence of genomic DNA contamination. Invitrogen SYBR Green/ROX, primers and 1 μg of isolated RNA from each mouse were subjected to the following PCR conditions and were run on an ABI 5700 (Applied Biosystems Inc., Carlsbad, CA): 95 ℃ for 10 min, followed by 40 cycles of amplification (95 ℃ for 10 s, 60 ℃ for 15 s). All cycle threshold (CT) values were greater than 30, and were acceptable for further use (data not shown). GAPDH oligonucleotide primers used were: 5'-TGTGTCCGTCGTGGATCTGA-3' and 5'-CCTGCTTCACCACCTTCTTGA-3'. RNA (1 μg) from each mouse was then converted to cDNA using QIAGEN RT^2 First Strand kit according to manufacturers' instructions. Resulting cDNA from individual mice was pooled into diseased and healthy groups of mice for each treatment group and each experiment, mixed with the kit's array master mix experimental cocktail preparation, and subjected to the same PCR conditions and equipment noted above. PCR array data was analyzed using QIAGEN RT^2 PCR array analysis software and fold changes were calculated relative to house-keeping genes by the software. Only 2-fold changes or greater were considered.

Statistical analysis

Following review by a biostatistician for appropriateness of the statistical methods used, all data, except survival curves, were evaluated by the Kruskal-Wallis test with Dunn's multiple comparisons test. Because the Kruskal-Wallis test has no analog of the ANOVA linear contrast that focuses attention on a specific pre-specified comparison of groups, differences in the mdr1a$^{-/-}$ groups were further evaluated by the Mann-Whitney test for ordinal data and unpaired *t*-test with Welch's correction for continuous data. Survival curves were evaluated by the Log-rank (Mantel-Cox) test. A *P*-value of < 0.05 was considered statistically significant. Prism 6 software was used for all statistical calculations.

RESULTS

The ethanolic extract of P. vulgaris decreases severity of macroscopic disease parameters and delays onset of severe colitis in mdr1a$^{-/-}$ mice

To determine the efficacy of *P. vulgaris* extract in the treatment of spontaneous colitis, mdr1a$^{-/-}$ and FVB^{WT} mice were gavaged daily with vehicle (5% ethanol) or 2.4 mg *P. vulgaris* extract. Previously published data shows that mdr1a$^{-/-}$ mice develop disease between 8 and 36 wk of age, with the average age of disease onset occurring at 20 wk[30]. As expected, FVB^{WT} mice treated with *P. vulgaris* were not adversely affected by the administration of the extract despite the long course (14 wk) of treatment (data not shown). As anticipated, many vehicle-treated mdr1a$^{-/-}$ mice developed severe colitis and weight loss and were removed from study prior to 20 wk of age. Out of 16 mdr1a$^{-/-}$ mice treated with vehicle, 7 required removal from study prior to 20 wk of age, compared to only 4 out of 13 *P. vulgaris*-treated mdr1a$^{-/-}$ mice. Treatment with the *P. vulgaris* extract was able to delay onset of severe colitis and reduce the number of mdr1a$^{-/-}$ mice that had to be removed from study, the difference was not significant when compared to vehicle-treated mdr1a$^{-/-}$ mice (Figure 1). In addition, the phlogistic nature of the resident microbiota contributes to the mucosal inflammation in mdr1a$^{-/-}$ mice as evidenced by the ability of metronidazole treatment to prevent the onset of clinical disease (Figure 1).

Representative photographs (Figure 2) show the extent of macroscopic and microscopic tissue damage in vehicle-treated mdr1a$^{-/-}$ mice. In these mice, ceca were atrophied with visibly enlarged cecal tonsils suggesting immune activation. The ceca of the vehicle-treated mdr1a$^{-/-}$ mice were almost devoid of contents, and both cecal and colonic tissues are notably thickened and rigid. Occasional blood was noted in cecal and colonic contents while no formed fecal pellets were noted in the vehicle-treated mdr1a$^{-/-}$ mice. Conversely, the ceca and colons of *P. vulgaris*-treated mdr1a$^{-/-}$ mice were markedly improved and more closely resembled the tissue appearance of healthy FVB^{WT} as well as metronidazole-treated mdr1a$^{-/-}$ mice with regard to all parameters assessed.

Macroscopically, mild to severe typhlocolitis (a score of 2 to 9, respectively) was observed in 100% of vehicle-treated mdr1a$^{-/-}$ mice while all of the *P. vulgaris*-treated mice presented with macroscopic scores below the average score of the vehicle-treated mdr1a$^{-/-}$ mice (Figure 2C). *P. vulgaris* prophylaxis significantly ($P < 0.05$) improved macroscopic parameters of disease when compared to vehicle treatment in mdr1a$^{-/-}$ mice. In addition, the median colon length for *P. vulgaris*-treated mdr1a$^{-/-}$ mice was longer than that for the vehicle-treated in mdr1a$^{-/-}$ mice indicating less severe epithelial injury (Figure 2). Regardless of the treatment, FVB^{WT} mice did not exhibit any signs of clinical disease or tissue damage (Figure 2). These results indicate that treatment with the *P. vulgaris* ethanolic extract

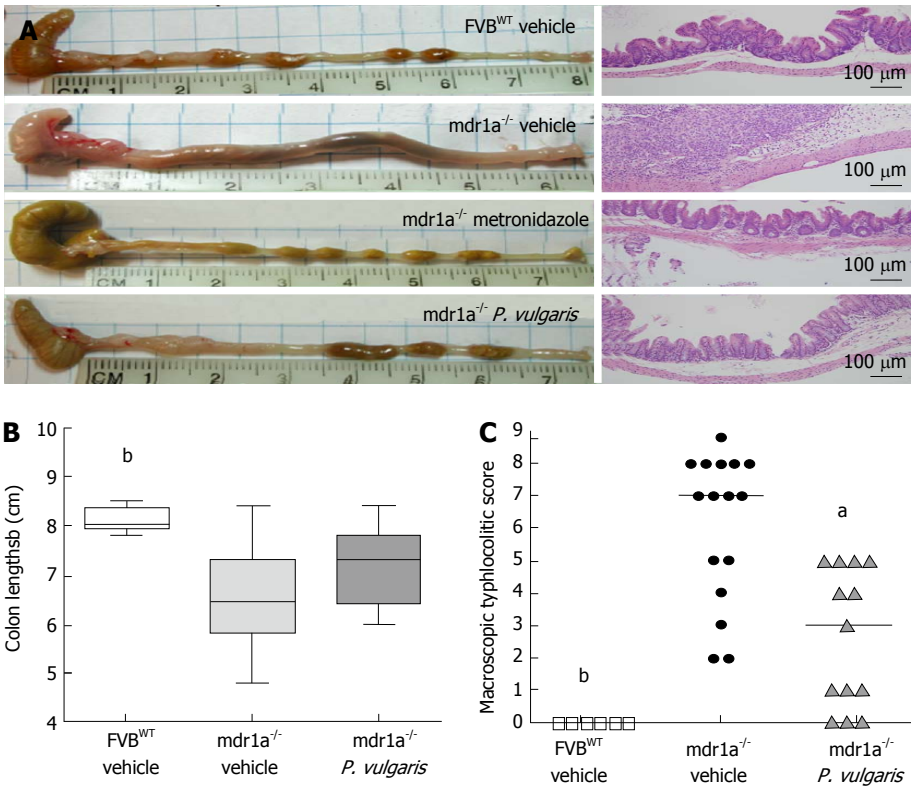

Figure 2 Oral administration of a *Prunella vulgaris* extract attenuated both microscopic and macroscopic cecal lesions in mdr1a$^{-/-}$ mice. A: Representative photographs of ceca and colons (left) and representative photomicrographs (200 ×) of histological sections of ceca (right) collected at necropsy from FVB^{WT} or mdr1a$^{-/-}$ mice treated with either vehicle or *Prunella vulgaris* (*P. vulgaris*) extract; B: Colon lengths were measured at necropsy and the group range is represented. Whiskers indicate minimum and maximum values, while the horizontal line represents the group median; C: Macroscopic typhlocolitic scores were assigned at necropsy as described in the Materials and Methods (Max/Severe = 9, Min/Healthy = 0). $^{a}P < 0.05$, $^{b}P < 0.01$ compared to mdr1a$^{-/-}$ vehicle as calculated by Kruskal-Wallis test. Vehicle-treated FVB^{WT} mice $n = 6$, vehicle-treated mdr1a$^{-/-}$ mice $n = 16$, *P. vulgaris*-treated mdr1a$^{-/-}$ mice $n = 13$.

Table 1 Histopathological scores of cecal tissue

Microscopic parameter	FVB^{WT} vehicle (n = 6)	mdr1a$^{-/-}$ vehicle (n = 10)	mdr1a$^{-/-}$ *P. vulgaris* (n = 7)
Mucosal height (μm)	3.5 ± 0.2^{a}	4.6 ± 0.2	3.9 ± 0.2
Ulceration	0.2 ± 0.2	1.9 ± 0.3	1.2 ± 0.4
Inflammation	1.3 ± 0.2^{b}	3.6 ± 0.2	2.5 ± 0.4^{c}
Edema	0.7 ± 0.5^{a}	2.4 ± 0.3	0.9 ± 0.3^{c}
Stromal collapse (necrosis)	0.0 ± 0.0^{a}	1.7 ± 0.4	0.5 ± 0.4
Gland hyperplasia	1.2 ± 0.2^{b}	2.8 ± 0.2	2.1 ± 0.3^{c}
Additive cecal score	6.9 ± 0.9^{b}	17.0 ± 1.2	11.1 ±1.5
Mice exhibiting cecal neutrophil infiltrate	17%^{b}	100%	38%^{c}

Average values are shown here ± standard error of the mean except where noted. $^{a}P < 0.05$, $^{b}P < 0.01$ compared to mdr1a$^{-/-}$ vehicle with Kruskal-Wallis test with Dunn's multiple comparisons test. $^{c}P < 0.05$ compared to mdr1a$^{-/-}$ vehicle with Mann-Whitney test.

attenuated macroscopic disease and delayed the onset of spontaneous colitis in mdr1a$^{-/-}$ mice.

Impact of P. vulgaris treatment on the severity of histopathological lesions

Histological inflammation of the cecum (Table 1) and colon (data not shown) was evaluated in the context of mucosal height, ulceration, extent and character of inflammatory cell infiltrate, edema, stromal collapse and glandular necrosis, and glandular hyperplasia. The ceca of vehicle-treated mdr1a$^{-/-}$ mice were characterized by crypt hyperplasia, extensive transmural ulceration and inflammatory cell infiltration, as well as submucosal edema and occasional stromal collapse (Figure 2 and Table 1). *P. vulgaris*-treated mdr1a$^{-/-}$ mice exhibited statistically significant ($P < 0.05$) improvement in inflammation, edema, gland hyperplasia, and neutrophil infiltration (Table 1). As expected, FVB^{WT} mice presented with no evidence of mucosal inflammation. While 100% of vehicle-treated mdr1a$^{-/-}$ mice exhibited extensive neutrophilic infiltration into the cecal lamina propria, neutrophils were only noted in cecal mucosa of 38% of *P. vulgaris*-treated mdr1a$^{-/-}$ mice (Figure 2 and Table 1). As a measure of the infiltration of granulocytes

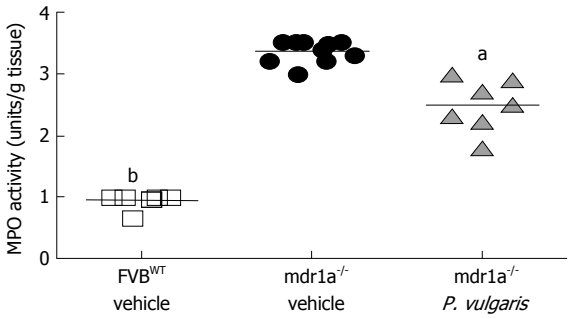

Figure 3 Administration of a *Prunella vulgaris* ethanolic extract reduced local myeloperoxidase activity in the colon of mdr1a$^{-/-}$ mice. Homogenates of colonic tissue were subjected to an assay for MPO activity. $^{a}P < 0.05$, $^{b}P < 0.01$ compared to mdr1a$^{-/-}$ vehicle as calculated by Kruskal-Wallis test. Vehicle-treated FVB^{WT} mice $n = 6$, vehicle-treated mdr1a$^{-/-}$ mice $n = 10$, *P. vulgaris*-treated mdr1a$^{-/-}$ mice $n = 7$. MPO: Myeloperoxidase; *P. vulgaris: Prunella vulgaris.*

into the mucosal tissue, MPO activity was assessed in tissue homogenates. In comparison to tissue samples from vehicle-treated mdr1a$^{-/-}$ mice, the associated MPO activity was significantly diminished in *P. vulgaris*-treated mdr1a$^{-/-}$ mice ($P < 0.05$) (Figure 3). In contrast to colon length, there was no histological evidence that the *P. vulgaris* treatment attenuated microscopic lesions when compared to vehicle-treated mdr1a$^{-/-}$ mice (data not shown), suggesting that the bioactive benefit of *P. vulgaris* localized in the cecum. Together, these data indicated that the benefits provided by the oral administration of *P. vulgaris* were to attenuate the severity of inflammation and injury in the cecal mucosa in association with a reduction of the presence or recruitment of inflammatory granulocytes.

Impact of the ethanolic extract of P. vulgaris on the induction of innate chemotactic and pro-inflammatory cytokines

To further investigate the mechanism(s) related to improved mucosal homeostasis and the associated reduction in neutrophils and MPO activity in the colons of *P. vulgaris*-treated mdr1a$^{-/-}$ mice, serum samples collected at necropsy were examined for the presence of chemokines and cytokines. Of those present in the kit, multiple cytokines/chemokines (Eotaxin, IL-13, IL-15, IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, LIF, LIX, M-CSF, MCP-1, MIP-1α, MIP-2, and RANTES) were not detectable in the serum of any treatment group (data not shown). However, several analytes were significantly elevated in mdr1a$^{-/-}$ mice compared to FVB^{WT} mice including G-CSF, IL-10, CXCL10, KC, CXCL9, and TNF-α ($P < 0.01$), and IL-9 ($P < 0.05$) (Table 2). When comparing *P. vulgaris* extract-treated to vehicle-treated mdr1a$^{-/-}$ mice, the levels of IL-10 ($P < 0.01$) and CXCL9 ($P < 0.05$) and TNF-α ($P < 0.05$) were significantly lower in the *P. vulgaris*-treated mdr1a$^{-/-}$ mice (Table 2). For the remainder of the cytokines/chemokines listed in Table 2, there was a trend for lower amounts in the serum of *P. vulgaris*-treated mdr1a$^{-/-}$ mice when compared to vehicle-treated mdr1a$^{-/-}$ mice. This data indicates that oral administration of the ethanolic extract of *P. vulgaris* is able to attenuate production of several innate chemokines and cytokines induced by the inflammatory response in mdr1a$^{-/-}$ mice.

Differential regulation of gene expression pathways by in vivo treatment with the ethanolic extract of P. vulgaris

To further characterize the attenuation of mucosal inflammation provided by *P. vulgaris* treatment, a microarray analysis for inflammatory gene expression was performed in order to identify differential gene regulation between disease phenotypes of botanical extract-treated mdr1a$^{-/-}$ mice (*e.g.*, healthy = macroscopic score < 2 ; colitic = macroscopic score ≥ 2) and between FVB^{WT} mice and mdr1a$^{-/-}$ mice treated with vehicle. At the extremes of microscopic and macroscopic lesion scores, it was observed that no vehicle treated mdr1a$^{-/-}$ mice were characterized as "healthy" and no FVB^{WT} mice were characterized as "colitic" (data not shown). Genes encoding *CCL2, CXCL1, CXCL9, IL-1α, MMP10, TNF-α, VCAM-1, CCL20,* and *IL-2* were all downregulated more than 2-fold by *P. vulgaris* treatment in mdr1a$^{-/-}$ mice that did not develop colitis (Table 3). *P. vulgaris* treatment appears to modulate the NF-κB pathway in the preservation of mucosal homeostasis in mdr1a$^{-/-}$ mice.

Influence of P. vulgaris on local T cell and B cell populations

Because T and B cells are activated as a consequence of inflammation, T cell and B cell populations in the cecal tonsils of mdr1a$^{-/-}$ and FVB^{WT} mice were analyzed to evaluate the effects of *P. vulgaris* treatment on local lymphocyte populations (Figure 4). Severe colitis in vehicle-treated mdr1a$^{-/-}$ mice resulted in 3-fold more CD4^{+} T cells (Figure 4A) and 6-fold more CD8^{+} T cells (Figure 4B) in the cecal tonsil as compared to vehicle gavaged FVB^{WT} mice. In *P. vulgaris*-treated mdr1a$^{-/-}$ mice, the numbers of CD4^{+} T cells in the cecal tonsils were significantly lower ($P < 0.05$) when compared to vehicle-treated mdr1a$^{-/-}$ mice (Figure 4A), and there was a trend indicating fewer CD8^{+} T cells in the *P. vulgaris*-treated mdr1a$^{-/-}$ mice (Figure 4B).

Vehicle-treated mdr1a$^{-/-}$ mice exhibited a 2.5-fold increase in the number of PNA^{+}B220^{+} germinal center B cells (Figure 4C) in the cecal tonsil as compared to vehicle gavaged FVB^{WT} mice. Remarkably, *P. vulgaris* treatment significantly ($P < 0.05$) decreased PNA^{+}B220^{+} germinal center B cells in mdr1a$^{-/-}$ mice (Figure 4C). Together, these data indicate that the expansion CD4^{+} T cell and PNA^{+}B220^{+} germinal center B cell populations were significantly lower in the cecal tonsils of *P. vulgaris*-treated mdr1a$^{-/-}$ mice.

P. vulgaris prevents antigenic responses to some members of the intestinal microbiota

Antibody responses to antigens derived from the gut

Table 2 Assessment of selected cytokines in the serum of mice

Cytokine/chemokine	FVB^{WT} vehicle (n = 6)	mdr1a$^{-/-}$ vehicle (n = 16)	mdr1a$^{-/-}$ *P. vulgaris* (n = 13)
G-CSF	140.1 ± 15[b]	9694.0 ± 2563	4597.0 ± 1931
GM-CSF	ND	21.1 ± 11	1.5 ± 1
IL-9	77.9 ± 27[a]	200.1 ± 38	117.8 ± 14
IL-10	ND[b]	19.4 ± 4	4.6 ± 2[a,d]
IL-17	4 ± 2	16 ± 4	1 ± 1
CXCL10	8.4 ± 5[b]	724.3 ± 136	397.2 ± 85
KC	69.5 ± 24[b]	527.1 ± 119	253.4 ± 58
CXCL9	108.7 ± 37[b]	3901.0 ± 858	1319.0 ± 277[c]
TNFα	9.3 ± 0.1[b]	14.8 ± 1	9.9 ± 3[c]

Serum samples were collected at the time mice were euthanized and analyzed as described in Materials and Methods. Average values (pg/mL serum) are shown here ± standard error of the mean except where noted. [a]P < 0.05, [b]P < 0.01 compared to mdr1a$^{-/-}$ vehicle with Kruskal-Wallis test with Dunn's multiple comparisons test. [c]P < 0.05, [d]P < 0.01 compared to mdr1a-/- vehicle with unpaired t-test with Welch's correction. ND: Not detectable; *P. vulgaris*: *Prunella vulgaris*.

Table 3 Attenuation of inflammatory gene expression in mdr1a$^{-/-}$ mice treated orally with an extract from *Prunella vulgaris*

Gene	Pathway affiliation	Fold change compared to vehicle-treated mdr1a$^{-/-}$ mice[a]		
		FVB^{WT} vehicle healthy	mdr1a$^{-/-}$ *P. vulgaris* healthy	mdr1a$^{-/-}$ *P. vulgaris* colitic
Ccl2	NF-κB, LDL	-7.3	-3.4	-1.1
Cxcl1	NF-κB	-11.3	-7.2	-1.0
Cxcl9	NF-κB, Jak/Stat	-23.9	-4.2	-1.1
Icam1	NF-κB, Phospholipase C	-3.2	-1.2	1.8
Il1a	NF-κB	-13.4	-8.0	1.3
Mmp10	NF-κB, Jak/Stat	-6.6	-8.5	1.2
TNFα	NF-κB	-10.2	-2.6	1.2
Vcam1	NF-κB, Phospholipase C, LDL	-3.8	-2.0	1.2
Ccl20	NF-κB	-3.6	-2.0	1.4
Il2	NF-κB, NFAT, Calcium, PKC	-3.7	-2.2	-1.5

[a]A negative value indicates that there was a lower level of gene expression when compared to the level of gene expression in vehicle-treated mdr1a$^{-/-}$ mice that developed severe colitis. *P. vulgaris*: *Prunella vulgaris*.

microbiota (*e.g.*, clostridial cluster group XIVa) have been noted in IBD patients and in murine models of IBD[31-33]. These antibody responses are indicative of a loss of epithelial integrity and immune tolerance to the microbiota and do not occur in healthy humans or mice. Pooled serum samples from mdr1a$^{-/-}$ mice treated with *P. vulgaris* were evaluated by immunoblot analysis against antigens (*i.e.*, whole cell sonicate) derived from select members of the clostridial cluster group XIVa within the microbiota (Figure 5). As anticipated, sera from FVB^{WT} mice did not display antibody reactivity against these bacterial antigens, suggesting that these mice maintained immunological tolerance to their gut microbiota. Conversely, sera from vehicle-treated mdr1a$^{-/-}$ mice did contain antibodies reactive to these bacterial antigens, indicating a loss of immunologic tolerance to these members of the microbiota. Sera from *P. vulgaris*-treated mdr1a$^{-/-}$ mice displayed little to no detectible antibody response to the three bacterial antigens (Figure 5).

DISCUSSION

As the long term safety and efficacy of current parenteral therapeutics for IBD are a concern and antibiotics are deemed unreliable for long-term use in IBD patients, there is a need for new therapies that may include complementary treatments[8,9,34]. Complementary and alternative therapy including nutraceuticals hold realistic potential in treating or supplementing treatment of inflammatory disorders, as the anti-inflammatory and antioxidant benefits of plant-derived components are becoming more extensively characterized[35-39]. *P. vulgaris*, already popular in Asian medicine, is a viable candidate for study as a therapeutic agent in the treatment of IBD as it contains several anti-inflammatory, immunomodulatory, and antioxidant flavonoids, polyphenols, and triterpenoids and has no documented toxic side-effects[37,40-44]. In this regard, rosmarinic acid, the most plentiful phenolic compound found in *P. vulgaris*, was found to protect mice against the deleterious effects associated with sepsis by downregulating inflammatory genes in the NF-κB pathway including the related pro-inflammatory cytokines TNF-α and IL-6[45].

The mdr1a$^{-/-}$ mouse model is ideal to use for studies of potential IBD therapeutics that are relevant to human medicine as mdr1a$^{-/-}$ mice are immunocompetent, develop spontaneous colitis in the context of a leaky intestinal epithelium, and exhibit cytokine profiles and

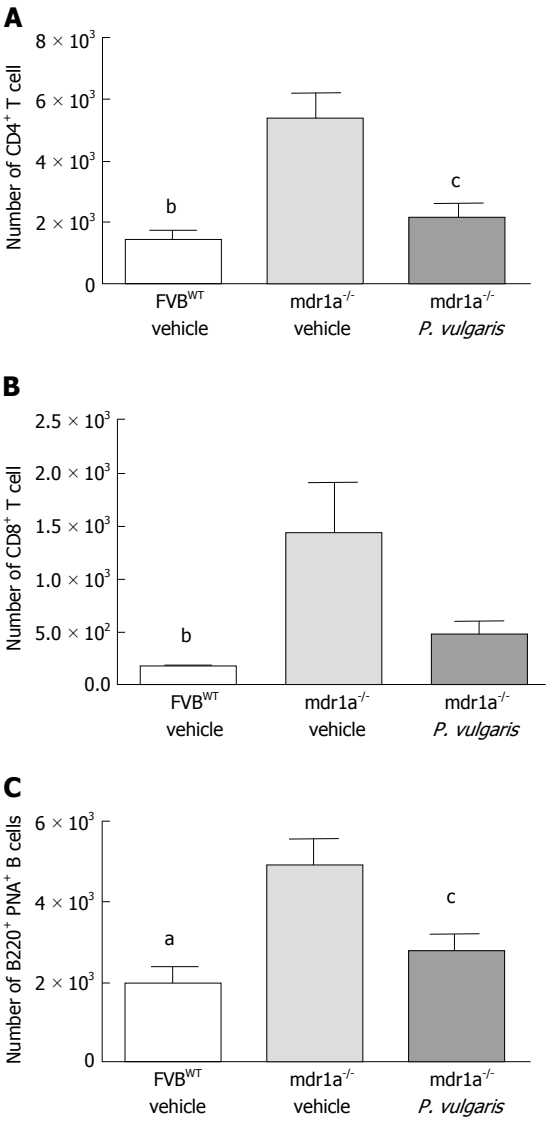

Figure 4 Evaluation of T cell and B cell subsets in the cecal tonsil of mdr1a$^{-/-}$ mice treated with *prunella vulgaris* ethanolic extract. Cecal tonsils were excised at necropsy, single cell suspensions prepared and labeled for flow cytometric analysis as described in Materials and Methods. Absolute numbers of (A) CD4+ T cells; (B) CD8ß+ T cells; and (C) B220+PNA+ germinal center B cells in the cecal tonsils of mice. $^{a}P < 0.05$, $^{b}P < 0.01$ compared to mdr1a$^{-/-}$ vehicle as calculated by Kruskal-Wallis test. $^{c}P < 0.05$ compared to mdr1a$^{-/-}$ vehicle as calculated by unpaired *t*-test. The *n* for each group is equal to that noted in Figure 1 and data are representative of two independent experiments. Vehicle-treated FVB^{WT} mice *n* = 5-6, vehicle-treated mdr1a$^{-/-}$ mice *n* = 8-10, *P. vulgaris*-treated mdr1a$^{-/-}$ mice *n* = 5-7.

immune responses similar to those documented in clinical IBD cases[30,46-51]. It was previously demonstrated that administration of curcumin attenuated mucosal inflammation in mdr1a$^{-/-}$ mice[52]. In these studies, similar to previously published studies, onset of clinical disease (*e.g.*, weight loss) in vehicle-treated mdr1a$^{-/-}$ mice was observed at roughly 10 wk of age (Figure 1)[30]. In contrast, onset of clinical disease was delayed by treatment with the *P. vulgaris* extract; in addition, markedly fewer of the mdr1a$^{-/-}$ mice treated developed severe clinical disease by 20 wk of age (Figure 1). *P. vulgaris* treatment of mdr1a$^{-/-}$ mice was also found

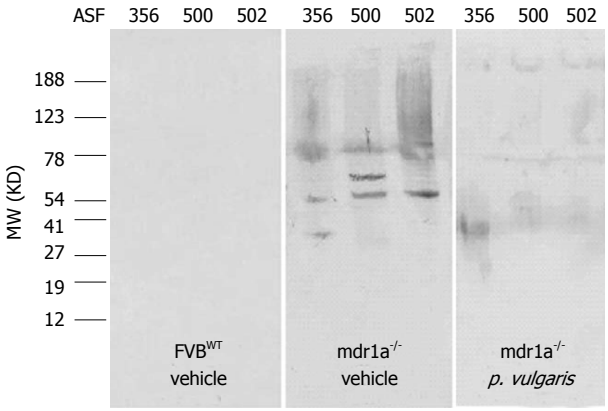

Figure 5 Vehicle-treated mdr1a$^{-/-}$ mice with severe colitic inflammation developed serum antibody to antigens derived from gut microbiota while those treated with *prunella vulgaris* extract do not. Whole cell sonicates of three separate clostridial species present as part of the intestinal microbiota (altered Schaedler flora members 356, 500 and 502) were subjected to SDS-PAGE. Western blot analysis was performed using sera collected at necropsy. Antigens in lanes represented in each panel are as follows from left to right: ASF502, ASF500 and ASF356.

to attenuate macroscopic lesions associated with the characteristic severe typhlocolitis observed in this murine model (Figure 2). In contrast to the vehicle-treated mdr1a$^{-/-}$ mice, ceca of mdr1a$^{-/-}$ mice treated with a *P. vulgaris* extract retained normal mucosal architecture, lacked enlarged lymphoid aggregates, and retained luminal contents devoid of blood or mucus. Macroscopically, the colons of botanical-treated mdr1a$^{-/-}$ mice were more similar in appearance to the colons of the FVB^{WT} control mice with regard to presence of formed feces, and lack of grossly visible tissue edema and rigidity (Figure 2). Mdr1a$^{-/-}$ mice treated with *P. vulgaris* extract also presented with normal colon lengths (Figure 2); microscopically, colonic lesions were less attenuated in the botanical extract-treated mdr1a$^{-/-}$ mice than those present in the cecum (data not shown). The microscopic lesions observed in the ceca of mdr1a$^{-/-}$ mice treated with *P. vulgaris* were markedly less severe when compared to those observed for vehicle-treated mdr1a$^{-/-}$ mice (Table 1). Together, these findings highlight the differences between colonic and cecal compartments in terms of the magnitude of the disease. Perhaps these differences arise from the more dense concentration of metabolically active microbes in the cecum as compared to the colon, which may lead to more efficient metabolism/degradation of the extract and greater health benefit at more proximal gastrointestinal sites.

Although the etiology of IBD is still ill defined, many recognize that the inductive phase of colitis involves a compromised intestinal epithelium and activation of innate immune responses, including neutrophil activation, transmigration across the mucosal epithelium, and enzymatic damage to host tissues[3,53-56]. Flavonoids from licorice have been shown to inhibit neutrophil infiltration into lung tissue after lipopolysaccharide-induced inflammation and reduce the severity of associated inflammatory damage to host pulmonary

tissues[57]. As a group, the mdr1a$^{-/-}$ mice treated with the *P. vulgaris* ethanolic extract presented with markedly less neutrophilic infiltration into the cecal mucosa when compared to vehicle-treated mdr1a$^{-/-}$ mice (Table 1). Reduction of the neutrophilic infiltrate in these mice was correlated with less severe microscopic scores and a significant decrease in tissue levels of MPO activity (Figures 2 and 3, Table 1). MPO enzymatic activity is a known correlate to intestinal damage and is often used as a marker of IBD severity in many animal models of colitis[58-62].

Since neutrophils are not resident in tissues, cytokine and chemokine signals produced by epithelial cells and local macrophages are responsible for the recruitment of neutrophils into the mucosal tissues[63,64]. Homeostatic production of these innate chemokines is central to mucosal health, while over-production contributes to the development of severe inflammation in colitis[55,56,65]. Debate regarding the role of NF-κB activation and the exacerbated recruitment innate immune cells in acute intestinal inflammatory models continues, with some research pointing to a protective effect by these components and others revealing a deleterious effect[23,66-68]. However, many agree that dysregulation of NF-κB signaling and the consequent innate cellular responses are causative factors in the inductive phase and maintenance of chronic inflammation associated with human CD and UC[69-71]. Pro-inflammatory mediators induced by activation of NF-κB are abnormally upregulated in CD and UC patients[72]. Moreover, it concludes nuclear translocation of NF-κB in epithelial cells and local monocytes upregulates production of pro-inflammatory cytokines and chemokines such as TNF-α, IL-1β, KC, and CXCL9. These cytokines increase expression of adhesion molecules (*i.e.*, VCAMs, ICAMs, and MadCAM) on endothelial cells, while chemokines create chemical gradients to attract neutrophils and other innate inflammatory cells to sites of injury[55,64,65,73]. Others have reported that the ability to regulate or attenuate cytokine production (*e.g.*, TNF-α, IL-1α, IL-1β, IFN-γ) decreases the expression of chemokines (*e.g.*, IL-8/KC and VEGF) and adhesion molecules on endothelial cells resulting in amelioration of inflammatory tissue damage in several disease models, including colitis[1,45,66,74]. The patterns of cytokine and chemokine production observed in aged-matched, vehicle-treated mdr1a$^{-/-}$ mice in the current study (Table 2) was consistent with that previously reported[51]. Serum samples from mdr1a$^{-/-}$ mice treated with the ethanolic extract of *P. vulgaris* had lower levels of cytokines that would contribute to the production of granulocytes and monocytes (G-CSF and GM-CSF) as well as the neutrophil chemotactic factor KC (Table 2). Importantly, treatment with the *P. vulgaris* extract reduced serum levels of TNF-α (Table 2), a cytokine known to be a key regulator of inflammatory responses in colitis[75]. These data indicate that *P. vulgaris* extract reduced the production of cytokines and chemokines central to the induction and maintenance of chronic inflammation.

Activation of the transcription factor NF-κB and the regulation of its target genes have well documented links to the chronicity of inflammation associated with IBD[29,69-71]. Recent studies have shown that flavonoids similar to those identified in *P. vulgaris* are capable of downregulating NF-κB and ultimately regulating the production of innate chemotactic factors and pro-inflammatory cytokines[76,77]. One such study showed that the flavonoid luteolin decreased NF-κB expression in the ceca and colons of IL-10$^{-/-}$ mice, and effectively ameliorated spontaneous colitis[23]. Similarly, the ethanolic extract of *P. vulgaris*, which is known to contain several flavonoids (data not shown)[15], downregulated expression of chemokine genes (*Ccl2, Cxcl1/KC, Cxcl9/CXCL9,* and *Ccl20*) and genes involved in the increased expression of adhesion molecules (*VCAM-1, ICAM, TNFα* and *IL-1α)* and tissue remodeling to allow for inflammatory cell transmigration (*MMP-10*) (Table. 3). All of these genes participate in the activation of or are regulated by NF-κB[78-83]. Based on our findings, the ethanolic extract of *P. vulgaris* likely attenuates neutrophil recruitment into the colonic tissues of mdr1a$^{-/-}$ mice by downregulating genes regulated by NF-κB signaling. The importance of regulating inflammation in mdr1a$^{-/-}$ mice prior to the onset of clinical disease is underscored by recent data showing that regulation of inflammatory gene expression is altered in mdr1a$^{-/-}$ mice and in mice treated with dextran sodium sulfate (DSS) prior to any histologic signs of inflammation[84,85]. Since defects in gene expression precede inflammation, prophylactic approaches to control mucosal inflammation rather than providing therapy at or after the onset of an inflammatory flare may prove advantageous. In the current study, therapeutic initiation of *P. vulgaris* treatment (*i.e.*, after colitic onset) was ineffective at reducing the severity of inflammation (data not shown). This observation supports the current hypothesis that the ethanolic extract of *P. vulgaris* modulates innate inflammatory gene expression, and that effective treatment should begin prior to the onset of clinical disease.

In addition to NF-κB signaling and innate immune activation, adaptive immune responses also play an integral role in mediating the chronicity and severity of colitic disease in experimental models and in humans with IBD. In particular, aberrant CD4$^+$ T cell responses to antigens derived from the resident microbiota have been implicated in the pathogenesis of IBD[31,86]. Pretreatment with the ethanolic extract of *P. vulgaris* decreased production of CXCL10 and CXCL9, two proteins that are induced by IFN-γ and are chemotactic for T cells (Table 2)[79,87]. These chemokines and other cytokines participate in inflammatory feedback loops that may be interrupted by treatment with the ethanolic extract of *P. vulgaris*. The observation of reduced numbers of CD4$^+$ and CD8$^+$ T cells in the cecal tonsils of mdr1a$^{-/-}$ mice (Figure 4) is consistent with the lower amounts of CXCL10 and CXCL9 in the serum. There were also lower levels of IL-9 in the serum of mdr1a$^{-/-}$ mice treated with

the ethanolic extract of *P. vulgaris* (Table 2), a cytokine known to enhance $CD4^+$ T cell proliferation and inhibit apoptosis[88]. Moreover, the decreased expression of *CXCL9, Ccl2, IL-1α, TNF-α,* and *Ccl20* genes in extract treated mdr1a$^{-/-}$ mice provides additional evidence that treatment with *P. vulgaris* extract impacted the robustness of the local T cell response (Table 3). CCL20 is strongly chemotactic for immature dendritic cells, which would mature upon collecting antigen in the tissues, present that antigen to T cells, and stimulate an adaptive immune response[89].

With respect to the induction of antibody specific to antigens derived from the resident microbiota, germinal centers will develop in lymphoid tissue upon B cell activation by T dependent antigens[90]. The results of this study demonstrated that the number of PNA^+B220^+ B cells present in *P. vulgaris*-treated mice was significantly less than that detected in the vehicle-treated mdr1a$^{-/-}$ mice (Figure 4). As a consequence of the attenuated germinal center B cell response, there was a lack of antibody production towards bacterial antigens derived from the resident microbiota in *P. vulgaris*-treated mdr1a$^{-/-}$ mice (Figure 5). Collectively, these data present evidence that the ethanolic extract of *P. vulgaris* acts to maintain mucosal homeostasis in mdr1a$^{-/-}$ mice by regulating gene expression associated with innate inflammatory responses and attenuating the activation of the adaptive immune response.

It has been recently reported that 40% to 50% of adults suffering with inflammatory bowel disease or irritable bowel disease utilize complementary and alternative medicine to treat their symptoms[34]. Because of the prevalence at which patients use complementary approaches to attenuate clinical symptoms, it is critical to evaluate the efficacy of nutraceuticals in pre-clinical controlled studies. The work highlighted in this study indicates that an ethanolic extract derived from *P. vulgaris* was safe when administered daily for 14 wk and markedly attenuated the severity of colitis in mice that are genetically prone to develop mucosal inflammation. The health benefits associated with consuming plant-derived nutraceuticals are likely associated with the richness and complexity of anti-inflammatory compounds present in botanical extracts. There is a need to further evaluate the underlying mechanism(s) that contributed to the anti-inflammatory activity of *P. vulgaris* extracts in order to provide a basis for their legitimate use as a prophylactic or supplementary option for the treatment of IBD and other chronic inflammatory disorders.

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COMMENTS

Background

Extracts of *Prunella vulgaris* (*P. vulgaris*) have been shown to contain anti-inflammatory components but there is limited information regarding the ability of these extracts to attenuate or prevent inflammation *in vivo*. Mice that are deficient in the expression of the multiple drug resistance gene (*i.e.*, mdr1a$^{-/-}$) develop spontaneous colitis by 12 to 15 wk of age. As opposed to chemically-induced models of colitis, these mice offer a excellent model to assess the anti-inflammatory capabilities of botanical extracts administered as an oral formulation.

Research frontiers

Treatment of inflammatory bowel disease is dominated by the use of drugs and biologicals that systemically target inflammatory processes. The development of effective treatment modalities that can be delivered orally and target the inflammatory response in the gastrointestinal mucosa would reduce the systemic side-effects observed with other treatment regimen. The studies presented herein demonstrate that botanical extracts can effectively attenuate the severity of colitis on a murine model of spontaneous colitis.

Innovations and breakthroughs

This is the first study to demonstrate the oral administration of an ethanolic extract derived from *P. vulgaris* can be used to delay the onset of and ameliorate the severity of spontaneously occurring colitis in mdr1a-deficient mice.

Applications

It is estimated that 30% to 70% of patients suffering from inflammatory bowel diseases use some form of complementary and alternative therapy to treat their symptoms. Many of the parenteral therapies (*e.g.*, steroids, 5-aminosalicylates, monoclonal antibodies) used to control gastrointestinal inflammation are associated side-effects. The development of extracts derived from medicinal plants, such as *P. vulgaris,* that can be delivered orally and ameliorate gastrointestinal inflammation may be useful adjunct treatments for IBD patients.

Terminology

Mice lacking the multiple drug resistance (*mdr1a*) gene fail to produce an epithelial cell transporter protein (a 107 kDa P-glycoprotein) responsible for pumping various compounds across the cell membrane. The pathological lesions and cytokine profiles observed in the colon of mdr1a-deficient mice resembles that noted in human ulcerative colitis patients.

Peer-review

This is a well written manuscript, investigating the ability of an orally-supplemented *P. vulgaris* extract to attenuate the clinical symptoms of colitis in an animal model of spontaneous colitis (mdr1a$^{-/-}$ mice). The authors used valid methodological approaches to compare the histopathological, biochemical and immunological profile of the supplemented and control animals, the presentation of the results was clear and the discussion was thorough.

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ORIGINAL ARTICLE

Retrospective Study

Typical and atypical symptoms of gastro esophageal reflux disease: Does *Helicobacter pylori* infection matter?

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Author contributions: Grossi L was the author involved in the conception of the study and drafting the manuscript; Grossi L and Ciccaglione AF performed the exams and obtained the clinical history of patients and interpreted the results; Marzio L supervised the report and gave final approval of the version submitted.

Institutional review board statement: It was not necessary to get an Ethics Committee approval as no drugs or therapeutic techniques have been used in this study. However, the work has been done with the permit of the Ethics Committee of Ospedale Spirito Santo, Pescara, which was fully informed about the research.

Informed consent statement: Patients were not required to give a complete informed consent. All patients recruited into the study had been referred to our Unit to perform a pH-monitoring. They were only interviewed about *H. pylori* status, without assuming any drugs or testing medical techniques. After receiving full information about the study and giving informed, written consent, each patient underwent C13 Urea Breath test. All patients were reassured about the anonymous characteristics of data recruitments.

Conflict-of-interest statement: We have no financial relationships to disclose.

Data sharing statement: No additional data are available.

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Abstract

AIM: To analyze whether the presence of *Helicobacter pylori* (*H. pylori*) infection could affect the quality of symptoms in gastro-esophageal reflux disease (GERD) patients.

METHODS: one hundred and forty-four consecutive patients referred to our Unit for suspected GERD were recruited for the study. All patients underwent esophageal pH-metric recording. For those with a positive test, C_{13} urea breath test was then performed to assess the *H. pylori* status. GERD patients were stratified according to the quality of their symptoms and classified as typical, if affected by heartburn and regurgitation, and atypical if complaining of chest pain, respiratory and ears, nose, and throat features. *H. pylori*-negative patients were also asked whether they had a previous diagnosis of *H. pylori* infection. If a positive response was given, on the basis of the time period after successful eradication, patients were considered as "eradicated" (E) if *H. pylori* eradication occurred more than six months earlier or "recently eradicated" if the therapy had been administered within the last six months. Patients without history of infection were identified as "negative" (N). χ^2 test was performed by combining the clinical aspects with the *H. pylori* status.

RESULTS: one hundred and twenty-nine of the 144 patients, including 44 *H. pylori*-positive and 85 *H. pylori*-negative (41 negative, 21 recently eradicated, 23 eradicated more than 6 mo before), were eligible for the analysis. No difference has been found between *H. pylori* status and either the number of reflux episodes (138 ± 23 *vs* 146 ± 36, respectively, $P = 0.2$, not significant) or the percentage of time with pH values < 4 (6.8 ± 1.2 *vs* 7.4 ± 2.1, respectively, $P = 0.3$, not significant). The distribution of symptoms was as follows: 13 typical (30%) and 31 atypical (70%) among the 44 *H. pylori*-positive cases; 44 typical (52%) and 41 atypical (48%) among the 85 *H. pylori*-negative cases, ($P = 0.017$ *vs H. pylori*+; OR = 2.55, 95%CI: 1.17-5.55). Furthermore, clinical signs in patients with recent *H. pylori* eradication were similar to those of *H. pylori*-positive ($P = 0.49$; OR = 1.46, 95%CI: 0.49-4.37); on the other hand, patients with ancient *H. pylori* eradication showed a clinical behavior similar to that of *H. pylori*-negative subjects ($P = 0.13$; OR = 0.89, 95%CI: 0.77-6.51) but different as compared to the *H. pylori*-positive group ($P < 0.05$; OR = 3.71, 95%CI: 0.83-16.47).

CONCLUSION: Atypical symptoms of GERD occur more frequently in *H. pylori*-positive patients than in *H. pylori*-negative subjects. In addition, atypical symptoms tend to decrease after *H. pylori* eradication.

Key words: Eradication; *Helicobacter pylori*; C_{13} urea breath test; Symptoms; Gastro-esophageal reflux disease; pH-metry

Core tip: This study aimed to investigate whether the presence of *Helicobacter pylori* (*H. pylori*) infection could affect the symptom pattern of patients with gastro-esophageal reflux disease (GERD). GERD patients with *H. pylori* were predominantly affected by atypical symptoms (chest pain, respiratory and ears, nose, and throat features) whilst patients without infection mainly referred typical GERD symptoms (heartburn, regurgitation). Therefore, it seems reasonable to assume that *H. pylori* infection may have a role in GERD pathogenesis or at least in the modulation of symptoms appearance.

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INTRODUCTION

Gastro-esophageal reflux disease (GERD) and *Helicobacter pylori* (*H. pylori*) infection represent two of the most common diseases affecting upper GI tract. GERD is clinically characterized by different patterns, generally identified as typical or atypical. Typical symptoms are heartburn and acid regurgitation[1], while atypical extraesophageal manifestations can include symptoms primarily attributable to other organs, such as chronic cough, non cardiac chest pain, chronic pharyngitis and laryngitis[2]. A small amount of patients refer symptoms (epigastric pain, nausea, belching, vomiting) that may overlap with other gastrointestinal conditions, such as dyspepsia, severe gastritis, peptic ulcer disease or hiatal hernia. Despite a huge amount of evidence on diagnosis and therapy of such different patients, limited information is available to explain why one can experience typical or atypical symptoms and whether the presence of *H. pylori* infection could affect the quality of symptoms in GERD patients. Whilst the role of *H. pylori* has been widely recognized in the pathogenesis of gastritis[3,4], peptic ulcer[5,6] and even gastric malignancies[7,8], there is conflicting evidence in the literature about a possible link between infection and natural history of GERD. Many authors proposed a "protective" role of *H. pylori* against acid refluxes, probably due to the reduced acid production in infected patients, but the exact mechanism is not well known[9]. On the contrary, other studies have demonstrated the lack of any influences between the two diseases[10]. Therefore, the question has been mainly limited to demonstrate whether *H. pylori* could protect from or facilitate the onset of pathological gastro-esophageal refluxes. In the present study we investigated whether *H. pylori* infection has a role in the clinical appearance of GERD.

MATERIALS AND METHODS

Selection of patients

We enrolled 144 consecutive patients undergoing esophageal 24-h pH-metric recording for suspected GERD on the basis of typical or atypical extraesophageal symptoms. To rule out overlapping with other upper GI diseases, we did not consider patients with epigastric pain, nausea, vomiting as predominant symptoms and scheduled for pH monitoring. The procedure was performed using a probe with a single glass electrode on the tip (Jubileum, Microbioprobe and Telemedicine srl, Marigliano-NA, Italy) connected to a portable data logger (pH-day, Menfis Biomedical, Bologna Italy). The main inclusion criteria was the confirmation of GERD by the pH monitoring, according to De Meester criteria[11]. All patients had a previous upper GI endoscopy within the last six months in order to exclude the presence of malignancies, Los Angeles grade C or D esophagitis, peptic ulcer, erosive gastritis, and hiatal hernia. In addition, recent full examinations by cardiologist, pneumologist and otolaryngologist were required to rule out specific diseases of their competence. All patients were required to complete a minimum four-week wash-out period of antisecretory drugs (PPI, H_2-

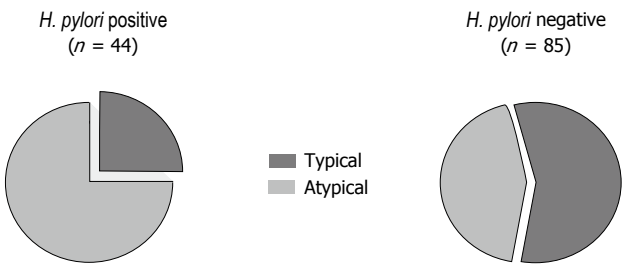

Figure 1 Distribution of symptoms in *Helicobacter pylori*-positive (on the left) and *Helicobacter pylori*-negative (on the right) patients. The data represent the percentage distribution of typical symptoms (in dark grey) and atypical extraesophageal symptoms (in light grey). *H. pylori: Helicobacter pylori.*

blockers) and medications potentially affecting upper GI motility (nitrates, calcium channel blockers, xanthines, benzodiazepines, and beta agonists), before entry into the study. Body mass index (BMI) was also calculated for all patients. Obese subjects (BMI > 30 kg/m^2) were considered not eligible for the study and were excluded. Smoking habits were also recorded for all eligible patients.

GERD symptoms

Patients were divided in two groups according to their GERD symptoms: "Typical" including patients with symptoms such as heartburn and regurgitation, and "Atypical" including subjects with symptoms including chest pain, chronic cough and chronic pharingytis/laryngitis. If both kinds of symptoms were present, patients were asked to indicate the predominant one in terms of its impact on their daily activities. The subjects were then assigned accordingly to group A or group B.

H. pylori infection

A C_{13} Urea Breath Test was performed on all patients on the same day of pH-metric recording. Patients were classified as *H. pylori*-positive (*H. pylori*+) or *H. pylori*-negative (*H. pylori*-). *H. pylori*-negative patients were also asked whether they had a previous diagnosis of *H. pylori* infection. If a positive response was given, on the basis of the time period after successful eradication, patients were considered as "eradicated" (E) if *H. pylori* eradication occurred more than 6 mo earlier or "recently eradicated" (RE) if the therapy had been administered within the last six months. Patients without history of infection were identified as "negative" (N).

Statistical analysis

After collecting the data, χ^2 test was performed to identify significant correlations between clinical aspects (*i.e.,* typical or atypical) and *H. pylori* status (*i.e.,* positive or negative; if negative: eradicated or recently eradicated). A *P*-value less than 0.05 ($P < 0.05$) was inferred significant. Odd ratio (OR) and 95%CI were also calculated. The primary endpoint was to find a correlation between symptoms pattern and *H. pylori* presence; then, we wanted to evaluate whether this correlation could change in relation of period of time after bacterial eradication.

RESULTS

pH profile and H. pylori status

Of the 144 initially enrolled subjects, 129 patients (73 male, 56 female, age: 19-65) resulted affected by GERD at 24-h pH monitoring. In this case series, 44 were *H. pylori*-positive and 85 were *H. pylori*-negative. No difference has been found between *H. pylori* status and either the number of reflux episodes (138 ± 23 *vs* 146 ± 36, respectively, $P = 0.2$, not significant) or the percentage of time with pH values < 4 (6.8 ± 1.2 *vs* 7.4 ± 2.1, respectively, $P = 0.3$, not significant). Among the negative group, 41 patients had no history of previous *H. pylori* infection (N), 21 patients had been recently *H. pylori*-eradicated (RE) and 23 patients had been successfully treated for *H. pylori* eradication more than six months earlier (E).

GERD symptoms

Typical symptoms of GERD were present in 57 patients (34 male, 23 female), whereas 72 patients (38 male, 34 female) resulted affected by atypical manifestations.

BMI and smoking habits

Among 129 patients with GERD, 12 subjects (7/72 patients of atypical group and 5/57 patients of typical group) had a BMI indicative of slight overweight, *i.e.,* between 25 and 30 (9.7% *vs* 8.3%, respectively, $P = 0.1$, not significant). Furthermore, there were 41 active smokers with 22 patients among atypical group and 19 patients among typical group (30.5% *vs* 33.3%, respectively, $P = 0.2$, not significant).

H. pylori infection and quality of symptoms

Group *H. pylori*+: 31 of the 44 patients (70%) with *H. pylori* infection were affected by atypical symptoms, whereas 13 of them (30%) referred typical clinical signs. Group *H. pylori*-: 41 of the 85 patients (48%) without infection had atypical manifestations and 44 of them (52%) showed typical pattern (Figure 1). Group N: 16 of the 41 patients (35%) without *H. pylori* infection were affected by atypical symptoms, whereas 27 of them (65%) referred typical clinical signs. Group RE: 13 of 21 patients (62%) with recent *H. pylori* eradication had atypical symptoms and 8 of 21 (38%) presenting typical GERD manifestations (Figure 2). Group E: 12 of the 23 patients (52%) with *H. pylori* eradication performed more than 6 mo earlier had atypical GERD symptoms. Typical signs occurring in 9 patients (48%) (Figure 2).

Statistical significance

Atypical GERD symptoms were significantly more frequent in *H. pylori*-positive than in *H. pylori*-negative patients ($P = 0.017$; OR = 2.55, 95%CI: 1.17-5.55). Also, patients with recent eradication of *H. pylori* infection had a predominance of atypical signs that resulted not significantly different from *H. pylori*-positive ($P = 0.49$; OR = 1.46, 95%CI: 0.49-4.37), but

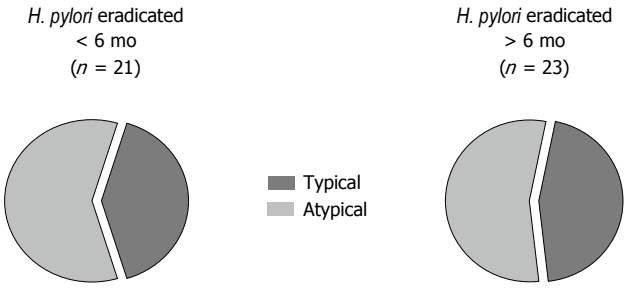

Figure 2 Distribution of symptoms in patients who eradicated *Helicobacter pylori* less than 6 mo before pH monitoring (on the left) and in patients who eradicated *Helicobacter pylori* more than 6 mo earlier (on the right). The data represent the percentage distribution of typical symptoms (in dark grey) and atypical extraesophageal symptoms (in light grey). *H. pylori: Helicobacter pylori.*

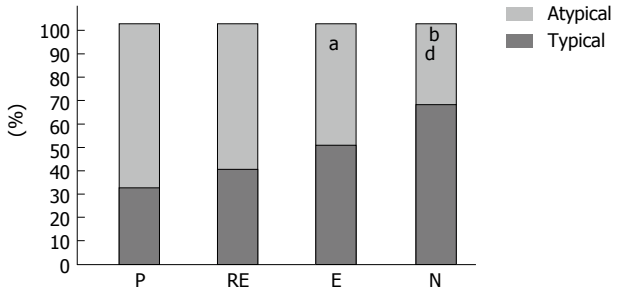

Figure 3 Percentage distribution of typical (dark grey) and atypical (light grey) symptoms in our four different groups of patients. From left to right, the bars indicate the group of patients *Helicobacter pylori (H. pylori)*-positive (P), eradicated less than 6 mo before pH monitoring (RE), eradicated more than 6 mo earlier (E), and *H. pylori*-negative (N). As shown, there is a progressive change in the percentage of symptoms related to the pattern of *H. pylori* infection. $^{d}P < 0.001$ between N and P; $^{b}P < 0.01$ between N and RE; $^{a}P < 0.05$ between E and P.

were different in comparison with *H. pylori*-negative patients ($P < 0.05$; OR = 2.36, 95%CI: 0.12-1.06). In patients with *H. pylori* eradication obtained more than 6 mo earlier, the clinical pattern was similar to the *H. pylori*-negative group ($P = 0.13$; OR = 0.89, 95%CI: 0.77-6.51) and their symptoms were mainly typical with a distribution significantly different as compared to the *H. pylori*-positive group ($P < 0.05$; OR = 3.71, 95%CI: 0.83-16.47) (Figure 3).

DISCUSSION

The results of our study indicate that the presence of *H. pylori* infection in patients affected by GERD is associated with a greater frequency of atypical extraesophageal manifestations. In addition, GERD patients without *H. pylori* infection are preferentially affected by typical heartburn and regurgitation.

The relationship between GERD and *H. pylori* infection has been widely analyzed over the years but the question is still controversial. There are data supporting a protective role of *H. pylori*[12,13] as a consequence of gastric atrophy and hypochlorhydria from parietal cells destruction due to chronic *H. pylori* infection[14]. In the meanwhile, mild antral gastritis could be associated with hyperchlorydria and more severe GERD by reduction in the number of somatostatin-secreting D-cells with loss of negative feedback on gastric acid secretion[15]. Discordant results are also referred to the need of *H. pylori* eradication in GERD patients. In fact, some evidence suggests that eradication of the infection may be a risk factor for *de-novo* endoscopic esophagitis[16], whereas other studies report a low risk of gastric atrophy in patients with successful *H. pylori* eradication and undergoing long-term acid suppression with PPI[17].

Our study tried to look at this relationship from another perspective. We analyzed data of patients with GERD to determine whether the status of *H. pylori* infection affects the clinical pattern of reflux disease, without considering whether *H. pylori* could determine or prevent GERD. We found that the presence of gastric infection seems to facilitate the insurgency of atypical extraesophageal manifestations of reflux. It is unlikely that this association arose by chance as clinical pattern in GERD patients shows a clear trend towards atypical manifestations, directly correlated to *H. pylori* status. In fact, the symptoms of *H. pylori*-positive patients were predominantly atypical, a condition confirmed in the cases with very recent eradication. On the other hand, clinical aspects of patients successfully treated for *H. pylori* infection long time earlier resulted similar to those of *H. pylori*-negative patients. This suggests that the presence of bacterial infection has an action on GERD clinical pattern which is progressively reduced by the time the infection is eradicated. The major criticism of our results is that other factors, *e.g.,* smoking, obesity and alimentary habits, could potentially affect the course of reflux disease and its clinical patterns[18]. However, these factors were unlikely to play a role in our population since we ruled out severe obese patients and recruited only slightly overweight patients, whose distribution was similar between subjects with typical and atypical symptoms. Furthermore, no correlation was found between smoking and clinical characteristics. Therefore, albeit considering the potential limit of a retrospective analysis, our data seem to indicate that life styles probably affect the characteristics of GERD less than *H. pylori* status.

In the present study, we did not identify the mechanisms through which *H. pylori* acts on the quality of reflux disease. Therefore, we can only make some speculations on the underlying conditions affecting different symptom patterns. First, the more severe degrees of esophagitis seem to be less correlated to *H. pylori* presence[19]; this could be one explanation for a major heartburn in our patients without infection. However, we can only partially confirm this assumption because in order to properly reduce the chance of confounding bias in our analysis, we excluded patients with Los Angeles grade C-D, known to have fewer reflux symptoms[20]. Second, the onset of symptoms (chronic cough, laryngitis or chest pain) seems strictly related to reflux episodes that extend proximally[21]. It could be that *H. pylori*-associated antral

gastritis increases the release of gastrin concomitantly with higher acidity and increased volume of refluxate that more easily reaches the proximal site of the esophagus. Our study did not directly analyze the extension of refluxate as a single-channel pH-metric probe was used; however, there is some evidence on a greater frequency of proximal reflux episodes in pediatric patients with *H. pylori* infection[22] in good accordance with our results. Another possible explanation of the relationship between *H. pylori* and clinical appearance of GERD may be found in the modulation of afferent neural signals by *H. pylori*[23] an effect that seems related to ghrelin, a peptide with intense prokinetic activity on LES region[24]. It is well known that *H. pylori*-positive patients show low levels of circulating ghrelin, which tend to increase after bacterial eradication[25]. It is also demonstrated that patients with *H. pylori* infection have a lower LES tone and a reduced esophageal body motility[26]; therefore, it seems likely that a low plasma ghrelin concentration limits the clearance of acid inside the esophagus, thus facilitating proximal extension of reflux episodes.

A final consideration is that our present findings were made on the Caucasian population so it is reasonably difficult to extrapolate data from our study to predict what might happen to patients from other countries. In fact, the literature shows that the relationship between GERD and *H. pylori* seems completely different in East Asia compared to Western countries[27,28]. It is also well known that genetic predisposition can alter acid secretion[29] as well as visceral sensitivity of esophageal wall to acid[30]. Therefore, the interaction between host genetic factors and other agents, such *H. pylori* itself, could lead to different expression of GERD, but this aspect needs to be further clarified.

In conclusion, our findings suggest a role for *H. pylori* infection in the clinical pattern of GERD. Further analyses are required to evaluate if our results represent a potentially new strategy to modulate symptoms occurrence or indicate a simple association with no roles in the pathogenesis of GERD. Since it is already known that *H. pylori* does not affect the pH profile of patients with GERD[31], it seems appropriate to say that the relationship between *H. pylori* and pH appears more complex than previously thought. In fact, *H. pylori* is likely to interact with GERD, but when these two entities coexist, *H. pylori* seems to change the way of GERD symptoms appear rather than promoting or facilitating it.

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COMMENTS

Background

Although gastro-esophageal reflux disease (GERD) and *Helicobacter pylori* (*H. pylori*) infection represent two of the most common diseases of upper gastrointestinal tract, the relationship between these two entities is still not completely elucidated. The majority of studies tended to investigate whether *H. pylori* could facilitate the onset of GERD or protect against the disease. Similarly, the eradication of *H. pylori* in GERD patients is questionable.

Research frontiers

The management of *H. pylori* infection is generally targeted to cure and prevent gastric and duodenal diseases. Diseases of the esophagus, reflux disease in particular, have been for decades considered to be of secondary importance in relation to *H. pylori* infection. In this study the presence of *H. pylori* is associated with a greater amount of atypical symptoms, whereas typical pattern is common in patients without the infection. Interestingly, the symptom pattern shows a trend from atypical to typical over time associated to *H. pylori* eradication, thus suggesting that the bacteria may have a role in the natural history of GERD.

Innovations and breakthroughs

To the authors' knowledge, this is the first study investigating the possible relationship between GERD and *H. pylori* from a different perspective, without considering the frequency of reflux disease after *H. pylori* eradication. In fact, the authors performed the study on a population of GERD patients for identifying two different symptom patterns that resulted related to *H. pylori* status.

Applications

This study could shed some light on why GERD patients refer different symptoms pattern. Hence there is a need for further investigation of *H. pylori* status not only in patients affected by gastroduodenal diseases, but also in patients with GERD.

Terminology

pH-metry or pH-monitoring is the gold standard in the diagnosis of gastroesophageal reflux disease. C_{13} urea breath test is the most accurate non-invasive test to diagnose *H. pylori* infection. Both tests could be affected by false negative results in case of concomitant use of antisecretory drugs.

Peer-review

This study is an important contribution to the literature regarding *H. pylori* and GERD, giving new information and possible explanations on the relationship between these two diseases.

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ORIGINAL ARTICLE

Observational Study

Prevalence of eosinophilic oesophagitis in adults presenting with oesophageal food bolus obstruction

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Abstract

AIM: To look at the relationship between eosinophilic oesophagitis (EO) and food bolus impaction in adults.

METHODS: We retrospectively analysed medical records of 100 consecutive patients who presented to our hospital with oesophageal food bolus obstruction (FBO) between 2012 and 2014. In this cohort, 96 were adults (64% male), and 4 paediatric patients were excluded from the analysis as our centre did not have paediatric gastroenterologists. Eighty-five adult patients underwent emergency gastroscopy. The food bolus was either advanced into the stomach using the push technique or retrieved using a standard retrieval net. Biopsies were obtained in 51 patients from the proximal and distal parts of the oesophagus at initial gastroscopy. All biopsy specimens were assessed and reviewed by dedicated gastrointestinal pathologists at the Department of Pathology, University Hospital Geelong. The diagnosis of EO was defined and established by the presence of the following histological features: (1) peak eosinophil counts > 20/hpf; (2) eosinophil microabscess; (3) superficial layering of eosinophils; (4) extracellular eosinophil granules; (5) basal cell hyperplasia; (6) dilated intercellular spaces; and (7) subepithelial or lamina propria fibrosis. The histology results of the biopsy specimens were accessed from the pathology database of the hospital and recorded for analysis.

RESULTS: Our cohort had a median age of 60. Seventeen/51 (33%) patients had evidence of EO on biopsy findings. The majority of patients with EO were male (71%). Classical endoscopic features of oesophageal rings, furrows or white plaques and exudates were

found in 59% of patients with EO. Previous episodes of FBO were present in 12/17 patients and 41% had a history of eczema, hay fever or asthma. Reflux oesophagitis and benign strictures were found in 20/34 patients who did not have biopsies.

CONCLUSION: EO is present in approximately one third of patients who are admitted with FBO. Biopsies should be performed routinely at index endoscopy in order to pursue this treatable cause of long term morbidity.

Key words: Oesophagitis; Eosinophilia; Food bolus obstruction; Endoscopy; Dysphagia

Core tip: Eosinophilic oesophagitis (EO) is a clinical entity that is becoming more frequent in patients seeking medical attention for food bolus obstruction (FBO). The main symptom in adult patients is dysphagia. Various studies have shown the presence of EO in 20% to 54% of the patients presenting with food bolus impaction. Approximately one in three patients who presents with FBO has EO. Biopsies should be performed routinely at index endoscopy in order to pursue this treatable cause of long term morbidity.

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INTRODUCTION

Eosinophilic oesophagitis (EO) is an emerging cause of food bolus impaction and dysphagia in adults and children[1]. According to Kerlin *et al*[2], acute food bolus obstruction (FBO) is frequently encountered as a gastrointestinal emergency. FBO is the third most common gastrointestinal emergency after gastrointestinal bleed in the upper and lower gastrointestinal tracts[2]. Approximately two thirds of FBO in adults of the Western world are caused by food bolus impaction with meat in comparison to fish bones in Asia[1]. The condition of the oesophagus and the type of food bolus play a role in food impaction. Risk factors for FBO include edentulous individuals and known oesophageal pathology amongst others[3]. With regards to the management of FBO, Ikenberry *et al*[4] recommended a time frame of 6 h to remove the food bolus due to the potential risk of ischaemia, necrosis and perforation.

There is a paucity of data on the epidemiological changes in FBO and its link to EO. According to a study, EO was found in 54% of patients presenting with oesophageal food bolus impaction[5]. The incidence of EO is rising as clinicians become more familiar with this condition. Currently, EO is a clinicopathologic entity defined by symptoms related to oesophageal dysfunction and eosinophil-predominant inflammation on oesophageal biopsies characterised by a peak value of ≥ 15 eosinophils per high power field[6,7].

MATERIALS AND METHODS

Our aim of this study was to evaluate the association of EO with FBO in adults. We retrospectively analysed medical records relating to 100 consecutive patients who presented to a tertiary hospital with oesophageal FBO between 2012 and 2014. There were a total of 96 adult patients and 4 paediatric patients (age < 16), whom were excluded from this study. Sixty-four percent of the adult patients were males. Out of this 96 patients, 11 of them required ear, nose and throat intervention or declined gastroscopy. These patients have also been excluded from this analysis. A total of 85 adult patients with FBO underwent gastroscopy and during this procedure, the food bolus was either advanced into the stomach using push technique or removed using retrieval net. Biopsies were obtained in 51 patients from proximal and distal parts of the esophagus.

Biopsies were analysed in the laboratory by experienced gastrointestinal pathologists and the cut off point of more than 20 eosinophils per high power field (hpf) was considered in cases of EO.

RESULTS

The median age of the cohort was 60. Out of the 51 patients who had biopsies, 19 (37%) patients had reflux oesophagitis confirmed histologically, which made up the majority of patients with FBO. This was followed by EO (33%), barrett's oesophagus (10%) and achalasia (2%). eighteen percent of these biopsies were attributed to other causes, which included benign changes and non-specific inflammation. The results are shown in Figure 1.

The majority of patients with EO were male (71%). Classical endoscopic features of oesophageal rings, furrows or white plaques and exudates were found in 59% of patients with EO. The median age for EO patients was 3O. Twelve out of the 17 patients (59%) had prior episodes of FBO and 41% had a history of atopy (hay fever, asthma and eczema).

Among the 34 patients who did not have biopsies, 20 of them had endoscopic changes consistent with gastro-oesophageal reflux disease or pre-existing benign strictures.

DISCUSSION

There is an increasing awareness towards EO. This condition refers to symptoms related to oesophageal dysfunction with evidence of eosinophil-predominant inflammation on oesophageal biopsies which is charac-

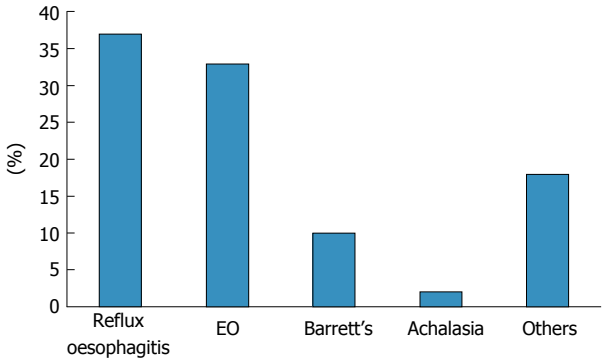

Figure 1 Aetiology of food bolus obstruction in the cohort. EO: Eosinophilic oesophagitis.

terised by a peak value of EO ≥ 15 eosinophils per high power field. The exclusion of secondary causes of oesophageal eosinophilia and the presence of mucosal eosinophilia being isolated to the oesophagus and persisting after a proton pump inhibitor trial are also included in the diagnostic criteria for EO[6]. Eosinophils are not normally present in the normal esophageal epithelia. The presence of eosinophilia indicates abnormality but unfortunately, this does not point towards EO but also other causes such as reflux esophagitis. EO usually affects young adults and in our study, the mean age of patients with EO was 3O. Our results have shown that one in three patients who presents with FBO has EO. Endoscopic evidence of EO was not present in all the patients which confirms findings from previous studies[8]. They demonstrated 10%-20% of EO patients can have an endoscopically normal appearing oesophagus. According to Kerlin *et al*[2], there is increased awareness of EO as a contributing factor to dysphagia and chest discomfort especially in young men. Our results are consistent with the Kerlin study which showed that up to one third of patients who presented with food bolus impaction have EO.

Besides being a retrospective study with its own intrinsic limitations, this is a small study with 100 patients' medical records analysed. Therefore, our analysis will have Type 1 error. Our laboratory uses the cut-off point of 20 eosinophils/hpf. According to the current guidelines, EO is defined histologically with eosinophils count of 15 or more eosinophils/hpf. The current recommendation is to take at least two to four biopsies from the distal and proximal oesophagus. We did not have a uniform protocol for the number of biopsies to be taken for each patient.

This study demonstrates that EO plays an essential role in food bolus impaction of the oesophagus. Patients who present with FBO have approximately a1 in 3 chance of having EO. This condition is not always associated with clear macroscopic changes or a history of atopy. In patients with no obvious cause for FBO, biopsies should be performed routinely at index endoscopy in order to pursue this treatable cause of long term morbidity.

COMMENTS

Background

Since the first description of eosinophilic oesophagitis (EO) in 1962 by Schreiber, there has been a growing recognition and understanding of this condition. Food bolus obstruction (FBO) in the esophagus is most commonly related to reflux-related oesophageal diseases and oesophageal dysmotility. EO has been reported as an uncommon cause of FBO. Epidemiological data on FBO and its relationship to EO remain limited.

Research frontiers

FBO in the oesophagus is becoming a common mode of presentation for EO. The authors recommend that biopsies should be performed at index endoscopy in order not to miss the diagnosis.

Innovations and breakthroughs

The authors' findings suggest that EO should be thought of in all cases of FBO and biopsies should be performed in all instances where there is no macroscopic abnormality on upper endoscopy. The results confirm the findings of a previous retrospective study done in Australia and highlight the need for large prospective studies to assess the demographics and aetiology of FBO.

Applications

The changes in the aetiology of FBO can be attributed to an increasing recognition and prevalence of EO especially in the younger population. Proximal and distal oesophageal biopsies should be attempted at index endoscopy in order to pursue this treatable cause of long term morbidity.

Terminology

The conceptual definition of EO is considered as a chronic, immune antigen-mediated oesophageal disease characterised clinically by symptoms related to oesophageal dysfunction and histologically by eosinophil-predominant inflammation.

Peer-review

This retrospective report focuses on patients presenting with food bolus impaction.

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CASE REPORT

Massive duodenal variceal bleed; complication of extra hepatic portal hypertension: Endoscopic management and literature review

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Abstract

Bleeding from duodenal varices is reported to be a catastrophic and often fatal event. Most of the cases in the literature involve patients with underlying cirrhosis. However, approximately one quarter of duodenal variceal bleeds is caused by extrahepatic portal hypertension and they represent a unique population given their lack of liver dysfunction. The authors present a case where a 61-year-old male with history of remote crush injury presented with bright red blood per rectum and was found to have bleeding from massive duodenal varices. Injection sclerotherapy with ethanolamine was performed and the patient experienced a favorable outcome with near resolution of his varices on endoscopic follow-up. The authors conclude that sclerotherapy is a reasonable first line therapy and review the literature surrounding the treatment of duodenal varices secondary to extrahepatic portal hypertension.

Key words: Extrahepatic portal hypertension; Duodenal varices; Sclerotherapy

Core tip: Bleeding from duodenal varices is a gastrointestinal emergency and focused patient history may help the clinician to suspect this life threatening diagnosis. Clinician's need to have a high degree of suspicion for bleeding varices even in the absence of known cirrhosis if certain clinical characteristics are present, such as history of crush injury. If duodenal varices are diagnosed on endoscopy, endoscopic injection sclerotherapy can be a highly successful definitive intervention. The authors suggest ethanolamine injection sclerotherapy, though multiple alternative sclerosants are also established in the literature as are other therapeutic alternatives including

endoscopic band ligation. With prompt endoscopic management, life threatening bleeding can be effectively mitigated and with the expectation of excellent long term outcomes.

Steevens C, Abdalla M, Kothari TH, Kaul V, Kothari S. Massive duodenal variceal bleed; complication of extra hepatic portal hypertension: Endoscopic management and literature review. *World J Gastrointest Pharmacol Ther* 2015; 6(4): 248-252 Available from: URL: http://www.wjgnet.com/2150-5349/full/v6/i4/248.htm DOI: http://dx.doi.org/10.4292/wjgpt.v6.i4.248

INTRODUCTION

Bleeding from duodenal varices is a rare but potentially fatal condition. A gastroenterologist will encounter these only rarely as they represent only about 0.4% of all variceal bleeding[1], yet the traditionally reported mortality approaches 40%[2], underscoring the importance of prompt and effective management. While there have been hundreds of cases reported, most describe bleeding varices secondary to cirrhosis or intrahepatic portal hypertension. This gap in the literature largely excludes the 25% of duodenal variceal bleeds which are caused by extrahepatic portal hypertension[3]. The varices that result from localized vascular hypertension may respond differently to endoscopic interventions and ultimately experience different outcomes given their normal central portal pressures and lack of underlying liver dysfunction. Proven endoscopic interventions include endoscopic band ligation (EVL) or endoscopic injection sclerotherapy (EIS). We present a case of profound gastrointestinal (GI) bleeding from duodenal varices in a patient with mesenteric vein obstruction, which was successfully managed with a single episode of EIS with ethanolamine, and review the present literature in an attempt to clarify the effectiveness of available endoscopic interventions for extrahepatic portal hypertension.

CASE REPORT

A 61-year-old male was referred for recurrent GI bleeding. The presentation to an outside hospital one day prior had been notable for sudden onset of melena and bright red blood per rectum (BRBPR). His medical history included coronary artery disease; gastroesophageal reflux disease and a remote crush injury with partial small bowel resection. There was no previous GI bleeding, liver disease, alcohol intake, or non-steroidal anti-inflammatory drug use and his medications were significant only for aspirin 81 mg daily. Initially, he had tachycardia to 123 beats per minute with a benign abdominal examination and gross blood on rectal exam. Hematocrit was 35% with platelet count of 99000/uL, and international normalized ratio 1.1. He underwent esophagogastroduodenoscopy (EGD), which revealed extensive duodenal varices. Follow-up angiography of the portal system had unremarkable arterial vasculature, but abnormal venous anatomy significant for occlusion of the superior mesenteric vein with extensive collateral formation.

Due to the complexity and risk of definitive intervention, the patient was transferred to a tertiary care center. At the time of transfer, he had received 7 units of packed red blood cells and was being medically treated with pantoprazole and octreotide drips. Intensive care unit (ICU) admission was required; at that time he remained tachycardic with continued BRBPR and his hematocrit had fallen to 22%. Repeat EGD visualized a deformed duodenum with large varices present in the second and third portion with stigmata of recent bleeding (Figure 1). The decision to intervene with injection sclerotherapy was made and 4 mL of 5% ethanolamine was injected with satisfactory hemostasis. There were no episodes of re-bleeding and he was discharged 5 d later. Follow-up EGD at one month showed near complete resolution of the duodenal varices, which was persistent at his follow up EGD 20 mo after the initial presentation (Figure 2).

DISCUSSION

Duodenal varices in extrahepatic portal hypertension are likely much more common than currently recognized considering that only about 65% of patients with the condition will present with GI bleeding[3]. In the absence of cirrhosis or active hemorrhage, patients with asymptomatic duodenal varices may never receive portal system imaging or upper endoscopy. Recognized etiologies of extrahepatic portal hypertension leading to duodenal varices include pancreatitis, omphalophlebitis, intra-abdominal tumors, previous surgery, retroperitoneal fibrosis and portal/splenic vein thrombosis among others[3]. Our case is the second noting the association of remote crush injury with a late presenting duodenal variceal bleed[4]. The vessels injured by this type of trauma may be especially prone to stenosis or obstruction during the healing process. Regardless of the inciting injury, the varices result from collateral venous vessel formation around the obstruction in an attempt to maintain hepatopedal blood flow[5]. Bleeding from this anomalous vasculature represents a GI emergency.

The acute bleeding episode from duodenal varices is massive; an average of 10.4 units of packed red cells is transfused during these bleeds[6]. Prompt endoscopic diagnosis and management is crucial to achieving a successful outcome. Deformity of the duodenum with radiography or endoscopy can provide clues to the presence of duodenal varices, thus prompting the endoscopist to carefully scrutinize this location. The duodenal bulb is the most common location for them to occur although varices can be present in any portion[7]. The location of the varices within the duodenum could depend on the inciting injury. Both our case and a

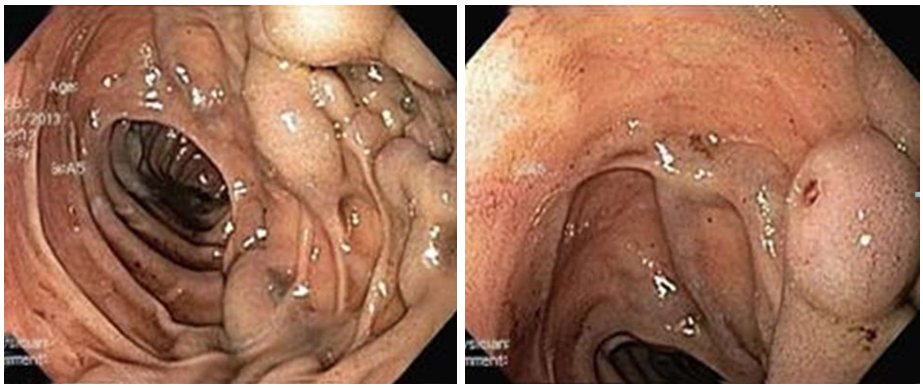

Figure 1 Isolated varices in the second portion of the duodenum with red wale sign.

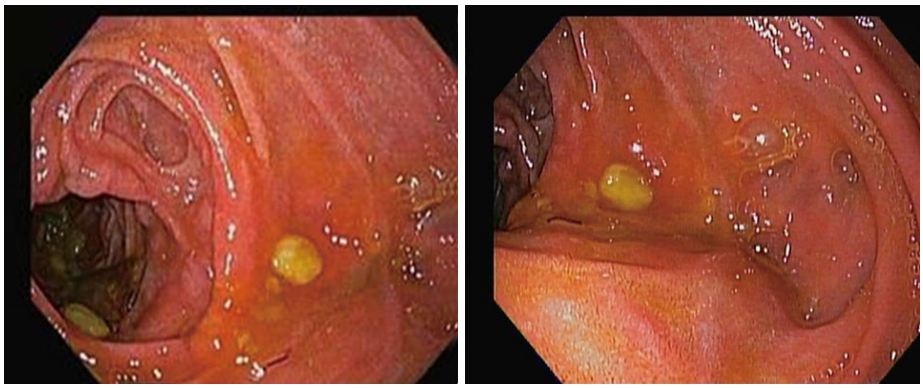

Figure 2 Follow up endoscopy at 20 mo post initial endotherapy showing near complete resolution of the duodenal varices.

previous case in the literature note bleeding from the second portion of the duodenum in a patient with history of crush injury[4]. Once the diagnosis is made and the patient is sufficiently stabilized, a number of interventional options exist.

Endoscopic therapy for bleeding duodenal varices emerged with sclerotherapy in the early 1980's[8] and EVL in the mid 1990's[9]. Since then, there have been only a handful of endoscopically managed duodenal variceal bleeds caused by extrahepatic portal hypertension (Table 1). These cases demonstrate a wide variety of etiologies, but vascular abnormalities underlie most of them. Duodenal varices in general have unique anatomy, which must be taken into account when choosing endoscopic therapy. The varix consists of a single afferent vessel, arising from either the superior or inferior pacreaticoduodenal vein, and a single efferent vessel, which drains into the inferior vena cava. Though visible, the varix is typically buried deep into the submucosa[1]. This anatomy may render the varices difficult to fully eradicate and explain some of the late re-bleeding noted in Table 1.

Our case was successfully managed with ethanolamine injection sclerotherapy and the patient did not experience re-bleeding. To our knowledge, this is only the third reported case of ethanolamine sclerotherapy for bleeding duodenal varices caused by extrahepatic portal hypertension.

There are potential concerns and complications to consider when choosing ethanolamine sclerotherapy in the duodenum. The thin duodenal wall and its risk of perforation are cited as potential complications. Indeed, there are case reports of duodenal perforation after multiple episodes sclerotherapy for a duodenal ulcer[17], but no similar reports exist for the use of sclerotherapy for duodenal varices. Another sclerotherapy option, cyanoacrylate, was carefully considered for treatment of these bleeding duodenal varices. Cyanoacrylate has been very effective for the treatment of gastric varices and a case series of bleeding duodenal varices caused by intrahepatic portal hypertension noted success in 4 out of 4 patients[18]. This agent has its own set of risks associated with its use, most notably being distant embolization during treatment of the duodenal varix[19]; for this reason, we typically invoke an informed consent discussion prior to cyanoacrylate injection which was not possible in this particular case. Outside of sclerotherapy, endoscopic band ligation is the other major endoscopic intervention used for the treatment of bleeding duodenal varices. Significant theoretical risks are also associated with EVL. In porcine models, duodenal banding demonstrated a 100% perforation rate[20]. Additionally, it is suggested that if the entire varix cannot be banded at the time of intervention, there is the risk of creating a wide defect with re-bleeding after sloughing occurs[21]. To mitigate these risks, some authors have previously recommended additional procedures such as balloon

Table 1 Summary of the reported cases of endoscopically managed duodenal variceal bleeds caused by extrahepatic portal hypertension

Ref.	Etiology/clinical history	Intervention	Outcome	Additional
Bosch *et al*[10]	Mesenteric vein thrombosis	EVL	Stable (11 mo)	
Goetz *et al*[11]	Post-trauma splenectomy	EVL	Stable (4 mo)	
Gunnerson *et al*[4]	Crush injury	EVL	Stable (2 yr)	
Gunnerson *et al*[4]	Anomalous venous vasculature	EVL	Re-bleed (8 mo)	EIS; (Sod mon)
Cottam *et al*[12]	Multiple surgical procedures	EIS; (Epi)	Re-bleed (wk)	Surgery
Osaka *et al*[13]	Vascular malformation	EIS; (Eth)	Re-bleed (unknown)	Surgery
Tsuji *et al*[14]	Motor vehicle accident	EIS; (Polid, Throm)	Stable (unknown)	Surgery
Sans *et al*[15]	Caroli's Dz, SMV thrombosis	EIS; (Thromb, Eth)	Stable (5 mo)	
Kao *et al*[16]	Pancreatitis; portal vein stenosis	EIS; (Cyano, Lip)	Stable (2 mo)	

Epi: Epinephrine; Eth: Ethanolamine; Throm: Thrombin; Cyano: Cyanoacrylate; Lip: Lipiodiol; Polid: Polidocanol; Sod mon: Sodium morrhuate; EVL: Endoscopic interventions include endoscopic band ligation; EIS: Endoscopic injection sclerotherapy.

occluded retrograde transvenous obliteration following EVL[9]. The cases reported in the current literature do not support these fears; from the few case reports available, EVL appears to be safe and effective with only one episode of late re-bleeding at 8 mo[4]. The ultimate choice of endoscopic intervention will depend on the clinical scenario, endoscopist expertise and local practice patterns.

Our patient's presentation was typical for duodenal variceal bleeding. He had a remote history of intra-abdominal injury and presented with a hemodynamically unstable GI bleed requiring ICU care. In the acute presentation, we contend that proceeding with a sclerosant such as ethanolamine is a reasonable and safe first line approach given its limited risk profile and proven success. With prompt stabilization and intervention, he experienced a good outcome, which appears typical for duodenal variceal bleeds secondary to extrahepatic portal hypertension. Given the resolution of his varices on repeat endoscopy, his prognosis is likely excellent. This case demonstrates that duodenal variceal bleeds caused by extrahepatic portal hypertension have unique clinical history and physiology. They respond well to intervention and good outcomes should be expected with prompt and effective management.

COMMENTS

Case characteristics

A 61-year-old man presented with melena and bright red blood per rectum.

Clinical diagnosis

On examination, he was noted to be tachycardic but with benign abdominal examination and gross blood on digital rectal examination.

Differential diagnosis

With the reported melena and hemodynamic instability, massive upper gastrointestinal bleeding was suspected and the differential diagnosis included peptic ulcer disease, esophageal/gastric varices or dieulafoy lesion.

Laboratory diagnosis

He was noted to have low Hematocrit of 35% which fell to a low of 22% with mild thrombocytopenia (platelet count 99000/μL), and normal international normalized ratio is 1.1.

Imaging diagnosis

Esophagogastroduodenoscopy revealed extensive duodenal varices and follow-up angiography of the portal system visualized abnormal venous anatomy significant for occlusion of the superior mesenteric vein with extensive collateral formation.

Treatment

The patient was stabilized with seven packed red blood cell transfusions, with initial medical therapy of pantoprazole and octreotide infusions; definite therapy was endoscopic sclerotherapy with injection of 4 mL of 5% ethanolamine into the varices with resolution of active bleeding.

Related reports

The patient had a history of remote crush abdominal injury and small bowel resection which may have resulted in his anomalous vasculature, thus predisposing him to extrahepatic portal hypertension and the resultant isolated duodenal varices.

Term explanation

Omphalophlebitis is inflammation of the umbilical vein.

Experiences and lessons

A patient medical history indicative of prior intraabdominal pathology such as crush injury should raise suspicion that duodenal varices could be present which allows the endoscopist to adequately prepare for appropriate therapeutic intervention; in this case sclerotherapy was an effective and long lasting definitive treatment.

Peer-review

This paper addresses an important and poor-investigated area dealing with the endoscopic treatment of bleeding from duodenal varices and provides a good review of the literature of the different endoscopic approaches in intrahepatic and extrahepatic portal hypertension causing massive duodenal variceal bleeding. The authors provide a very detailed report of their experience in treating patients affected by this clinical condition and discuss the endoscopic approach and the clinical outcome.

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CASE REPORT

Preoperative detection of intrahepatic venovenous shunt treated by microwave precoagulation during right hepatectomy

Jean-Baptiste Delhorme, Riccardo Méméo, Jacques Marescaux, Patrick Pessaux

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Abstract

A 53-year-old woman underwent a 2-stage right hepatectomy for bilobar metastasis of an ileal neuroendocrine carcinoma. Preoperative three-dimensional computed tomography reconstruction helped to diagnose an intrahepatic venovenous shunts from the right and middle hepatic veins to the left hepatic vein, which could cause a intraoperative bleeding. Hemostasis was performed by means of precoagulation with microwave-assisted coagulation.

Key words: Three dimensional modeling; Microwave; Hepatectomy; Precoagulation; Bleeding; Venovenous shunt

Core tip: Detection of anomalies in hepatic vascularization before liver surgery is crucial in order to prevent intraoperative difficulties such as massive bleeding, which needs to be controlled. Surgical planning is now well-known as a major step in liver surgery. From a general standpoint, three-dimensional computed tomography (3D-CT) is not used to this purpose but can be very useful to identify vascular structures. We report a case where intrahepatic venovenous shunts were preoperatively diagnosed by means of 3D-CT reconstruction and were managed with precoagulation microwave-assisted coagulation.

Delhorme JB, Méméo R, Marescaux J, Pessaux P. Preoperative

detection of intrahepatic venovenous shunt treated by microwave precoagulation during right hepatectomy. *World J Gastrointest Pharmacol Ther* 2015; 6(4): 253-256 Available from: URL: http://www.wjgnet.com/2150-5349/full/v6/i4/253.htm DOI: http://dx.doi.org/10.4292/wjgpt.v6.i4.253

INTRODUCTION

Detection of anomalies in hepatic vascularization before liver surgery is crucial in order to prevent intraoperative difficulties such as massive bleeding, which needs to be controlled. Surgical planning is now well-known as a major step in liver surgery[1], and the absence of agreement between a preoperative planning and the realized operating techniques has been shown as risk factor of morbidity. From a general standpoint, three-dimensional computed tomography (3D-CT) is not used to this purpose but can be very useful to identify vascular structures. As blood loss represents a major complication prognostic factor in liver surgery, several bloodless techniques are available. In case of persistent bleeding, tissue precoagulation with microwave energy can be a very attractive tool to achieve tissue hemostasis.

We report a case where intrahepatic venovenous shunts were preoperatively diagnosed by means of 3D-CT reconstruction and were managed with precoagulation microwave-assisted coagulation.

CASE REPORT

Lower limb edema made it possible to detect a voluminous intrahepatic lesion compressing the retrohepatic vena cava in a 53-year-old woman. This lesion located in segments Ⅳ and Ⅶ of the liver measured 16 by 13 by 15 cm. The patient's past medical history included thyroidectomy, appendectomy, arterial hypertension and hypercholesterolemia. Percutaneous liver biopsy confirmed the diagnosis of liver metastasis of a grade 2 neuroendocrine tumor (Ki-67 index = 5%). Preoperative examination including CT-scan, MRI, and a somatostatin receptor scintigraphy showed a primitive tumor at the last ileal loop with bilobar synchronous liver metastasis (voluminous lesion in the right liver and another one in liver segment Ⅱ). After discussion in our oncological multidisciplinary committee, a 2-stage surgical strategy was put forward.

The first step consisted in a right colectomy with radiofrequency ablation of the liver metastasis in liver segment Ⅱ. The postoperative outcome was uneventful. Histopathological examination confirmed an ileal grade 2 well-differentiated neuroendocrine carcinoma, classified pT3N1 (11 lymph nodes involved out of 21 retrieved) M1 according to the 7th edition of the AJCC/UICC system TNM classification (Ki-67 index = 5% and the mitotic index was less than 1 mitosis/10HPF).

One month later, a percutaneous right portal embolization was performed in order to induce left liver hypertrophy. Six weeks later, the future remnant liver (segments Ⅰ, Ⅱ, Ⅲ and Ⅳb) represented 29.5% of the total liver (*vs* 22.5% prior to embolization). The 3D virtual anatomical model was obtained from thoraco-abdominal CT-scanning using a customary software [VR-RENDER®, IRCAD (Institut de Recherche contre les Cancers de l'Appareil Digestifs)]. The 3D modeling confirmed the compression of the right and middle supra-hepatic veins as well as the presence of intrahepatic venovenous shunts from the right and middle hepatic veins to the left hepatic vein crossing through segments Ⅳ and Ⅴ of the liver (Figure 1). The model was then processed using a VR-RENDER® plug-in application, the Virtual Surgical Planning (VSP®, IRCAD), in order to delineate surgical resection planes, including elective ligature of vascular structures and precoagulation of venovenous shunts. We discussed the possibility of a preoperative endovascular embolization of the shunt, but it was retained due to the risks of the procedure.

A right hepatectomy was initiated with an intraoperative ultrasound examination, which corroborated the presence of venovenous shunts. The right branch of the portal vein and the hepatic artery were dissected, clamped, and then ligatured. As anticipated, parenchymal transection using ultrasonic tissue ablation system (CUSA®) induced bleeding, caused by the difficulty to control the venovenous shunts. We decided to completely isolate the liver from blood flow by clamping hepatoduodenal ligament, infrahepatic vena cava, and suprahepatic cava. Despite this total liver vascular exclusion, bleeding was still difficult to control. Consequently, as preoperatively planned, it was decided to perform a precoagulation of the multiple shunts by means of a microwave-assisted coagulation. We used a 2 cm microwave needle applied onto the shunts crossing through liver segment Ⅳ transection line (Figure 2). This maneuver allowed to control bleeding and right hepatectomy was then completed in adequate conditions (Figure 3). The postoperative outcome was uneventful and the patient was discharged on postoperative day 12.

Histopathological examination confirmed the completeness of resection of two right liver metastases from a G2 well-differentiated neuroendocrine carcinoma with Ki-67 index comprised between 5% and 10% and less than 1 mitosis/10HPF. The multidisciplinary committee opted for an oncological follow-up. At one year, the patient was disease-free.

DISCUSSION

Preoperative evaluation of hepatic vascularization can be very useful in order to anticipate potential intraoperative difficulties, in particular when hepatic vein resection is required. Several detection techniques have been described in the literature in order to analyze intrahepatic venovenous shunts. Sakaguchi *et al*[2] showed that hepatic occlusion venography allowed for the detection of venovenous shunt in half of patients while it was unpredictable from CT-scan or laboratory data. The main drawback of this technique lies in its invasiveness.

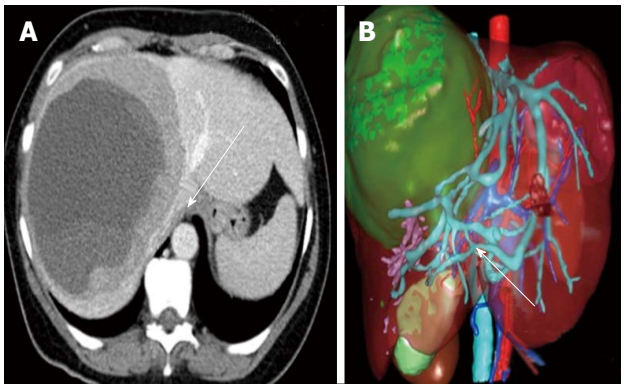

Figure 1 Preoperative three-dimensional computed tomography reconstructed from conventional two-dimensional computed tomography; the tumor is represented in green. A: Compression of the right and middle hepatic veins (arrow); B: Venous intrahepatic shunt (arrow).

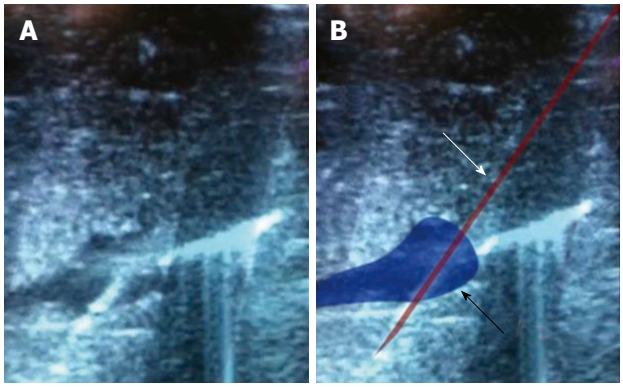

Figure 2 Intrahepatic venovenous shunt and insertion of the microwave needle. A: Ultrasound view of the intrahepatic venovenous shunt with insertion of the microwave needle; B: Diagram showing in blue the intrahepatic venovenous shunt (black arrow) and in red the microwave needle (white arrow).

Intrahepatic venous anastomosis has also been evaluated in living donor liver transplantation with Doppler ultrasonography and pulse-inversion ultrasonography by Kaneko *et al*[3]. This procedure seems to be efficient for detection of venovenous shunts between the middle and the right hepatic vein but it is operator-dependent and not often available. Detection of venovenous shunts is also efficient with 3D-venography reconstructed from multidetector-row computed tomography during angiography[4]. Three-dimensional computed tomography, as shown in our case report, appears to be an appropriate tool to evaluate hepatic vascularization prior to surgery. As demonstrated by Kamiyama *et al*[5] in a cohort, 3D-CT provided key information that could not be obtained with two-dimensional computed tomography (2D-CT).

From CT or MRI images, it's often difficult for the surgeon to plan precisely the technical procedure. These imaging exams provide images in 2D, but surgeons work in 3D. It seems fundamental to offer surgeons patient-specific 3D model of the liver for easy interpretation of the anatomy. Volume rendering available on all radiology consoles does not make independent each organ, and therefore does not allow performing a virtual surgery. To overcome this limitation, we created a software allowing the identification and delimitation of each anatomical and pathological structure with different colours (Figure 1). This 3D model is optimal to obtain preoperative surgical planning and intraoperative guidance. In our case report, the venovenous shunt was known before surgery and difficulties to perform hemostasis were anticipated.

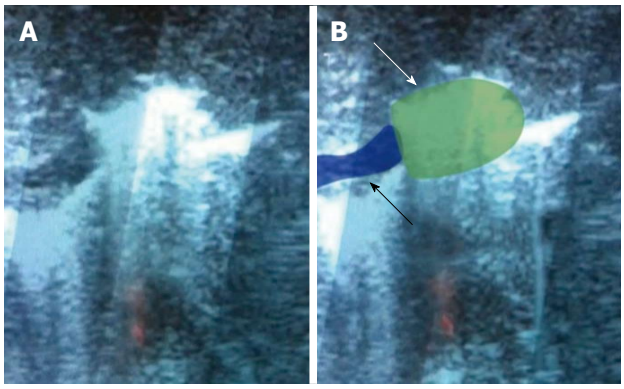

Figure 3 Thrombus at the end of the procedure. A: Ultrasound view of the thrombus at the end of the procedure; B: Diagram showing in blue the intrahepatic venovenous shunt (black arrow) and in green the thrombus (white arrow).

In our case, microwave was successfully used not to obtain tissue coagulation but to achieve elective precoagulation of the shunt. The needle was electively and precisely inserted with ultrasound control into the shunt to obtain a thrombus. Microwave coagulation is also less time-consuming as compared to radiofrequency coagulation. In case of difficulty to control bleeding during hepatectomy and to limit blood loss, especially in cirrhotic liver, it might be better to perform tissue precoagulation using microwave coagulation.

In conclusion, the presentation of this case report underscores two major key topics concerning liver surgery. First and foremost, a thorough knowledge of liver anatomy preoperatively is essential as it gives the surgeon the opportunity to anticipate hemostatic difficulties in liver surgery where blood loss is a major complication and prognostic factor. This can be easily evaluated by means of preoperative 3D-CT. Secondly, tissue precoagulation using radiofrequency or microwave coagulation should remain available for the surgeon as they can be important tools to control persistent bleeding in spite of conventional bloodless hepatectomy techniques.

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COMMENTS

Case characteristics

A 53-year-old woman underwent a 2-stage right hepatectomy for bilobar metastasis of an ileal neuroendocrine carcinoma.

Clinical diagnosis

Lower limb edema made it possible to detect a voluminous intrahepatic lesion compressing the retrohepatic vena cava.

Differential diagnosis

Malignant tumors (hepatocarcinoma or other liver metastases) and benign lesions (focal nodular hyperplasia, hemangioma and adenoma).

Imaging diagnosis

Preoperative examination including computed tomography (CT)-scan, magnetic resonance imaging, and a somatostatin receptor scintigraphy showed a primitive tumor at the last ileal loop with bilobar synchronous liver metastasis. The 3D virtual anatomical model was obtained from thoraco-abdominal CT-scanning using a customary software (VR-RENDER®, IRCAD).

Pathological diagnosis

Percutaneous liver biopsy confirmed the diagnosis of liver metastasis of a grade 2 neuroendocrine tumor (Ki-67 index = 5%).

Treatment

Right hepatectomy with precoagulation and microwave.

Related reports

Few papers report the preoperative detection and intraoperative management of intrahepatic veno-venous shunt.

Term explanation

The precoagulation of the shunt avoided major bleeding during the parenchymal transection.

Experiences and lessons

First and foremost, a thorough knowledge of liver anatomy preoperatively is essential as it gives the surgeon the opportunity to anticipate hemostatic difficulties in liver surgery where blood loss is a major complication and prognostic factor. This can be easily evaluated by means of preoperative 3D-CT. Secondly, tissue precoagulation using radiofrequency or microwave coagulation should remain available for the surgeon as they can be important tools to control persistent bleeding in spite of conventional bloodless hepatectomy techniques.

Peer-review

This video clip and the case report are well presented.

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