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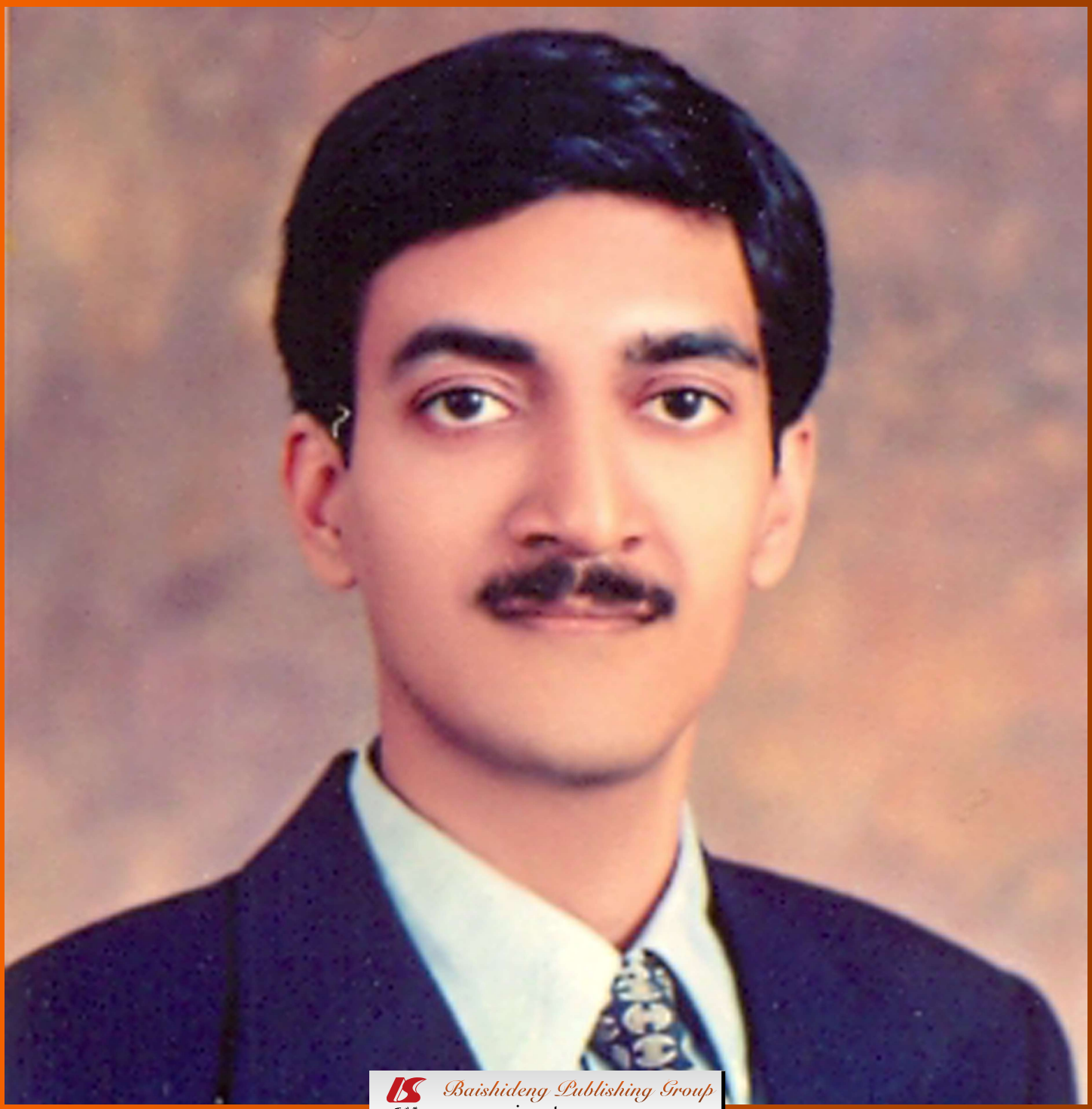

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EDITORIAL

Barriers to colorectal cancer screening in the developing world: The view from Pakistan

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Abstract

Colorectal cancer screening has become a defining concern of current gastroenterological practice in many Western nations. This same focus does not exist in many developing countries, including Pakistan. There is a need to develop a model for the developing world. Here are several areas that need to be pursued: (1) epidemiological research; (2) physician and public education; (3) training of gastroenterologists, especially female ones; (4) less expensive and more culturally acceptable screening options (fecal occult blood testing); and (5) cost-effectiveness analyses. Gastroenterologists in developing countries need to step up to educate people and promote, where possible and in keeping with local conditions, the prevention and early diagnosis of colorectal cancer.

Key words: Colon cancer; Cancer screening; Pakistan; Cancer

Core tip: Gastroenterologists in developing countries need to step up to educate people and promote, where possible and in keeping with local conditions, the prevention and early diagnosis of colorectal cancer.

Ahmed F. Barriers to colorectal cancer screening in the developing world: The view from Pakistan. *World J Gastrointest Pharmacol Ther* 2013; 4(4): 83-85 Available from: URL: http://www.wjgnet.com/2150-5349/full/v4/i4/83.htm DOI: http://dx.doi.org/10.4292/wjgpt.v4.i4.83

INTRODUCTION

Colorectal cancer screening has become a defining concern of current gastroenterological practice in many Western nations. This same focus does not exist in many developing countries, including Pakistan.

LACK OF EPIDEMIOLOGICAL DATA

A basic prerequisite for any screening program is knowledge of the incidence and prevalence of the disease in question. In the absence of this information any screening process is unjustifiable. Until as recently as last year, there was no useful incidence data on colorectal cancer in Pakistan. Recently a study has shown that Pakistan falls into a low incidence region/category for colorectal cancer[1]. The crude incidence rate is 3.2% in both males and females. Most significantly, however, the incidence appears to be rising, particularly in males. This study also suggested that given an aging population, a strong tradition of consanguineous marriages, and a high prevalence of colorectal cancer risk factors, including a trend towards a more "westernized" dietary intake, this low incidence may, in fact, be an artifact. This data may also be an underestimation of colorectal cancer in Pakistan because the registry is voluntary and some cases may have gone unreported.

FINANCIAL LIMITATIONS

Implementation of Western models of large scale colonoscopic screening programs would place an insurmountable burden on already struggling health care systems in many developing countries. As a reflection of the state of healthcare in Pakistan, data on life expectancy

Table 1 Life expectancy, healthcare expenditure per capita in Pakistan compared with other countries

Country	Life expectancy (M/F, yr)	Expenditure on health per capita ($, 2011)
Pakistan	66/68	69
India	64/67	141
Bangladesh	69/70	67
Sri Lanka	71/78	191
Singapore	80/85	2787
United Kingdom	79/82	3322
United States	76/81	8608

Available from: URL: http://www.who.int/countries/, accessed on May 16, 2013.

and healthcare expenditure per capita are given in Table 1. In Pakistan there is no health insurance system and the burden of any investigation rests solely with the patient. Given that the average annual income in Pakistan is $650, the cost of different screening options is of paramount consideration. A colonoscopy costs $100 here and fecal occult blood testing costs $1.30. Regardless, it is still cheaper to diagnose colorectal cancer early than treat advanced malignancies.

LACK OF RESOURCES

Even if money to support a large scale colorectal cancer screening process were to be suddenly available, many trained gastroenterologists would be required which are already in short supply in many developing countries. In Pakistan, a country of 180 million people, there are limited number of gastroenterologists and endoscopy units and these are mostly concentrated in urban areas leaving the majority of the population without any access to gastroenterologic facilities.

LACK OF PHYSICIAN AND PUBLIC AWARENESS

There is a great lack of awareness about many malignancies, including colorectal cancer, in Pakistan. Even amongst physicians, there is a lack of awareness about the symptoms of colorectal cancer. For example, many physicians do not know that the presence of blood in the stool, especially in someone older than 50, needs to be investigated further and can't simply be attributed to hemorrhoids and ignored. Risk factors for colorectal cancer need to be highlighted, in particular the genetic aspects of colorectal cancer risk. First degree relatives of patients with colon cancer are rarely told that they are at increased risk for developing this malignancy and, therefore, need to be screened appropriately. Beyond this, the concept of screening asymptomatic persons, at average risk for colorectal cancer needs to be introduced and promoted here.

CULTURAL BARRIERS

There are many cultural barriers that exist in Pakistan and would impede the implementation of a colon cancer screening program. Patients are wary of talking about even the possibility of cancer, there is widespread fear of endoscopic procedures due to concerns about potential complications and rumors of excruciating procedure-induced pain, and there is a widespread misconception that biopsying a malignant lesion invariably leads to spread of cancer. Finally, with Pakistan being a conservative Muslim country, female patients here are reluctant to have colonoscopy exams performed by male doctors and in this country of 180 million people, there are only a handful of female gastroenterologists.

The Asia Pacific consensus recommendations for colorectal cancer have focused primarily on data from East and Southeast Asia and have overlooked the Indian Subcontinent (Pakistan, India, Bangladesh) which together comprise more than one billion people[2]. A prospective multinational colonoscopy screening study found that the prevalence of advanced colorectal neoplasms in asymptomatic Asians is comparable to that in the West[3]. Again, the Indian Subcontinent was under-represented. Finally, cost-effective analyses conducted in other parts of the world are not necessarily directly applicable to our setting.

For all the reasons mentioned above, the implementation of more well-established cancer screening protocols (for breast cancer, cervical cancer, prostate cancer) have also not yet occurred in Pakistan. Is colon cancer screening a luxury of developed nations, unaffordable in the developing world? There are possible solutions to these obstacles. There is a need to develop a model for the developing world. Here are several areas that need to be pursued: (1) epidemiological research; (2) physician and public education; (3) training of gastroenterologists, especially female ones; (4) less expensive and more culturally acceptable screening options (fecal occult blood testing); and (5) cost-effectiveness analyses.

In a country beset by terrorism, militancy, and political uncertainty, it is easy to lose sight of issues relating to cancer screening. The initiation and implementation of any large-scale cancer screening program requires careful thought. Before starting a colon cancer screening program in Pakistan, efforts must be made to increase physician and public awareness regarding colon cancer, in particular, and the philosophy behind cancer screening, in general. Gastroenterologists in Pakistan and other developing countries need to step up to educate people and promote, where possible and in keeping with local conditions, the prevention and early diagnosis of colorectal cancer.

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REVIEW

Eosinophils and mast cells as therapeutic targets in pediatric functional dyspepsia

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Abstract

There is an increasing appreciation for the importance of inflammation as a pathophysiologic entity that contributes to functional gastrointestinal disorders including functional dyspepsia (FD). Importantly, inflammation may serve as a mediator between psychologic and physiologic functions. This manuscript reviews the literature implicating two inflammatory cell types, mast cells and eosinophils, in the generation of dyspeptic symptoms and explores their potential as targets for the treatment of FD. There are a number of inciting events which may initiate an inflammatory response, and the subsequent recruitment and activation of mast cells and eosinophils. These include internal triggers such as stress and anxiety, as well as external triggers such as microbes and allergens. Previous studies suggest that there may be efficacy in utilizing medications directed at mast cells and eosinophils. Evidence exists to suggest that combining "anti-inflammatory" medications with other treatments targeting stress can improve the rate of symptom resolution in pediatric FD.

Key words: Eosinophils; Mast cells; Functional dyspepsia; Abdominal pain; Stress

Core tip: Current evidence implicates gastric mast cells and duodenal eosinophils in the pathophysiology of functional dyspepsia and as mediators between psychologic and physiologic factors. Increased antral mast cell density is associated with anxiety, electromechanical dysfunction, and the postprandial distress syndrome (PDS) subtype of functional dyspepsia. Likewise, increased duodenal eosinophil density is associated with anxiety and the PDS subtype, however, effects on electromechanical function are more indirect. More importantly, mast cells and eosinophils appear to be therapeutic targets offering newer options for treating functional dyspepsia.

Friesen CA, Schurman JV, Colombo JM, Abdel-Rahman SM. Eosinophils and mast cells as therapeutic targets in pediatric functional dyspepsia. *World J Gastrointest Pharmacol Ther* 2013; 4(4): 86-96 Available from: URL: http://www.wjgnet.com/2150-5349/full/v4/i4/86.htm DOI: http://dx.doi.org/10.4292/wjgpt.v4.i4.86

INTRODUCTION

A majority of children with chronic abdominal pain presenting to pediatric gastroenterology practices fulfill criteria for a functional gastrointestinal disorder (FGID), with the two most common being functional dyspepsia (FD) and irritable bowel syndrome (IBS)[1-4]. Prevalence estimates for FD are 3.5%-27.0% in children/adolescents and 20%-30% in adults, highlighting the pervasive nature of this disorder[5,6]. FD is defined as persistent or recur-

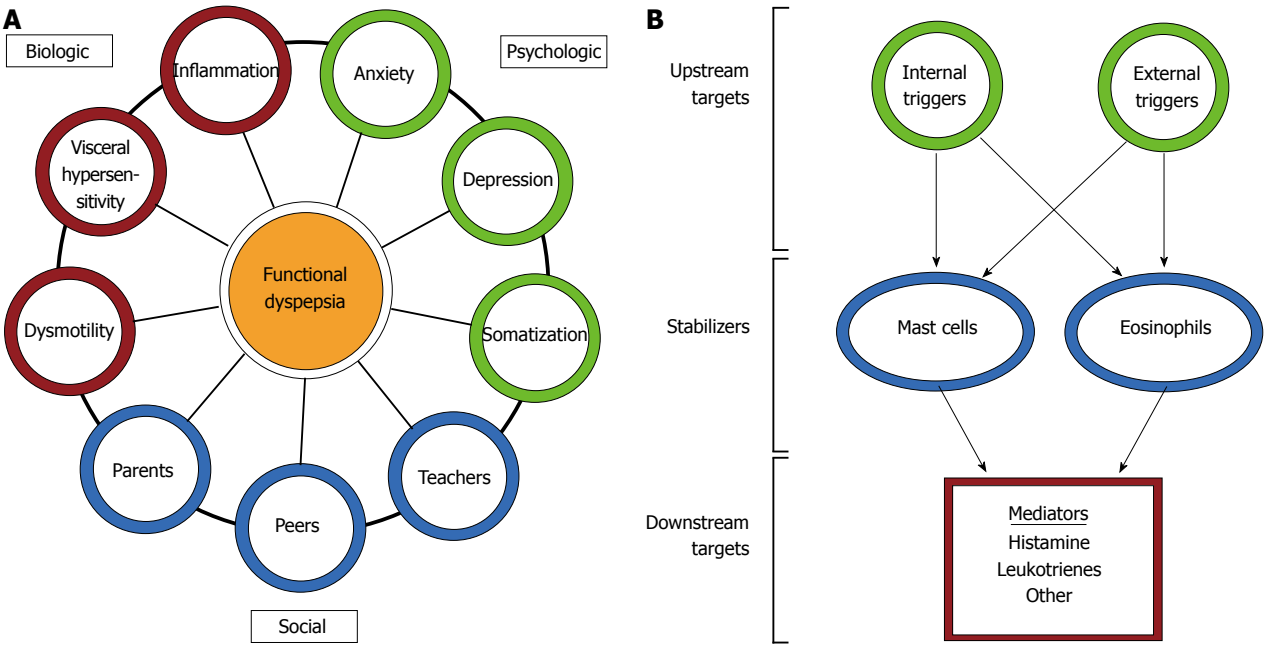

Figure 1 Overview of symptom contributors and points of intervention. A: Overview of symptom contributors in the biopsychosocial model of abdominal pain; B: Overview of the points of intervention in treatments targeting mast cells and eosinophils in functional dyspepsia.

rent pain or discomfort centered in the upper abdomen (above the umbilicus) that is unrelated to a change in stool frequency or form and not exclusively relieved by defecation. A diagnosis of FD is accompanied by the lack of evidence for an inflammatory, anatomic, metabolic, or neoplastic process that explains the patient's symptoms; however, mild, chronic inflammatory changes on mucosal biopsies do not preclude the diagnosis[5,6].

In adults, there are two recognized FD subtypes based on studies utilizing factor analysis, postprandial distress syndrome (PDS) and epigastric pain syndrome (EPS). PDS is defined as bothersome postprandial fullness occurring after ordinary sized meals and/or early satiation that prevents finishing a regular meal. EPS is defined as intermittent pain or burning of at least moderate severity localized to the epigastrium. The Rome pediatric subcommittee did not adopt the adult subtypes because of a lack data to support their existence in children and adolescents. However, there is now some data to suggest that these adult subtypes may have meaningful associations with mucosal inflammation and psychosocial functioning in children with FD[7]. For example, pediatric dyspepsia is associated with lower quality of life, increased functional disability, and increased likelihood of meeting criteria for an anxiety disorder, however, the association with anxiety appears to predominate in patients experiencing symptoms consistent with PDS[7,8]. A similar relationship between PDS and anxiety has been described in adults[9].

FGIDs, including FD, are probably best understood through a biopsychosocial model. This model suggests that interactions between biological/physiological factors (*e.g.,* inflammation, mechanical disturbances, hypersensitivity), psychological factors (*e.g.,* anxiety, depression, somatization), and social factors (*e.g.,* interactions with parents, teachers, or peers) collectively contribute to the symptoms of FD (Figure 1). The biological factors most often implicated in FD include motility disturbances, such as delayed gastric emptying, gastric electrical disturbances, and impaired accommodation, and visceral hypersensitivity to distension, acid, and/or lipids[10-12]. Delayed gastric emptying and gastric electrical disturbances have been demonstrated in a substantial proportion of children with FD[13,14]. Similarly, water load volume, as an indicator of visceral sensitivity, differs between children with FD and healthy controls[15,16]. Consequently, electromechanical disturbances and visceral hypersensitivity represent frequent targets for therapeutic intervention in FD.

Recently, there is an increasing appreciation for the importance of inflammation as a pathophysiologic entity that contributes to FGIDs including FD. Importantly, inflammation may serve as a mediator between psychologic and physiologic functions. This manuscript reviews the literature implicating two inflammatory cell types, mast cells and eosinophils, in the generation of dyspeptic symptoms and explores their potential as targets for the treatment of FD.

MAST CELLS

In the context of FGIDs, mast cells have been studied primarily in adults with IBS where their numbers are generally elevated in the ileum and colon[17,18]. In addition, adult IBS has been associated with an increase in the density of degranulating mast cells and mast cells in close proximity to nerves which correlate with abdominal pain severity and frequency[19]. Increased mucosal mast cell density has also been demonstrated in the gastric corpus and antrum of adults with FD[20,21]. Increased mast cell

density is generally isolated to the stomach of adults with FD, while increased mast cell density in the duodenum is generally associated with IBS[21,22].

Due to a lack of normal control data, it is unclear whether gastric mast cells are elevated in pediatric FD, however, antral mast cells do appear to be actively degranulating in children with FD. The mean reported degranulation index is 67% with more than 80% of patients demonstrating degranulation indices of greater than 50%[23]. Mast cells in the proximal stomach have been shown to degranulate with balloon distension of this region, demonstrating possible hypersensitivity in adults with FD[24]. Although equivalent data are not available for children, mast cell density is positively correlated with slower gastric emptying and pre-prandial dysrhythmia in children with FD[23]. Of note, the presence of pre-prandial dysrhythmia appears to be associated with increased post-prandial pain[13].

EOSINOPHILS

Ethical considerations preclude tissue sampling for evaluation of eosinophil density in otherwise healthy children. Existing pediatric data provide suggestive evidence of eosinophil densities that may be abnormal in the absence of established "norms". In a pediatric autopsy study (for which presence of gastrointestinal symptoms could not be evaluated), eosinophil density was < 10/hpf in the antrum and ≤ 20/hpf in the duodenum in 100% and 82% of samples evaluated, respectively[25]. Review of biopsies from 682 presumably symptomatic children referred for endoscopy found eosinophil density was ≤ 10/hpf in the antrum and ≤ 20/hpf in the duodenum of 90% and 93% of children, respectively[26]. Maximum eosinophil density was 8/hpf in the antrum and 26/hpf in the duodenum. Thus, eosinophil density cut points of 10/hpf in the antrum and 20/hpf in the duodenum seem reasonable, but may need to be considered in tandem with measures of activation.

Eosinophil biologic activity results from mediator release with most mediators active in a concentration-dependent fashion. Thus, eosinophil effects are not just dependent on cell density, but on the extent of degranulation. However, these events may not be tightly correlated[27]. In a previous study involving 20 children with a diagnosis of FD, an eosinophil density > 20/hpf was present in only 15%, but moderate to extensive degranulation was demonstrated by electron microscopy in 95% of those evaluated[28].

Location of eosinophils also may be important to consider. Dyspeptic adults have demonstrated increased eosinophil density in the duodenum as compared with controls; however, the quantity of antral eosinophils did not differ between groups[29,30]. Duodenal biopsies from adult dyspeptics also revealed more extensive degranulation, with enhanced extracellular major basic protein (MBP)[30]. This is consistent with observations of degranulation and MBP release in pediatric patients with FD[28]. In adults, an increased eosinophil density and a higher prevalence of duodenal eosinophilia has been specifically associated with the PDS subtype of FD[30]. Eosinophilia within the upper gastrointestinal tract has been evaluated in children with unspecified (by Rome criteria) abdominal pain, as well as children with FD, providing some pediatric information regarding the association between eosinophil location and patient symptoms. In a study of 1191 children with unspecified chronic abdominal pain, eosinophilia was identified in the antrum or duodenum in 11.4%[31]. Another study found gastric eosinophilia in 19% and duodenal eosinophilia in 32% of children with unspecified chronic abdominal pain[32]. In contrast, in children specifically fulfilling FD criteria, duodenal eosinophilia has been demonstrated in 79% of patients, which closely mirrors adult findings[33].

INTERACTIONS BETWEEN MAST CELLS AND EOSINOPHILS

Mast cells and eosinophils exert their biologic functions almost exclusively by the release of mediators after activation. The effects of specific mediators depend, to some extent, on the local biochemical milieu of the involved tissue[34]. As a consequence of this paracrine activity, mast cells and eosinophils interact highly with each other. In addition, mast cells and eosinophils demonstrate self-sustaining autocrine activity. For example, both eosinophils and mast cells produce interleukin (IL)-5 which augments mast cell cytokine production and is critical for the growth, chemotaxis, and activation of eosinophils[35,36]. Mast cells and eosinophils both produce eotaxin which, in conjunction with mast cell-produced histamine, serves as a chemoattractant for eosinophils[35,37]. Mast cells and eosinophils both also produce and express receptors for leukotrienes and tumor necrosis factor-α (TNF-α) which effect chemotaxis, survival, and activation of these two cell types[35,37]. Given the countless mediators that these cells produce, it is likely that activation of either cell will result in alteration of function of the other.

SPECIFIC CONDITIONS ASSOCIATED WITH MUCOSAL INFLAMMATION

There are a number of triggers or inciting events which may initiate an inflammatory response, and the subsequent recruitment and activation of mast cells and eosinophils, in the gastrointestinal tract. These include internal triggers such as stress and anxiety, as well as external triggers such as microbes and allergens.

Internal triggers

Anxiety and stress are the most highly implicated internal triggers in the development and/or maintenance of FGIDs, including FD. Children with FGIDs tend to have more concurrent symptoms of anxiety and depression than do their peers[38]. Approximately 50% of children

with FD demonstrate elevated anxiety scores either in isolation or as part of more global psychosocial dysfunction[39]. Further, mucosal eosinophil density, as well as antral mast cell density, correlates with anxiety scores in children with FD[7,40].

Thus, the role of inflammation in the biopsychosocial model is probably best illustrated by examining the stress response. Corticotropin releasing hormone (CRH), produced by the hypothalamus (as well as immune cells including lymphocytes and mast cells) is a major mediator of the stress response in the hypothalamic-pituitary-adrenal (HPA) axis and, subsequently, within the brain-gut axis. The stress response results in physiologic effects which appear relevant to FGIDs including inflammation, altered gastric accommodation, gastric dysmotility, and visceral hypersensitivity. CRH also has CNS effects which may alter central processing of nociceptive messages including anxiogenic effects. Of note, the relationship between the CNS and gastrointestinal pathophysiology is bidirectional. In a rodent model, gastric irritation in the neonatal period induces a long lasting increase in depression- and anxiety-like behaviors, as well as an increased sensitivity of the HPA axis to stress[41]. CRH stress systems may be activated by afferent nerves from inflamed sites or *via* cytokines including TNF-α, IL-1, IL-6 and IL-12[42].

CRH receptors are widely expressed within the gastrointestinal tract and immune cells. Mast cells express both CRH1 and CRH2 receptor subtypes at their surface. Most of the inflammatory cell actions, including those on mast cells, occur *via* CRH-R2 receptors[43]. Once mast cells are activated, they release mediators which recruit and activate eosinophils, with both cell types interacting in a bi-directional fashion with T helper cells (Th). There also may be a direct effect for CRH on eosinophils. In a rodent model, psychologic stress results in eosinophilic expression of CRH[44]. CRH is not expressed by eosinophils in the intestines of the mice except under psychologic stress and decreases after the stress is removed[44].

Once activated by CRH, mast cells may release pro-inflammatory cytokines[45]. Adults have demonstrated selective luminal release of tryptase and histamine from jejunal mast cells under cold stress at a magnitude similar to that induced by antigen exposure in food allergic patients[46]. Once released, mast cell and eosinophil mediators can stimulate afferent nerves signaling pain, can sensitize afferent nerves resulting in visceral hypersensitivity, and can alter electromechanical function. Histamine can stimulate afferent sensory nerves *via* H2 receptors[47]. CRH has been shown to activate gastrointestinal mast cells with resultant mediator sensitization of afferent sensory enteric nerves[48-50]. Low grade inflammation may lead to visceral sensitivity and motility disturbances; the key appears to be a shift from a TH1 to a TH2 response, with eosinophils and mast cells as the key effector cells[51]. Stress has been shown to shift the relative proportion and trafficking of T helper lymphocytes towards a Th2 or "allergic" phenotype[42]. This shift is driven by central and peripheral CRH, catecholamines, and histamine *via* H2 receptors. The Th2 phenotype is associated with release of IL-4, IL-10, and IL-13 which stimulate growth and activation of mast cells and eosinophils[42].

External triggers

A number of external triggers have been identified, such as microbes and allergens, which may result in eosinophil and/or mast cell recruitment and activation. The immune system may be activated by an acute infection and continue to generate symptoms after the infection resolves, resulting in so-called post-infectious FD (PI-FD). *Helicobacter pylori* (*H. pylori*) colonization represents a unique situation where symptoms may result from chronic infection and, in many patients, persist after eradication.

FD has been reported at a higher prevalence following both bacterial and parasitic infections[52]. It seems likely that FD may also be induced by viral gastroenteritis in a manner similar to that of IBS. In a study of 88 children with a previous positive bacterial stool culture, FD was present in 24% and IBS in 87%[53]. Fifty-six percent of the patients reported the onset of abdominal pain after the acute infection. Another study identified 82 adults with persistent abdominal symptoms following *Giardia* infection, with FD in 24.3% and IBS in 80.5%[54]. Over half of these patients reported exacerbation due to specific foods and, consistent with the biopsychosocial model, nearly half reported exacerbations with physical or mental stress[54].

PI-FD appears to represent an impaired ability to terminate the inflammatory response after elimination of the offending pathogen. It may also be associated with neuroplastic changes in visceral and central afferent pathways[55]. Duodenal eosinophilia has been described in PI-FD and gastric mast cells are significantly increased in PI-FD as compared to healthy controls[51,56]. PI-FD is associated with increased gastric release of histamine and 5-hydroxytryptamine as well as increased number of mast cells in close proximity to nerve fibers as compared to healthy controls or non-PI-FD[57].

The role of *H. pylori* in FD remains incompletely defined. Multiple studies have demonstrated a moderate reduction in FD symptoms with eradication of this organism while others have shown no clinical benefit[57-60]. A Cochrane review concluded that eradication was significantly better than placebo[61]. However, a large number of patients continue to experience symptoms following eradication. These may be patients in whom *H. pylori* had no pathologic role or may represent patients with PI-FD and prolonged submucosal inflammation[62].

H. pylori colonization in children is associated with a mixed inflammatory infiltrate including eosinophils which decrease with eradication[63]. *H. pylori* colonization also may be associated with increased antral mast cell density, though this appears to be *H. pylori* strain specific[64]. In the setting of *H. pylori*-associated nodular gastritis, eosinophils may be of particular significance. Patients with nodular gastritis have a higher incidence of dyspeptic symptoms which resolve with eradication therapy[62].

Nodularity is associated with an increased density of eosinophils[65]. Even in the absence of nodularity, *H. pylori* colonization is associated with increased antral eosinophils, as well as increased gastric fluid eosinophil cationic protein indicating that the eosinophils are actively degranulating[26,63,66]. These findings would suggest a possible pathophysiologic role for eosinophils in contributing to symptoms in patients with *H. pylori* colonization prior to and following eradication.

The role of allergies in the development of FD has not been well studied; however, their potential to contribute given the observed increases in and activation of mast cells and eosinophils in FD is certainly plausible. FGIDs occur more commonly in children with a history of cow's milk allergy as infants[67]. In these children, mucosal application of cow's milk is associated with increased eosinophils and mast cells and rapid degranulation within 10 min of application[68]. In addition, cow's milk exposure is associated with increased mast cells in close proximity to nerves[68]. A history of allergy is associated with increased duodenal eosinophil density in adults with FD[30]. Whether food allergy accounts for a substantial portion of children with FD is not clear. We previously found no significant increase in immunoreactivity to common food allergens in FD children with duodenal eosinophilia, though it is possible that the reactions were localized to the mucosa and thus missed in our assessment[69]. It is also possible that environmental allergens may play a role in FD. Antigen exposure in adults with birch pollen allergy results not only in an increase in symptoms of FD but also an increase in mucosal MBP+ eosinophils and IgE-bearing cells in the majority of patients[70].

"ANTI-INFLAMMATORY" THERAPY

Treatments with the potential to impact symptoms related to inflammatory cells would primarily act by three mechanisms: (1) controlling upstream factors which recruit or activate inflammatory cells; (2) controlling the release of mediators from inflammatory cells; and (3) antagonizing the downstream effects of mediators once released from inflammatory cells (Figure 1).

Treatment directed at upstream factors

Treatments directed at upstream factors would include those which interfere with activation of mast cells or eosinophils by internal triggers (*e.g.,* CRH antagonists, selective serotonin reuptake inhibitors (SSRI) anti-depressants, anti-anxiety treatments) or external triggers (*e.g.,* corticosteroids, anti-TNF-α, anti-IL-5 and anti-IgE).

The biopsychosocial model and CRH physiology would suggest a potential role for antagonizing CRH, or controlling its secretion by modulating anxiety and the stress response either through the use of SSRIs, anti-anxiety medications, or relaxation techniques. Though there are no previous controlled studies evaluating CRH-antagonists or SSRIs in pediatric FD, some evidence exists for the role of relaxation *via* biofeedback-assisted relaxation training (BART). Biofeedback is a technique whereby individuals are trained to relieve physical or emotional symptoms using signals from their bodies that are displayed visually or aurally. Biofeedback can be paired with relaxation training to yield BART. BART paired with fiber supplementation has been shown to be superior to fiber alone in children with non-specific abdominal pain[71]. The effect of BART directly on inflammation has not been studied. However, BART has been studied as an adjunctive treatment to medications directed at inflammation in children with FD in association with duodenal eosinophilia[72]. Children receiving medication plus BART demonstrated better outcomes with regard to pain intensity, duration of pain episodes, and global clinical improvement as compared to children receiving medications alone[72].

Corticosteroids represent another group of agents which may be used in the setting of mucosal eosinophilia to block upstream activation and upstream effects, although they have not been studied directly in patients with FD. Prednisone has long been considered the mainstay in the treatment of eosinophilic gastroenteritis though there are no placebo-controlled studies evaluating efficacy. The less than favorable side effect profile represents a significant draw back in considering its use as a long term agent. Budesonide may represent a safer alternative. Budesonide is a synthetic corticosteroid with high topical activity, substantial first pass elimination and relatively low systemic bioavailability. Among the commercially available preparations is an oral enteric-coated capsule formulated to optimize delivery to the ileum and colon[73]. The delivery pattern would suggest that budesonide may be less effective for proximal small bowel disease. However, the budesonide granules dissolve at an alkaline pH normally present in the proximal small bowel. Although acid suppression with omeperazole does not affect absorption, acid suppression in combination with delayed gastric emptying, as might be expected with mucosal inflammation, has not been evaluated[73]. The literature regarding budesonide and eosinophilic gastroenteritis consists of case reports where budesonide therapy has been reported to be effective against eosinophilia in the duodenum and jejunum[74-76].

TNF-α represents another theoretical "upstream" treatment target for FD. CysLTs induce TNF-α production which has been demonstrated to recruit and prolong survival of eosinophils, as well promote a TH2 response depending on other chemokines present in the microenvironment[77-79]. In a variety of allergic mouse models, anti-TNF antibodies have been shown to decrease eosinophilic infiltration and local Th2 cytokine transcription and secretion[80-82]. Pre-treatment serum TNF-α concentrations correlate negatively with the clinical response to montelukast in pediatric FD in association with duodenal eosinophilia indicating that mediation by TNF-α may represent an alternative pathway for symptom generation in these patients. Although there are no controlled studies, anti-TNF-α antibody has been reported to be

effective in a series of children with resistant eosinophil disease including patients with FD[83].

Eosinophils and/or mast cells exhibit a number of cell surface markers which also serve as potential therapeutic targets in blocking upstream activation. These have been well reviewed elsewhere[84]. However, there are two of these, IL-5 and IgE, which have been targeted in humans with gastrointestinal eosinophilia and, thus, warrant specific mention.

IL-5 serves to stimulate the expression of eosinophils. In general, most clinical studies evaluating anti-IL-5 antibodies have demonstrated decreases in eosinophil density but little clinical benefit[85]. There are limited reports on the use of anti-IL-5 in patients with gastrointestinal eosinophilia and none specifically in patients with FD. In a small pilot study of adults with eosinophilic gastroenteritis, a single dose of anti-IL-5 resulted in a 50%-70% decrease in mucosal eosinophil density in 3 of the 4 patients but with minimal symptom improvement[86]. The effect of anti-IL-5 on duodenal eosinophil density was assessed in 11 adult patients treated for eosinophilic esophagitis[87]. While esophageal density decreased significantly, there was no significant effect on duodenal eosinophil density. This may simply indicate that the normal physiologic duodenal eosinophil population is unaffected.

Anti-IgE antibody has also been evaluated in a small study of adults with eosinophilic gastroenteritis but not specifically in patients with FD. In an uncontrolled, open-label study of 9 patients, anti-IgE resulted in a non-statistically significant reduction in eosinophil density in the antrum (69%) and duodenum (59%)[88]. Symptoms significantly improved but improvement had no direct relation to the decrease in mucosal eosinophil density.

Treatment directed at controlling mediator release

Mast cell stabilizers, including cromolyn and ketotifen, represent an attractive potential therapy given data implicating mast cells in the generation of dyspeptic symptoms as previously discussed. These agents inhibit the release of mast cell mediators and, consequently, their pathophysiologic effects.

There have been no adult studies on the use of mast cell stabilizers in patients with FD. Benefit has been demonstrated in adults with IBS where it is suggested that the response may be related to blocking allergic or immunologic reactions to foods[89-91]. In an open-label observational study of oral cromolyn in children with FD in association duodenal eosinophilia, resolution of pain was demonstrated in 89% of patients who had previously failed to respond to H2 and combined H1/H2 antagonism[92].

Ketotifen, which antagonizes the H1-receptor, in addition to stabilizing mast cells has been shown to significantly decrease pain in adults with IBS and to increase the threshold for discomfort in patients with visceral hypersensitivity though this effect could not be correlated with pain improvement[93]. Whether the observed response to this drug is related to H1-receptor antagonism or mast cell stabilization is unclear.

Treatment directed at downstream mediators

In general, treatments directed at antagonizing the downstream effects of mediators released by mast cells and/or eosinophils are associated with a more rapid onset of action and fewer side effects. Therefore, they should probably be viewed as first line agents in treatment directed at mast cells and eosinophils in FD. It should be noted that the two most common downstream targets, histamine and leukotrienes, also have pro-inflammatory effects that may result in further upstream activation. Further, the simple experience of symptoms may cause physical and/or emotional stress that promotes upstream activation through the pathway previously described. Thus, addressing these downstream treatment targets may have direct effect on reducing symptoms in the short-term, while also indirectly serving to reduce activation of inflammatory cells in the long-term.

Acid reduction remains the most common treatment prescribed empirically by pediatric gastroenterologists for children with dyspepsia[1]. While there are numerous adult studies to support this practice, pediatric studies are limited. In adults, H2 antagonism has been shown to improve at least some symptoms associated with FD (abdominal pain, indigestion, belching, and gastroesophageal reflux symptoms) and appear to be superior to prokinetic medications and short term use of anxiolytics[94-97]. In adults with dyspepsia, proton pump inhibitors (PPIs) are superior to placebo in symptom reduction although this appears limited to patients with ulcer-like or reflux-like dyspepsia[98-101]. Whether PPIs are superior to H2 antagonism is not completely clear. Omeperazole was found to have a modest increase in efficacy as compared to ranitidine at 4 wk (51% *vs* 36%) but there was no benefit at 6 mo[97].

In children with abdominal pain, famotidine was superior to placebo in global improvement with clear benefit in those with dyspepsia[102]. In a large pediatric study, omeperazole was shown to have a very modest advantage in the relief of all symptoms as compared to either famotidine or ranitidine but there was no significant difference between the three with regard to resolution of abdominal pain, epigastric pain, nausea or vomiting[103].

Given the response to PPIs, it would appear that at least some of the clinical improvement from H2 antagonism or PPIs is related directly to acid suppression. A significant portion of responders may derive benefit from treatment of overlap GER or possibly from peptic gastritis or duodenitis, however, the benefit may also be due to limiting acid exposure in patients with acid hypersensitivity. With H2 antagonism, the benefit may also be unrelated to acid reduction as histamine has direct gastric myogenic actions, modulates afferent enteric nerve excitability, and acts as an immunomodulating agent[104-108]. H2 receptors affect not only acid secretion but influence neurotransmission and immune responses[47].

There may also be additional benefit from H1 antagonism. Combining an H1 antagonist with an H2 antagonist has been reported to relieve symptoms in 50% of

children with FD in association with duodenal eosinophilia and in 79% of adults with FD in association with increased antral mast cell density who had previously failed to respond to acid reduction therapy[92,109]. H1 receptors have direct affects on smooth muscle contraction and visceral sensitivity[47]. Some benefit from H1 antagonism may also be due to an anxiolytic effect. Immune modulators, such as suplatast sodium, may also indirectly inhibit H1 receptor expression by suppressing IL-4 and IL-5 production from TH2 cells[110]. Shirai *et al*[111], reported successful treatment with suplatast sodium in an adult with eosinophilic gastroenteritis. It has not been specifically used in patients with FD.

H4 antagonists are currently in development and may represent a treatment option in the future. H4 receptors are abundant in the small intestine, largely on hematopoietic cells including eosinophils and mast cells, as well as endocrine cells[112,113]. H4 receptor activation results in eosinophil and mast cell chemotaxis (but not degranulation) as well as T cell cytokine production[112]. Current H1 antagonists do not inhibit H4 receptors but they do share common ligands[112].

CysLTs also are a potential downstream therapeutic target. The pattern of eosinophil degranulation in pediatric FD is consistent with the release of major basic protein, which is known to enhance the synthesis of cysLT. CysLT, in turn, stimulates smooth muscle contraction and recruitment of eosinophils[114]. CysLTs have been shown to alter mast cell function *via* induction of IL-5 and TNF-α production in primed mast cells, an effect blocked by cysLT inhibition[115]. Leukotrienes have the potential to increase intestinal sensory nerve sensitivity during inflammation as LT receptors are expressed on spinal nerve terminals and cysLTs have been shown to increase excitability of enteric neurons and to have a procontactile effect on the esophagus, stomach, small intestine, colon, and gallbladder[116-123].

In a double-blind, placebo-controlled, cross-over trial of children with FD in association with duodenal eosinophilia, montelukast, a cysLT receptor antagonist, was found to be superior to placebo with regard to relief of pain[124]. The response rate was 84% in patients with eosinophil density between 20 and 29/hpf as compared to a 42% response rate with placebo. This high response rate was confirmed in a second study which also determined that the short term positive clinical response was unrelated to a decrease in eosinophil density or activation[33]. This would suggest that the effect may be mediated through an enteric nerve effect on motility or sensitivity though that remains to be demonstrated. Other leukotriene antagonists (*e.g.,* pranlukast, zafirlukast) have not been evaluated in FD or eosinophilic gastroenteritis.

CONCLUSION

Current evidence implicates gastric mast cells and duodenal eosinophils in the pathophysiology of FD and as mediators between psychologic and physiologic factors. Increased antral mast cell density is associated with anxiety, electromechanical dysfunction, and the PDS subtype of FD. Likewise, increased duodenal eosinophil density is associated with anxiety and the PDS subtype, however, effects on electromechanical function are more indirect.

While empirical data is limited, previous studies suggest that there may be efficacy in utilizing medications directed at mast cells and eosinophils. Most current data regarding treatment response consists of case series utilizing H1/H2 antagonists, mast cell stabilizers, and anti-TNF-α, as well as a controlled trial demonstrating clinical efficacy for the use of montelukast. Evidence exists to suggest that combining "anti-inflammatory" medications with other treatments targeting stress can improve the rate of symptom resolution in pediatric FD.

FUTURE DIRECTIONS

There remains a need for placebo-controlled trials of the various medications and other treatments targeting mast cells and eosinophils which have been suggested to have efficacy, either alone or in combination. Likewise, there is a need to better define the upstream and downstream mediators for both mast cells and eosinophils as potential therapeutic targets for future drug development or as potential targets for agents currently available, such as lipoxygenase inhibitors, prostaglandin synthetase inhibitors, or newer drugs targeting eosinophil adhesion or Siglec-8[125,126].

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REVIEW

Metabolomics as a diagnostic tool in gastroenterology

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Abstract

Metabolomics has increasingly been applied in addition to other "omic" approaches in the study of the pathophysiology of different gastrointestinal diseases. Metabolites represent molecular readouts of the cell status reflecting a physiological phenotype. In addition, changes in metabolite concentrations induced by exogenous factors such as environmental and dietary factors which do not affect the genome, are taken into account. Metabolic reactions initiated by the host or gut microbiota can lead to "marker" metabolites present in different biological fluids that allow differentiation between health and disease. Several lines of evidence implicated the involvement of intestinal microbiota in the pathogenesis of inflammatory bowel disease (IBD). Also in irritable bowel syndrome (IBS), a role of an abnormal microbiota composition, so-called dysbiosis, is supported by experimental data. These compositional alterations could play a role in the aetiology of both diseases by altering the metabolic activities of the gut bacteria. Several studies have applied a metabolomic approach to identify these metabolite signatures. However, before translating a potential metabolite biomarker into clinical use, additional validation studies are required. This review summarizes contributions that metabolomics has made in IBD and IBS and presents potential future directions within the field.

Key words: Metabolomics; Microbiota; Inflammatory bowel diseases; Irritable bowel syndrome

Core tip: Metabolic profiling is a powerful exploratory tool for understanding interactions between nutrients, the intestinal metabolism and the microbiota composition in health and disease and, to gain more insight in metabolic pathways. Metabolomics may advance our understanding, diagnosis and treatment of inflammatory bowel disease and irritable bowel syndrome. Metabolic reactions initiated by the host or gut microbiota can lead to "marker" metabolites present in different biological fluids that allow differentiation between health and disease. Disease-related mechanisms may be uncovered and verified, and candidate diagnostic biomarkers in biological samples are characterized.

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INTRODUCTION

Different "Omic" approaches are currently applied to identify novel diagnostic targets and disease specific markers, and to characterize the link between gut microbiota or host metabolism and functional alterations in the pathophysiology of different diseases. Genomics, transcriptomics and proteomics provide extensive information regarding the genotype but convey limited information about the phenotype (Figure 1). Gene expression and protein data mainly indicate the potential for specific

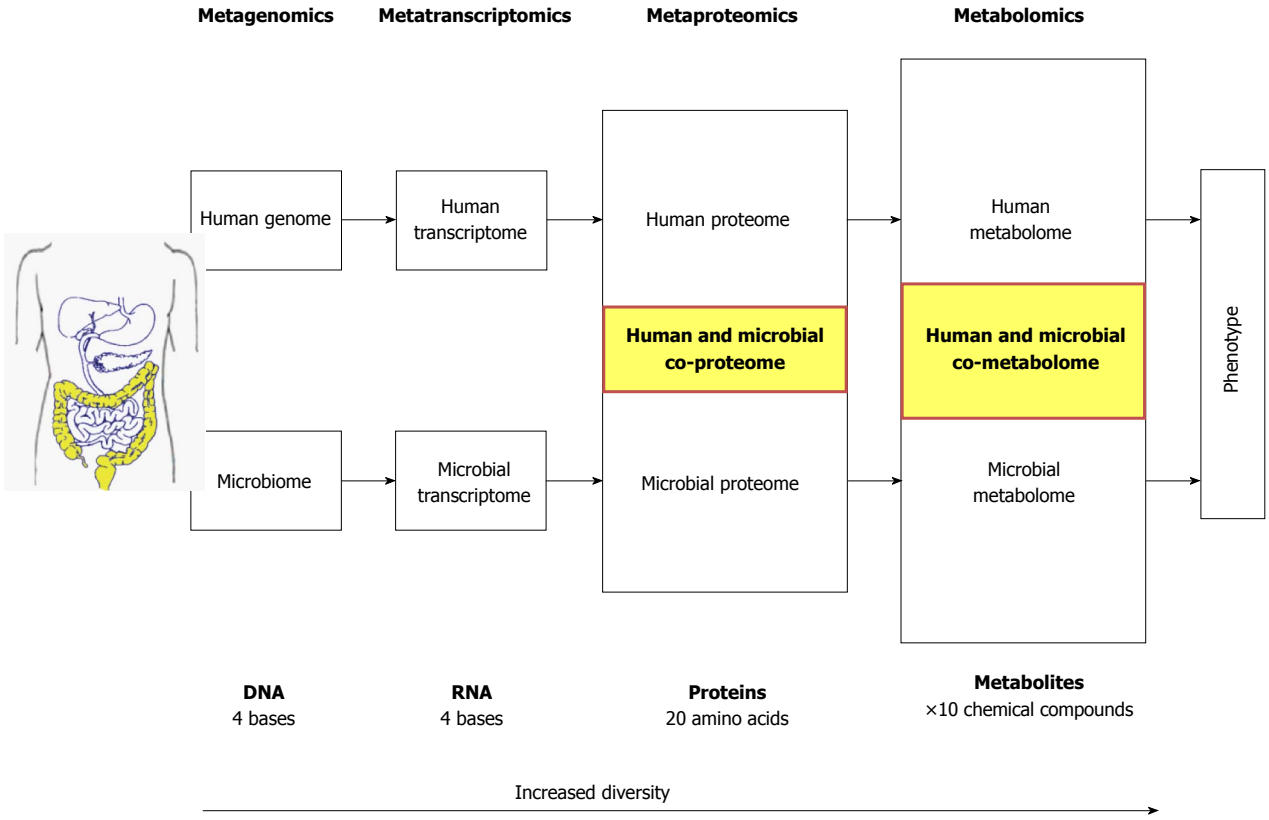

Figure 1 Overview of different "Omic" approaches (metagenomics, metatranscriptomics, metaproteomics and metabolomics) determining an individual's "Genotype to phenotype".

metabolic functions and do not always reflect the effective physiological processes as several downstream regulatory mechanisms are involved. As compared to other "omics", metabolic profiling or metabolomics, integrates the effects of gene regulation, post-transcriptional regulation and pathway interactions. This downstream synthesis of diverse signals ultimately makes metabolites direct molecular readouts of cell status that reflect a meaningful physiological phenotype (Figure 1)[1,2]. In addition, changes in metabolite concentrations are also induced by exogenous factors such as environmental and dietary factors which do not affect the genome. Metabolomics is defined as "the non-biased identification and quantification of all metabolites in a biological system"[3]. For the quantitative analysis of metabolites in response to disease, Nicholson and colleagues introduced the term metabonomics or "the quantitative measurement of the multiparametric metabolic responses of a living system to pathophysiological stimuli or genetic modification"[4]. In practice, within human disease research, both terms are used indifferently.

Metabolic profiling is a powerful exploratory tool for understanding interactions between nutrients, the intestinal metabolism and the microbiota composition in health and disease and, to gain more insight in metabolic pathways. Metabolomic studies allow evaluation of metabolites by a top-down approach bypassing the need for an *a priori* hypothesis. Generally, metabolomic analysis has in view two major opportunities. First, untargeted analysis of a large number of metabolites enhances the chance to discover metabolites that are associated with the disease and might serve as biomarkers. In this respect, biomarker models are designed to discriminate with optimal sensitivity/specificity between groups, but do not presume biological understanding as an absolute prerequisite for biomarker development. However, understanding of the biological pathways can certainly support an assay[5]. Second, the profile of metabolites affected by the disease may provide new insights into the pathogenesis and eventually reveal new therapeutic targets.

Until now, genomic and proteomic methodologies have often been applied to uncover gastrointestinal related pathophysiological processes[6-11]. However, currently, metabolomics technologies are increasingly used for discovery of gastrointestinal disease signatures and have been applied for the screening of different pathological conditions that are linked with a metabolic imbalance. This review focuses on the contribution of metabolic profiling in advancing research in the field of inflammatory bowel disease (IBD) and irritable bowel syndrome (IBS).

THE MICROBIOTA AND ITS METABOLIC ACTIVITY

The microbiota residing in the human gastrointestinal tract, especially the large intestine, is recognized as one of the most metabolically active organs of the human

body. This microbial ecosystem is extremely complex and dynamic with high densities of living bacteria consisting of approximately 500-1000 different species[12,13]. In healthy adults, 80% of the identified fecal microbiota can be classified into three dominant phyla: *Firmicutes*, *Bacteroidetes* and *Actinobacteria*, but there is substantial variation in the species composition between individuals[14]. A total of about 10^{14} bacterial cells are present in the adult intestine, which is ten times the number of cells in the human body[15]. This microbiome outnumbers the host's genetic potential by two orders of magnitude[16] and provides a diverse range of biochemical and metabolic activities to complement the host's physiology. The presence and metabolic activities of a specific bacterial community play an important role in maintaining the host's overall health and well-being, and has been shown to respond to metabolic challenges and dietary factors. This complex microbial system varies with the host's age, diet and health status[17].

Through the process of fermentation, colonic bacteria produce a wide range of compounds that may influence the physiological processes in the colon. The human microbiota is characterized by a significant degree of functional redundancy, meaning that different bacteria can perform similar functions and metabolize the same substrate, thereby producing similar metabolites[18]. Therefore, not only the composition but also the functional capacity of the intestinal microbiota is highly important regarding the clinical end points. Nevertheless, metabolic insights remain limited due to the inaccessibility of the intestinal habitat and the complexity of the microbiota composition[12]. A number of factors, such as nutrient availability, physicochemical nutrient properties, colonic transit time, and age of the host, influence the composition and the metabolic activity of the colonic microbiota. Nutrient availability is believed to be the most important regulator of bacterial metabolism. Especially the ratio of available carbohydrate to nitrogen determines the degree of saccharolytic *vs* proteolytic fermentation[19]. Colonic fermentation of carbohydrates results in the generation of short-chain fatty acids (SCFA) which are generally assumed to be beneficial for the host[20]. Protein fermentation gives rise to a variety of metabolites such as phenolic compounds, branched-chain fatty acids, S-containing compounds, amines and ammonia[21,22].

HOST AND INTESTINAL MICROBIOTA CO-METABOLOME

The intestinal microbiota produces a number of compounds during the metabolism of nutrients and xenobiotics (compounds of non-host origin that enter the gut with the diet or are produced by the microbiota). Some of these metabolites are excreted in feces whereas others are absorbed through the colonic mucosa and enter the systemic circulation where they can be further modified by human metabolism. For instance, p-cresol is a bacterial fermentation product produced in the colon from tyrosine that is effectively absorbed. It is conjugated in the colon mucosa or liver to p-cresol sulfate or p-cresol glucuronide which improves the water solubility and facilitates its urinary excretion[23]. These metabolites are called human commensal co-metabolites. Also the opposite occurs. A number of metabolites that are derived from host metabolism are returned to the gut *via* biliary excretion where they can be further metabolized by the microbiota. For instance, bile acids that have escaped absorption in the terminal ileum can be deconjugated and converted to secondary bile acids by microbial metabolism[24].

These host-microbiota metabolic interactions complicate the interpretation of metabolite profiles. In addition, this co-metabolism explains that the outcome of metabolome analyses clearly depends on the biomatrix chosen. The contribution of the microbial metabolism is more likely reflected in the fecal metabolome than in urinary, serum of breath profiles. Urinary profiles contain human and human-microbial co-metabolites whereas serum profiles seem less influenced by bacterial metabolism.

METABOLOMICS-BASED METHODS

Analytical strategies

Fiehn *et al*[1] defined metabolomic analysis as "a comprehensive and quantitative analysis of the metabolome" with the metabolome defined as the whole of metabolites produced by an organism. However, due to the chemical diversity and different physicochemical properties of the metabolites and the large dynamic range of metabolite concentrations in different biological samples, it is virtually impossible to measure the complete metabolome. By selecting a specific analytical platform and a biofluid in which metabolites will be measured, the metabolome will be reduced to those specific conditions. Serum, plasma, urine, feces and tissue are the most studied biological matrices[25]. An overview of the different steps involved in the analytical process is shown in Figure 2.

Multiple analytical techniques have been used for the analysis of the metabolome. Gas chromatography (GC), liquid chromatography (LC) and high/ultra performance liquid chromatography (H/UPLC) coupled to mass spectrometry (MS), and nuclear magnetic resonance spectroscopy (NMR) enable detection, identification and quantification of metabolites[26,27]. Other analytical options consist of Fourier transform infrared spectroscopy (FTIS) or capillary electrophoresis (CE) coupled to MS[28,29].

The applicability of these analytical techniques differs. GC-MS provides an extraordinary resolution, permitting the separation of structurally similar compounds. However, this technique requires the compounds to be volatile and thermally stable. A chemical derivatization step can be applied prior to the chromatographic separation to render polar metabolites more volatile. Purge-

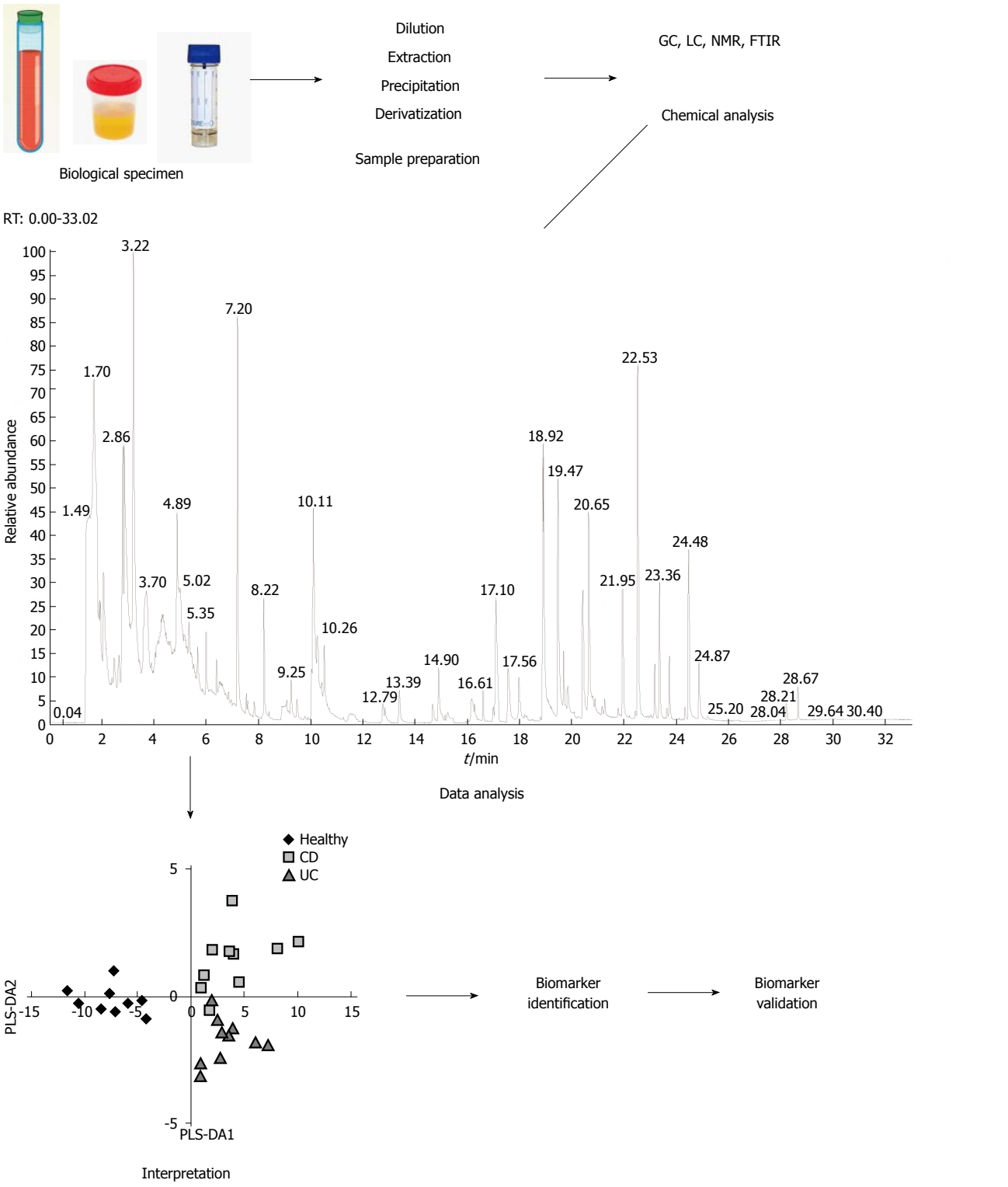

Figure 2 Overview of the different steps involved in the analytical process of metabolomics. GC: Gas chromatography; LC: liquid chromatography; NMR: Nuclear magnetic resonance; FTIR: Fourier Transform infrared spectroscopy; PLS-DA: Partial least squares discriminant analysis; CD: Crohn's disease; UC: Ulcerative colitis.

and-trap and solid phase micro-extraction are sample preparation techniques often used in combination with GC. For metabolites that are not volatile and which cannot be derivatized, LC-MS is applied. LC-MS can detect a relatively broad spectrum of (polar and non-polar) metabolites with ample selectivity and sensitivity[30]. CE-MS is a rather new technique in metabolomics that is more sensitive and detects a wider spectrum of (polar) compounds than LC-MS[31,32]. ^{1}H-NMR is a non-destructive technique that does not require prior separation of the compounds in the biofluid. It provides detailed information on molecular structure and requires only minimal

sample preparation, but has a lower sensitivity than the MS based techniques[33]. Often, a combination of different techniques is applied as none of the individual methods will cover the full metabolome[27]. Several on-line databases for identifying metabolites from experimental NMR and/or MS data are available, as summarized in Table 1. These databases contain chemical, spectral, clinical and molecular data about metabolites found in different human biofluids.

Depending on the technique, detection limits vary: detection limits for NMR and FTIS (mM sensitivity) are much higher as compared to GC-MS (< mM sensitivity) and LC-MS (nM sensitivity). As a consequence, MS-based techniques are preferably applied for quantification of specific metabolites.

Data handling

The analysis and interpretation of complex metabolomics data is facilitated by the application of chemometric and bio-statistical tools. Commonly used tests in metabolomic studies include principal component analysis (PCA) and partial-least squares discriminant analysis (PLS-DA). PCA is an unsupervised classification method, since the variation in the data is analyzed without a priori designation of samples into their classes. In contrast, PLS-DA is considered a supervised classification method, as the samples are designated into their classes for comparison.

In metabolomics, typically the number of variables or metabolites largely exceeds the number of samples measured. This can lead to the discovery of a number of variables that randomly, *i.e.,* by chance, correlate to the outcome variable and in this way give the impression of a good predictive ability. However, if such a set of variables is chosen and the model is applied to new samples, the predictive ability might be very poor. This is known as over-fitting or fitting to the noise and can be avoided by careful cross-validation of the model. Cross-validation implicates that the data set is split in a training set and a test set. The biomarker model is discovered using the training set and the performance of the model is evaluated using the test set. In case of a relatively low sample number (< 100), multiple rounds of cross validation are performed using different partitions of the data in training set/test set and the performance results are averaged[5].

METABOLIC SIGNATURE AND GASTROINTESTINAL PHENOTYPES

Dysbiosis in gastrointestinal disorders

In gastrointestinal diseases such as IBD and IBS, there is an emerging consensus hypothesis that a dysbiosis of the microbiota is involved in initiating the disease or maintaining it. Several studies identified a disproportion of the predominant bacteria in fecal samples of IBD patients[18,42] and IBS patients[43]. For example, a reduction in the abundance and diversity of Firmicutes is frequently associated with IBD and IBS. At present, studies comparing the metabolic activity of the microbiota of IBD and IBS patients as compared to normal individuals are emerging thereby investigating whether eventual differences could be related to the pathogenesis of the disease[44,45] or whether they could be used as a classification tool in clinical diagnosis.

Metabolomics in IBD

Inflammatory bowel diseases comprise Crohn's disease and ulcerative colitis as the two major phenotypes. Although both phenotypes share similar pathophysiological and clinical features, they require different therapeutic management and display different prognosis. Both manifestations are influenced by genetic predispositions as well as microbial and environmental factors. At present, the diagnosis of IBD mainly relies on clinical, endoscopic, radiologic and histologic examination which implicates that diagnosis is only possible at a relatively advanced stage of the disease. Less invasive methods such as analysis of biomarkers from urine, serum, or feces, however, would be of significant advantage and useful for primary diagnosis, surveillance, and early detection of relapses.

Several biomarkers or sets of biomarkers have been tested in clinical trials including acute phase proteins such as C-reactive protein, fecal markers (lactoferrin, calprotectin, and PMN-elastase) and serological markers (antibodies against luminal antigens and anti-glycan antibodies)[46]. Recently, the exploration of metabolomics in IBD rose from the need to improve diagnosis and to allow better stratification of patients into IBD subtypes.

Several studies have applied a metabolomic approach to discriminate IBD patients from healthy controls, to discriminate CD from UC and patients with active disease from patients in remission. An overview of the studies in humans is presented in Table 2.

The growing acceptance of the involvement of the gut microbiota in the chronic mucosal immune activation underlying the pathogenesis of IBD has led to an interest in the use of fecal extracts or fecal samples as biofluids to apply metabolite profiling.

Marchesi *et al*[47] was the first to differentiate IBD patients from healthy controls based on ^{1}H-NMR analysis of fecal water extracts and to discriminate CD patients from UC patients. Fecal water extracts from IBD patients were characterized by a depletion of bacterial degradation products such as SCFA, dimethylamine and trimethylamine suggesting a disruption of the normal bacterial ecology, called dysbiosis, either as the cause or consequence of the disease. In a study in identical twins including healthy twins and twins with inactive CD, either concordant or discordant, fecal extracts were analyzed using ICR-FT/MS with ultrahigh mass resolution. Healthy subjects could nicely be discriminated from CD patients. In addition, it was possible to separate patients with predominantly ileal involvement from patients with predominantly colonic involvement of the disease. Inter-

Table 1 Overview of web-based databases for metabolite identification

Database	URL or web address	Extra information
Human Metabolome Database (HMDB)	http://www.hmdb.ca/	Wishart *et al*[34]
Madison Metabolomics Consortium (MMC) Database	http://mmcd.nmrfam.wisc.edu/	Cui *et al*[35]
Biological Magnetic Resonance Data Bank (BMRB)	http://www.bmrb.wisc.edu/	Ulrich *et al*[36]
Golm Metabolome Database	http://csbdb.mpimp-golm.mpg.de/csbdb/gmd/gmd.html	Kopka *et al*[37]
BiGG (a knowledgebase of Biochemically, Genetically and Genomically structured genome-scale metabolic network reconstructions)	http://bigg.ucsd.edu/	Schellenberger *et al*[38]
SetupX and BinBase	http://fiehnlab.ucdavis.edu/	Skogerson *et al*[39]
MassBank	http://www.massbank.jp/	Horai *et al*[40]
METLIN	http://metlin.scripps.edu/	Smith *et al*[41]

estingly, the separation of the groups was higher for the metabolite profiles than for the microbial community profiles, analyzed on the same samples with T-RFLP fingerprints generated using general bacterial and *Bacteroides* group-specific primers[48]. The higher discrimination of the metabolite data was attributed to a direct link of the metabolites to function.

In another study, ^{1}H-NMR profiling of fecal extract allowed to discriminate patients with UC from healthy controls. Elevated levels of taurine and cadaverine in UC patients were the major discriminative findings. Samples were also analyzed for microbiota composition using DGGE. Canonical correlation analysis between NMR and DGGE data sets, based on PC scores accounting for 90% of the original variance, revealed a good correlation ($r = 0.85$, $P < 0.002$) between gut microbiota profile and metabolite composition suggesting a direct link between both parameters[49].

A recent study analyzed metabolite profiles in feces of chronic gastrointestinal disorders using GC-MS with headspace sample preparation. Samples of CD patients showed significant higher levels of ester and alcohol derivatives of SCFA and indole as compared to healthy controls, UC and IBS patients. Following treatment, the metabolite profile was altered to more closely resemble that of healthy volunteers[50]. As many microbial metabolites are absorbed and excreted in urine, either as such or after further metabolism by human enzymes, metabolite profiles of urine samples may also reflect the impact of the microbiota composition[51]. Several studies were able to discriminate IBD patients from healthy controls based on metabolite profiling of urine samples[52-54]. In all these studies, hippurate levels were lower in IBD patients as compared to controls suggesting hippurate as a biomarker of IBD. Hippurate or N-benzoylglycine is a mammalian-microbial co-metabolite that originates from bacterial fermentation of dietary aromatic compounds (polyphenols, purines or aromatic amino acids) to benzoic acid which is further conjugated to glycine in the liver[55]. Remarkably, urinary metabolite profiling allowed to differentiate between UC and CD in only one study[51], whereas two other studies failed to do so[53,54]. This discrepancy may highlight the fact that IBD is a multifactorial disease with a high variety in phenotypes and severity[56]. Indeed, the notion that IBD is actually a syndrome comprising several disease subtypes, is gaining more and more acceptance[46].

Metabolite profiles in serum or plasma or in colonic mucosal biopsies rather reflect changes in the host's metabolism and provide less information on the impact of the gut microbiota composition and/or activity. As compared to urinary and fecal profiles, metabolite profiles in serum or plasma may be less affected by environmental factors and are subject to less inter-individual variation[57].

Results from studies that analyzed serum/plasma or colonic mucosa cells indicate that both CD and UC have an impact on the amino acid metabolism[53,58-63]. Several amino acids occurred in lower levels in colonic mucosal cells from IBD patients as compared to controls. As higher amino acid levels were found in fecal extracts[47,64], this may be the result of malabsorption due to inflammation. An alternative explanation is that inflammatory conditions induce large energy requirements to repair the damaged mucosa leading to enhanced protein catabolism. Specifically, the role of decreased levels of glutamine in the pathogenesis of IBD has been studied. Besides butyrate, glutamine is an important energy source for the colonocytes and accounts for about 30% of their energy needs. In a mouse model of DSS-induced colitis, similar reductions in glutamine levels in serum and colonic tissue were observed and supplementation with glutamine attenuated the DSS-induced colitis[65].

In a recent study, Hisamatsu *et al*[66] calculated an AminoIndex based on multivariate analysis of amino acid profiles in serum of IBD patients which allowed to distinguish between CD and UC and also reflected disease activity.

Zhang *et al*[67] specifically recruited patients with active UC and short disease duration (2.7 years) and evaluated the efficacy of ^{1}H-NMR metabolic profiling of serum for early stage diagnosis of UC. Although active UC patients could be discriminated from healthy controls in a multivariate OPLS-DA model, the number of altered metabolites in this study was rather limited.

Metabolomics in IBS

Irritable bowel syndrome is a multifactorial functional disorder of the gastrointestinal tract that affects about 10%-15% of the adult population. IBS patients have symptoms of pain and bowel dysfunction. A subset of patients that developed IBS after an infection, so-called

Table 2 Overview of the studies that applied metabolomics to discriminate inflammatory bowel diseases and/or irritable bowel syndrome patients from controls

Reference	Analytical platform	Biofluid	Samples	Observations
Marchesi *et al*[47]	^{1}HNMR	Faecal extracts	CD (n = 10), UC (n = 10), HC (n = 13)	Depletion of SCFA and methylamine and trimethylamine in CD patients Higher amounts of amino acids in UC and CD compared to healthy controls
Jansson *et al*[64]	ICR-FT/MS	Faecal water	10 twin pairs with CD, 7 healthy twin pairs	Discrimination based on disease location (ileal or colonic CD) significant differences in the types and number of metabolites within specific pathways, including tyrosine and phenyl-alanine metabolism and bile acid and fatty acid biosynthesis
Le Gall *et al*[49]	^{1}HNMR	Faecal water	UC (n = 13; 31 samples), IBS (n = 10; 21 samples), HC (n = 22; 72 samples)	Discrimination between UC and HC; no classification of IBS Increased taurine and cadaverine in UC
Walton *et al*[50]	GC-MS	Faeces	UC (n = 20), CD (n = 22), IBS (n = 26), HC (n = 19)	Increased concentrations of ester and alcohol derivates of short-chain fatty acids and indole in CD After treatment, metabolite patterns are more similar to those of HC
Williams *et al*[52]	^{1}HNMR	Urine	CD (n = 86), UC (n = 60), HC (n = 60)	Discrimination between CD, UC and HC Significantly different metabolites include hippurate, p-cresol sulfate and formate Clustering independent of diet and medication
Schicho *et al*[53]	^{1}HNMR	Urine, serum, plasma	CD (n = 20), UC (n = 20), HC (n = 40)	IBD patients could be discriminated from HC, differences between CD and UC less pronounced Discriminating metabolites include amino acids, creatine, creatinine, metabolites of urea cycle, monosaccharides, hippurate (urine)
Stephens *et al*[54]	^{1}HNMR	Urine	CD (n = 30), UC (n = 30), HC (n = 60)	Metabolites for distinguishing IBD from HC: TCA cycle intermediates, amino acids metabolites derived from gut microflora (methanol, formate, hippurate, acetate, and methylamine); as well as the other metabolites trigonelline, creatine, urea, and taurine No discrimination between UC and CD after removal of patients with surgical intervention confounder
Ooi *et al*[58]	GC-MS	Colonic biopsies, serum	Colonic biopsies: UC (n = 22), serum: UC (n = 13), CD (n = 21), HC (n = 17)	Reduced levels of amino acids resulting in reduced levels of TCA cycle related downstream molecules in colonic tissue of UC Serum amino acid profiling enabled discrimination between UC and CD
Bjerrum *et al*[60]	^{1}HNMR	Colonic biopsies, colonocytes, lymphocytes, urine	Active UC (n = 35), quiescent UC (n = 33), HC (n = 25)	No discrimination between active UC, inactive UC and HC based on urine or lymphocyte profiles Inactive UC could not be differentiated from HC Active UC characterized by higher antioxidants and amino acids and lower levels of lipid, myo-inositol, betaine and glycerophosphoglycine 20% of inactive UC had similar profile as active UC
Bezabeh *et al*[63]	^{1}HNMR	Colonic biopsies	UC (n = 26; 45 samples), CD (n = 21; 31 samples), controls (38 non-inflamed IBD, 25 cancer patients)	Accurate classification of UC *vs* CD Some non-inflamed tissues from IBD had abnormal NMR-spectra
Balasubramanian *et al*[61]	^{1}HNMR	Colonic biopsies	Active UC (n = 20), Inactive UC (n = 11), Active CD (n = 20), Inactive CD (n = 6), HC (n = 26)	Higher α-glucose and lower amino acids, membrane components, lactate and succinate in active UC and CD compared to HC Lower lactate, glycerophosphorylcholine and myo-inositol in inactive UC and lower lactate in inactive CD compared to HC Lower formate in active UC *vs* active CD
Sharma *et al*[62]	^{1}HNMR	Colonic biopsies (inflamed and non-inflamed)	UC (n = 12), CD (n = 9), controls (n = 25)	No differentiation between inflamed and non-inflamed samples Lower levels of amino acids, membrane components, lactate and formate in IBD *vs* controls and higher levels of glucose
Hisamatsu *et al*[66]	AA analyzer	plasma	CD (n = 165), UC (n = 222), HC (n = 210)	Multivariate indexes established from plasma aminograms distinguish CD or UC from HC Other indexes distinguish active UC and CD from each remission patients and correlate with disease activity indices
Zhang *et al*[67]	^{1}HNMR	Serum	Active UC (n = 20), HC (n = 19)	Active UC displayed increased 3-hydroxybutyrate, β-glucose, α-glucose and phenylalanine and decreased lipid compared to healthy controls
Ponnusamy *et al*[71]	GC-MS	Faeces	IBS (n = 11) *vs* non-IBS (n = 8)	Elevated levels of amino acids and phenolic compounds that were highly correlated with abundance of lactobacilli and Clostridium

Ohman *et al*[69]	GC-MS	Faeces	IBS-D (n = 30), CD (n = 62), UC (n = 48), HC (n = 109)	Significantly more esters in IBS-D, association of aldehydes with IBD Accurate separation of IBS-D from active CD, UC and HC

SCFA: Short-chain fatty acids; IBD: Inflammatory bowel diseases; IBS: Irritable bowel syndrome; TCA: Tricarboxylic acid; HNMR: Nuclear magnetic resonance; ICR-FT/MS: Fourier transform ion cyclotron resonance mass spectrometry; GC-MS: Gas chromatography-mass spectrometry.

post-infectious IBS, may have microscopic inflammation but with normal mucosal appearance on endoscopy. Recently, a new IBS entity, IBD-IBS, has been described: these patients have pain and diarrhea similar to IBS in association with minimal or no evident intestinal inflammation[68]. The exact etiology has not been identified. Yet, environmental, psycho-social, physiological and genetic factors are believed to play a role. Also the role of an abnormal microbiota composition is supported by clinical and experimental data[69]. Disruption of the balance between the host and the intestinal microbiota results in changes in the mucosal immune system that range from overt inflammation, as seen in Crohn's disease, to low-grade inflammation without tissue injury, as seen in a subset of IBS patients[70].

Surprisingly, very little work has been conducted on metabolite profiling of IBS patients. ^{1}H-NMR metabolite profiling of fecal extracts allowed to separate IBS patients from healthy controls with moderate success (sensitivity = 57%, specificity = 76%)[49]. Two other studies applied GC/MS to analyze fecal samples although with different sample preparation. Ahmed *et al*[71] analyzed the volatile organic compounds (VOC) in the headspace of the fecal samples, *i.e.*, compounds with a vapour pressure that is sufficiently high to enable them to move from the solid or liquid phase into the gaseous phase. Those VOC comprise hydrocarbons, alcohols, aldehydes, ketones, esters and organic acids[72]. In contrast, the volatility of polar compounds in the fecal samples was increased by trimethylsilyl derivatization in the study by Ponnusamy *et al*[73]. Although both studies were able to discriminate IBS patients from healthy controls, the metabolites responsible for discrimination were different which is inherent to the different sample preparation. Ahmed *et al*[71] found increased abundance of esters in diarrhea predominant IBS-patients compared with healthy controls. Of the 28 VOCs positively associated with IBS, 22 belonged to the class of esters. Ponnusamy *et al*[73] highlighted higher levels of specific amino acids (alanine and pyroglutamate) and phenolic compounds (hydroxyphenyl acetate and hydroxyphenyl propionate) and associated these metabolic changes to alterations of specific gut microbial populations including lactobacilli and *Clostridium*.

Translating metabolites in gastrointestinal biomarkers

Metabolites are promising biomarkers because they can be easily measured from non-invasive breath, urine, feces or blood samples. Several studies have identified such metabolite signatures that should allow classification of samples as healthy or diseased. To translate these models into the clinic, additional validation studies are required. First of all, experiments to validate the biomarker model need to be performed. This level of validation includes (1) lab repeatability studies where the same samples are analyzed in the same laboratory by the same observer; (2) lab replication studies, where independent samples are analyzed in the same lab by the same observer; (3) inter-lab repeatability studies, were the original samples are analyzed in a different laboratory by a different observer; and (4) inter-lab replication studies, were independent samples are analyzed in a different laboratory.

Secondly, most studies mentioned above have compared metabolite profiles obtained from patients with a distinct diagnosis of either IBD or IBS to healthy subjects. To ensure clinical utility, it might be necessary to include additional control groups including patients with other gastrointestinal or inflammatory disorders like patients with infectious GI disease or neoplastic disease.

Thirdly, the influence of potential confounders on the performance of the models needs to be established. Confounding factors might be related to the subject (gender, age, disease location, comorbidities) or might be of environmental origin (diet, diurnal variation, medication, smoking).

CONCLUSION

Metabolomics may advance our understanding, diagnosis and treatment of inflammatory bowel disease and irritable bowel syndrome. Using this approach, disease-related mechanisms may be uncovered and verified, and candidate diagnostic biomarkers in biological samples are characterized. Before usage as clinical diagnostics, metabolites must be verified and validated in large clinical trials. To translate metabolomics data into a more profound biological understanding of the disease, more knowledge on the relevance of a decrease or increase in certain metabolites is warranted. Metabolomic profiling mainly detects associations between profiles and specific phenotypes which may not always be meaningful. In addition, it often remains unknown whether changes in metabolites are the cause or a consequence of the disease. Integration of other "Omics" with metabolomic may enable a further understanding of gastrointestinal related pathophysiological processes.

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MINIREVIEWS

Non-dietary forms of treatment for adult celiac disease

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Abstract

At present, treatment for celiac disease includes a strict gluten-free diet. Compliance, however, is difficult and gluten-free food products are costly, and, sometimes very inconvenient. A number of potential alternative measures have been proposed to either replace or supplement gluten-free diet therapy. In the past, non-dietary forms of treatment were used (*e.g.*, corticosteroids) by some clinicians, often to supplement a gluten-free diet in patients that appeared to be poorly responsive to a gluten-free diet. Some of new and novel non-dietary measures have already advanced to a clinical trial phase. There are still some difficulties even if initial studies suggest a particularly exciting and novel form of non-dietary treatment. In particular, precise monitoring of the response to these agents will become critical. Symptom or laboratory improvement may be important, but it will be critical to ensure that ongoing inflammatory change and mucosal injury are not present. Therapeutic trials will be made more difficult because there is already an effective treatment regimen.

Key words: Adult celiac disease; Gluten-free diet; Non-dietary treatment of celiac disease; Tight junction inhibition; Protease; Immunotherapy; Vaccination

Core tip: Non-dietary forms of treatment for adult celiac disease are currently being evaluated and some have reached clinical trials. Some novel approaches being investigated include hydrolysis of gliadin peptides, inhibition of intestinal permeability, blockade of T lymphocytes and transglutaminase 2/human leukocyte antigen-DQ2 functions as well as induction of immune tolerance. Future evaluations will need to define effects on specific endpoints and ensure an improvement in symptoms, laboratory test results and, most important, mucosal inflammatory changes. Therapeutic trials with novel agents will be difficult from an ethical perspective as the current form of management with a gluten-free diet already provides an excellent result for most compliant patients with celiac disease. Finally, effects on other known superimposed diseases will need close evaluation (*i.e.*, lymphoproliferative and other malignancies).

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INTRODUCTION AND DIAGNOSIS

Celiac disease is a small bowel disorder that appears to respond clinically and histopathologically to a strict gluten-free diet. Indeed, the only universally accepted form of effective therapy for celiac disease is a gluten-free diet for life after the diagnosis has been accurately established.

Diagnosis involves demonstration of the following, ideally in a sequential fashion: (1) classical histopathological features of celiac disease shown in biopsies from the proximal small bowel; and (2) a response to a gluten-free diet[1]. A very recent review and update on the prevalence, diagnosis, pathogenesis and treatment of celiac disease has appeared[2]. Some, but not all clinicians, particularly those evaluating the pediatric age group, believe that serological testing (especially with tissue transglutaminase

antibodies) coupled with definition of human leukocyte antigen (HLA)-DQ2 and HLA-DQ8, rather than biopsy may be sufficient for diagnosis[3,4].

Most patients present with diarrhea and weight loss. However, in recent years, more and more patients are now being detected with limited or no intestinal symptoms. In part, this reflects a greater appreciation by physicians for a widening spectrum of extra-intestinal changes associated with celiac disease and increased performance of screening using widely available serological markers (*e.g.*, antibodies to tissue transglutaminase, or tTG). In addition, however, some recent studies have also suggested that there may be a very real increase in celiac disease even over the past decade or so, possibly related to some, as yet, unrecognized environmental factor[5,6]. Typical biopsy changes include "flattening" of the villi with extension of the crypt epithelial cell compartment, increased numbers of plasma cells and lymphocytes in the lamina propria region, and increased numbers of intraepithelial lymphocytes. Although typical, these changes are not, in themselves, diagnostic as several disorders may mimic the changes of celiac disease[7]. Only celiac disease responds to a gluten-free diet, although some symptoms, incorrectly attributed to celiac disease, may also respond to removal of gluten from the diet.

GLUTEN-FREE DIET AND COMPLIANCE

It is well known that life-long compliance to a gluten-free diet is difficult and expensive. In reality, a major problem underlying this form of prescribed diet therapy in celiac disease is complete removal of gluten since this substance is ubiquitous and present in many foods[8]. Even foods that some authorities consider as safe, such as oats, may be contaminated with other grains that contain the injurious peptide sequences. The Food and Drug Administration in the United States has arbitrarily established a limit of < 20 ppm gluten (*i.e.*, about 10 ppm gliadin) to be established as a "gluten-free" food. Total daily consumption of gluten also appears to be critical and some experts have estimated a threshold for some individuals with celiac disease to be lower than 50 mg daily[9]. Even with these numerical considerations though, some patients with celiac disease may be even more sensitive, after only single ingestion of minute amounts of gluten. Even small amounts may provoke increased circulating levels of tissue transglutaminase antibodies and induce inflammatory changes in small bowel biopsies.

In recent years, a number of alternative dietary (*e.g.*, genetically-modified gluten) and non-dietary approaches have been considered[10-12]. Some are further detailed here including those already studied in some clinical trials as well as some that have not yet been evaluated. These might potentially serve, at least in part, in the future horizon for treatment of celiac disease. It is unlikely that any of these will be designated for independent treatment alone since the gluten-free diet, in spite of being difficult, costly and, often inconvenient, remains a highly effective management approach.

GLIADIN PEPTIDE HYDROLYSIS

Some plants and micro-organisms express endoproteolytic enzyme activities that can hydrolyze the proline-containing gluten in foods to amino acids and smaller length oligopeptides that might permit later hydrolysis by human intestinal brush border enzymes. The prolyl-endopeptidases (PEP) are a family of enzymes with the ability to cleave internal proline residues in a proline-containing peptide[13]. Even though PEP activity is expressed in the human small intestine, a gliadin peptide (*i.e.*, 33-mer) that appears to be highly immunogenic is poorly hydrolyzed by human PEP[14]. Other species, including some bacteria and fungi, express PEP activities and may, in theory, be very effective.

Aspergillus niger PEP can hydrolyze a number of gliadin peptides and its activity has been shown to inhibit the gliadin-induced immunologic response by gluten-specific T-cells[15]. In a gastrointestinal model system, most hydrolysis appeared to occur in the stomach compartment with little activity required in the small intestine[16]. Alternative PEPs from other microbial species (*Flavobacterium meningosepticum, Sphingomonas capsulata, Myxococcus Xanthus*) can hydrolyze gliadin peptides *in vivo* in the rat[17,18], and pretreatment of gluten with PEP appeared to reduce malabsorption of fat or carbohydrate in patients with celiac disease[19].

Use of enzymes that involve other mechanisms could provide different treatment approaches. For example, specific proteases cleave storage proteins during germination of different grains and, as a result, may increase the rate of gluten degradation. A barley proteinase that hydrolyzes wheat gluten in rats has been reported to potentially provide protection against ingested gluten in gluten-sensitive rhesus monkeys[20,21].

Additional studies have also suggested that different hydrolytic enzyme activities may be used in combination to improve efficiencies. For example, ALV003 consisting of PEP from *Sphinogomonas capsulata* and a barley protease may prevent the T-cell response in patients with known celiac disease[19]. In early clinical studies, orally-administered ALV003 was well tolerated without significant adverse effects[22]. Phase 2 trials are in process and, have appeared in abstract form, suggesting possible benefit.

Alternative approaches to hydrolyze toxic gluten peptides have also employed enzyme mixtures isolated from germinating *Triticeae*, including wheat, rye and barley. *In vitro* studies using intestinal epithelial cells and organ cultures of intestinal biopsies from untreated patients with celiac disease have demonstrated a reduction in markers of epithelial cell injury[23].

Another suggested alternative to facilitate gluten degradation includes use of whole cultured bacteria. Normally, a complex microbial population is present in the intestinal lumen. A number of studies from different groups[24,25] have described substantial quantitative and qualitative dif-

ferences in the intestinal microbiome of patients with celiac disease. More specifically, *bifidobacteria*, among several bacterial species, are reportedly abnormal in patients with celiac disease. *In vitro* cell culture studies as well as studies in animals have demonstrated reduced gluten toxicity and results of clinical trials are anticipated[26,27].

Sequestration of gluten by polymeric binders acting in the intestinal lumen of patients with celiac disease could be a further alternative approach. Gluten may complex with linear co-polymers of hydroxyethylmethracylate and sodium-4-styrene sulfonate to reduce toxic changes of gliadin induced in intestinal epithelial cells[28]. In addition, this agent also reduced gliadin-induced alterations in barrier function and the numbers of immunoreactive cells, including intra-epithelial lymphocytes, in mice[29]. Human effects of polymeric binders are not known, but the apparent limitation in side effects, low cost and potential for improved compliance compared to gluten-free diets is attractive.

INHIBITION OF INTESTINAL PERMEABILITY

The small intestinal mucosa in celiac disease is "leaky" with increased permeability. One of the proteins that contributes to permeability is zonulin. Larazotide acetate (*i.e.*, AT-1001) is a synthetic peptide derived from zonula occludens toxin of *Vibrio cholera*[30]. It has been hypothesized to inhibit zonulin receptor binding to reduce the gliadin-induced increases in intestinal permeability. A phase 1 evaluation in treated celiac patients suggested that the medication was well tolerated, reduced intestinal permeability, decreased pro-inflammatory cytokine production and symptoms in celiacs after gluten exposure[31]. A phase 2 evaluation showed a reduction in symptoms and autoantibodies. Added studies are needed[32].

T-CELL LYMPHOCYTE BLOCKADE AND INHIBITION

Another broad category of agents being explored include agents that function to block key lymphocyte effects on the small intestinal mucosa. Specific antagonists as well as monoclonal antibodies that affect specific lymphokines are being explored[33,34].

For example, gluten effector T-cells may be directed, at least in part, to the small intestinal mucosa by chemokine 25 and its receptor CC chemokine receptor 9. Blockade of this interaction by a selective antagonist has been hypothesized as a potential clinical approach in celiac disease.

Another suggested approach involves development of monoclonal antibodies, including anti-CD3, anti-CD20, anti-interleukin (IL)-10 anti-IL-15 antibodies[33,34]. For example, reversal of mucosal damage in the small intestine of mice with overexpression of IL-15 could provide an avenue for further evaluation.

TG2 AND HLA-DQ2 BLOCKADE

Several approaches may emerge for blockade of the adaptive immune response in celiac patients. One involves blockade of TG2 effects. TG2 enhances the binding of gliadin peptides to HLA-DQ2 and enhances T-cell activation in the small intestinal mucosa[35]. Inhibition of *in vitro* TG2 activity inhibits gliadin-specific T-cell clones from celiacs. Similar inhibition occurs in the gliadin-induced proliferations of some, but not all (*e.g.*, CD8-positive lymphocytes) lamina propria lymphocytes and epithelial cells. Although TG2 is found in other tissues, TG2 inhibitors could theoretically provide a potential avenue for future therapy.

Another area of focus has been related to development of HLA-DQ2 blocking agents using gluten peptide analogues. These include both cyclic and dimeric gluten peptide analogues as well as gluten peptides with azidoproline residues substituted for proline. By changing the gliadin T-cell stimulatory sequence, conversion to an agonist or antagonist may result[36].

IMMUNE TOLERANCE INDUCTION

In celiac disease, antigen-based therapy specific for a specific peptide sequence in gliadin might be an important future avenue of treatment. A peptide vaccine could promote tolerance by altering the effects of some immune-mediated cells involved in celiac disease pathogenesis. To date, however, definition of the precise antigen involved may not be sufficiently precise, to permit development of an effective vaccine for all celiac patients. A clinical phase 1 trial with Nexvax 2 peptide vaccine-containing a mixture of immunotoxic gliadins has been initiated[37].

CONCLUSION

A number of avenues of treatment for celiac disease have been proposed as alternatives to a strict gluten-free diet. Some of these appear to be already advanced at the level of the bench in the laboratory, and even at the bedside in some clinical trials. At this time, there are still difficult issues that need to be addressed. First, the endpoint of any treatment regimen will require detailed evaluation. The gold standard is mucosal biopsy, but other forms of non-invasive evaluation require assessment to precisely define, not only the degree of responsiveness to a specific treatment regimen, but also the quality of the treatment response. For example, improved symptoms or improved laboratory parameters may signal an improved state, but if there is ongoing inflammatory change and mucosal injury, the treatment may not be a real advance in management and may still carry the long-term risks of only partially-treated celiac disease. Second, therapeutic trials will be difficult and, by necessity from an ethical

perspective, still require that patients with celiac disease be treated in both a treatment arm and the "placebo" arm with a known effective therapy, *i.e.*, gluten-free diet. At best, in spite of the burdens imposed on the celiac patient at present, the goal of these potentially new forms of therapy in celiac disease may predictably be to supplement the gluten-free diet in long-term management of celiac disease. Finally, the long-term effects of these therapies may not be immediately evident and require many years to define. In celiac disease, there appears to be an increased risk for some malignant diseases, including lympho-proliferative diseases, such as T-cell lymphoma[38-40]. It is conceivable that some of these novel non-dietary forms of therapy may actually alter this background risk, especially over an extended period.

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ORIGINAL ARTICLE

Validation of methods to assess potential biomarkers in pediatric patients with esophageal eosinophilia

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Abstract

AIM: To validate methods for determining mast cell density, extracellular major basic protein content, and presence of fibrosis in esophageal eosinophilia.

METHODS: Twenty specimens with > 20 eosinophils/high-power field (hpf) classified as high eosinophil density (HE) and 20 specimens with < 5 eosinophils/hpf classified as low esophageal density (LE) were identified. All 40 specimens underwent immunohistochemical staining and trichrome staining. Mast cell density, extracellular major basic protein (MBP) density, and presence of subepithelial fibrosis were assessed in a standardized manner. All specimens were evaluated by two separate observers and by a single observer on two separate occasions to evaluate reproducibility of the methods.

RESULTS: A strong inter-observer correlation was noted for both peak and mean mast cell counts (r = 0.725, P < 0.0001 and r = 0.823, P < 0.0001). A strong intra-observer correlation also was noted for both peak and mean mast cell counts (r = 0.752, P < 0.0001 and r = 0.878, P < 0.0001). A very strong inter-observer correlation was noted for both peak (τ = 0.867, P < 0.0001) and mean extracellular MBP densities (r = 0.925, P < 0.0001). A very strong intra-observer correlation was noted for both peak (τ = 0.875; P < 0.0001) and mean extracellular MBP densities (r = 0.956, P < 0.0001). Excellent inter-rater reliability was found for fibrosis (κ = 0.887). Mast cell and MBP densities, as well as presence of fibrosis, were significantly increased in HE *vs* LE. The HE group had significantly higher intraepithelial mast cell peak (29.35 ± 21.61 *vs* 12.45 ± 8.26, P = 0.002) and mean (19.84 ± 15.81 *vs* 6.35 ± 4.5, P = 0.001) densities than the LE group. The HE group had significantly higher peak extracellular MBP (2.35 ± 0.67 *vs* 0.45 ± 0.61, P < 0.001) and mean extracellular MBP (1.95 ± 0.76 *vs* 0.20 ± 0.29, P < 0.0001) densities than the LE group. Seventy-three percent of patients with HE (11/15) had fibrosis, whereas only 10% of patients with LE (1/10) had fibrosis (P < 0.01). MBP performed the best in predicting classification of HE *vs* LE, with mean MBP demonstrating 100% sensitivity and 95% specificity at the optimal cut point.

CONCLUSION: This study provides methodology and proof-of-concept for future evaluation of these biomarkers for differentiating esophageal eosinophilic diseases such as reflux esophagitis and eosinophilic esophagitis.

Key words: Eosinophilia; Immunohistochemical staining; Tryptase; Major basic protein; Subepithelial fibrosis

Core tip: Esophageal mucosal eosinophilia challenges many clinicians and researchers. Biomarkers have been

proposed to help clarify and better characterize eosinophil driven disease. This study provides validation of methods used previously to differentiate low eosinophil density from high eosinophil density. Eosinophilic major basic protein appears to be the best predictor for classification of eosinophilia at both extremes. This lays the ground work for future studies to examine varying degrees of eosinophilia using biomarkers.

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INTRODUCTION

It is well known that the presence of eosinophils in esophageal mucosa denotes pathology; however, the basis for eosinophilic infiltration is not always clear and has been a topic of numerous studies in both children and adults[1]. Mucosal eosinophils are increased in both reflux esophagitis (RE) and eosinophilic esophagitis (EoE) as well as Candidal esophagitis, viral esophagitis and Crohn's esophagitis to name a few. The two most common causes of increased eosinophils without other disease features are reflux esophagitis and eosinophilic esophagitis. Determining the density of eosinophils (*i.e.*, cells per high-power field, hpf) present on biopsy specimens is one factor considered in distinguishing between these two diagnoses[1-6]. Most studies suggest that a significantly higher number of eosinophils are found in patients with EoE; however, the appropriate number needed to make a diagnosis of EoE is unclear[1]. This is problematic as the two forms of esophagitis have markedly different treatments and prognosis. Thus, finding additional biomarkers to aid in diagnosis is warranted.

Examination of the pathway through which eosinophils exert their influence in EoE may shed light on potential biomarkers. First, eosinophils, which are normally absent in the esophagus, may infiltrate the esophageal mucosa and become activated. Once activated, eosinophils release their granule proteins causing inflammation and tissue damage. Granule proteins, such as major basic protein (MBP), eosinophilic cationic protein, and eosinophilic peroxidase, have cytotoxic effects on esophageal epithelium[7-9]. Their pro-inflammatory properties continue the cycle of inflammation. Eosinophils elaborate fibrogenic growth factors and induce fibrogenesis through secretion of granule proteins such as MBP. MBP also triggers degranulation of mast cells which, in turn, release inflammatory mediators such as cytokines and histamine[7,8].

Mucosal mast cells may have a role as a biomarker in that they are increased in pediatric patients with EoE as compared to RE, or gastroesophageal reflux disease (GERD), which is often used interchangeably with RE[9]. Mast cells are present at higher densities in GERD patients exhibiting > 7 eosinophils/hpf[10]. Moreover, no intraepithelial esophageal mast cells are present in controls[10]. Further, mast cell density has been documented to decrease with treatment of EoE[11].

Extracellular MBP also shows promise as a biomarker for EoE. Chehade *et al*[12] classified eosinophil degranulation as either absent/mild or extensive based on the pattern of extracellular MBP. Extensive degranulation was more prevalent in EoE patients and the extent of degranulation was unrelated to eosinophil density. Mueller *et al*[5] studied EoE in adults, evaluating MBP by a semi-quantitative method, and found degranulation in 72%.

Fibrosis also may be a potential biomarker, as indicated by the increased frequency of esophageal stricture in EoE[1]. In children, esophageal subepithelial fibrosis has been demonstrated in 89% of EoE patients as compared to 37.5% of GERD patients[13]. Although fibrosis was found in a significant portion of the GERD patients, the pattern was clearly different than with EoE, as the fibrosis was associated with lymphoid tissue in GERD. Chehade *et al*[12] documented fibrosis in 57% of EoE pediatric patients and in no patients with GERD. Moreover, the presence of fibrosis in the EoE group was associated with a higher proportion of patients demonstrating extensive eosinophil degranulation[12].

Evaluation of mast cell density, extracellular MBP, and/or fibrosis may be useful in making a diagnosis of EoE, particularly in patients with eosinophil counts placing in the mid-range between typical reflux-associated esophagitis and EoE on biopsy. While mast cell density, extracellular MBP, and fibrosis all appear to be potential markers, the methods for histologic evaluation of these have varied and, further, reproducibility and reliability of these markers in differentiating RE and EoE has not yet been established.

The current study was performed in two steps. In part one, the aim was to establish reproducible methods for determining mast cell density, extracellular MBP content, and presence of fibrosis, respectively. In part two, the primary aim was to determine whether the reproducible markers validated in the first step would reliably differentiate between patients with high esophageal density and patients with low esophageal density. A secondary aim was to explore relationships between eosinophil density and the potential biomarkers.

MATERIALS AND METHODS

The study was approved by the institutional review board at Children's Mercy Hospital, Kansas City, Missouri.

Selection

Archived distal esophageal specimens from patients previously undergoing endoscopy by a pediatric gastroenterologist during a one year period were identified from the pathology database at Children's Mercy Hospital. No medical chart review was performed for this methodology study. Specimens with esophageal eosinophilia were reviewed. Twenty cases classified as low esophageal den-

sity (LE; 1-4 eosinophils/hpf) and twenty cases classified as high esophageal density (HE; > 20 eosinophils/hpf) were chosen. All biopsy specimens, which were previously fixed in formalin, embedded in paraffin, sliced (5-μm sections), and stained with haematoxylin and eosin, were independently evaluated by a pediatric gastroenterologist who confirmed the number of eosinophils/hpf prior to inclusion of the specimen in this study. The entire specimen was scanned to identify the subjective area of greatest eosinophil density. Eosinophils were counted in five consecutive hpfs. Peak and mean (the average of the 5 hpfs) eosinophil counts were recorded.

Sample processing

Mast cells: The archival tissue blocks of esophageal mucosa were evaluated by immunohistochemical (IHC) staining using the labeled streptavidin-biotin (LSAB) method. The paraffin-embedded tissue specimens were de-paraffinized using a xylenes-substitute followed by 100% ethanol then rehydrated in aqueous buffer. No antigen retrieval was performed on tissue for anti-tryptase staining. The primary antibody fixation occurred using mouse monoclonal anti-human mast-cell tryptase (Clone AA1) diluted to 1:1000. Tissues were incubated with anti-tryptase for 1 h at room temperature (20 ℃-25 ℃). After washing in aqueous buffer, a secondary antibody using biotin-anti immunoglobulin G (IgG) was incubated for 20 min at room temperature. The specimens again were washed in aqueous buffer. Specimens were incubated with streptavidin-horseradish peroxidase at room temperature for 20 min, washed in aqueous buffer, followed by the addition of diaminobenzidine (DAB) hydrogen peroxide solution for 5 min at room temperature. Specimens were washed in running tap water for 5 min. All specimens were counterstained with haematoxylin followed by bluing solution and then mounted for microscopic exam.

Major basic protein: The archival tissue blocks of esophageal mucosa were evaluated by IHC staining using the LSAB method. The paraffin-embedded tissue specimens were de-paraffinized using a xylenes-substitute followed by 100% ethanol then rehydrated in aqueous buffer. Tissue was digested with 0.5% pepsin for 30 min at 37 ℃ for antigen retrieval. Peroxidase activity was inactivated using 3% hydrogen peroxide solution for 5 min at room temperature. To reduce background staining, normal goat serum block (5% goat serum in distilled water) and avidin/biotin blocking systems were used. The primary antibody fixation occurred using mouse monoclonal anti-human e-MBP (Clone BMK13) diluted to 1:30. Tissues were incubated with anti-MBP for 24 h at 4 ℃. After washing in aqueous buffer, a secondary antibody using biotin-anti IgG was incubated for 20 min at room temperature. The specimens were washed in aqueous buffer. Specimens were incubated with streptavidin-horseradish peroxidase at room temperature for 20 min, washed in aqueous buffer, followed by the addition of DAB hydrogen peroxide solution for 5 min at room temperature. Specimens were washed in running tap water for 5 min. All specimens were counterstained with haematoxylin followed by bluing solution and then mounted for microscopic exam.

Fibrosis: The archival tissue blocks of esophageal mucosa were subjected to trichrome staining using Gomori's one-step method. The paraffin-embedded tissue specimens were de-paraffinized using xylenes and then rehydrated in distilled water. Slides were pretreated with hot Bouin's solution for 1 h. After washing well in running water to remove all yellow color, slides were placed in Gill's Haematoxylin for 5 min. Slides were washed in tap water then stained with Gomori's trichrome for 10 min. They were rinsed briefly in 1% acetic acid solution then rinsed quickly in distilled water. The tissue specimens were dehydrated in 100% ethyl alcohol and then mounted for microscopic exam.

Sample evaluation

Mast cell density: Mast cell density was assessed by subjectively identifying the area of greatest involvement after scanning the entire specimen and then counting tryptase-positive cells in 5 consecutive hpfs (Figure 1A). Mast cell enumeration was performed by 2 blinded observers and by one of the observers (Observer 1) on 2 separate occasions separated by at least one week to establish inter-rater and intra-rater reliability for the method. Peak and mean mast cell densities were recorded.

Major basic protein: Specimens initially were subjectively evaluated in a blinded fashion by a pathologist and two observers to determine a stratification strategy for extracellular MBP. MBP has been previously been evaluated by semi-quantitative methods with classification into either 2 or 4 categories[5,12]. A decision was made to employ a 4-point scale as follows: 0, none; 1, mild (< 5% involvement of MBP granules); 2, moderate (5%-25% involvement of MBP granules); and 3, severe (> 25% involvement of MBP granules) (Figure 1B-E). We elected to evaluate with 4 categories because our pre-decision specimen evaluation seemed to indicate that this was a feasible solution and because 4 categories gave us the possibility of a more robust method if reproducible. Extracellular MBP density was assessed by subjectively identifying the area of greatest involvement after scanning the entire specimen and then rating MBP density on the 4 point scale in 5 consecutive hpfs. MBP evaluation was performed by the same 2 blinded observers and by one of the observers (Observer 1) on 2 separate occasions separated by at least one week to establish inter-rater and intra-rater reliability for the method. Peak and mean densities of extracellular MBP were recorded.

Fibrosis: Subepithelial fibrosis was assessed by the trichrome stain and rated as normal (no fibrosis seen) or abnormal (increased collagen deposition) (Figure 1F). The entire specimen was scanned to determine the presence of collagen deposition. Specimens were evaluated

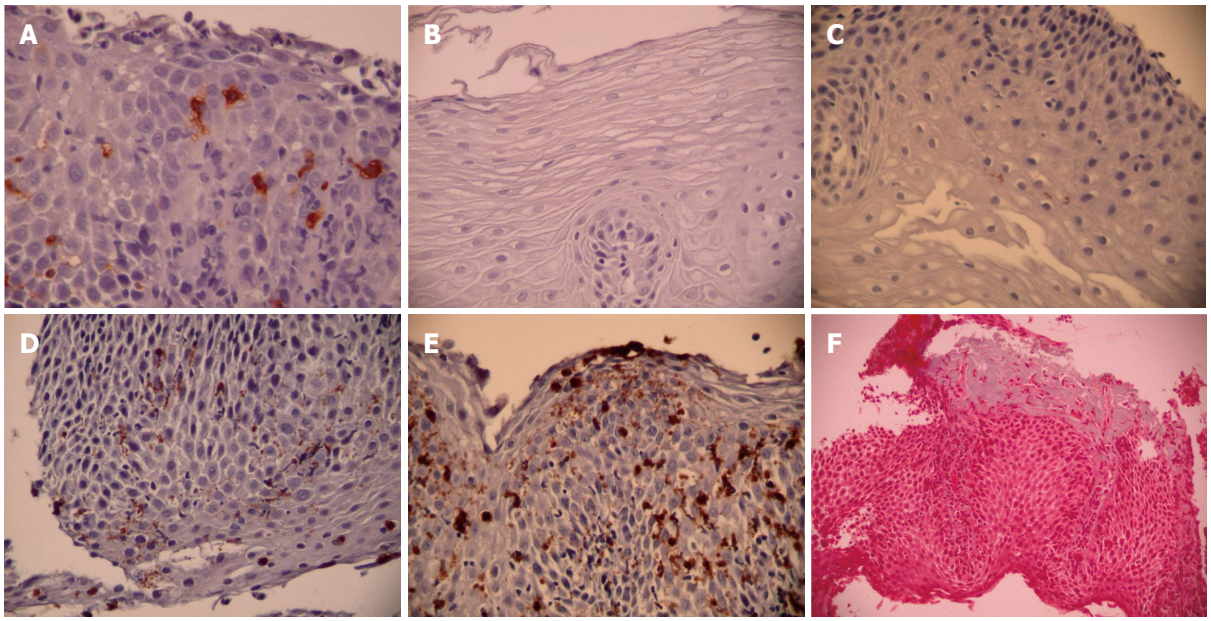

Figure 1 Sample evaluation. A: Tryptase staining for mast cells; B: Grade 0 major basic protein (MBP) involvement (none); C: Grade 1 MBP involvement (< 5%); D: Grade 2 MBP involvement (5%-25%); E: Grade 3 MBP involvement (> 25%); F: Trichrome staining for fibrosis.

by the same 2 blinded observers and by one of the observers (Observer 1) on 2 separate occasions separated by at least one week to establish inter-rater and intra-rater reliability for the method.

Statistical analysis

Measures of agreement were calculated to establish reliability between the two observers for each of the three markers. Measures of agreement also were calculated for mast cell density and extracellular MBP to establish reproducibility on the two separate evaluations by Observer 1; no reproducibility evaluation was conducted for fibrosis given the simple dichotomous nature of this variable. Pearson's correlation was employed for mast cell density and mean extracellular MBP, while Kendall's tau was used for peak extracellular MBP and kappa was used for fibrosis.

Once reproducible methods were obtained, differences in mast cell density, extracellular MBP, and presence of subepithelial fibrosis were compared between HE and LE by a combination of Student's *t* test and χ^2. Receiver operating characteristic (ROC) curves were used to determine the sensitivity and specificity of different mast cell and extracellular MBP densities, respectively, in predicting classification group membership (*i.e.*, HE *vs* LE) based on eosinophil counts completed at the time of biopsy. Correlations between eosinophil, mast cell, and extracellular MBP densities were evaluated by Pearson's correlation. Statistical analysis was performed with SPSS version 16.0. A *P* value of 0.05 was considered significant.

RESULTS

The HE group had significantly more eosinophils/hpf (peak: 96.45 ± 45.6; mean: 63.07 ± 27.99) than the LE group (peak: 2.10 ± 1.07; mean: 0.86 ± 0.61, $P < 0.0001$). Peak eosinophil density ranged from 39-201/hpf in the HE patients and from 1-4/hpf in the LE group. In all cases, the original classification was confirmed and served as the gold standard for group assignment (HE *vs* LE).

Step 1: Reliability and reproducibility

Mast cell density: A strong inter-observer correlation was noted for both peak and mean mast cell counts ($r = 0.725$, $P < 0.0001$ and $r = 0.823$, $P < 0.0001$). A strong intraobserver correlation also was noted for both peak and mean mast cell counts ($r = 0.752$, $P < 0.0001$ and $r = 0.878$, $P < 0.0001$).

Major basic protein: A very strong inter-observer correlation was noted for both peak ($\tau = 0.867$, $P < 0.0001$) and mean extracellular MBP densities ($r = 0.925$, $P < 0.0001$). A very strong intra-observer correlation was noted for both peak ($\tau = 0.875$, $P < 0.0001$) and mean extracellular MBP densities ($r = 0.956$, $P < 0.0001$).

Fibrosis: Excellent inter-rater reliability was found for fibrosis ($\kappa = 0.887$).

Step 2: Biomarker comparison between HE and LE

Mast cell density: The HE group had significantly higher intraepithelial mast cell peak (29.35 ± 21.61 *vs* 12.45 ± 8.26, $P = 0.002$) and mean (19.84 ± 15.81 *vs* 6.35 ± 4.5, $P = 0.001$) densities than the LE group (Figure 2A). Peak mast cell density ranged from 3-89 in the HE group and from 4-32 in the LE group. Mean mast cell density ranged from 1.4-65.0 in the HE group and from 2.0-17.8 in the LE group. ROC curve analysis indicated that both mean (AUC = 0.839, $P < 0.0001$) and peak (AUC = 0.795, $P < 0.001$) mast cell density differentiate between HE and LE, but no specific cut point could be identified with adequate sensitivity and specificity. The best performer in accurately classifying HE was a cut-off 11.2 mast cells/hpf (*i.e.*, aver-

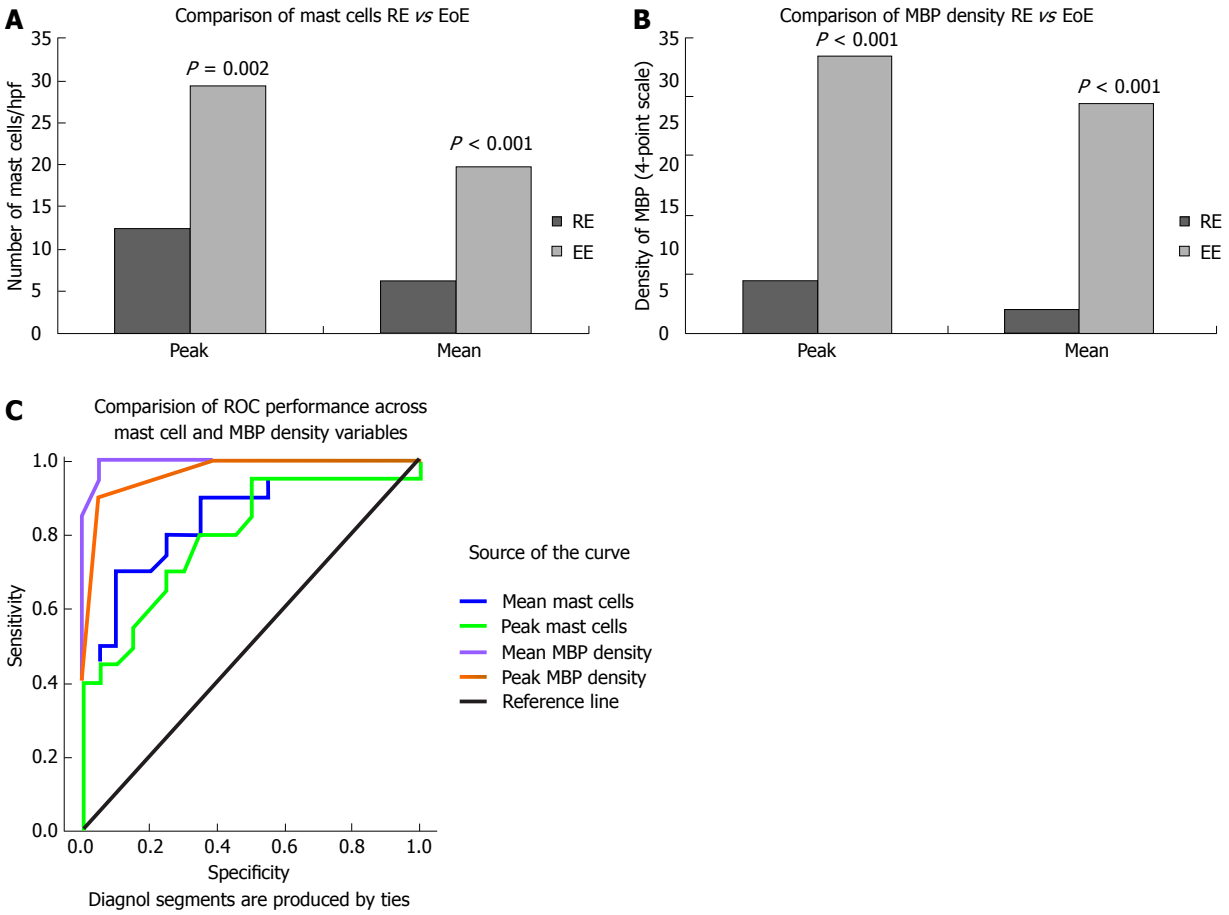

Figure 2 Biomarker comparison between high eosinophil and low esophageal density. A: Comparison of mast cells reflux esophagitis (RE) *vs* eosinophilic esophagitis (EoE); B: Comparison of major basic protein (MBP) density RE *vs* EoE; C: Comparison of receiver operating characteristic (ROC) performance across mast cell and MBP density variables.

age across 5 consecutive hpfs in the area deemed to have greatest involvement after visual scan), which detected a true positive classification for HE 70% of the time and a false positive classification for HE 10% of the time.

Major basic protein: The HE group had significantly higher peak extracellular MBP (2.35 ± 0.67 *vs* 0.45 ± 0.61, $P < 0.001$) and mean extracellular MBP (1.95 ± 0.76 *vs* 0.20 ± 0.29, $P < 0.0001$) densities than the LE group (Figure 2B). Ninety percent of patients with HE (18/20) had moderate to severe peak staining with extracellular MBP, whereas 95% of patients with LE (19/20) had none to mild peak staining for extracellular MBP. ROC curve analysis indicated that both mean (AUC = 0.995, $P < 0.0001$) and peak (AUC = 0.966, $P < 0.0001$) extracellular MBP density differentiate between HE and LE. The best performer in accurately classifying HE was a cut-off of 0.7 for mean extracellular MBP (*i.e.*, average across 5 consecutive hpfs in the area deemed to have greatest involvement after visual scan), which detected a true positive classification for HE 100% of the time and a false positive classification for HE only 5% of the time (Figure 2C).

Fibrosis: Fifteen specimens were excluded because of the lack of lamina propria. Seventy-three percent of patients with HE (11/15) had fibrosis, whereas only 10% of patients with LE (1/10) had fibrosis ($P < 0.01$).

Relationships between markers in HE patients: Mean eosinophil density was correlated with both peak ($r = 0.495$, $P = 0.03$) and mean ($r = 0.610$, $P = 0.004$) extracellular MBP density, while peak eosinophil density was correlated with mean ($r = 0.539$, $P = 0.01$), but not peak, extracellular MBP density. There was no correlation between eosinophil density and mast cell density or between mast cell density and extracellular MBP density, respectively. There was no relationship between the presence of fibrosis and densities of eosinophils, mast cells, or extracellular MBP, respectively.

DISCUSSION

When attempting to establish clinically meaningful biomarkers, the process includes several challenging steps. Establishing reproducible and valid methods for measuring or identifying the markers, demonstrating that the markers differ, noting adequate sensitivity and specificity of the markers, and demonstrating that the marker prospectively predicts treatment response, disease progression, and/or prognosis are all necessary components. The current study was undertaken to evaluate the first of these steps for three potential biomarkers supported by

previous studies as differing in patients with HE *vs* LE, namely: mast cell density, extracellular MBP density, and the presence of fibrosis. The esophageal specimens in this study were chosen at extremes (LE < 5 eosinophils/hpf and HE > 20 eosinophils/hpf) in an attempt to validate each of the biomarkers individually. A future direction would apply this validated methodology to varying degrees of eosinophilia and diagnostic dilemmas.

The methodology of immunohistochemical staining to differentiate extreme degrees of eosinophilia is valid based on our study. Applying this method to future studies with less extreme degrees of eosinophilia is warranted. If this method can accurately distinguish varying degrees of eosinophilia on initial biopsies, then follow-up biopsies which are often necessary to clarify diagnoses can be eliminated. For example, distinguishing reflux esophagitis from eosinophilic esophagitis has important implications for the patient, as the treatment and natural history vary greatly for each disorder. Currently, an initial biopsy with determination of the number of eosinophils is performed on patients with clinical symptoms of dysphagia, vomiting, acid reflux, and/or abdominal pain. If a patient is not taking proton pump inhibitor, a trial of high dose proton pump inhibitor is usually followed by a second endoscopy with biopsy to clarify or confirm the diagnosis and hence distinguish between severe RE or GERD and EoE.

Basal cell hyperplasia, papillary elongation, and eosinophilic infiltration are all non-specific findings of esophagitis, even though they tend to be more prominent in patients with EoE[3,5,6,13-15]. The actual number of eosinophils per hpf is the only current histologic parameter used, in part, to establish the diagnosis of EoE. This might be simple enough for the most mild cases of RE and the most severe cases of EoE; however, clear cut-offs are not established and in the subtle, indeterminate cases, it is clearly not enough. Identifying biomarkers capable of differentiating EoE and RE has the potential to decrease the diagnostic burden of additional tests, the costs of evaluation, and sequelae associated with treatment delay in EoE.

We also sought to provide proof-of-concept for the second step by determining whether the markers differed between HE and LE. We were able to demonstrate excellent intra-and inter-observer reproducibility for the methodologies employed, with reliabilities above 0.80 for all methods except peak mast cell density counts, which fell only slightly below this threshold (0.73-0.75).

After demonstrating reproducibility, we undertook the next step of evaluating whether the particular markers could predict group assignment for HE *vs* LE. Mast cell density, density of extracellular MBP, and presence of fibrosis were all significantly greater in the HE group when compared to the LE group. These findings are consistent with previous studies evaluating histological differences between RE and EoE in both children and adults[5,10,12,16,17]. However, no particular cut point could be identified for mast cell density which demonstrated both good sensitivity and specificity. The presence of fibrosis appeared to be more useful in differentiating between EoE and RE. Fibrosis was present in 73% of HE patients and 10% of LE patients. However, the biggest challenge with using fibrosis as a marker is having biopsies deep enough to be evaluable. We had to exclude 38% of our specimens because we did not have adequate lamina propria. This difficulty has been previously reported[2]. In the current study, quantifying extracellular MBP appeared to be the most promising method for differentiating HE and LE (and potentially EoE from RE), with mean MBP density, in particular, yielding excellent sensitivity and specificity in the diagnostic classification of HE.

Extracellular MBP is a marker of eosinophil activation and degranulation. Previous electron microscopic studies of esophageal eosinophils in esophagitis have demonstrated eosinophil activation indicated by inversion of core-to-matrix densities and lucency of core protein[18]. This core lucency corresponds to the release of MBP. From a practical standpoint, the evaluation of extracellular MBP can be performed on the routine biopsies obtained during endoscopy but does require immunohistochemical staining, as MBP or extracellular granules are not sufficiently detected by routine staining[5]. The cost and feasibility of IHC staining for extracellular MBP granules appear reasonable; however, this would need to be confirmed with future prospective studies. Although there was moderate correlation between eosinophil density and MBP density, eosinophil enumeration alone does not predict MBP density; thus, while related, these two measures appear non-redundant and may add unique information helpful in the diagnosis of EoE.

We have identified reproducible methodologies for evaluating three potential biomarkers in differentiating LE from HE. Of the three, semi-quantitative assessment of extracellular MBP appears to be the most promising, with both mean and peak values performing well in terms of sensitivity and specificity on a group of patients with HE based on ROC curve analysis. It remains to be seen whether extracellular MBP can add to diagnostic differentiation in prospective studies, particularly histologically indeterminate cases, but this is certainly an important direction for future research. This is a very important topic for future studies. It would be necessary to correlate a sensitive and specific biomarker with clinical symptoms, disease activity, pathology findings, and treatment response. This initial validation of methodology study provides evidence for future studies. Future work in this area will help establish whether extracellular MBP, or other potential biomarkers for EoE, will be able to predict responses to treatment or prognosis in a prospective fashion to reduce diagnostic burden, evaluation costs, and sequelae associated with treatment delay in EoE.

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COMMENTS

Background

Inflammation in the esophagus can cause clinical symptoms of pain in the chest or upper abdomen, difficulty swallowing, vomiting or regurgitation. The most common type of inflammation in the esophagus is due to eosinophilic inflammation. While a number of disorders can cause eosinophilic inflammation, the two most common are reflux esophagitis and eosinophilic esophagitis. These two disorders have similar clinical complaints and are challenging to differentiate. These disorders have different treatment pathways so it is important to make not only an accurate, but also a timely diagnosis. Using biomarkers to help distinguish these two disease entities will allow for more efficient and accurate diagnoses. There have not been any established or validated biomarkers for these diseases to date.

Research frontiers

Identifying biomarkers for diagnosis and management of eosinophilic disorders of the esophagus is a current hotspot for research. A biomarker that is reproducible, inexpensive, non-invasive, and corresponds to the severity of disease would be a major advantage in the treatment of children with eosinophilic disorders of the esophagus.

Innovations and breakthroughs

To date, biomarkers for eosinophilic disease of the esophagus have been described; however, not validated for their reliability or reproducibility. This study is the first step in validation of methods to assess for potential biomarkers. Being able to differentiate between low eosinophil density *vs* high eosinophil density will aid in timely diagnosis of disease and decrease the need for multiple biopsies on separate occasions to better characterize the disorder.

Applications

This study demonstrated that extracellular major basic protein is the best predictor of determining the degree of esophageal eosinophilia at two extremes. This was both reliable and reproducible. This provides a basis for future studies to examine varying degrees of eosinophilia using extracellular major basic protein as a biomarker for eosinophilic disease of the esophagus.

Terminology

Eosinophils: Eosinophils are white blood cells that are normally produced in the body in response to allergic or parasitic conditions; Major basic protein: Major basic protein (MBP) is a granule protein released by the activated eosinophil. MBP also triggers degranulation of mast cells which, in turn, release inflammatory mediators such as cytokines and histamine. Immunohistochemical staining: Immunohistochemical staining is a process for detecting proteins by using antibodies that bind to specific antigens.

Peer review

The present study is an interesting study for validation of methods for assessing potential biomarkers for eosinophilic disease of the esophagus. Future work should include correlation of the validated methods with symptoms, disease severity and treatment response.

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BRIEF ARTICLE

Four-year follow-up of endoscopic gastroplication for the treatment of gastroesophageal reflux disease

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Abstract

AIM: To evaluate the long-term effect of Endocinch treatment for gastroesophageal reflux disease (GERD).

METHODS: After unblinding and crossover, 50 patients (32 males, 18 females; mean age 46 years) with pH-proven chronic GERD were recruited from an initial randomized, placebo-controlled, single-center study, and included in the present prospective open-label follow-up study. Initially, three gastroplications using the Endocinch device were placed under deep sedation in a standardized manner. Optional retreatment was offered in the first year with 1 or 2 extra gastroplications. At baseline, 3 mo after (re) treatment and yearly proton pump inhibitor (PPI) use, GERD symptoms, quality of life (QoL) scores, adverse events and treatment failures (defined as: patients using > 50% of their baseline PPI dose or receiving alternative antireflux therapy) were assessed. Intention-to-treat analysis was performed.

RESULTS: Median follow-up was 48 mo [interquartile range (IQR): 38-52]. Three patients were lost to follow-up. In 44% of patients retreatment was done after a median of 4 mo (IQR: 3-8). No serious adverse events occurred. At the end of follow-up, symptom scores and 4 out of 6 QoL subscales were improved (all $P < 0.01$ compared to baseline). However, 80% of patients required PPIs for their GERD symptoms. Ultimately, 64% of patients were classified as treatment failures. In 60% a post-procedural endoscopy was carried out, of which in 16% reflux esophagitis was diagnosed.

CONCLUSION: In the 4-year follow-up period, the subset of GERD patients that benefit from endoscopic gastroplication kept declining gradually, nearly half opted for retreatment and 80% required PPIs eventually.

Key words: Endoscopic therapy; Endocinch; Gastroesophageal Reflux; Gastroplication; Follow-up studies

Core tip: The long-term efficacy of the first commercially available endoluminal suturing device for the treatment of gastroesophageal reflux disease (GERD), Endocinch, was evaluated. In the 4-year follow-up period, the subset of GERD patients that benefit from endoscopic gastroplication kept declining gradually. Up to 80% of patients again required acid-suppressive medication, making this endoscopic treatment procedure unsuccessful for the majority of GERD patients.

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INTRODUCTION

Gastroesophageal reflux disease (GERD) is a common disorder with a substantial impact on quality of life and healthcare resources. Often, it is a chronic ailment that requires maintenance medical therapy. Important in the pathophysiology of GERD are a low basal tone of the lower esophageal sphincter (LES), transient LES relaxations (TLESRs), and the presence of a hiatal hernia[1]. Laparoscopic fundoplication normalizes esophageal acid exposure by tightening the LES and repairing the hiatal hernia. Five years after surgery the overall patient satisfaction rate is still as high as 88% and only 14% requires daily antisecretory medication[2]. Indications for laparoscopic fundoplication are medication side-effects, incomplete relief of GERD symptoms despite medical treatment, or reluctance to take lifelong maintenance medication.

In the last two decades, the drawbacks inherent to a surgical procedure led to the development of endoscopic therapies for the treatment of GERD. These endoscopic procedures aimed to include the advantages of surgery, *i.e.*, cure of the disease, while avoiding the complications of a surgical procedure. Various types of endoscopic procedures (thermal ablation, injection or implantation techniques and suturing devices) were designed and implemented. Several have already been withdrawn from the market because of safety concerns or lack of efficacy. In general, suturing devices raised highest expectations. They were designed to place stitches at or just below the esophagogastric junction, thereby creating gastroplications that tightened the LES. The Endocinch device is one of the first generation devices that is still available for commercial use.

Only a few studies have published long-term results (18-41 mo)[3-6]. In 2007 we completed a 3-mo, randomized, sham-controlled trial with a total follow-up duration of one year[7]. During short to medium-term follow-up, symptoms improved moderately, with no significant effects on acid exposure compared to sham. Like others, we had concerns about the durability of the sutures[8]. In a recent systematic review of randomized controlled trials and comparative studies on endoscopic treatments for GERD it was concluded that there is still not sufficient evidence to determine the long-term efficacy and safety of Endocinch[9]. The present study followed up on the 3-mo sham-controlled trial and aimed to prospectively evaluate its long-term efficacy and safety.

MATERIALS AND METHODS

We conducted a prospective follow-up study until December 2008, which included all patients that were treated with Endocinch in the initial single-center, randomized trial that started in August 2003.

Approval for these studies was granted by the medical ethics committee of the University Medical Center Utrecht, the Netherlands. It was obtained prior to the start of the original study and once more during the long-term follow-up period.

Study design

The single-center, double-blind, randomized, placebo-controlled trial included 60 patients that were allocated to 3 groups (Figure 1). Each group contained 20 patients. After 3 mo, the remaining untreated patients in the sham and observation groups were offered Endocinch treatment. 30 patients that agreed to cross over were successfully treated with Endocinch. Within the first year, patients that were classified as failures (defined as: unsatisfactory symptom response and/or < 50% reduction of their baseline dose of antisecretory medication) were offered retreatment. A total of 50 patients were included in the present follow-up study.

Endpoints were prospectively defined and included: GERD symptoms (heartburn and regurgitation), use of antisecretory medications, quality of life, adverse events, retreatment with Endocinch, and other reflux treatments. Patients were assessed on a yearly basis and these data were compared with pretreatment (baseline) and 3 mo post-treatment data.

The study objective was to establish whether the short-term effects would last. The hypotheses were that in the long-term: (1) symptoms; and (2) use of acid-suppressive medication would no longer be reduced compared with baseline values.

Patients

All randomized patients met the following inclusion criteria: persistent heartburn and/or regurgitation, and at least partial response to anti-secretory drugs and dependence on them for at least 1 year, unwillingness to take drugs lifelong, and esophageal pH results compatible with GERD diagnosis (> 5% of the time a pH < 4 or a 95% symptom association probability). Exclusion criteria were: < 18 years of age, severe esophageal motility disorder on manometry, hiatus hernia > 3 cm in length, history of thoracic or gastric surgery, reflux esophagitis grade C or D (LA classification), Barrett's epithelium, severe comorbidity (cardiopulmonary disease, portal hypertension, collagen diseases, morbid obesity and coagulation disorders), use of anticoagulant or immunosuppressive drugs, or a history of alcohol or drug misuse.

Before enrollment in the 3-mo trial each patient's eligibility was assessed. Patients underwent an upper endoscopy. The required daily dose of acid-suppressive medication to achieve optimal symptom control was recorded during a 1-mo run-in period. Subsequently, an esophageal manometry and ambulatory 24-h pH monitoring were performed after discontinuation of medication for 1 whole week (the results were published in a previous article)[7]. Prior to randomization, written informed consent was obtained from all patients.

Baseline and follow-up assessment

The pre-treatment (baseline) questionnaire was completed at the end of the run-in period of the initial trial

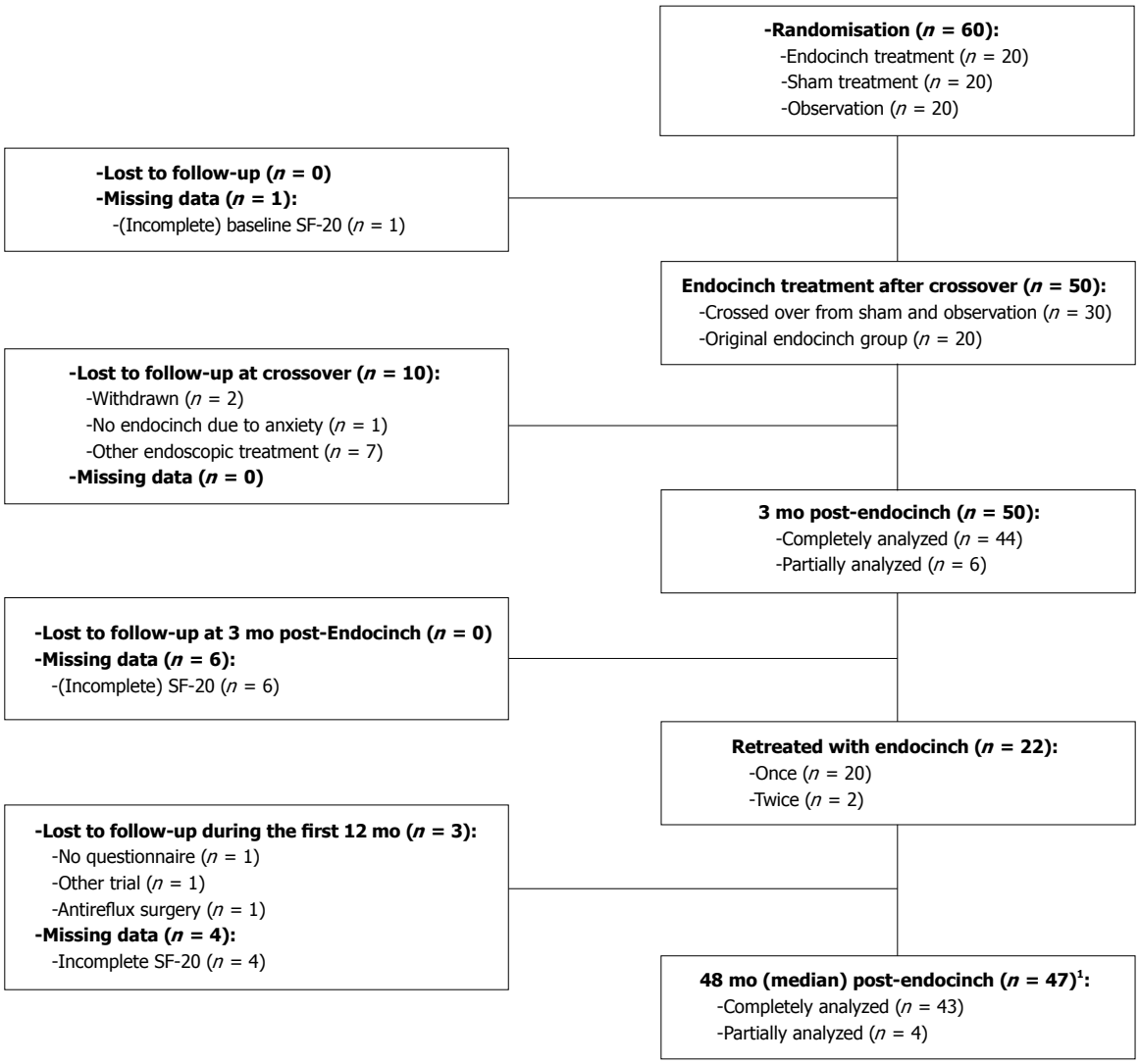

Figure 1 Flow diagram of follow-up. [1]Between 12 and 48 mo, 9 patients had antireflux treatment: 8 patients underwent antireflux surgery and 1 an alternative endoscopic treatment.

while patients were still off acid-suppressive medication. At 3 mo after randomization a second questionnaire was filled out (off medication). Esophageal manometry and ambulatory 24-h pH monitoring were repeated in the active and sham groups. Patients that were retreated underwent the same follow-up procedure. Additional reassessment took place at 6 and 12 mo. From 12 mo on, patients were reassessed with a yearly questionnaire. In accordance with intention to treat analysis, questionnaires from treatment failures were included.

Treatment procedure

Endoscopic suturing was carried out with the Endocinch suturing device (BARD Endoscopic Technologies, CR Bard, Billerica, MA, United States). The initial treatment aimed to create three gastroplications. During the first year of follow-up, patients with failure of therapy were offered retreatment consisting of one or two extra plications. All procedures were performed by the same endoscopist (MPS). The procedure was carried out using two video gastroscopes (type GIF-160, Olympus Nederland BV, Zoeterwoude, The Netherlands) and the endoscopic suturing device. After endoscopic placement of an esophageal overtube, two stitches were placed adjacent to one other, using the same thread. The first gastroplication was positioned about 1.5-2.0 cm below the squamocolumnar line along the lesser curvature. The second endoscope was used to create a plication by tightly pulling the sutures together and placing a suture anchor. A second plication was placed 1 cm above the first, and a third plication was placed at the level of the second along the greater curvature. All procedures were carried out under deep sedation with a combination of midazolam and pethidin administered intravenously. Oxygen saturation was monitored during the procedure. Afterwards, patients were observed for a period of 4 h during which blood pressure and heart rate were measured hourly.

Questionnaire

GERD symptoms were scored utilizing a 6-point numeric scale measuring heartburn and regurgitation frequency

(< 1 per month, once monthly, once weekly, once daily, 25 times per day, > 5 times daily) and a 4-point numeric scale measuring severity (not, mildly, moderately and very severe)[10]. The heartburn and regurgitation symptom scores were calculated by multiplying the frequency with the severity score. Quality of life assessments were done using the 20-item Short-Form Health Survey (SF-20). The SF-20 consists of 20 items that assess the following 6 aspects: limitations in physical, role and social activities due to health problems, perception of mental and general health, and bodily pain. Each individual scale was transformed to a 100-point scale, 0 being the worst and 100 the best score, except in bodily pain perception in which a higher score stands for experiencing more pain.

Statistical analysis

The mean SD is provided when data are normally distributed, the median [interquartile range (IQR)] when the distribution is not normal.

The Wilcoxon signed-ranks test (nonparametric two-related samples) was used to test whether post-treatment values differed from baseline values. Whether two variables were related to each other was explored with logistic regression analysis. Differences and relations were considered significant if $P < 0.05$.

RESULTS

Follow-up

Baseline characteristics are provided in Table 1. Follow-up and missing data are presented in Figure 1. The median duration of follow-up was 48 mo (IQR: 38-52). Three patients were lost to follow-up in the first 12 mo. Between 12 and 48 mo nine patients reached the following predefined endpoints: 8 patients underwent antireflux surgery and one patient received an alternative endoscopic treatment. Four SF-20 questionnaires were either incomplete or missing. Therefore, 43 patients were analyzed completely, and 4 partially. Twenty-two (44%) patients were retreated with a mean of 1.4 plications, after a median period of 4 mo (IQR: 3-8). The median follow-up period after retreatment was 33 mo (IQR: 15-48). Twenty-eight patients (60%), all with persistent complaints, underwent a postprocedure upper gastrointestinal endoscopy. A mean of 0.73 stitches per patient were judged as functional. In twelve patients (24%) erosive reflux esophagitis was diagnosed (grade A, $n = 7$; grade B, $n = 4$; grade C, $n = 1$).

Table 1 Baseline patient characteristics[1] *n* (%)

Characteristic	Study group ($n = 50$)
Age (yr)	46 (11)
Male sex	64
Body mass index (kg/m^2)	27 (4)
GERD symptom score[2]	
Heartburn	18 (15-20)
Regurgitation	15 (12-18)
SF-20 score[3]	
Physical function	50 (17-75)
Role function	100 (0-100)
Social function	80 (60-100)
Mental health	76 (60-88)
General health	40 (18-70)
Bodily pain perception	75 (50-75)
PPI dose (mg)[4]	40 (38-54)
Time pH < 4, %	8.4 (6.1-12.7)
LES pressure (kPa)	0.9 (0.3-1.5)
Hiatal hernia length (cm)	1 (1-2)
Esophagitis grade A/B with or without Barrett's metaplasia, *n* (%)	38

[1]Data are presented for all patients included in the analyses, values enclosed in parentheses are means (SD) or medians (IQR); [2]Frequency multiplied with severity; Scores range from 0 to 24 and were scored while off antisecretory drugs; [3]Short-Form General Health Survey scores range from 0 to 100, with higher scores indicating better function, except for the bodily pain perception score; [4]Proton pump inhibitor dose per day. GERD: Gastroesophageal reflux disease; PPI: Proton pump inhibitor; LES: Lower esophageal sphincter.

Use of acid-suppressive medication and alternative treatment

Compared with baseline, acid-suppressive medication use decreased significantly. At 3 and 48 mo the median doses were 31% ($P < 0.001$) and 97% ($P < 0.001$) of the baseline dose (Figure 2). Twenty percent were completely off acid-suppressive medication at the end of follow-up. At the end of follow-up, 18 (36%) of the 47 remaining patients used 50% or less of their baseline dose and had not received another treatment (Figure 3). As such, 64% were classified as treatment failures. If retreated patients were also considered to be treatment failures, irrespective of the effect of retreatment, the number of failures amounted to 36 patients (72%).

Symptoms

Compared with baseline, heartburn and regurgitation symptom scores were significantly decreased both at 3 mo and at the end of follow-up (Table 2). At 3 mo, heartburn and regurgitation scores decreased with 41% and 37%, respectively. At 48 mo, the heartburn score was reduced by 32% ($P < 0.001$) and the regurgitation score by 34% ($P < 0.001$).

Quality of life

At 3 mo, the SF-20 quality of life scores significantly improved in 5 of 6 subscales ($P < 0.026$, Table 2). Only the mental health subscale score had not changed significantly. At the end of follow-up, the same subscales remained significantly improved compared with baseline with the exception of role function.

At the end of follow-up, 17% of patients indicated that their GERD had completely been cured, 30% indicated that it had improved, 46% that it was unchanged and 7% that it had worsened.

Adverse events

One patient had a major hemorrhage immediately after the procedure and was hospitalized. After receiving endoscopic injection therapy to stop the bleeding and a

Table 2 Outcome at 3 mo post-treatment and at the end of follow-up (median period of 48 mo) *n* (%)

Variable	Baseline	3 mo		End of follow-up		*P* values[1]	
		Absolute values	%[2]	Absolute values	%[2]	3 mo	End
GERD symptom scores							
Heartburn score[3]	16.4 (5.8)	9.7 (7.6)	-41	11.2 (8.5)	-32	< 0.001	< 0.001
Heartburn frequency	5.0 (1.3)	3.2 (2.1)	-35	3.4 (2.2)	-31	< 0.001	< 0.001
Heartburn severity	3.1 (0.9)	2.3 (1.3)	-26	2.5 (1.4)	-20	< 0.001	< 0.003
Regurgitation score[3]	15.6 (4.8)	9.9 (7.8)	-37	10.2 (7.3)	-34	< 0.001	< 0.001
Regurgitation frequency	5.0 (1.1)	3.3 (2.2)	-33	3.4 (2.0)	-32	< 0.001	< 0.001
Regurgitation severity	3.1 (0.8)	2.2 (1.4)	-28	2.5 (1.2)	-20	< 0.001	< 0.002
SF-20 scores[4]							
Physical health	46.3 (34)	64.0 (35)	38	60.1 (35)	30	< 0.009	< 0.02
Role function	59.2 (48)	76.7 (39)	31	73.9 (42)	25	< 0.023	< 0.09
Social function	71.4 (32)	81.3 (29)	14	83.2 (28)	16	< 0.026	< 0.006
Mental health	74.0 (18)	73.2 (17)	-1	76.1 (15)	3	NS	NS
General health	43.2 (27)	56.2 (28)	30	62.6 (28)	45	< 0.001	< 0.001
Bodily pain perception	67.4 (26)	48.9 (35)	-27	44.8 (34)	-34	< 0.003	< 0.001

[1]Baseline data compared with 3 mo and end of follow-up data (median of 48 mo); [2]Change in percentage compared with baseline, to calculate the change, the absolute values rounded to 2 decimal places instead of 1 were used; [3]Frequency multiplied with severity. Scores range from 0 to 24; [4]Short-Form General Health Survey scores range from 0 to 100, with higher scores indicating better function, except for the bodily pain perception score. GERD: Gastroesophageal reflux disease; NS: Not significant.

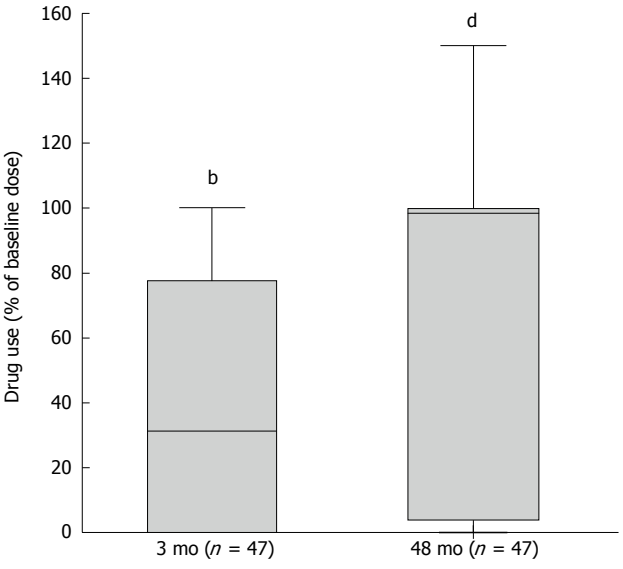

Figure 2 Medication use at 3 mo after endocinch and at the end of follow-up. Presented as percentage of the baseline dose. Interquartile range (IQR) , range and median are shown; Median values were 31% and 97% for 3 mo and end of follow-up, respectively ([b]*P* < 0.001, [d]*P* < 0.001, both compared with baseline).

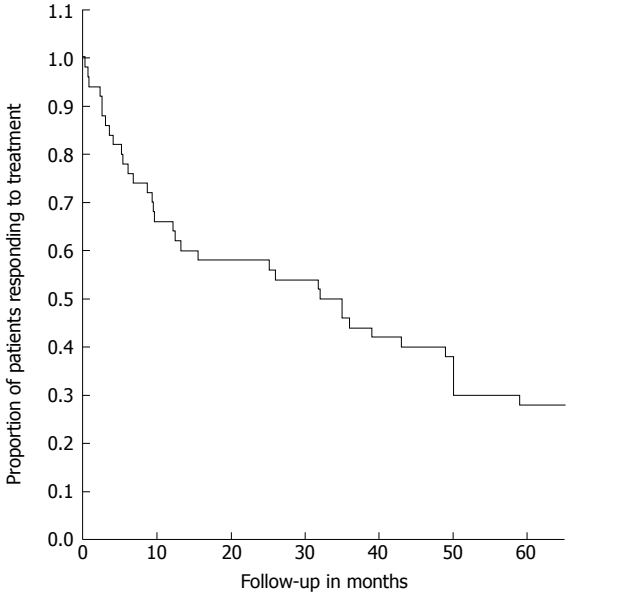

Figure 3 Kaplan-Meier survival curves. Analysis of proportion of patients responding to Endocinch during follow-up; All treatment failures (*i.e.*, > 50% of baseline acid-suppressive dose or other antireflux treatment) were included.

blood transfusion, the patient made a speedy recovery. No other adverse events occurred either during treatments or follow-up.

DISCUSSION

Endoscopic treatment of GERD using Endocinch may improve GERD symptoms, decrease medication use and increase quality of life. However, after 4 years the treatment effect persists in less than half of treated patients. During the first follow-up year, the success rate progressively declined. This decline continued during the subsequent three years and it is likely that it will continue to do so. The percentage of treatment failures rose to 64%. At the end of follow-up, 47% of patients indicated that GERD was still improved compared to baseline and the GERD symptom scores and quality of life scores remained significantly improved. Yet, 80% required PPIs.

The limited number of adverse events in this follow-up study demonstrated that the procedure is safe in the long-term.

One study reported that treatment failure occurred in 80% of the patients during the 18-mo follow-up study (*n* = 70)[3]. Treatment failures were defined as in our study (unimproved heartburn symptoms or PPI dose exceeding 50% of the baseline dose), but patients were not re-treated. Only 6% of patients succeeded to stay off PPIs

after two years. The rate of 80% is comparable to the 72% found in the present study, when all retreated patients were also counted as treatment failures. However, in our study follow-up duration was much longer (48 *vs* 18 mo). Two other, multicenter studies from the US (n = 85) and Japan (n = 48) showed slightly better results with rates of 40% and 30%, respectively, of patients staying off PPI after two years[4,5]. These results indicate that 6% is relatively low and that our 20% accurately reflects the percentage of patients that remain off medication in the long-term (≥ 4 years).

The loss of functional plications, due to too superficial (*i.e.*, not transmural) suturing, is generally accepted as the cause of loss of treatment effect[11,12]. In the present study no systematic endoscopic evaluation of the functionality of sutures was performed, because only treatment failures underwent a follow-up endoscopy. Hence, the actual proportion of patients with functional sutures is unknown. Among treatment failures a mean of only 0.73 sutures were still considered to be functional. Increasing the initial number of plications will probably only temporarily prolong the treatment effect, comparable to performing a second procedure, as was shown by the present study and others[6]. Also measurements to improve endurability, such as cauterization of the mucosa of a gastroplication, as evaluated in a 2-year follow-up study (n = 18), will not have a beneficial long-term effect[13]. Only modifications of the suturing technique can improve the depth of stitches, but up until now no improvements of the device have been made.

One can question whether the observed effect of treatment after 4 years is really the result of diminished esophageal acid exposure. After all, pH measurements were not repeated. Our initial study already showed that the improvement of esophageal acid exposure was not significantly greater than after sham treatment[7]. This is consistent with the results in other studies that found no significant differences or only marginal differences between baseline and post-procedure pH-values[3,6,14,15]. On the other hand, in our initial study a subgroup analysis (responders *vs* non-responders) made likely that the observed treatment effect was largely due to the reduction of esophageal acid exposure. We therefore believe that, although no repeated pH-metry was done, the effect after four years can also be attributed to esophageal acid reduction. It is highly unlikely that a placebo effect would still persist.

In summary, this is the longest prospective follow-up study after treatment with the endocinch procedure. It shows that GERD symptoms, medication use and quality of life improve in a subset of patients that gradually becomes smaller during follow-up. Endocinch can be carried out safely in an outpatient setting under conscious sedation. However, 44% had to undergo retreatment and eventually 80% needed PPIs again. In conclusion, in the long-term this procedure is not beneficial for the majority of GERD patients.

COMMENTS

Background

Gastroesophageal reflux disease (GERD) is a common, often chronic ailment with an important impact on quality of life and healthcare resources. Many patients require daily acid-suppressive medication or ultimately antireflux surgery. In recent years, several less invasive, endoscopic procedures have been developed. Endocinch is one of the first generation antireflux suturing devices, creating a barrier at or just below the gastro-esophageal junction against reflux.

Research frontiers

Many studies, including a sham-controlled trial of our group, have demonstrated the short-term safety and efficacy of the Endocinch procedure on symptoms, but often failed to show significant improvements in esophageal acid exposure. Several follow-up studies have raised questions about medium-term efficacy. Prospective data exceeding two years of follow-up are scarce.

Innovations and breakthroughs

Loss of treatment effect could be due to loss of functional sutures, as was suggested by some researchers. Retreatment has been shown to (at least temporarily) improve results. In the present study, retreatment was also offered in case of early treatment failure and prospective follow-up duration was extended up to a median of four years.

Applications

The results of this study suggest that the endocinch procedure improves GERD symptoms, quality of life and reduces the use of acid-suppressive medication in only a subset of patients. A subset that gradually becomes smaller during four years of follow-up. Percent of 44 patients had to undergo retreatment and eventually 80% required acid-suppressive medications again. The study can conclude that in the long-term this procedure is not beneficial for the majority of GERD patients.

Terminology

Gastroesophageal reflux disease is a chronic disease in which the lower esophageal sphincter (LES) allows gastric acids to reflux into the esophagus leading to symptoms as heartburn and acid indigestion. Endocinch is a commercially developed endoscopic device designed to place stitches at or just below the esophagogastric junction, creating so-called 'gastroplications' that tighten the LES.

Peer review

This is a well-designed prospective follow-up study in which the authors evaluated the long-term effect of endoluminal gastroplication with the Endocinch system for GERD. New important data have been provided that demonstrate that this procedure is not beneficial in the long-term for the majority of GERD patients.

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3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

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6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

Volume with supplement

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9 Outreach: Bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

Books

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10 **Sherlock S**, Dooley J. Diseases of the liver and billiary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

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15 Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: http://www.cdc.gov/ncidod/eid/index.htm

Patent (list all authors)

16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

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