Visceral hypersensitivity in Irritable Bowel Syndrome:
pathophysiological mechanisms

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Visceral hypersensitivity in Irritable Bowel Syndrome: pathophysiological mechanisms

Viscerale hypersensitiviteit in Prikkelbaar Darmsyndroom: pathofysiologische mechanismen

(met een samenvatting in het Nederlands)

Proefschrift

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Voor opa Kerckhoffs

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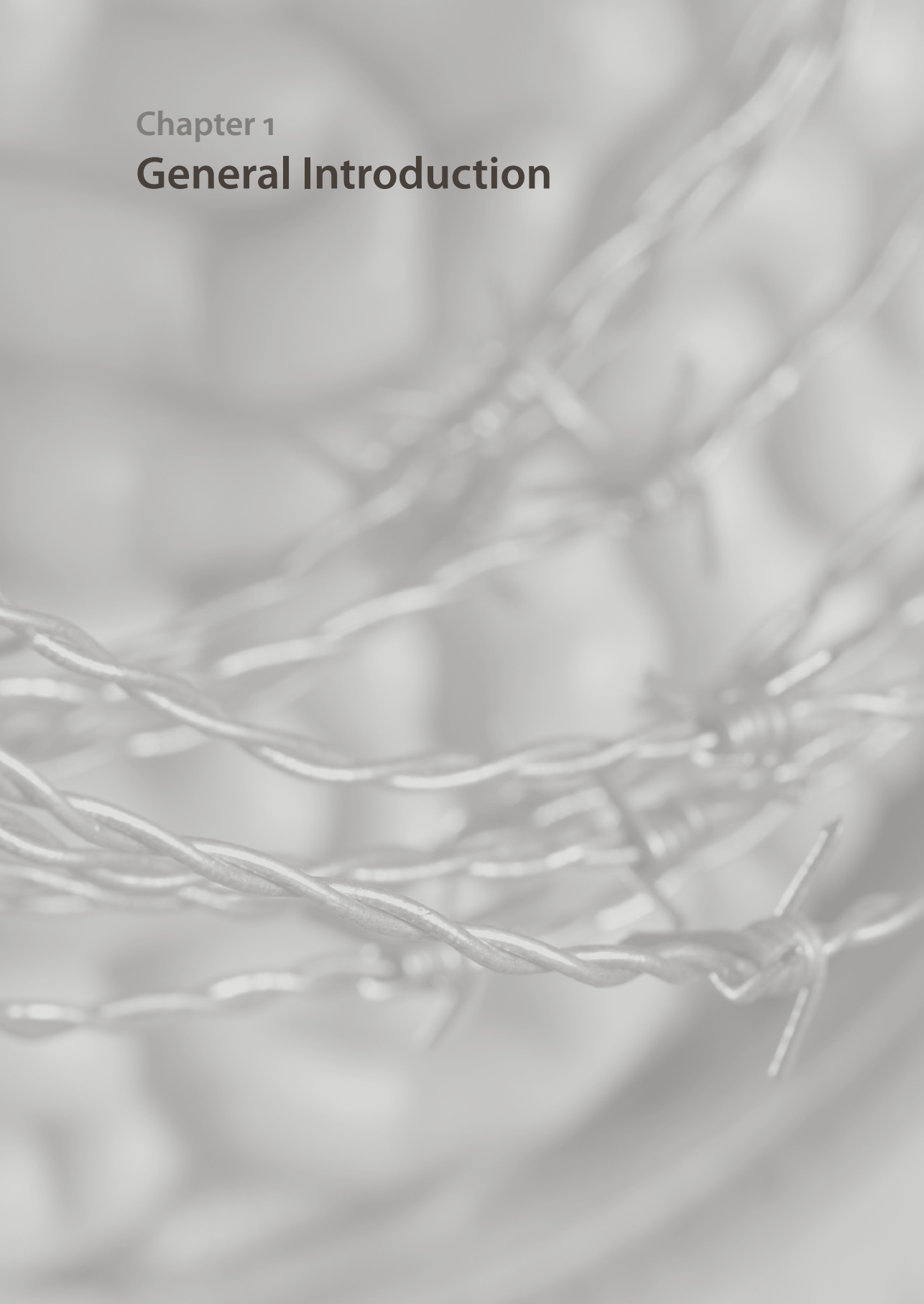

Chapter 1

General Introduction

Irritable Bowel Syndrome

Irritable Bowel Syndrome (IBS) is a functional bowel disease in which abdominal pain or discomfort is associated with disordered defecation. In the general Western population IBS symptoms are present in about 3%-22% at any point in time.(1-3) In the Netherlands the prevalence of IBS is estimated at 9%.(4) The prevalence of IBS is higher in women. In absence of biological markers, symptom-based criteria are used to define IBS. Nowadays the Rome criteria are the most applied and accepted criteria for IBS.(5) In the Rome II criteria, abdominal discomfort or abdominal pain needs to be present during at least 12 weeks in the previous year, but these weeks are not necessarily consecutive.(5) Furthermore, abnormal stool frequency, abnormal stool form, abnormal stool passage, passage of mucus and bloating or feeling of abdominal distension cumulatively support the diagnosis of IBS. Using the Rome II criteria, IBS patients are categorised according to their predominant bowel habit as constipation predominant IBS (IBS-C), diarrhea predominant IBS (IBS-D) or IBS with alternating constipation and diarrhea (IBS-A). Rome II criteria were applied in this thesis. Rome III criteria, published in 2006, differ from the Rome II criteria at several points. Symptoms need to be present 6 months and must be currently active for 3 months. Furthermore IBS patients are subtyped exclusively on their stool form using the Bristol Stool chart.(6) IBS patients are divided into one of four subgroups: IBS-C, IBS-D, IBS-A or unsubtyped IBS. Constipation is defined as hard or lumpy stools and diarrhea as loose, mushy or watery stools.

Pathophysiology of IBS

Many pathophysiological explanations have been suggested for IBS; however no unique mechanism has been identified. Available studies indicate that IBS symptom generation is related to disturbances in brain-gut interactions. The dysregulation of the brain-gut axis involves abnormal function and interplay between the enteric, autonomic and/or central nervous systems. The brain-gut axis may be modulated by abnormal central processing, autonomic and hormonal events, genetic and environmental factors, psychological disturbances and postinfectious after effects. Peripheral alterations probably dominate in some patients and disturbed central processing of signals from the periphery dominates in others. In this thesis we focus on the evidence for peripheral alterations in the pathophysiology of IBS, such as intestinal microbiota composition, intestinal mucosal barrier, serine protease and serotonergic signalling components and inflammatory changes.

IBS and visceral hypersensitivity

Visceral hypersensitivity is difficult to measure but by inflating a bag in the gastrointestinal tract discomfort or pain to distension of the intestine can act as a marker for visceral perception. IBS patients in comparison to healthy subjects have a lower pain threshold to bag distension of the bowel (visceral hyperalgesia), or they have increased sensitivity even to normal intestinal function (allodynia), and there may be an increased area of somatic referral of visceral pain. Hypersensitivity and sensitization may initiate peripherally, centrally or a combination of peripherally and centrally. Peripheral sensitization may occur through altered receptor sensitivity at the gut mucosa and myenteric plexus. Central sensitization may also increase excitability by altered central downregulation of visceral afferent transmission.(7) In this thesis we focus on the peripheral factors influencing visceral hypersensitivity. The peripheral factors underlying the pathophysiological mechanisms and/or symptom generation in IBS seem to be multifactorial. Hypersensitivity may occur through altered receptor sensitivity, which may be enabled by mucosal inflammation, degranulation of mast cells close to enteric nerves, or altered serotonin activity, possibly enhanced by alterations of the microbiota composition or infection.(8-15)

IBS and microbiota

Indirect support for the involvement of bacteria in IBS is derived from the observation that antibiotic therapy could alleviate IBS symptoms in patients with bacterial overgrowth, while, in contrast, the use of antibiotics for non-gastrointestinal complaints increased the likelihood for the development of abdominal symptoms.(16;17) Secondly, in up to 30% of IBS patients the first symptoms occur after a gastrointestinal infection.(18;19) At last, probiotics can be used to therapeutically modulate the gastrointestinal microbiota in IBS. In particular, *Bifidobacterium infantis* and a mixture of *L. rhamnosus*, *B. breve* and *Proprionibacterium freudenreichii* spp. *shermanii* have demonstrated significant benefit in global symptom score reduction in IBS patients.(15;20;21)
Direct support for the involvement of bacteria in IBS is derived from studies on gastrointestinal microbial fermentation patterns and composition in both IBS patients and healthy subjects. Distinct microbial fermentation patterns, associated with an increased hydrogen production, were observed in the bowel content of IBS patients.(22) Furthermore, it has been addressed that bacterial overgrowth plays a role in IBS using breath testing techniques.(23) Controversies in literature exists on the usefulness of breath tests to diagnose

bacterial overgrowth. Currently culture based methods are considered the gold standard for diagnosing bacterial overgrowth. However in the last decade molecular based techniques have shown that only a small part of bacteria can be cultured. In this thesis we evaluate the usefulness of lactulose breath tests, culture and molecular based methods to diagnose bacterial overgrowth. To evaluate faecal microbial composition in IBS patients the most recent studies used molecular based techniques such as profiling, sequencing, and qPCR analyses of bacterial small subunit ribosomal RNA genes.(15;24-26) These studies revealed a temporal instability of the microbiota, increase of total anaerobes, a decrease of *Bifidobacteria* spp or lactobacilli and coliforms or an increase of species belonging to the Clostridium cluster in IBS patients. Altogether, these observations suggest an altered composition and activity of the gastrointestinal microbiota in relation to IBS.
The composition of gastrointestinal microbiota can be divided into luminal bacteria, which are dispersed in liquid faeces or bound to food particles, and the mucosa-associated bacteria, bound to a mucus layer adjacent to the intestinal epithelium. The luminal bacteria form the majority of the microbiota in the gastrointestinal tract and might play a key role in bloating and flatulence in IBS through carbohydrate fermentation and gas production. The mucosa-associated bacteria have the potential to influence the host via immune-microbial interactions. Unlike the luminal bacteria which can be examined in faeces the mucosa-associated bacteria require invasive examination. Studies on mucosa-associated bacteria in IBS patients are currently limited to one.(27) This study focussed on mucosa-associated bacteria in the distal small intestine and large intestine. Since the number of bacteria is lower in the small intestine in comparison to the large intestine, differences in microbiota composition in the proximal small intestine might affect the host more than in the large intestine and might play a more important role in IBS symptom generation. Different invasive and non-invasive sampling techniques are available to access the small intestinal microbiota and will be reviewed in this thesis. Data on (mucosa-associated) bacterial composition in the proximal intestine in IBS patients in comparison to healthy subjects are currently lacking and will be investigated in this thesis.

IBS and intestinal permeability

The single layer of epithelial cells of the intestinal tract has the difficult task to regulate the passage of fluids, electrolytes and nutrients but to prevent the access of potentially harmful entities such as bacteria, antigens and toxins. The

mucus layer, the enterocytes and the intercellular tight junctions amongst others contribute to this selective permeation. Enteric pathogens increase mucosal permeability, mainly through disruption of epithelial tight junctions.(28) In animal models for IBS, also stress leads to increased gut permeability and bacterial translocation mediated via mast cell activation.(29;30) Altered intestinal permeability has been identified in IBS-D or postinfectious IBS patients.(31-33) On the other hand, in some studies containing relatively large groups of IBS patients, no indication of enhanced intestinal permeability was found.(34;35) The presence of increased intestinal permeability in postinfectious IBS patients reflects a lack of recovery of tight junction disruption that occurred during the acute infection.(32) Increased intestinal permeability may play an important role because it exposes the mucosa to an abnormal challenge of luminal antigens of dietary and bacterial origin promoting and maintaining mucosal immune activation. Immune activation, caused by increased intestinal permeability, contributes to mucosal barrier disruption allowing the passage of luminal contents into the mucosa, thereby creating a vicious circle. In this thesis we investigate the effect of a low dose NSAIDs on intestinal permeability in IBS patients. NSAIDs are known to increase intestinal permeability. Other factors enhancing intestinal permeability such as stress, lipopolysaccharides, bile salts or the activation of the protease activated receptors (PAR), initiate a long-term hypersensitivity of the gut to distension. Furthermore, pharmacological blockade of increased permeability prevents hypersensitivity to colorectal distension which indicates moreover the relationship between intestinal permeability and visceral hypersensitivity.(36;37) Since a subset of IBS patients is visceral hypersensitive it's interesting to evaluate the effect of a low dose NSAID on intestinal permeability in IBS patients.

Role of serine protease and serotonergic signalling on the pathophysiology of IBS

Several studies indicate that abnormalities in serine protease and serotonergic signalling pathways play a role in the pathophysiology of IBS. Serine proteases are the signalling molecules that activate protease activated receptors (PAR). From the four members of PAR's identified in humans, PAR-2 is highly expresses on intestinal epithelial cells. Activation of PAR-2 by cleaving the tethered ligand evokes visceral hypersensitivity, hyperexcitability of enteric neurons and intestinal permeability.(38-40) This makes PAR-2 an important receptor in the pathophysiology of IBS and mediating the symptoms of IBS.

The primary activating protease of PAR-2 is trypsin. Recently, it has been found that the serine protease trypsin IV, produced by intestinal epithelial cells, can activate PAR-2.(41) Serine proteases of other endogenous origin such as mast cell tryptase and/or bacterial origins are also able to cleave the receptor. IBS patients have an increased serine protease activity in colonic mucosa. Moreover, the mucosal supernatant activates murine extrinsic primary afferent neurons in culture and induces hyperalgesia and allodynia in mice upon colorectal distension. These responses are diminished after pre-incubation with serine protease inhibitor.(42) Furthermore, faecal serine protease activity appeared to be significantly higher in patients with diarrhea-predominant IBS than in both controls and IBS patients with either constipation or alternating bowel habits.(43) Expression of trypsinogens, zymogens of active trypsins, is elevated in colonic mucosa from IBS patients.(42) Besides, the pronociceptive effects of colonic mucosal supernatant and faecal samples are PAR-2 mediated. (42;44) In contrast to the large intestine the mucosal serine protease signalling in the small intestine of IBS patients and healthy subjects is currently unknown and will be studied in this thesis. Furthermore the relationship between altered large intestinal mucosal serine protease signalling and rectal visceral hypersensitivity will be studied. Tryptase released from activated mast cells in close proximity to mucosal nerve endings is correlated with abdominal pain. (10) However whether or not these nerve endings are PAR-2 immunoreactive is currently unknown. In this thesis, the spatial interaction between immunoreactive PAR-2 neurons and mast cells will be assessed.

Serotonin (5-HT) also plays a role in the pathophysiology of IBS. The major source of 5-HT in the human body is located in enterochromaffin (EC) cells in the mucosa of the intestine. Serotonin is released from the EC cells after luminal stimuli, including mechanical distortion. Once released, serotonin acts on receptors located on sensory neurons. Tryptophan hydroxylase (TPH) is the rate-limiting enzyme in the serotonin synthesis. After release from the EC cells, 5-HT acts on 5-HT receptors situated on the mucosal projections of primary afferent neurons. 5-HT receptors are also situated on EC cells, modulating 5-HT release by autoregulatory mechanisms. From the seven identified 5-HT receptors the 5-HT3 and 5-HT4 receptor play an important role in gastrointestinal sensory and motor functions.(45;46) Serotonin has to be removed rapidly from the neuroendocrine cell-sensory nerve junction to terminate responses and to prevent desensitisation of the receptors. A specific 5-HT transport protein (SERT), expressed by enterocytes, is responsible for this uptake. It is becoming increasingly clear that changes in serotonin signalling occur in IBS.

(12;13;33;47-49) These changes include EC cell numbers, TPH message levels, 5-HT content, 5-HT release, SERT immunoreactivity, SERT message levels and platelet-free serum 5-HT levels. However, the results of these investigations are not entirely in agreement. For example, various combinations of changes in EC cell populations and 5-HT content have been reported in different forms of IBS. (12;33;47;50) In this thesis we will investigate the serotonergic signalling components in the large intestine and the relationship between altered serotonergic signalling and rectal hypersensitivity in IBS patients to validate the findings that have been reported and to resolve the discrepancies that exist in EC cell numbers, SERT and TPH message levels. Also little is known on alterations in serotonergic signalling components in the small intestine and this will be investigated in this thesis as well.

Outline and aims of this thesis

This thesis focuses on several pathophysiological mechanisms of visceral hypersensitivity: (1) microbiota composition in IBS patients, (2) the role of intestinal permeability in the pathogenesis of IBS, (3) serine protease and serotonergic signalling in small and large intestine of IBS patients. We investigated the pathophysiology of IBS using several techniques. Culturing, breath testing, FISH, Q-PCR and Denaturing Gradient Gel Electrophoresis (DGGE) are used to study the intestinal bacterial composition in IBS patients. The intestinal barrier function was investigated by measuring intestinal permeability using polyethylene glycols of increasing molecular weight as probes.

In our attempt to identify molecular factors underlying sensory abnormalities observed in IBS patients we focused on serine protease and serotonergic signalling in several regions of the small and large intestine. Firstly, mRNA expression levels of genes encoding proteins responsible for 5-HT synthesis and inactivation, or affecting release of 5-HT and levels of genes activating the serine protease signalling cascade are quantified by real-time PCR in mucosal biopsy specimens. Secondly, by using ELISA, 5-HT, 5-HIAA and substance P content are quantified. In addition 5-HT positive cells, intraepithelial lymphocytes, mast cells and PAR-2 positive neurons are counted by using immunohistochemistry.

The aim of the current thesis is to further elucidate the pathophysiology of IBS. For that purpose, we have addressed the following major questions:

1 What are the current views on invasive and non-invasive sampling techniques to collect information about microbiota in the small intestine?

2 Are lactulose breath tests, culture and molecular based methods useful in diagnosing bacterial overgrowth?

3 What is the difference in composition of both faecal and duodenal mucosa-associated microbiota in IBS patients and healthy subjects?

4 Do NSAIDs increase intestinal permeability in IBS patients more than in healthy subjects?

5 What is the role of altered mucosal serine protease and serotonergic signalling in small and large intestine of IBS patients and are they related to rectal visceral hypersensitivity?

6 What is the role of PAR-2 in the submucosal mast cell-nerve interactions in the small intestine of IBS patients?

Chapter 2

Sampling of bacteria in the human gastrointestinal tract

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Gastrointestinal Microbiota, 2006, 25-50

Antonie van Leeuwenhoek (born 24 October 1632 and died 1723) was the first to observe numerous micro-organisms ("animalcules") from the gastrointestinal tract with his microscope. He was also the first to make glass lenses powerful enough to observe and describe bacteria. His curiosity brought him to investigate samples taken from his own mouth and from other people who never brushed their teeth and compared these findings with people who brushed their teeth daily and used large amounts of alcohol. In addition, he even investigated his own faecal samples in a period of diarrhea and compared these findings with faecal samples of animals. He reported these observations to the Royal Society in London.(51)

We now know that the mucosal surface of the human gastrointestinal tract is about 300 m^2 and is colonized by 10^{13}-10^{14} bacteria consisting of hundreds of different species. The prevalence of bacteria in different parts of the gastrointestinal tract depends on pH, peristalsis, oxidation-reduction potential within the tissue, bacterial adhesion, bacterial cooperation, mucin secretion containing immunoglobulins, nutrient availabilty, diet and bacterial antagonism. The composition of the Gram-negative, Gram-positive, aerobic and anaerobic microflora has been extensively studied by culturing methods and shown to change at the various sites of the gastrointestinal tract. (**Figure 1**)

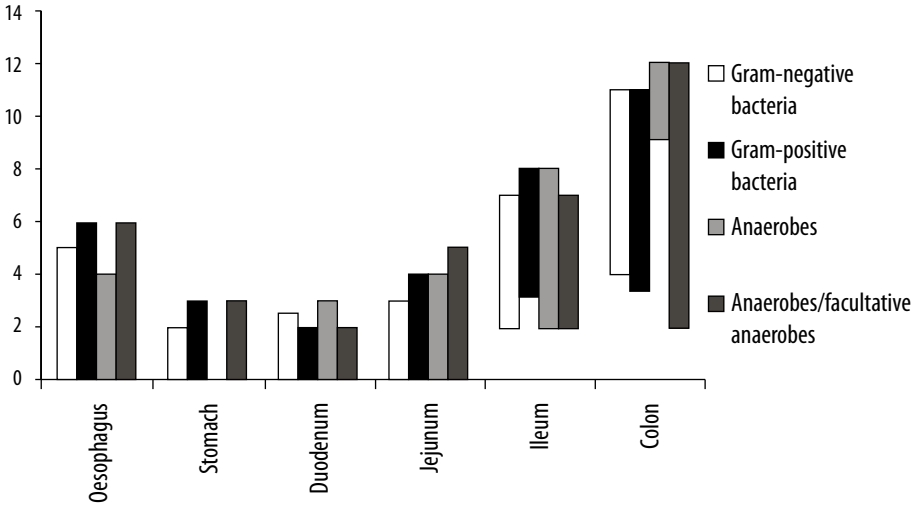

Figure 1 *Ranges of ^{10}log numbers of Gram-negative bacteria, Gram-positive bacteria, anaerobes and aerobes and facultative/anaerobes per gram intestinal material in the human intestinal tract.(22;54;61;66;68;76;153)*

The stomach and proximal small bowel normally contain relatively small numbers of bacteria because of peristalsis and the antimicrobial effects of gastric acidity. An intact ileocecal valve is likely to be an important barrier to backflow of colonic bacteria into the ileum. The intestinal microflora play a prominent role in gastrointestinal physiology and pathology. A bacterial population is essential for the development of the gastrointestinal mucosal immune system, for the maintenance of a normal physiological environment, and for providing essential nutrients.(52) Using culturing techniques dietary changes seemed to have a negligible effect on the intestinal microflora composition.(53)(54) More recently molecular techniques show that diet can alter the microflora composition, but the predominant groups are generally not substantially altered.(55;56) In contrast, antibiotics can dramatically alter the composition of intestinal microflora.

1.2 Physiology of microflora host interaction in humans

Normal gastrointestinal tract microflora is essential for the physiology of its host. The microflora in the gastrointestinal tract have important effects on nutrient processing, immune function and a broad range of other host activities some of which are briefly described below.(57)

Pasteur (1822-1895) suggested that the intestinal microflora might play an essential role in the digestion of food. We now know that bacteria harbour unique metabolic capabilities which enable an otherwise poorly utilizable nutrient to be metabolized.(58) The intestinal microflora possesses enzymes that can convert endogenous substrates and dietary components, such as fibers, to provide short-chain fatty acids and other essential nutrients, which are absorbed by the host.(53) The above-described interaction of host and bacteria, when one or both members derive specific benefits from metabolic capabilities, is defined as mutualism. Bacteria also produce a number of vitamins, especially those of the B-complex.(59)

Microflora also affords resistance to colonization by potential pathogens that cannot compete with entrenched residents of the microbial community for nutrients.(57) Autochthonous microorganisms colonize particular habitats whereas transient bacteria can only colonize particular habitats under abnormal conditions. Metabolic waste products help to prevent the establishment of other species. Normal microflora also prevents colonization by potential pathogens by releasing bacteriocins and colicins which have antibacterial activity. A pathogenic relationship results in damage to the host. Most pathogens are allochthonous microorganisms. However, some

pathogens can be autochthonous to the ecosystem. They live in harmony with the host unless the system is disturbed. Antibiotic therapy can drastically reduce the normal microflora and the host may then be overrun by introduced pathogens or by overgrowth of organisms normally present in small numbers. An example of a disturbed system is that after treatment with clindamycin, overgrowth by *Clostridium difficile*, which survives treatment, can give rise to pseudomembranous colitis.(53;60)

Microbial factors are known to influence host postnatal development. Commensals acquired during the early postnatal life are essential for the development of tolerance, not only to themselves but also to other luminal antigens. Development of B-and T-cell responses depends on microbiota. The natural antibodies that arise in response to the antigens of the normal gut microflora are of great importance in immunity to a number of pathogenic species.

Somatic hypermutation of immunoglobulin (Ig) genes in intestinal lymphoid follicles plays a key role in regulating the composition of the microbial community.(58)

Bacteria participate in bile acid metabolism. In the colon, bacterial enzymes convert cholic acid and chenodeoxycholic acid into the secondary bile acids deoxycholic acid and lithocholic acid, respectively, which in general are poorly reabsorbed; most of these are then eliminated in the stool. In patients with small bowel bacterial overgrowth (SBBO), bile acids are deconjugated and metabolized more proximally in the small bowel and removed from further participation in the normal enterohepatic circulation, resulting in bile acid malabsorption and steatorrhea. Steatorrhea is defined as excessive loss of fat in the stool, greater than 7 grams or 9% of intake for 24 hours.(61)

The effects of having a normal enteric microflora can also be determined by comparing the characteristics of germ-free and conventionally reared animals. In the small bowel of germ free animals there are dramatic reductions in leukocytic infiltration of the lamina propria and the size and number of Peyer's patches. Besides, the intraluminal pH is more alkaline and the reduction potential more positive. Colonization of the intestinal tract of germ free animals with even a single strain of bacteria is followed by the rapid development of physiologic inflammation of the mucosa resembling that of conventional animals. The migrating motor complex (MMC) is a cyclic pattern of motility that recurs during fasting and is an important mechanism in controlling bacterial overgrowth in the upper small bowel. Gut transit is slow in the absence of the intestinal microflora. The effect of selected microbial

species in germ free rats on small intestinal myoelectric activity is promotion or suppression of the initiation and migration of the MMC depending on the species involved. Anaerobes, which have a primitive fermenting metabolism, emerge as important promoters of regular spike burst activity in the small intestine. After introduction of *Micrococcus luteus* and *Escherichia coli* in germ-free rats the MMC period prolongs. *Clostridium tabificum*, *Lactobacillus acidophiles* and *Bifidobacterium bifidum* introduction in germ-free rats reduces significantly the MMC period and accelerates small intestinal transit. Intestinal microflora accelerates transit through small intestine in the fasting state compared to the unchanged intestinal myoelectric response to food. Concluding, promotive influence of the conventional intestinal microflora on MMC reflects the net effect of bacterial species with partly opposite effects. (62-64)

In general one can conclude that the bacterial microflora has a number of specific functions in transport, absorption of nutrients and in the modulation of the immune system of the gastrointestinal tract. The introduction of pathogen bacteria can disturb the normal physiological functions of the gastrointestinal tract to a great extent. A number of function test for the detection of pathogen bacteria have been developed, as will be explained in the next paragraphs.

1.3 Importance of sampling the gastrointestinal tract

The current knowledge of the human intestinal microflora using faeces and gastrointestinal fluids or biopsies is mostly based on culture techniques and recently on molecular biology techniques. There are various methods of obtaining material to study the microflora. The various methods of investigating microflora in the gastrointestinal tract will be discussed in this chapter specified for different compartments of the gastrointestinal tract. We will also describe the advantages and disadvantages of the methodology of sampling. Sampling of the gastrointestinal tract is clinically necessary for the diagnosis of *Helicobacter pylori*, and the etiology of diarrhea.

One could also sample the gastrointestinal tract for research questions on small bowel bacterial overgrowth (SBBO) or on the host-bacterial relationships in the gut. Research or diagnosis of bacteria anywhere in the GI tract can be performed using invasive or non-invasive methods.

2 *Esophagus: Microflora and sampling techniques*

2.1 *Common microflora*

The mouth and the oropharynx predominantly harbor Gram positive organisms.(65) The largest number of bacterial microflora consists of streptococci, *Neisseria*, *Veillonella* and *Fusobacteria*. Bacteroides, lactobacilli, staphylococci, yeasts and *Enterobacteria* are also present in smaller amounts.(66) The esophagus is covered with stratified squamous epithelium, which is a mechanical barrier (coated with saliva and mucus) to prevent infection, has high peristalsis and has a high mucus secretion which contains immunoglobulins. Because of the lack of absolute anatomic or known physiological barriers, bacteria can be introduced into the esophagus by the swallowing of food, by resident oral microflora or by reflux from a colonized stomach.(67) Little is known about the presence of bacterial biota in the esophagus.(67) The esophagus provides a potential environment for bacterial colonization. It has a large mucosal surface downstream of the bacterial species-rich oropharynx. Using molecular biology techniques one could find members of 6 phyla. *Streptococcus* (39%), *Prevotella* (17%) and *Veilonella* (14%) are most prevalent. Most esophageal bacteria are similar or identical to residents of the upstream oral biota.(67) Quantitative cultivation-based studies indicate that aerobic organisms are present in all subjects and obligate anaerobes in 80% of the subjects. No differences in frequencies of isolation or composition of the microflora are found between different subjects.(68;69)

2.2 *Pathologic microflora*

A pathogen is a microorganism that is disease producing. Commensals may become pathogenic and cause disease if host defense mechanisms are compromised or if endogenous organisms are introduced into normally sterile body sites.

The esophagus of people with deficient immune systems (HIV, post-transplantation) may become infected with *Candida albicans*, cytomegalovirus, herpes simplex virus, *Histoplasma capsulatum*, *Mycobacterium avium*, *Cryptosporidium*. Those organisms are usually not seen in immunocompetent people.

With the exception of mycobacterium species, bacterial etiologies for inflammation involving the distal esophagus have not been explored.(70) Mycobacterial involvement of the esophagus is rare (incidence 0.14%) in both immunocompromised and immunocompetent hosts with advanced pulmonary tuberculosis.(70)

2.3 Luminal washes

Luminal washes to sample esophageal bacteria yield poor responses. The washes demonstrate transient bacteria of oropharyngeal origin, are either sterile or contain an average of 16 colony forming units/ml, with no common species found.(71;72) Either intestinal contents are passed through the alimentary canal with high peristalsis and prevent bacteria from residing in the esophagus, or the bacteria present in the washes are not cultivatable. One hypothesis is that the bacteria are more closely associated with the esophageal mucosa and can not be removed by simple washes. This technique is not commonly used for research questions and is clinically irrelevant.

2.4 Biopsy

Esophageal mucosal biopsy specimens from the distal esophagus can be obtained during upper endoscopy. The endoscope passes orally into the esophagus and the biopsy forceps can be shielded from oral microflora. The forceps consists of a pair of sharpened cups. Forceps with a central spike make it easier to take specimens from lesions which have to be approached tangentially (e.g. in the esophagus). The maximum diameter of the cups is limited by the size of the operating channel. The length of the cups is limited by the radius (73) of curvature through which they must pass in the instrument tip. Patients are instructed not to eat or drink for at least 4-6 hours before endoscopy (small sips of water are permissible for comfort).(74) The channel of the endoscope can also harbor bacteria if secretions have inadvertently been suctioned while advancing the endoscope. Oropharyngeal and gastric bacteria can contaminate the biopsy. Chlorhexidine or acidified sodium chlorite mouth rinse have been used to decontaminate the oropharynx.
To compare biopsy samples of two individuals or to compare the reproducibility in one subject the biopsies have to be taken at the same level.(75)

3 Stomach: Microflora and sampling techniques

3.1 Common microflora

The human stomach is lined with columnar secreting epithelium. Normally most of the bacteria in the stomach are killed because of low pH levels. The concentration of bacteria in the stomach is less than 10^3 colony forming units per ml.(54) (76) (73) Lactic acid bacteria are commonly isolated from the human gastric acid contents, especially when good anaerobic techniques are used. Candida and some other yeast species are also isolated. Bacteria isolated from gastric contents are considered transients. These bacteria have been passed

down from habitats above the stomach or have been present in ingested materials.(77) The normal resident microflora of the stomach consists mainly of Gram-positive aerobic bacteria, such as streptococci, staphylococci and lactobacilli.(54;73;76;78) **Table 1** describes the microflora isolated from gastric contents. (**Table 1**)

Table 1 Microorganisms isolated from the stomach.(54;59;69;147)

Microbial type
Lactobacilli
Streptococci
Bifidobacteria
Clostridia
Veillonella
Coliforms
Peptostreptococcus, *Bacteroides*,
Staphylococcus, *Actinobacillus*
Candida albicans
Torulopsis
Unidentified yeasts
Neisseria
Micrococcus

Most prevalent bacteria underlined

In healthy fasting patients large numbers of *Enterococcus*, *Pseudomonas*, *Streptococcus*, *Staphylococcus* and *Rothia (Stomatococcus)* may be isolated in culture when acidity is physiologically reduced, as occurs at night and during phase I (motor quiescence) of the migrating motor complex.(79-81)

3.2 Pathologic microflora

Bacteria closely associated and attached to the epithelium like *H. pylori* may be sampled from gastric contents with difficulty.(77) *H. pylori* is a Gram-negative bacterium that resides below the mucous layer next to gastric epithelium. *H. pylori* is rarely found before age 10 but increases to 10% in those between 18 and 30 years of age and to 50% in those older than age 60.(82) In developing nations the majority of children are infected before age 10 and adult prevalence peaks at more than 80% before age 50. Thus *H. pylori* ranges depend on age and socioeconomic differences.(83) *H. pylori* produce urease, an enzyme that breaks down urea into ammonium and bicarbonate. Ammonium provides an alkaline environment, which helps the bacterium protect itself from gastric acid injury. Most infected subjects do not have symptoms of *H. pylori* infection. However, *H. pylori* may induce acute gastritis (epigastric pain, bloating, nausea and vomiting), and/or chronic gastritis. Furthermore, it may also be associated with ulcer disease and gastric carcinomas.

Other gastric bacteria besides Helicobacter species only become apparent in patients with reduced acidity (achlorhydria, elderly).(84) Colonization of the gastric lumen occurs in patients on antisecretory medication meant to reduce gastric acid secretion. Many subjects regularly use these antisecretory drugs. Acid suppression may allow bacteria to survive in the stomach which results in gastric bacterial overgrowth. The degree of bacterial overgrowth depends upon the elevation of the pH.(65)

Infectious gastritis is rarely caused by *Mycobacterium tuberculosis*, *Mycobacterium avium*, *Actinomyces israellii* and *Treponema pallidum*.(61)

3.3 Biopsy

To investigate gastric microflora, tissue is generally obtained by endoscopic biopsy. Slightly less invasive methods are available such as the use of a small bowel biopsy tube or capsule, or biopsy forceps that can be passed through a modified nasogastric tube positioned either in the gastric body or antrum to obtain a specimen. Biopsy is clinically unnecessary to diagnose *H. pylori* microbiological unless one wishes to isolate the organism for antibiotic susceptibility testing.

Recommendations to maximize the diagnostic yield of endoscopic biopsies include the use of large-cup biopsy forceps, obtaining at least two samples from the lesser curvature and the greater curvature (the prepyloric antrum and the body), proper mounting and preparation of the samples. Special

stains (H&E, Giemsa and Warthin-Starry staining) are often used to help detect the presence of *H. pylori*.(85)
The rapid urease test (by agar gel slide tests) involves placing a biopsy specimen from the antrum of the stomach on a test medium that contains urea.(86) The biopsy specimens for the rapid urease test have to be removed from the sterilized biopsy forceps with a sterile toothpick and have to be placed immediately into a tube. The urea is hydrolyzed by urease enzymes of *H. pylori* and the ammonium formed increases the pH. A phenol indicator that changes the color from yellow at pH 6.8 to magenta at pH 8.4 can detect the pH alteration. The color change read off 1 hour after and 24 hours after the introduction of the gastric biopsy is an indication for the presence of *H. pylori*. Recommendations to maximize the rapidity and sensitivity of rapid urease tests are to warm the slide and use two regular or one jumbo biopsy specimen(s).(87) Increasing the number of biopsies to more than two biopsies from the antrum may increase the sensitivity, given that this probably increases the *H. pylori* load and therefore the amount of urease. However, this will prolong the endoscopy time and add to the discomfort of the patient. The agar gel test may take up to 24 hours to turn positive, particularly in the presence of a low bacterial density. Recent use of antibiotics, bismuth or proton pump inhibitors may render rapid urease tests falsely negative. Compared with histology as the gold standard in the diagnosis of *H. pylori* infection, the sensitivity of the test is 70-99% and the specificity is 92-100% in untreated patients.(87) Mucosal biopsies can be fixed in neutral buffered formaldehyde and if the rapid urease test is negative, sent in the next day for histologic assessment. The presence or absence of *H. pylori* can be established by examining three sets of tissue levels within 12 consecutive sections. On microscopic examination of the tissue obtained by biopsy, the bacteria may be seen lining the surface epithelium. The sensitivity for histologic examination is 70-90%. Giemsa staining is required for *H. pylori* diagnosis.
Culture for *H. pylori* is insensitive. Biopsies should be plated within 2 hours (or transported in a special medium) on nonselective media enriched with blood or serum, and incubated in a moist and microaerobic atmosphere. The identity of any colonies grown can be confirmed using Gram's stain and biochemical tests.

3.4 Aspiration

To sample gastric fluid one can use a Shiner tube. This is a polyvinyl tube with a stainless steel sampling capsule at the end. The specimens are obtained by

suction. This tube can be sterilized in the autoclave or by boiling.(76) Sampling the lumenal content of the stomach may lead to underestimation of the size or even misinterpretation of the composition of gastric microbial communities.(77) Estimates per unit weight of material of the population levels of microbes attached to an epithelium surface made from samples of the mucosa itself have been found to be higher than estimates made from luminal content in the region.(77) This technique is not clinically relevant and is hardly ever used in research models.

3.5 Urea breath test

The urea breath test is a non-invasive test that detects radio-labeled carbon dioxide excreted in the breath of persons with *H. pylori* infection; orally administered urea is hydrolyzed to carbon dioxide and ammonium in the presence of the enzyme urease, which is present in *H. pylori*. In non-infected subjects, urea leaves the stomach unchanged, unless there is urease activity from bacteria in the oral cavity or in situations of gastric bacterial overgrowth. The urea breath test is a highly sensitive (93.3%) and specific (98.1%) method.(88) There are 2 breath tests available: ^{14}C-urea breath test (radioactive) and ^{13}C-urea breath test (stable isotope). The ^{13}C-urea breath test avoids radioactivity and is the test of choice for children and pregnant women. The major disadvantage is the need for a gas isotope mass spectrometer to analyze the breath samples and calculate a ratio of ^{12}C to ^{13}C. A 4-hour fast is generally recommended before the urea breath test. A test meal is given before the solution of labeled urea. This test meal delays gastric emptying and increases contact time with the bacterial urease. It is relatively inexpensive compared to the "gold standard" (endoscopy with biopsy and histological examination). The urea breath test avoids sampling errors, which can occur with random biopsy of the antrum. False positive results can occur if gastric bacterial overgrowth is present and involves urease-producing bacteria other than *H. pylori*. False positive results can also occur if the measurements are taken too soon after the urea ingestion because then the action of oral microflora on the urea is measured. False negative results can be seen if the patients recently had antibiotics, bismuth preparations or acid suppression therapy, because the test is dependent on the number of *H. pylori*.(89) Performance of the urea breath test has been associated with several disadvantages especially in infants, toddlers or handicapped children because one needs active collaboration. False positive results in infants affect the accuracy of the test but

correction for the CO_2 production of the tested individual will improve the specificity.(90;91)

Other tests that do not require a mucosal biopsy include serologic tests and stool antigen tests. Chronic *H. pylori* infection elicits a circulating IgG antibody response that can be quantitatively measured by enzyme-linked immunosorbent assay (ELISA). ELISA is based on specific anti *H. pylori* immune response. This serologic tests is as sensitive (95.6%) and specific (92.6%) as biopsy-based methods.(88)
The presence of IgG does not indicate an active infection. IgG antibody titers may decrease over time (6-12 months) in patients who have been successfully treated.
ELISA or immunochromatographic methods can be performed on the faecal samples to detect *H. pylori* antigen. The limit of sensitivity of the test is 10^5 *H. pylori* cells/ gram of faeces.(92) Sensitivities and specificities of 88-97% and 76-100% have been reported.(93) (88;91;91;91;94) The stool antigen test is not usable for follow-up evaluation of the *H. pylori* eradication as it gives false positive results.
One can conclude that the non-invasive tests are sufficiently accurate for the diagnosis of *H. pylori* infection.

4 Small intestine: Microflora and sampling techniques

4.1 Common microflora

The small intestine can be divided into proximal, mid and distal areas called the duodenum, jejunum and ileum. The velocity of the intraluminal content of the small intestine decreases from the duodenum to the ileum. Microbes that can be isolated from the small intestine pass down through the intestine with chyme and in the fasted state by the MMC or descend from habitats above the small intestine. MMC interdigestive motility also prevents SBBO with colonic microflora from entering the proximal small intestine. **Table 2** describes the microorganisms isolated from the small intestine.
The density of microflora increases towards the distal small intestine. The upper two thirds of the small intestine (duodenum and jejunum) contains only low numbers of roughly the same microorganisms, which range from 10^3 to 10^5 bacteria/ml.(54) These bacteria are mainly acid-tolerant lactobacilli and streptococci. The aerobic and Gram-positive species dominate in the proximal part while more distally, Gram-negative and anaerobic bacteria increasingly outnumber Gram-positive aerobic species. Whipple's disease a rare

Table 2 Microorganisms isolated from the small intestine(54;59;69)

Microbial types in the small intestine	Most prevalent bacteria in duodenum, proximal jejunum	Most prevalent bacteria in distal jejunum, ileum
Lactobacilli	Lactobacilli	
Streptococci	Streptococci	
Bifidobacteria		*Bifidobacteria*
Clostridia		*Clostridia*
Coliforms		
Bacteroides		*Bacteroides*
Veillonellae	*Veillonellae*	
Gram-positive nonsporing anaerobes		
Staphylococcus	Staphylococcus	
Actinobacillus	Actinobacillus	
Yeasts	Yeasts	
	Candida albicans	
	Haemophilus	
		Fusobacterium

multisystemic bacterial infection is caused by *Tropheryma whipplei*. In the small bowel, the *Tropheryma whipplei* could not be cultured for decades and was diagnosed by histopathology. Nowadays *Tropheryma whipplei* can be detected using polymerase chain reaction (PCR) or rRNA techniques on duodenal biopsies or faecal samples.(95) Facultative bacteria (oxygen-tolerant organisms) (predominantly streptococci, lactobacilli and enterobacteria) and anaerobes are present. The rich microflora of the cecum find their way through the ileocecal valve into the distal small intestine (ileum). The microflora begins to resemble that of the colon with around 10^7 to 10^8 bacteria/ml of the intestinal contents. With decreased intraluminal transit, decreased acidity and lower oxidation-reduction potentials, the ileum maintains more diverse and a greater number of bacterial populations.(77) Factors that compromise the oxidation-reduction potential within the tissues are obstruction and stasis, tissue anoxia, trauma to tissues, vascular insufficiency and foreign bodies.(96)

Decreased oxidation-reduction potential specifically predisposes to infection with anaerobes.(97)

4.2 Pathologic microflora

Pathologic bacteria of the small intestine, which cause severe diarrhea, are enterotoxic *Escherichia coli* (ETEC) and *Vibrio cholerae*. *Vibrio cholerae* is diagnosed when it is present in faecal material. Enterotoxic ETEC is a common cause of traveler's diarrhea. *Escherichia coli* produces enterotoxins that cause intestinal secretion and diarrhea.

In SBBO, the proximal small bowel is populated by a substantially higher number of microorganisms than usual. These are frequently anaerobic bacteria, which are normally not present in large amounts in the duodenum and the proximal jejunum. A total count of microorganisms exceeding 10^5 colony forming units/ ml in duodenal or jejunal aspirate is generally accepted as small bowel bacterial overgrowth.(98) Some authors also accept a concentration of colonic microorganisms above 10^3 CFU/ml as positive for SBBO. A profound suppression of gastric acid may facilitate the colonization of the upper small intestine.(65)

To diagnose SBBO the quantitative culture of a small intestinal aspirate (considered to be the gold standard) is used. Fluid aspirated from the descending part of the duodenum may be cultured to detect bacterial overgrowth in diffuse small bowel disorders.

4.3 Biopsy

To obtain biopsy samples one has to perform upper endoscopy. Upper endoscopy is performed after an overnight fast of at least 10 hours. An endoscope has a length of approximately 1 meter and has a biopsy channel. During endoscopy the esophagus, stomach and duodenal wall can be systematically inspected. To allow a good view one needs air insufflation; the patient may complain of bloating during the endoscopy. When the endoscope reaches the site of interest, the biopsy from the small intestinal mucosa is rapidly taken by standard biopsy forceps. **Figure 2** shows the size (in centimeters) of the tip of an endoscope and a biopsy forceps. With endoscopy one cannot sample the distal part of the jejunum and the ileum because it can not be reached using a standard endoscope.

Endoscopic biopsies are an adequate substitute for jejunal suction biopsies. The advantage over capsule biopsy is that the site of interest can be inspected before the biopsy is taken.(99-101) Adequacy of mucosal biopsies is a function

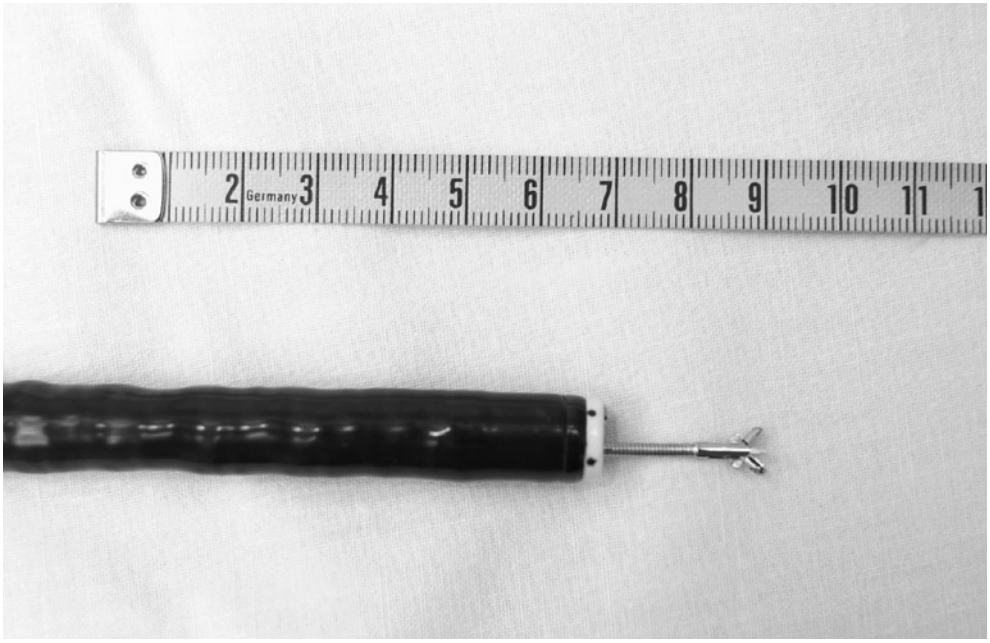

Figure 2 Tip of a standard endoscope and biopsy forceps with needle (tape measure in centimeters).

of size and numbers of biopsies obtained.(101) Alligator-type forceps obtain larger specimen pieces than oval-shaped forceps.(102) Forceps with a needle, or the multibite forceps, allow more biopsies to be taken per passage and improve the quality of tissue obtained.(102) Biopsy forceps without a needle can be used to obtain two samples per passage through the endoscope that are quantitatively as good as when only one sample is collected. This approach can save time, and causes no significant damage to the biopsy specimens.

Because air insufflation may distort the intraluminal anaerobic environment one could use nitrogen if one wants to culture anaerobic bacteria. There is also the risk of contamination with microflora from more proximal habitats, which were passed along via the endoscope. The biopsies have to be taken at a certain distance from the endoscope to prevent sampling contaminated parts of the intestine.
Intestinal biopsies taken from living persons may not yield satisfactory results because the biopsies are only a minimal part of the total bowel wall.(103) The number of persons sampled must be large to be reliable. The best source of information of microflora in the small intestine is samples taken from autopsy

studies in victims of accidents. As slow cooling of the gastrointestinal tract can cause alterations in bacterial localization the samples have to be taken immediately after death.(104) The number of individuals sampled must still be quite large.

4.4 Full thickness biopsy

Full thickness biopsy is a peroperative or laparoscopic biopsy (muscularis-containing biopsy) used to diagnose motility disturbances. One incision is situated below the umbilicus and one in the left fossa. The bowel loop is identified laparoscopically and will then be exteriorized through the incision below the umbilicus. The full-thickness biopsy of at least 10 × 10 mm will then be taken with a surgical knife. The bowel loop is closed with absorbable suture and repositioned into the abdomen.(103) Drawbacks of biopsies taken at surgery are the manipulation of the patients' diet (fasting) and the bowel preparation or preoperative treatment with antibiotics.(77;105) Using molecular typing of bacteria in intestinal tissues, it is better to use biopsies taken at surgery than endoscopic biopsies because of the larger sample size.(106)

4.5 Mucosal brushings

One passes the cytology brush, protected by a sheath, through the instrument channel of the endoscope. The brush is advanced from its sleeve within sight of the mucosal surface and rubbed and rolled across the surface after the endoscope is placed at the location of interest. Thereafter, the brush is pulled back into the sleeve. Normally, cytology brushes are only covered with a plastic sleeve to protect the specimen during withdrawal. This sleeve, however does not protect against contamination; the use of suction of saliva and gastric fluid during endoscopy contaminates the suction channel of the endoscope and the subsequent passage of the brush without a sheath through the suction channel causes loss of sterility of the brush.(74) These brushes cannot be used for sampling bacteria in the lumen of the gastrointestinal tract. Avoidance of any suction during endoscopy is extremely difficult. To obtain small bowel samples without contamination one could utilize a catheter with a specimen brush plugged with sterile Vaseline. Brushes cannot be protected from contact with air, so it is not useful for the isolation of anaerobes for culture. To determine the concentration of bacteria obtained by the brush present per milliliter, one has to standardize the loading capacity of the brush used. Brushing is a highly reproducible technique (92%).(107)

4.6 Peroperative needle aspiration

Peroperative needle aspiration is useful for relatively inaccessible locations. The microflora may be influenced by pre-operative fasting, antibiotic prophylaxis and anesthesia. Furthermore, this technique is only applicable for patients with an underlying disease who will undergo laparotomy or laparoscopy. Until 1959 one used peroperative needling technique performed at operation regularly.(108;109) The advantages of this technique are asepsis and the lack of contamination from other regions of the gastrointestinal tract. Nowadays, however, it is not performed routinely anymore.

4.7 Self opening capsule

Before the introduction of the endoscope the Crosby capsule was used to obtain biopsies from the small intestine. A Crosby capsule is a metallic capsule of 19 to 11 mm with a round opening of 4 mm first used in 1957.(100) A long tiny tube is attached to the capsule. A Crosby capsule is muscle loaded through an endoscope and the endoscope passes into the second part of the duodenum. Mucosa is sucked into the tube by suction and cut off. Failure of obtaining biopsies is 6%. The mucous membrane is very mobile with respect to the muscular layer so only mucosa is sucked into the capsule and the risk of perforation is very small. Muscularis propria is never cut. The risk of bleeding (0.14%) and intestinal perforation is very small.(110)
Every part of the stomach and the small intestine can be reached.(111) Sizes of the biopsies are 8 to 5 mm. Stomach biopsies are usually smaller.
There are also capsules which can be opened electronically. They have the disadvantage of a long interval between sample collection and culturing. In this period, bacteria inside the capsule can replicate and influence growth of other bacteria in the capsule. It is a very imprecise method. The advantage of this technique is that, like the Crosby capsule, every part of the small intestine can be examined. The disadvantage of the suction biopsy capsule used to provide specimens from the proximal jejunum is the need for radiological screening for the location of the capsule. This makes it unsuitable for repeated use in young children and women who are or might be pregnant. There may be some discomfort when the procedure is prolonged. The technique fails in up to 10% of the cases. To overcome the problem of determining the sampling location with the capsule biopsy, specimens taken had better be taken with endoscopic forceps. Capsule biopsies are not common in the current clinical gastroenterology practice.(99)

4.8 Aspirate

Small bowel aspiration for quantitative and qualitative culture specimen is still regarded as the gold standard for diagnosis of SBBO. The sample should be properly harvested with respect to sterile technique and accurate location. The exact composition of the microflora is not important for the diagnosis of SBBO if one uses the definition that more than 10^5 colony forming units/ml small intestinal fluid represents SBBO, but it is of use when antibiotic therapy is being considered. It should be realized that cultures of randomly harvested samples can produce false-negative results if the sample is not taken from the actual site of bacterial overgrowth.

Culturing is not necessary if one uses gas chromatographic detection and analysis of volatile fatty acids in the aspirates. The volatile fatty acids are produced by the metabolism of microorganisms such as *Bacteroides* and *Clostridia*. This is a rapid test for the presence of anaerobic bacteria. When gas chromatography of volatile fatty acids is compared with cultures of jejunal aspirates, it shows a sensitivity of 56% and specificity of 100%.(98) When results of testing for volatile fatty acids in jejunal aspirates are positive, this always indicates the presence of bacterial overgrowth. This procedure would avoid the more complicated, time-consuming and expensive bacteriological analysis of jejunal samples.(98;112;113)

The numbers of bacteria per milliliter of intestinal juice taken at two different levels of the proximal jejunum show highly significant correlations (r_s=0.90, $P < 0.001$); thus one does not have to obtain the aspirate from the exact same location in the proximal jejunum.(98)

Aspirate can be acquired by intestinal intubation (with sterile or non-sterile tubes), the capsule method, direct needle aspiration of the gut contents, peroral intubation and by the string test. (see below)

Intubation with sterile or non-sterile tubes

This endoscopic method for collection of proximal gastrointestinal fluid for culture is simple and can be performed during routine endoscopy. When the endoscope reaches the descending part of the duodenum, the polyethylene tube will pass through the biopsy channel into the intestinal lumen. Intestinal intubation seems to be the most suitable and reliable method for studying small intestinal microflora, because of the short sample collection time and minimal disturbance of physiological conditions. Care must be taken to prevent contamination with upper respiratory tract microflora during the passage of the tube and to maintain oxygen-free conditions for anaerobic

culturing. A closed polyethylene tube filled with water through the suction channel of the endoscope is therefore recommended, as it is not necessary to keep the suction channel sterile. The water has to have been boiled for sterilization and the removal of dissolved oxygen. The distal end is closed with a plug of agar. Because the innertube remains sterile even after the passage through the unsterile suction channel of the endoscope, the use of an overtube eliminates the possibility of contamination. The proximal end can be attached to a double way stopcock connected to a syringe containing boiled water. In the duodenum the agar plug can be expelled from the tube by injection of the water in the syringe. After several minutes the expelled water has gone through and the duodenal contents can be aspirated into the tube, after which the tube is removed from the endoscope. Precision of the sample site and proven absence of contamination are the main advantages. Since fresh aspirate is known to tolerate oxygen fairly well for an exposure time of at least eight hours, it is a good method for obtaining aerobic and anaerobic samples.(107;109;114)
Highly significant correlations ($r_s=0.84$, $P < 0.001$) were found between the numbers of bacteria per milliliter of jejunal aspirate obtained from the closed and open tubes confirming that the intubation method is highly reproducible. (98)
The use of suction during endoscopy contaminates the suction channel of the endoscope. One could discard the first milliliter of aspirate. This is very difficult in the duodenum, where at best only a few milliliters of aspirate will be found. (114) Using an open tube for collection of small bowel juice can theoretically lead to contamination; but according to previous studies this does not seem to be the case.(115) (116)

Duodenal string test (Enterotest)
The duodenal string test capsule is a cheap and simple device used for sampling the contents of the upper gastrointestinal tract (diagnosis typhoid fever; sampling duodenal contents by a "string" test yields a positive culture in 70% of patients).(117) The weighted gelatin capsule contains a silicone rubber bag and a 140 cm highly absorbent nylon string. After a 10-hour fast the device is administered. The first 10 cm of the nylon line is pulled out from the capsule by the protruding loop. The capsule is then swallowed with water while the loop is held outside the mouth. The loop is then taped to the face to secure the line. After approximately 3.5 hours the thread has moved into the duodenum. The volume of the duodenal fluid absorbed by the distal end of

the thread is calculated by subtracting the dry weight of the segment. The distal end is squeezed out between sterile gloved fingers in order to collect the intestinal content. Its major applications in pediatrics are the diagnosis of enteric parasitic infestations, confirmation of contaminated small-bowel syndrome, and the diagnosis of *Salmonella* infection, *Giardia lamblia* and assessment of duodenal bile salts in the diagnosis of neonatal cholestasis in duodenal contents. A drawback of the Enterotest is that when the string is pulled out of the gastrointestinal tract, the intestinal contents adhering to it are exposed first to the sterilizing effect of gastric acid and afterwards to contamination with microflora present in the esophagus and pharynx. The Enterotest is not useful for the isolation of anaerobes because samples can not be protected from contact with air. The clinical value of the string test compared with a sterile endoscopic method for sampling small bowel secretions is limited by poor sensitivity, specificity and positive predictive value. Thus the string test is not an adequate substitute for oro-duodenal intubation for the detection of SBBO.(107;118)

Peroral intubation

Peroral intubation and aspiration of luminal contents can be achieved by Miller-Abbott or Levin tubes. These tubes were modified to suit the special needs for culture studies. A capsule as the headpiece of a Miller-Abbott tube was designed, which opened and closed at will and was operated by hydraulic pressure. Despite the advantage of the large size of the capsule (44.5 × 12 mm) it has been proven possible for bacteria to gain access into the closed capsule in vitro. A Levin tube is clinically used as a gastroduodenal feeding tube with a length of 125 centimeters. A long radio-opaque tube is used, marked for accurate placement, either single- or double-lumened, with or without balloons, perforated by one or more holes at its distal end. These perforations were either left free or were protected by means of a collodion membrane, a thin rubber sheath, or by plugs, which could be either dissolved or dispelled by positive pressure at the moment of taking samples for culture.
Contamination depends on the degree of contamination of the surrounding fluid, the exposure time and the static environment.
The small intestine contains only a very small quantity of fluid in contrast to gastric juice, which may be aspirated in large quantities.
A disadvantage of peroral intubation is the lack of certainty that the specimen obtained from the desired level of the intestine has not been contaminated by bacteria from a higher position during its passage.

4.9 Noninvasive methods

Because small intestinal intubation for quantitative culture is inconvenient, expensive, and not widely available, a variety of surrogate tests for bacterial overgrowth in the small intestine have been devised based on the metabolic actions of enteric bacteria rather than on increases in the number of bacteria. Several indirect methods have been developed to overcome the problem of location-dependence of aspirates for culturing. **Table 4** compares small intestinal non-invasive tests with culture of material obtained with invasive methods for diagnosis of SBBO. Most of these indirect tests lack sensitivity for reliable detection of SBBO. The main reason for this is the great variability of the microflora and its metabolic profile. The tests are based on a specific bacterial metabolic activity. Thus, if this particular activity is not present in the microflora of a SBBO patient, the test will yield a false-negative result. For this reason urinary excretion tests (e.g. indican excretion, D-xylose, conjugated para-aminobenzoic acid) and analysis of intestinal aspirates for bacterial metabolic products (e.g. deconjugated bile acids in serum) lack the required reliability for detection of SBBO and have become obsolete.(119-122) These tests will not be described further.

Table 4 Small intestinal non-invasive tests compared to jejunal culture (gold standard) Comparison of sensitivity, specificity and simplicity of the various non-invasive tests for detection of small bowel bacterial overgrowth.(89;98;125;127;128;131;149-152)

Test	Sensitivity	Specificity	Simplicity
^{14}C-xylose BT	42-100%	85-100%	Excellent
Lactulose H_2 BT	68%	44%	Excellent
Glucose H_2 BT	62-93%	78-83%	Excellent
^{13}C and ^{14}C- glycocholate BT	20-70%	76-90%	

BT: breath test, H_2: hydrogen

To diagnose bacterial overgrowth breath tests are used, including the ^{14}C-glycocholate breath test, the ^{14}C-D xylose breath test, the lactulose H_2-breath test and the glucose-H_2 breath test. The rationale for the breath test is the production by intraluminal bacteria of volatile metabolites; that is, CO_2, H_2 or CH_4, from the administered substances, which can be measured in the exhaled air. The most successful and popular methods analyze either expired

isotope-labeled CO_2 after timed oral administration of ^{14}C-or ^{13}C-enriched substrates or breath hydrogen following feeding of a non-labeled fermentable carbohydrate substrate.
The ^{14}C- and ^{13}C- breath tests measure the pulmonal excretion of labeled carbon dioxide produced by the fermentation of labeled substrates, using either a radioactive or a stable isotope. The increasing availability of methods for analyzing stable isotopes has raised interest in replacing the radioactive ^{14}C by non-radioactive ^{13}C. The use of radioactive isotopes is not recommended for study of children or women who are or might be pregnant. $^{13}CO_2$ can be measured by mass spectrometry. Because of concerns about diagnostic accuracy, costs of the substrates and equipment, and limited availability, these tests have not gained widespread acceptance.
The first breath test to diagnose SBBO was the hydrogen breath test described by Levitt in 1969(123). Hydrogen is a constituent of human breath derived exclusively from bacterial fermentation reactions in the intestinal lumen. Bacteria produce hydrogen out of carbohydrates. Human tissue does not generate hydrogen. The colon is considered to be the only place in the human body where hydrogen is produced, because of the high amount of hydrogen-producing bacteria. In cases of SBBO, hydrogen is also produced in the small intestine.
Detection of hydrogen in expired breath is considered a measure of the metabolic activity of enteric bacteria. Part of the produced hydrogen is reabsorbed from the intestine into the blood and is exhaled. Measurement of breath hydrogen could circumvent the administration of a radioactive isotope in testing for bacterial overgrowth. The conditio-sine-qua-non for this test is the presence of a hydrogen-producing microflora, but in 15-20% of humans the microflora of the subject does not meet this condition. Hydrogen breath analysis is therefore not sufficiently reliable as a diagnostic tool in SBBO.

4.10 ^{14}C-glycocholate breath test

^{14}C-glycocholate breath test or bile acid test is based on the bile salt deconjugating capacity of bacteria in the proximal small bowel. Conjugated bile acids are excreted through the bile in the duodenum and they are reabsorbed in the terminal ileum. Conjugated bile acids are in the enterohepatic circulation. Physiologically, less than 5% of the conjugated bile acids reach the colon. After excretion in the duodenum, bile acids stimulate micellization of dietary lipids. After oral administration of glycocholic bile acid (a normal component of bile) this is normally reabsorbed in the terminal ileum. In cases of SBBO some

bacteria split off glycine on the amide bond of cholylglycine. Glycine is absorbed and fermented in the liver to CO_2, H_2O and NH_4; the CO_2 produced is exhaled. When using ^{14}C glycocholate, the $^{14}CO_2$ in the exhaled air can be measured.
The sensitivity is too low (20-70%) to allow SBBO to be demonstrated without additional intestinal culturing. A rise in labeled carbon dioxide does not differentiate bile salt wastage from bacterial overgrowth. This is a disadvantage given that a significant number of small bowel bacterial overgrowth patients may have had ileal resection. Ruling out bile salt malabsorption as an explanation of a positive breath test can be done with stool collection.(89;124) The false negative rate is 30%-40%. There are three reasons for false negative outcomes. Firstly, one needs anaerobic organisms to deconjugate bile salts. Secondly, not all cases of bacterial overgrowth involve bile salt deconjugation. Lastly, the fatty meal (usually a polymeric supplement) given with the cholylglycine may, in theory, affect the ratio of labeled and unlabeled carbon dioxide absorbed, diluting the labeled carbon dioxide with that produced from the metabolism of the meal. False positive results are possible in case of ileal pathology, ileal resection and increased intestinal transit. In those cases bile acids are deconjugated by the (anaerobic) colonic microflora. The disadvantage of using radioactivity in ^{14}C-substrate breath tests can be overcome by using the stable ^{13}C-isotope, which is measured by mass spectrometry in breath samples. However, the use of ^{13}C-isotope does not improve the sensitivity.

4.11 ^{14}C-D-Xylose breath test

The ^{14}C-D-xylose breath test was considered to be the only breath test for the detection of bacterial overgrowth with high sensitivity (95-100%) and 100% specificity but these promising results have not been sustained.(89) Compared with cultures of the duodenal aspirates, the sensitivity and specificity are 60% and 40%, respectively.(125)
This test is based on the assumption that aerobic Gram-negative overgrowth microflora ferment D-xylose. D-xylose is a monosaccharide which can be labeled with ^{14}C.(89) Produced $^{14}CO_2$ and unmetabolized xylose are absorbed by the proximal small bowel, which thus avoids confusion of results caused by metabolism of substrate by colonic bacteria. Subjects have to fast at least 8 hours before the test, and no smoking or exercise is permitted 12 hours before the breath test. Following a one gram oral dose of ^{14}C-D-xylose in water,

elevated $^{14}CO_2$ levels are detected in the breath within 60 minutes in 85% of patients with SBBO.
False negative rates are 35-78%. False negative results cannot be entirely attributed to the absence of D-xylose fermentation of the microflora (overgrowth bacteria in 81.8% of SBBO patients are capable of D-xylose fermentation); body weight is correlated to endogenous CO_2 production and should therefore also be taken into account.(126) Disturbed gastric emptying and small intestinal motility can also contribute to a false-negative result of the ^{14}C-D-xylose breath test because of delayed delivery of the labeled substrate to the metabolizing microflora. Refinement of the ^{14}C-D-xylose breath test to include a transit marker for intestinal motility increases its specificity. With the transit marker one can determine whether the site of metabolism is in the small intestine or in the colon.(127)

4.12 Lactulose hydrogen breath test

Lactulose is an easily fermented disaccharide and is used for the detection of bacterial overgrowth and for determination of the orocecal transit time. The lactulose hydrogen breath test is a simple, inexpensive and noninvasive technique to diagnose small bowel bacterial overgrowth. The lactulose breath test is performed after 12 hours fasting previous to the test. Hydrogen breath samples are taken at baseline and subsequently every 10-30 minutes after the test meal. The test meal contains 10-12 gram lactulose. The hydrogen breath samples are analyzed gas chromatographically.(128) Baseline samples average 7.1 ± 5 parts per million (ppm) of H_2 and 0 - 7 ppm for CH_4.(129) Values of the baseline sample over 20 ppm H_2 are suspect for bacterial overgrowth. Values between 10 and 20 ppm H_2 suggest incomplete fasting before the test or ingestion of slowly digested foods the day before the test, the colon being the source of the elevated levels.(129) Slowly digested foods like beans, bread, pasta, and fiber must not be consumed the night before the test because these foods produce prolonged hydrogen excretion.(129) The patient is not allowed to eat during the complete test. Antibiotics and laxatives must be avoided for weeks prior to breath hydrogen testing. Cigarette smoking, sleeping and exercise must be avoided at least a half hour before and during the test because these may induce hyperventilation.(89) Chlorhexidine mouthwash must be used before the test to eliminate oral bacteria, which might otherwise contribute to an early hydrogen peak after the substrate is given. Lactulose, which reaches the colon, shows peaks usually more than 20 ppm above baseline after 2-3 hours of testing. Lactulose is not absorbed in the

small intestine so every patient should have a colonic peak, assuming the colonic microflora has not been altered. Peaks associated with SBBO occur within 1 hour and are less prominent. Some laboratories measure H_2 and methane simultaneously whereas others test methane selectively after flat lactulose tests.(89)
The lactulose hydrogen breath test is positive for small intestinal bacterial overgrowth if there is an increase in breath hydrogen of >10 parts per million above basal that occurs > 15 minutes before the cecal peak. Strict interpretative criteria, such as requiring two consecutive breath hydrogen values more than 10 ppm above the baseline reading, and recording a clear distinction of the small bowel peak from the subsequent colonic peak (double peak criterion), are recommended. Application of the double peak criterion alone for interpretation of the lactulose hydrogen breath test is inadequately sensitive, even with scintigraphy, to diagnose bacterial overgrowth. Twenty-seven % of normal subjects have no peak due to organic acid reduction or dilution from voluminous diarrhea.(89) (**Figure 3**)

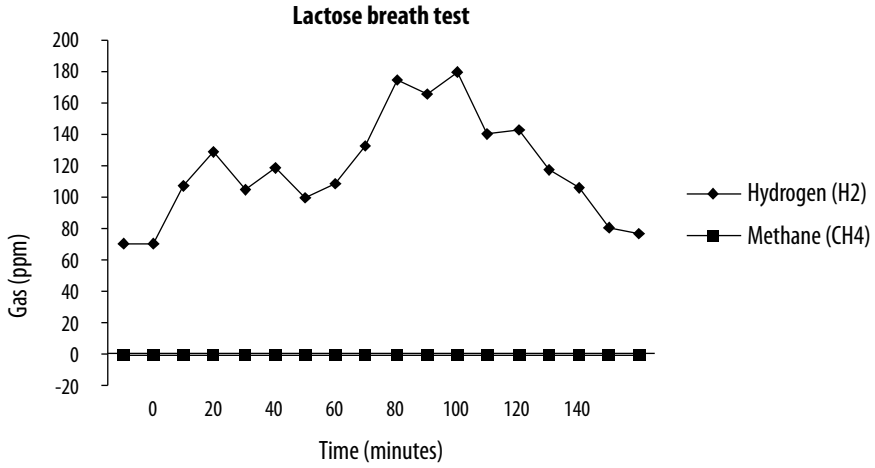

Figure 3 *Production of hydrogen (H_2) and methane (CH_4) in a patient with bacterial overgrowth of the small bowel. Fasting H_2 and CH_4 production at –10 and 0 minutes. 10 gram lactulose is administered at 0 minutes.*

The disadvantage of this test is that it is not always easy to distinguish breath hydrogen arising from small bowel colonization from that resulting from cecal fermentation in patients with an exceptionally rapid orocecal transit time.

A comparison with the jejunal culture sensitivity of 68% and specificity of 44% has been described.(98) A sensitivity of 16% for SBBO has been decribed.(130) Despite the attractive aspects of ease of performance and avoidance of a radioactive tracer, breath hydrogen tests are not sufficiently sensitive or specific to justify their substitution for the ^{14}C-D-xylose breath test for noninvasive detection of intestinal bacterial overgrowth.

4.13 Glucose hydrogen breath test

Glucose hydrogen breath tests can also be used to detect small bowel bacterial overgrowth. Glucose is completely absorbed before reaching the colon even in patients with previous gastric surgery, who have faster than normal transit.

Patients receive a solution containing 50-80 g glucose dissolved in 250 ml water after fasting for 12 hours. Breath hydrogen concentrations are analyzed with a H_2 monitor after direct expiration through a Y-piece that prevents air from mixing with the exhaled hydrogen.(131) Hydrogen concentration is determined every 10-15 minutes for two hours. Results of the hydrogen breath test are considered positive when the hydrogen concentration increases 14-20 ppm.(132) Smoking and exercise are not allowed during the test and the day previous to the test.(133) The hydrogen breath test shows stable intra-individual results in healthy people. However, in patients with high values there is a large day to day variation.(134) The coefficient of variation is 5-10%.(131;135) Sensitivity of 93% and specificity of 78% have been described (132)

The glucose hydrogen breath test has a sensitivity of 62% and a specificity of 83% compared with jejunal culture.(98) Poor sensitivity due to rapid absorption of glucose substrate in the proximal small bowel, which inhibits hydrogen generation, can be explained by a washout effect of concomitant diarrhea, loss of bacterial microflora because of recent antibiotic therapy or an acidic bowel lumen.

5 Large intestine: Microflora and sampling techniques

5.1 Common microflora

Table 3 represents the microorganisms isolated from large intestine samples and from faecal samples.

The large intestine including the cecum, colon and the rectum harbors probably over 400-500 species of bacteria. Novel molecular methods are aiding better understanding of the microflora which is challenging to culture due to the anaerobic nature of the most of the microflora and insufficient

Table 3 Microflora isolated from the large intestine and from the faeces(53;54;59;59;69;136;148)

Microbial types in the large intestine	**Microbial types in the faeces**
Lactobacilli	Lactobacilli
Streptococci	Streptococci
Bifidobacteria	*Bifidobacteria*
Clostridia	Clostridia
Propionibacterium	*Propionibacterium*
Eubacterium	*Eubacterium*
Bacteroides	*Bacteroides*
Fusobacterium	*Fusobacterium*
Veillonella	*Veillonella*
Staphylococcus	*Staphylococcus*
Coliforms	Coliforms
Bacillus sp	Bacillus sp
Yeasts	Yeasts
spiral shaped microbes	spiral shaped bacteria
Actinobacillus	
Enterobacteriaceae	
Enterococci	
	Peptococcus
	Ruminococcus
	Coprococcus
	Acidaminococcus, Succinivibrio, Butyrivibrio, Megasphaera,
	Gemminger
	Catenabacterium
	Peptostreptococcus

Most prevalent bacteria underlined

knowledge of the culturing conditions.(136;137) In large intestine samples contain 10^{11} to 10^{12} colony forming units/ml.(53)(54) Knowledge about the mucosa-associated bacterial communities in different parts of the colon is limited as most attention has been focused on bacteria present in faeces. Enormous microbial populations can develop in the lumen of the large bowel, and especially in that of the cecum because these areas have a relative stagnation in the flowing stream (up to 60h) and very low oxidation-reduction potentials. The transit time of the lumenal content exceeds the doubling times of bacteria.
99.9% of colonic microflora are obligate anaerobes. *Bacteroides*, *Bifidobacteria*, *Eubacteria*, *Clostridia* and *Enterobacteriaceae* can predominantly be found in the colon. Whether the microflora is transient or truly autochthonous to habitats in the region remains a main concern. Bacteria in food are known to pass into human faeces at high population levels. Bacteria from habitats above the large bowel pass down into the lumen of that region. The population levels of transients probably do not contribute significantly to the level in the region. Bacteria in the colon are important in processing maldigested carbohydrates.(138)

5.2 Pathologic microflora

Yersinia enterocolitica, *Salmonella*, *Shigella*, *Campylobacter*, *Clostridium difficile*, enterohemorragic *Escherichia coli* (EHEC) and enteropathogenic *Escherichia coli* (EPEC) are the most common pathologic bacteria to cause diarrhea. Diarrhea can also occur after oral antibiotic treatment. Poorly absorbed antibiotics change the normal composition of the microflora in the colon.(139) Suppression of the normal microflora may lead to reduced colonization resistance with subsequent overgrowth of resistant microflora, yeasts and *Clostridium difficile*. This organism produces a protein toxin which causes necrosis and ulceration of the colonic mucosa, called antibiotic associated hemorrhagic colitis.

5.3 Biopsy

A standard colonoscope has a length of 1.30 m to 1.60 m, so that the colon and the distal ileum can be evaluated. Long colonoscopes (165-180 cm) are able to reach the cecum even in overly long and tortous colons.(74) Biopsy specimens can be collected with a flexible colonoscope and flexible biopsy forceps. Patients are given a laxative solution to drink the day before the examination. The object of full preparation is to cleanse the entire colon of faecal material,

especially the proximal parts, to allow a clear view.(74) So it is very likely that the bacteria in the biopsy sample are mucosa-associated as the luminal bacteria will have been washed away.(140) 10^5-10^6 bacteria are present in biopsy samples.(141) The predominant mucosa-associated bacterial community is host specific and uniformly distributed along the colon but differs significantly from the faecal community.
Biopsy samples are very small in size and therefore more easily exposed to oxygen during sampling; therefore, the number of viable strict anaerobes might be reduced easily. Relatively high levels of facultative anaerobes are reported to be present in intestinal biopsy samples. To minimize contamination during sampling, the colonoscope jaws will have to be washed in tap water after each biopsy is performed.

5.4 Pyxigraphy

Pyxigraphy is a technique, which makes use of a capsule that can be swallowed, and by which contents of the gastrointestinal tract can be sampled under remote control. Pyxigraphy is a simple and safe sampling method that allows the microbial population of the proximal colon to be studied.(142)

5.5 Faecal samples

Faeces are a complex microbial habitat, with many niches to be filled by bacteria. It is estimated that bacteria make up 40-55% of faecal solids. All of the bacteria in faeces are exposed to the influences of dehydrating and concentrating mechanisms of the colon and rectum and intense biochemical activity of the organisms living in the material. When the samples consist of only faeces, the composition and localization of communities anywhere in the tract cannot be revealed. Bacteria account for about 30% of the faecal mass. *Bacteroides* accounts for nearly 20% of the species that can be cultivated from faeces.(53) The *Bacteroides* and *Prevotella* group (Gram negative anaerobes) and *Eubacterium rectale* and *Clostridium coccoides* species (Gram positive anaerobes) are predominantly present in the faecal samples.(136;138) In the colon of healthy humans, anaerobic species outnumber aerobic ones by at least 10-fold. The predominant bacterial community in faeces is stable in time, host specific, affected by ageing and not altered after consumption of probiotic strains.(143)
Stool specimens or rectal swabs can be used for the diagnosis of cholera. Dipsticks in rectal swabs are used for the rapid diagnosis of cholera caused by *Vibrio cholerae*. Dipstick analysis uses colloidal gold particles and is based on a

one-step immunochomatography principle. The sensitivity and the specificity of the dipsticks is greater than 92% and 91% respectively. This rapid test (diagnosis within 10 minutes) requires minimal technical skills.(144;145)
Most knowledge of the gastrointestinal microflora stems from colon or faeces bacteriology. A major limitation in studying the proximal human colonic microflora is the lack of suitable sampling methods. Studies in which only faeces are sampled can never reveal the composition and localization of epithelial and cryptal communities anywhere in the tract. Such studies reveal little about the composition of lumenal communities in any area except perhaps the large bowel.(77)
Faecal samples have to be collected in sterile bags and kept at low temperature (+4 degrees Celsius to minus 80 degrees Celcius) before processing.(138)
Low faecal pH is caused by ingestion of poorly absorbed carbohydrates or carbohydrate malabsorption due to bacterial fermentation in the colon. Faecal pH of less than 6 is highly suggestive of carbohydrate malabsorption. A breath hydrogen test with lactose can confirm carbohydrate malabsorption. In this test a fasting patient is given 25 g of lactose dissolved in water, and exhaled breath is assayed for hydrogen content at baseline and at intervals for several hours as described in **Figure 4**. Because hydrogen is not a normal product of human metabolism, any increase in breath hydrogen concentration represents bacterial fermentation and indicates that unabsorbed lactose has reached the colon.

6 Conclusion

The different methods of investigating the intestinal microflora in humans all have their advantages and their drawbacks as described above. If one desires information about the gastrointestinal tract one should also weigh the benefits of the (research) question and their financial consequences.
Sampling of the gastrointestinal tract in humans is far more difficult than in animal models. The sampled area is relatively small in comparison with the total area. In animal models the animal can be sacrificed so that the complete intestinal tract can be sampled and investigated. Individuals who are killed in accidents are the best source of complete information about microflora in the gastrointestinal tract.(77)
In general, the patient prefers the non-invasive method. Non-invasive methods are of particular importance for very young pediatric patients, pregnant women and elderly, as well as for research purposes. The difficulties of sampling the entire gastrointestinal tract are reduced by the non-invasive

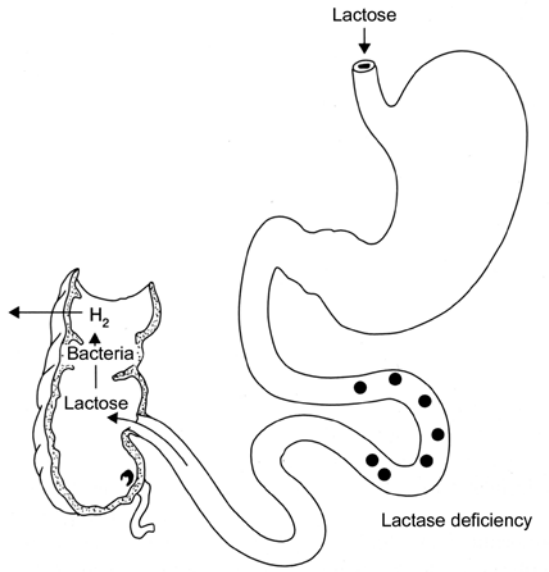

Figure 4 Carbohydrate malabsorption

tests. However, non-invasive methods are often less sensitive and less specific. Invasive methods, such as endoscopy, are extremely unpleasant but are highly sensitive and specific. The conditions that have to be satisfied in obtaining an uncontaminated specimen from anywhere in the gastrointestinal tract have to include:

1 strict asepsis of method, which necessitates that the instrument used must be suitable for sterilization by heat or gas
2 prevention of contamination of the internal channels in which the culture specimen is to be lodged, until the site of sampling is reached, and protection against further contamination on withdrawal of the instrument.
3 verification of the location from which cultures have been obtained.

Invasive methods have the advantage of sampling at the accurate location. As the development of molecular biology techniques increase, the current sampling techniques can be revised. The condition of anaerobic sampling is becoming less important. Possible improvement of the current sampling methods only seems possible in small details. Nanotechnology is one of the promising techniques for possible improvement of sampling and analysis of bacteria in the human gastrointestinal tract.(146)

Chapter 3

Critical evaluation of diagnosing bacterial overgrowth in the proximal small intestine

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Abstract

Introduction
Clinical small bowel bacterial overgrowth syndrome (SBBO) can be objectified by bacterial overgrowth tests. Since direct culture of jejunal aspirates has disadvantages, non-invasive tests such as breath tests (BT) are used. Major drawback of lactulose BT might be rapid lactulose transit to the colon. We evaluated diagnosing bacterial overgrowth using experimental and standard BT, and culture and molecular based methods.

Material and methods
Bacterial overgrowth was analysed in 11 controls and 15 SBBO predisposed subjects. During experimental breath testing an occlusive balloon limited lactulose to the small intestine. Jejunal fluid was analysed using culture and molecular based methods. Bacterial overgrowth was diagnosed on the basis of 20 ppm hydrogen or methane increase above baseline within 90 minutes or more than 10^3CFU/ml excluding lactobacilli and streptococci and furthermore using all published definitions.

Results
Experimental and standard BT showed no changes in timing of hydrogen excretion between controls and SBBO subjects. Using standard BT, 3 out of 11 controls and 8 out of 15 SBBO subjects were bacterial overgrowth positive. Total counts showed no significant differences between controls and SBBO subjects using culture and molecular based methods. Bacterial overgrowth was diagnosed in 0 out of 9 controls and 4 out of 12 SBBO subjects using culture based methods. Other definitions used in literature revealed no significant differences between controls and SBBO subjects.

Conclusion
In a small group of subjects the experimental BT did not improve the ability of lactulose BT to diagnose bacterial overgrowth. Culturing showed less bacterial overgrowth in controls compared to BT. Remarkably, current diagnostic criteria do not seem to be accurate in discriminating between SBBO subjects and controls.

Introduction

Bacterial overgrowth can be asymptomatic but small bowel bacterial overgrowth (SBBO) syndrome is characterized by steatorrhea, diarrhea, flatulence, abdominal pain and bloating. Symptoms of macrocytic anemia, weight loss, malabsorption of nutrients and vitamins and bone loss are related to severe bacterial overgrowth syndrome. This clinical SBBO syndrome is a condition associated with the proliferation of colon-type bacteria within the small intestine. SBBO syndrome should be distinguished from bacterial overgrowth. Besides an abnormal composition of bacteria, bacterial overgrowth in the small intestine might also reflect an increased number of bacteria in the small intestine. To demonstrate bacterial overgrowth to support the diagnosis SBBO syndrome, direct culture of jejunal contents is considered the gold standard.(98;154) However, this requires orointestinal intubation and proper specimen handling. Besides the fact that sampling aspirates is impractical, the scope of sampling leaves cases with isolated distal bacterial overgrowth undiagnosed.(155) SBBO syndrome was confirmed by bacteriologic analysis in 57-87% of patients with SBBO symptoms.(98;156) Cultures from several different jejunal sites revealed that the overgrowth flora may be non-continuous in the upper gastrointestinal tract, leading to false negatives when only one culture site is assessed.(157) Cultures may be false negative, particularly in cases of overgrowth by obligate anaerobes.(115) False positives due to contamination mainly from the oral microbiota are believed to be common as well.(158) Moreover, culturing reveals only a fraction (estimated at 20%) of microbiota compared to the molecular based methods.(159)

Because of these disadvantages, non-invasive tests such as the lactulose breath test, have been developed. The lactulose breath test relies on the fermentation of lactulose by bacteria with release of hydrogen and methane. Hydrogen and methane are subsequently absorbed and expired. Since lactulose is poorly absorbed, it is a suitable substrate for diagnosing bacterial overgrowth even in the distal small intestine.

Hydrogen breath tests have poor sensitivity and specificity in comparison with culture of small bowel aspirate.(98;160) This might be due to high false negative rates of aspirate cultures or delayed gastric emptying or false positive testing due to rapid transit of the lactulose.(125;157) One study combining the lactulose breath test with scintigraphy showed that early hydrogen rise was associated with increased transit time.(130) This supports the idea that the

poor sensitivity and specificity are due to increased transit time. Bacterial overgrowth in the small intestine might be diagnosed irrespective of the transit time when transit of lactulose to the large intestine is prevented. The aim of this investigation was to evaluate the effect on diagnosing bacterial overgrowth when lactulose intake is limited to the proximal small intestine by means of blocking the passage to the large intestine. The results of this experimental breath test were compared with the results of the standard breath test. In the same subjects culturing of jejunal aspirate was performed. Since culturing does not detect all bacteria we also applied molecular based methods to detect bacterial overgrowth. Bacterial overgrowth was diagnosed using all published definitions for culturing and lactulose breath testing results.

Material and methods

Subjects

Eleven healthy subjects and 15 subjects with diseases predisposing to SBBO syndrome were included in the study. The group of SBBO syndrome predisposed subjects consisted of 10 IBS patients, diagnosed according to Rome II criteria(5) and 5 patients with clinical suspicion of SBBO syndrome (**Table 1**). Pimentel et al. reported a prevalence of 78-84% bacterial overgrowth in IBS patients using the lactulose hydrogen breath test.(17;23) Breath tests were

Table 1 *Subject characteristics*

	Healthy subjects	IBS	Clinical SBBO syndrome
Number	11	10	5
Female/male ratio	7/4	7/3	3/2
Mean age (years±SEM)	26 (3.6)	39 (5.0)	58 (4.7)
Clinical diagnoses	NA	IBS-A (n=3) IBS-D (n=1) IBS-C (n=6)	Blind loop after surgery (n=1) Jejunal diverticulosis (n=1) Polyneuropathy (n=1) CIIP (n=1) Ileocecal resection (n=1)

NA: not applicable; IBS-A: alternating diarrhea and constipation predominant IBS; IBS-D: diarrhea predominant IBS; IBS-C: constipation predominant IBS; CIIP: chronic idiopathic intestinal pseudo-obstruction

abnormal irrespective of subgroups of IBS.(17;23;66) Patients with clinical suspicion of SBBO syndrome had symptoms of diarrhea, abdominal pain and weight loss. The conditions predisposing to bacterial overgrowth of the small intestine in the patients were blind loop after surgery, jejunal diverticulosis, ileocecal resection, polyneuropathy and chronic idiopathic intestinal pseudo-obstruction. Antibiotics or probiotics were not allowed for at least 2 weeks prior to the study. Medication known to affect the motility of gastrointestinal tract and proton pump inhibitors or antacids were not allowed the day before the study. All subjects gave informed consent. The study was approved by the ethics committee of the University Medical Center Utrecht, Utrecht, The Netherlands.

Lactulose breath testing

Subjects were asked to follow a low fibre diet the day before the test. After an overnight fast the subjects used an antimicrobial mouth rinse (Chloorhexidine digluconaat 0.2%, Corsodyl, 2 mg/ml, GlaxoSmithKline BV, Zeist, the Netherlands) to prevent metabolism of lactulose by the oral flora.
Subjects were instructed to collect air at the end of expiration. Two baseline breath samples were obtained. After intake of 12 grams of lactulose (Bertek Pharmaceuticals Inc., Milan, Italy) in 200 ml water, breath samples were analysed every 10 minutes for 2 hours. The air was analysed simultaneously for hydrogen and methane concentrations with a QuinTron SC MicroLyzer (QuinTron Instrument Company, Milwaukee, WI, USA). The instrument corrected differences in end expiratory breath samples. The minimal detectable concentration for both hydrogen and methane analysis was 1 part per million (ppm).
Smoking, sleeping and physical exercise were forbidden during the test.

Jejunal aspiration and experimental lactulose breath testing

A 3-lumen 400 cm silicone catheter (Dentsleeve Pty Ltd, Wayville, South Australia) was used to sample jejunal fluid and infuse lactulose. Tip weights and a balloon at the tip facilitated transport through the upper intestine. A second occlusive bag (length 10 cm, height 5 cm, diameter 3 cm) was attached to the catheter from 6 to 11 cm from the tip. The first channel was used to inflate the tip balloon, the second to inflate the occlusive bag and the third to sample jejunal fluid and infuse lactulose in the jejunum through three side holes at 14, 15 and 16 cm from the tip.

The catheter was introduced through the nose or mouth. On reaching the jejunum (10 cm distal of the Ligament of Treitz), confirmed by fluoroscopic examination, the occlusive bag was inflated to a pressure of 30 mmHg using an electronic Barostat. Jejunal fluid was collected after infusion of 10 ml 0.9% NaCl solution. Thereafter a baseline breath testing was performed and lactulose (12 grams dissolved in 20 ml water) was inserted through the catheter into the jejunum. Hydrogen and methane were measured every 10 minutes for 90 minutes as described above. Jejunal occlusion was confirmed using 20 mL Ultravist-150 (Schering-Plough) infusion through the side holes 14,15 and 16 cm from the tip, and X-ray at the end of the study.

Cultured-based microbiological analysis

The jejunal fluid samples were sent immediately to the microbial laboratory and five series of 10-fold dilutions were made. For bacterial determination and quantitative analysis, 100 µl of undiluted jejunal fluid or 100 µl of each dilution was cultured on different plates. The samples were cultured on blood agar, MacConkey-agar (for Gram-negative strains), Columbia CAN agar (for staphylococci and streptococci), Man-Rogosa-Sharpe-agar (for lactobacilli) and Brucella agar (for anaerobes). After 48 hours of incubation at 37°C under aerobe and anaerobe conditions, the micro-organisms were identified using standard microbiological techniques. Quantification of the microbiota was performed by counting the number of colony forming units per ml.

Molecular based methods

From the jejunal fluid, 1 ml was washed with 1 ml (0.2 µm filtered) sterile PBS and rigorously vortexed. The sample was centrifuged at 16,000 G. The pellet was washed twice with (0.2 µm filtered) sterile PBS and finally suspended in 1 ml (0.2 µm filtered) sterile PBS. Of this solution, 800 µl was used for DNA isolation according to the DNeasy Tissue protocol provided by the manufacturer (Qiagen, Venlo, The Netherlands). Quantitative PCR was performed using a Smart Cycler (Cepheid, USA). All qPCR reaction mixtures for the optimized SYBR Green I-based assay consisted of 0.1 µl 1:200 dilution of SYBR Green I (Molecular Probes, The Netherlands), 12.5 µl Smart Cyclerkit (Eurogentec, Netherlands), 6.9 µl nuclease free water (Promega, USA), 0.25 µl (50µM) forward primer and 0.25 µl (50µM) reverse primer.(24;161) The reaction mixtures contained either 5 µl template or water (no-template control). The thermal cycling conditions used were an initial DNA denaturation step at 95°C for 5 minutes followed by 35 cycles of denaturation at 95°C for 15 seconds, primer

annealing at 55°C for 20 seconds and extension at 72°C for 30 seconds. Finally, melt curve analysis was performed by slowly increasing the temperature from 60°C to 95°C (0.3°C per cycle) with simultaneous measurement of the SYBR Green I signal intensity. Primers used were previously described by Malinen *et al*. and Bartosch *et al*.(24;161)

Bacterial overgrowth diagnosis

In the literature 15 different lactulose breath test definitions for bacterial overgrowth are described (**Table 2**). These include 3 definitions based on fasting hydrogen or methane levels, 7 based on an increase above baseline values within a time period and 5 definitions using a double peak criterion. In the experimental test double peak criteria definitions are not applicable since the lactulose cannot reach the cecum. In this study bacterial overgrowth was diagnosed on the basis of an increase of 20 ppm hydrogen or methane above baseline within 90 minutes.

There is also no consensus in literature on the definition of a positive culture for bacterial overgrowth. The bacteriological definitions for bacterial overgrowth are shown in **Table 3**. These definitions range from the presence of specific bacteria, $>10^4$ CFU/ml to $>10^8$ CFU/ml to the total counts of all bacteria or only colonic-type flora or salivary-type flora. Moreover, the definition of colonic-type flora also varies in the literature.

Since colon-type bacteria (anaerobic and Gram negative species) in the small intestine are considered to be pathogenic and associated with SBBO symptoms, bacterial overgrowth was diagnosed as $> 10^3$ CFU/ml with the exclusion of lactobacilli and streptococci in the present study.(78;162;163)

Calculations and statistical analysis

In the lactulose breath test the baseline value was the average of two basal hydrogen or methane concentrations. The increase in hydrogen or methane concentration was calculated by subtracting the baseline value from the test value.

Jejunal fluid samples with bacterial growth lower than the cut off range of 10 CFU/ml were assigned a number of 1 $\log_{10}$ CFU/ml for statistics purposes. Molecular based and culture based total counts were compared between healthy subjects and SBBO syndrome predisposed subjects using the nonparametric Mann Whitney U test.

Changes in hydrogen and methane over time between healthy subjects and SBBO syndrome predisposed subjects were analysed using repeated measures

analysis of variance (ANOVA). The number of bacterial overgrowth positives in the healthy subjects and SBBO syndrome predisposed subjects were compared using the Chi square test. Statistical analysis was performed using commercially available software (SPSS 12.0.1 for Microsoft windows).

Table 2 Subjects with bacterial overgrowth using different lactulose breath test (LBT) definitions

			Standard LBT	
			Subjects	
No	**Definition bacterial overgrowth**	**References**	**HS (n=11)**	**Predisposed SBBO syndrome (n=15)**
1	Fasting $H_2 \geq 20$ ppm	(89;168)	3	3
2	Fasting $H_2 > 42$ ppm	(129)	2	1
3	Fasting $H_2/CH_4 \geq 12$-15 ppm	(169;170)	6	5
4	Fasting $H_2/CH_4 \geq 20$ ppm and $H_2/CH_4 \geq 12$-15 ppm < 90 min	(171)	4	9
5	$H_2 > 10$ ppm on 2 consecutive measures	(89;168)	9	12
6	$H_2 > 10$ ppm on 2 consecutive measures < 90 min	(172)	3	6
7	$H_2 \geq 15$ppm ≤ 120 min	(163)	8	11
8	$H_2 > 20$ ppm < 90 min and ≤ 180 min	(17;173-177)	7	9
9	$CH_4 > 20$ ppm < 90 min and ≤ 180 min	(17;173-175;177)	0	1
10	$H_2/CH_4 > 20$ ppm < 90 min and ≤ 180 min	(17;173-175;177)	3	8
11	2 distinct peaks; 1st $H_2 > 20$ ppm ≤ 90 min & 2nd > 15 min first peak	(178;179)	1	3
12	2 distinct peaks; 1st $H_2/CH_4 > 20$ ppm & 2nd > 15 min first peak	(169;171)	1	5
13	2 distinct peaks; 1st peak > 5 ppm & decrease at ≥ 2 consecutive measures ≤ 90 min & 2nd $H_2 \geq 20$ ppm	(173)	2	4
14	2 distinct peaks; 1st peak $H_2 \geq 10$ppm ≤ 90 min on 2 consecutive measures & 2nd peak $H_2 \geq 20$ ppm	(98;130;160;180;181)	1	4
15	2 distinct peaks; 1st peak $H_2 \geq 10$ ppm ≤ 20 min & 2nd peak > 15 min first peak	(130;182)	1	3

HS: healthy subjects, IBS: Irritable Bowel Syndrome, SBBO: small bowel bacterial overgrowth, H_2: hydrogen, CH_4: methane, ppm: parts per million, min: minutes.

Table 3 Subjects with bacterial overgrowth using bacteriological definitions

			Subjects	
No	**Definition bacteriological bacterial overgrowth**	**References**	**HS (n=9)**	**Predisposed SBBO (n=12)**
1	$> 10^3$ CFU/ml (lactobacilli & streptococci excluded)	(128)	0	4
2	$\geq 10^3$ colonic-type flora CFU/ml	(125;154;183)	0	1
3	$\geq 10^4$ CFU/ml	(107;127;184)	1	5
4	$> 10^4$ CFU/ml bifidobacteria, clostridia, *B.fragilis*	(152)	0	1
5	$\geq 10^5$ CFU/ml	(76;125;126;130;132;149;154;156;172;183-192)	0	5
6	$> 10^5$ colonic-type flora CFU/ml	(149;184;193-195)	0	2
7	$\geq 10^5$ CFU/ml salivary-type flora (yeasts, *Streptococcus mitus*, *Streptococcus salivarius*, *Staphylococcus aureus*, *Lactobacillus spp*)	(130;187)	0	4
8	$\geq 10^6$ CFU/ml	(89;98;158;181;196-199)	0	2
9	$\geq 10^7$ CFU/ml	(170)	0	1
10	$> 10^8$ CFU/ml	(196)	0	0
11	Colonic-type flora (*Enterobacteriaceae*, *Bacteroides*, *Clostridia genera*)	(98)	0	1
12	Colonic-type flora; *Enterobacteriaceae* $\geq 10^3$ CFU/ ml, *Bacteroides spp* $\geq 10^2$ CFU/ml, *Clostridium spp* $\geq 10^2$ CFU/ml	(130;187;188)	0	1
13	$\geq 10^3$ CFU/ml *Enterobacteriaceae* or enterococci	(107;152;200)	0	1
14	Gram negatives or anaerobes	(127)	0	4

CFU: colony forming units. Predisposed SBBO syndrome includes 7 IBS patients and 2 clinically suspected SBBO syndrome patients. In definition number 2 colonic-type was defined as at least 10^3 anaerobic organisms/ml or at least 10^3 *Enterobacteriaceae*, *Pseudomonas spp* and *Bacteroides spp*/ml. In definition number 5 colonic-type was defined as at least 10^5 Gram negatives, strictly anaerobes and enterococci. In the literature more definitions for colonic-type flora exist. (127;130;187;188;198)

Results

Subjects

SBBO syndrome predisposed patients (46 ± 4.2 years) were significantly older (P=0.002) than the healthy subjects (26 ± 3.6 years). There was no difference in the female/male ratio.

Experimental lactulose breath test

In 2 healthy subjects and 3 SBBO syndrome predisposed subjects the catheter did not reach the jejunum and therefore the experimental lactulose breath test could not be performed. Except for 1 healthy subject all subjects excreted hydrogen. Two out of 9 healthy subjects and none of the 12 SBBO syndrome predisposed subjects excreted methane. Lactulose remained in a limited area for a longer time period which resulted in increasing hydrogen production in all hydrogen producing subjects. We expected that in cases of small bowel bacterial overgrowth patients more bacteria are present in the proximal small intestine and the hydrogen peak would occur earlier than in healthy subjects. However, no changes in timing of hydrogen excretion could be seen in the SBBO syndrome predisposed group compared to the healthy subjects (**Figure 1**).

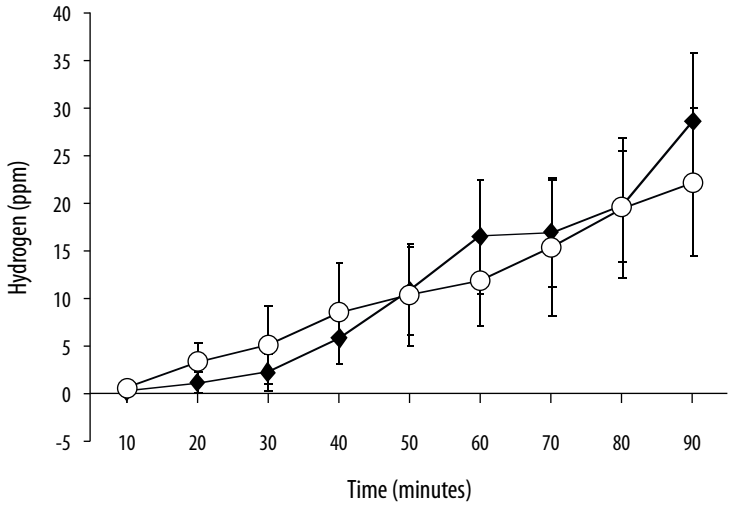

Figure 1 *Mean (± SEM) of hydrogen excretion in experimental breath test over time in 10 healthy subjects (◆) and 12 SBBO syndrome predisposed subjects (○). No significant differences in hydrogen excretion are seen between the two groups over time.*

Nevertheless, both in SBBO syndrome predisposed patients and healthy subjects hydrogen excretion increased over time.

Standard lactulose breath test

All subjects excreted hydrogen. However, in 3 healthy subjects and 5 SBBO syndrome predisposed subjects hydrogen did not increase 20 ppm above baseline within 2 hours. Methane was excreted in 3 healthy subjects (of whom 2 low hydrogen producers) and 2 SBBO syndrome predisposed subjects (of whom 1 low hydrogen producer). When the increase in ppm of hydrogen and methane over time was compared between the groups of healthy subjects and SBBO syndrome predisposed subjects, no significant differences were observed. (**Figure 2**) However, 5 out of 15 SBBO syndrome predisposed subjects showed a hydrogen increase greater or equal to 20 ppm above baseline within 90 minutes and this was observed in 2 out of 11 healthy subjects. Increase of at least 20 ppm methane above baseline within 90 minutes was observed in 1 out of 2 SBBO syndrome predisposed methane producers and in none of the 3 healthy subjects who were methane producers. In this study 3 out of 11 healthy subjects and 8 out of 15 SBBO syndrome predisposed subjects were diagnosed with bacterial overgrowth using the lactulose breath test. The results of the standard and experimental lactulose breath test were both positive or negative for bacterial overgrowth in 5 out of 10 healthy subjects and 7 out of 12 SBBO syndrome predisposed subjects.

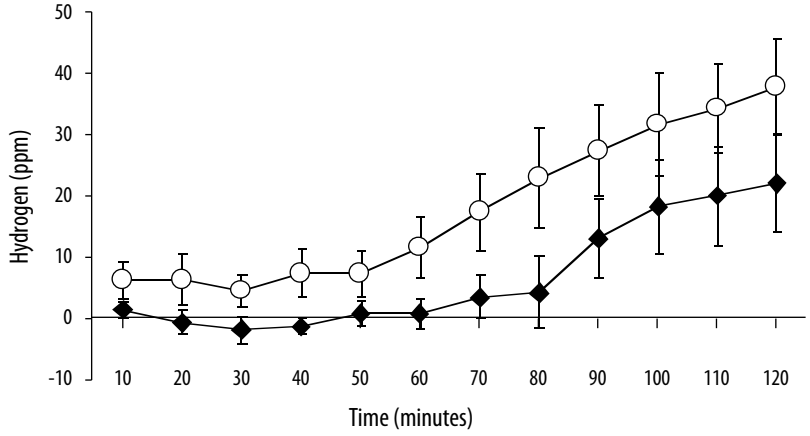

Figure 2 *Mean (± SEM) of hydrogen excretion in standard breath test over time in 11 healthy subjects (◆) and 15 SBBO syndrome predisposed subjects (○). No significant differences in hydrogen excretion are seen between the two groups over time.*

Using the standard breath test, applying the different lactulose breath test definitions for bacterial overgrowth (**Table 2**), 0 to 9 out of 11 healthy subjects were tested positive for bacterial overgrowth, which implies a high spread of specificity of the different definitions. In the predisposed SBBO syndrome patients group, applying the different definitions, 1 to 12 out of 15 patients were tested positive for bacterial overgrowth which implies a large range for sensitivity of the different definitions.

Jejunal aspirate

Cultured-based microbiological analysis

No jejunal aspirate could be obtained from 2 healthy subjects and 3 subjects predisposed to SBBO syndrome. No significant differences in median of log_{10} total counts of healthy subjects and SBBO syndrome predisposed subjects were found (**Figure 3**). Bacterial overgrowth (>10^3 CFU/ml with the exclusion of lactobacilli and streptococci) was diagnosed in none of the 9 healthy subjects and in 4 out of 12 SBBO syndrome predisposed subjects. Of these 4

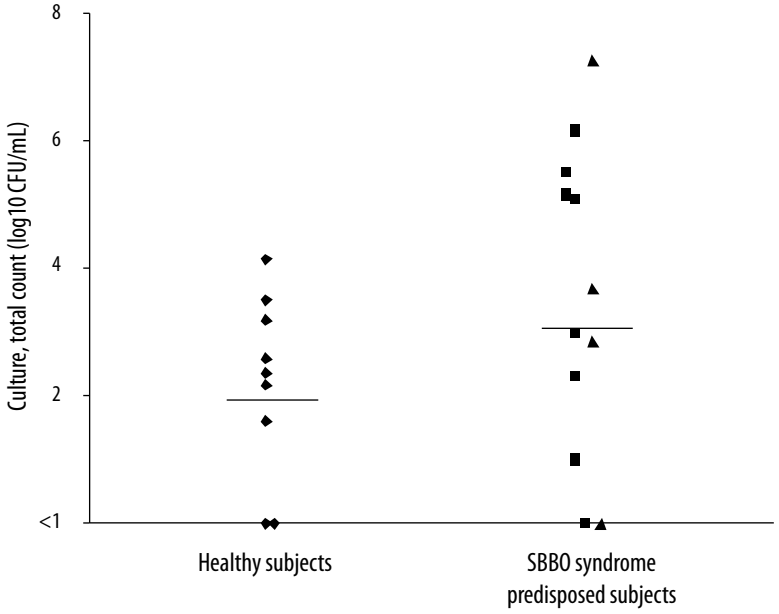

Figure 3 *Culture based total count in 9 healthy subjects (◆), 8 IBS patients (■) and 4 clinical SBBO syndrome predisposed patients (▲). Each dot represents log_{10} bacterial count of one subject. Bacterial counts lower than the cut off range of 10 CFU/mL are presented as <1. Horizontal lines show the medians of healthy subjects and the predisposed SBBO syndrome subjects (IBS patients and clinical SBBO syndrome predisposed patients)*

bacterial overgrowth diagnosed subjects, 3 subjects were also diagnosed as bacterial overgrowth positive with the lactulose breath test (2 IBS patients and 1 clinically predisposed SBBO syndrome subject).
The different definitions in the literature (**Table 3**) show that only one of the 9 healthy subjects had a diagnosis of bacterial overgrowth using a score of $>10^4$ CFU/ml; using the other definitions none of the healthy subjects tested positive for bacterial overgrowth. This implies high specificity for culture based definitions. In the predisposed SBBO syndrome patients the diagnosis bacterial overgrowth ranged from 0 to 5 out of 12.

Molecular based methods

Figure 4 shows $\log_{10}$ total counts of bacterial DNA of healthy subjects and SBBO syndrome predisposed subjects. Medians of $\log_{10}$ total count of bacterial DNA of healthy subjects and SBBO predisposed subjects showed no significant differences. PCR detects not only viable, but also non-viable bacteria.

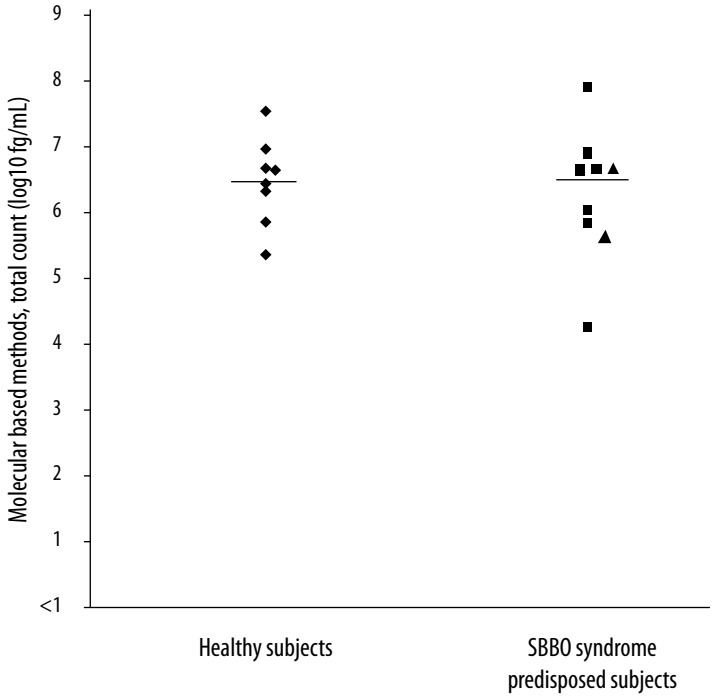

Figure 4 *Molecular based total count in 8 healthy subjects (◆), 7 IBS patients (■) and 2 clinical SBBO syndrome predisposed patients (▲). Each dot represents $\log_{10}$ count of one subject. Horizontal lines show the medians of healthy subjects and the predisposed SBBO syndrome subjects (IBS patients and clinical SBBO syndrome predisposed patients)*

Therefore, total bacterial load (**Figure 4**) estimated by real-time PCR is higher than culture counts of viable bacteria only (**Figure 3**). *Bacteroides fragilis* and *Clostridium coccoides* group could not be detected in the jejunal aspirates (data not shown).
No significant difference in the number of *Enterobacteriaceae* and *Faecalibacterium prausnitzii* between healthy subjects and SBBO syndrome predisposed subjects could be detected (data not shown).
Two out of 12 SBBO syndrome predisposed subjects and no healthy subjects showed $\geq 10^3$ CFU/ml *Enterobacteriaceae* using molecular based techniques. One of the two SBBO syndrome predisposed subjects also showed $\geq 10^3$ CFU/ml *Enterobacteriaceae* in the culture-based microbiological analysis.

Discussion

This study shows that limiting lactulose to the small intestine did not improve the lactulose breath test for diagnosing bacterial overgrowth in a small group of subjects. No differences in timing of hydrogen increase between healthy subjects and SBBO syndrome predisposed subjects could be found. Even so, in SBBO syndrome predisposed subjects as well as in healthy subjects, hydrogen producing bacteria are present in the proximal small intestine. Higher hydrogen excretion in SBBO syndrome predisposed patients, as expected, could not be demonstrated. In experimental breath testing an increase of 20 ppm above baseline occurs earlier than in the standard breath test. This difference in time might be due to the method of lactulose administration. In the standard breath test oral ingestion requires transport to bacteria after which hydrogen or methane can be produced. In the experimental test lactulose is infused in the small intestine and is immediately in contact with the local bacteria. Hydrogen appears in breath samples within about 2 minutes of lactulose coming into contact with bacteria.(164) In conclusion the experimental breath test did not improve the ability of lactulose breath testing to diagnose bacterial overgrowth which implies that transit time might not be cause of false testing.
Culturing and the experimental breath test might both be false negative since bacterial overgrowth might be patchy and both the experimental test and the aspiration of jejunal fluid are performed at the same site. Besides, the sampling might be too proximal in the small intestine perhaps occlusion of

the ileum and sampling ileum secretions could shed some light on the diagnosis of bacterial overgrowth.
Irrespective of the definition used, standard lactulose breath testing also showed no differences in bacterial overgrowth diagnosis between healthy subjects and SBBO syndrome predisposed subjects (**Table 2**). Approximately 8 to 15% of all subjects do not produce hydrogen and harbour methanogenic bacteria in the colon that convert hydrogen to methane.(89;165;166) Both hydrogen and methane were measured to eliminate false negatives due to lack of hydrogen production. False negatives may be due to high baseline hydrogen or methane levels which make it hard to increase to 20 ppm above baseline. In our study four healthy subjects and four SBBO syndrome predisposed subjects had baseline hydrogen or methane values above 20 ppm. This high baseline value might result from bacterial flora in the small intestine acting on the previous meal or passage of the previous meal from the small intestine to the large intestine. Furthermore, delayed gastric emptying might lead to false negatives.(125) False positives might be due to increased transit time, entry into the colon of residues from a previous meal stimulated by the ingestion of lactulose or the subject having failed to adhere to a low fibre diet the day before the test.
As mentioned above culturing, independent of the definition used, showed no differences in the number of subjects diagnosed with bacterial overgrowth between the healthy subjects and SBBO syndrome predisposed subjects (**Table 3**). However, culturing results show less bacterial overgrowth in healthy subjects compared to lactulose hydrogen breath tests. False negative results might be due to sampling at one site, low quantity of aspirated fluid and the need for 10 ml 0.9% NaCl –solution infusion. Dilution of the jejunal aspirate might lead to a lower count of bacteria in both SBBO syndrome predisposed and healthy subjects. However, there is a risk of inhomogeneous mixing in the SBBO syndrome predisposed subjects due to motility disorders.
Whether more bacterial overgrowth positives would be detected with more distal cultures and/or multiple cultures remains to be determined.
Since approximately 20% of the gut microbiota can be cultured, a total count analysis was performed using molecular based methods. Nevertheless, no differences in total count between healthy subjects and SBBO syndrome predisposed could be detected. Even so, not the number but the composition of microbiota might be more relevant in bacterial overgrowth. Therefore the number of colonic-type flora in literature as *Enterobacteriaceae*, *F.prausnitzii*,

B.fragilis and *C.coccoides* were determined using Q-PCR (**Table 3**). However, no differences could be revealed.

The present study could not reveal differences in bacterial overgrowth diagnosis between healthy subjects and SBBO syndrome predisposed subjects. The current diagnostic criteria do not accurately discriminate between SBBO syndrome predisposed subjects and healthy subjects as Quigley also recently discussed.(167)

In summary, this study demonstrates that the lactulose breath test limited to the small intestine did not shed more light on diagnosing bacterial overgrowth. Culture of jejunal aspirates seems to be more specific than lactulose breath testing although sensitivity of both culturing and lactulose breath testing remains doubtful.

Chapter 4

Lower *Bifidobacteria* counts in both duodenal mucosa-associated and faecal microbiota in Irritable Bowel Syndrome patients assessed by molecular analysis

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Submitted

Abstract

Introduction
To determine the composition of both faecal and duodenal mucosa-associated microbiota in Irritable Bowel Syndrome (IBS) patients and healthy subjects using molecular-based techniques.

Material and methods
Faecal and duodenal mucosa brush samples were obtained from 41 IBS patients and 26 healthy subjects. Faecal samples were analysed for the composition of the total microbiota using fluorescent in situ hybridization (FISH) and both faecal and duodenal brush samples were analysed for the composition of bifidobacteria using real time PCR.

Results
The FISH analysis of faecal samples revealed a 2-fold decrease in the level of bifidobacteria ($P<0.01$) in IBS patients compared to healthy subjects whereas no major differences in other bacterial groups were observed. At the species level, *Bifidobacterium catenulatum* was significantly lower ($P<0.001$) in the IBS patients in both faecal and duodenal brush samples that in healthy subjects.

Conclusion
Decreased bifidobacteria levels in both faecal and duodenal brush samples of IBS patients compared to healthy subjects indicate a role for microbiota composition in the IBS pathophysiology.

Introduction

Irritable Bowel Syndrome (IBS) is a common gastrointestinal disorder characterized by abdominal pain or discomfort and altered bowel function. Alterations in psychosomatic factors, gastrointestinal motility, visceral hypersensitivity and microbiota composition have been suggested to play a role in the pathophysiology of IBS.(201) Alterations in faecal and small intestinal microbiota composition in IBS patients have been reported and revealed a somewhat higher bacterial count in jejunal juice of IBS patients and lower numbers of faecal coliforms, lactobacilli and bifidobacteria than in healthy subjects.(184;202) More specifically, molecular-based methods showed that in IBS patients levels of members of the *Clostridium coccoides* subgroup, *Lactobacillus*, *Colinsella* and *Bifidobacterium catenulatum* group are different from that of healthy subjects.(24;26) These differences in the faecal microbiota composition may underlie symptom generation by promoting abnormal colonic fermentation.(22) However, mucosa-associated bacteria might be more relevant in the symptom generation of IBS, since faecal and jejunal juice samples are only representing the composition of luminal microbiota. Alterations in luminal bacteria composition may change the commensal microbiota and affects the microbiota adhering to the mucosa. Microorganisms adhering to the intestinal wall are more likely to affect the host's immune, physiological or neuronal system or vice versa. The composition of luminal and mucosa-associated bacteria are not the same since the micro-environments are different at the surface of the intestinal epithelium and the lumen.(141;203)

Therefore, we aimed to determine the composition of faecal luminal and mucosa-associated microbiota in IBS patients using molecular identification and quantification techniques.

Material and methods

Subjects

Twelve male and 29 female IBS patients included in this study fulfilled the Rome II criteria for IBS and were categorized in diarrhea predominant (IBS-D), constipation predominant (IBS-C) or alternating IBS subgroup (IBS-A).(5) The IBS population consisted of 14 IBS-D subjects, 11 IBS-C subjects and 16 IBS-A subjects. The control group consisted of eight male and 18 female healthy subjects from the general population, devoid of GI symptoms or major abdominal surgery. The healthy subjects were significantly ($P<0.001$) younger (31 years ± 2.06) than the group of IBS patients (age 42 years ± 2.12). Subjects taking medication known to influence bacterial composition and gastrointestinal motility, especially antimicrobial medications and/or probiotics were excluded from the study. The Human Ethics Committee of the University Medical Center Utrecht approved the study and all subjects gave written informed consent.

Sampling

To obtain small intestinal mucosa-associated material a sterile cytology brush (Uno-Brush, Prince Médical, Ercuis, France) sheathed in a sterile catheter was placed through the endoscope biopsy channel and advanced under direct vision out beyond the endoscope tip.(107) The duodenal mucosa was brushed three times and then pulled back into the sheath of the catheter. The catheter was removed and the brush was immediately cut off the catheter and placed into a sterile tube in liquid nitrogen and stored at -80°C until analysis.
Faecal samples, obtained before endoscopy, were collected and stored at -80°C until further handling.

Fluorescent in situ hybridization (FISH) analysis of faecal samples

The total number of bacteria present in the faecal samples was determined with the EUB 338 probe which targets all bacteria.(204) FISH analysis was essentially performed as described previously with the genus-specific probes listed in **Table 1**.(205) Approximately 0.5 grams of homogenized faeces was suspended in 4 ml of 0.2-mm-pore-size-filtered PBS and 0.5 ml 37% formaldehyde and thoroughly mixed by vortexing for 3 minutes. After incubation for 4 hours at 4°C the suspension was vortexed again for 2 minutes. Debris was removed by a short spin at 80 *g* for one minute. In an eppendorf tube 300 µl of the supernatant was collected and the fixed cells were washed twice with PBS.

For FISH analysis of the *Lactobacillus-Enterococcus* group the cells were first permeabilized by resuspending the pellet in 100 µl Proteinase-K solution (180 U/ml) (Sigma-Aldrich, Zwijndrecht, The Netherlands) and incubation for 10 minutes at 37°C. The cells were washed as described above and resuspended in 300 µl PBS/ethanol (1:1 vol/vol). After one hour of storage at -20°C, the cell suspension was diluted 1:10 in hybridization buffer at the required temperature for hybridization, and 5 ng labelled probe was added. Cells were hybridized for 16 hours at the prescribed hybridization temperature. After resuspension in 4 ml washing buffer, cells were filtered on a 0.2-mm-pore-size Isopore polycarbonate membrane filter (Millipore Corporation) and washed with 5 ml of 50°C washing buffer. Filters were mounted on microscope slides with Vectashield (Vector Laboratories, Burlingame, CA, USA), and hybridized cells were counted visually using an Olympus BX60 epifluorescence microscope using a FITC or Cy3-specific filter. All microscopic counts were determined in duplicate, with a minimum of 300 cells counted per assay.

Table 1 *Genus-specific probes used for FISH analysis*

Probe	Target group	Reference
EUB338	Total bacteria	(228)
Bac303	*Bacteroides-Prevotella* group	(229;230)
Bif164	*Bifidobacterium*	(205)
Erec482	*Clostridium coccoides-Eubacterium rectale* group	(231)
Chis150	*Clostridium histolyticum* group	(231)
Clit135	*Clostridium lituseburense* group	(231)
Cld73	*Clostridium difficile*	This study: cgccgctctttaccgaagt
Fprau645	*Faecalibacterium prausnitzii*	(230;232)
Lab158	*Lactobacillus-Enterococcus* group	(233)

DNA Extraction and PCR amplification of faecal and duodenal brush samples

Brush and faecal samples were thawed on ice cooled water. DNA was extracted using the DNeasy Tissue kit (Qiagen, Venlo, The Netherlands) or the Fast DNA Spin kit (Qbiogene, Irvine, USA) from the brush and faecal sample respectively. The eluted DNA samples were stored at -20°C. The integrity of the

isolated DNA was determined visually after electrophoresis on a 1.0% agarose gel containing ethidium bromide.

Real-time PCR

Quantification of Bifidobacteria genus and species specific belonging to bifidobacteria was performed using a 5'nuclease (TaqMan) assay as described previously.(206;207) Briefly, a 20 µl PCR amplification mixture containing 10 µl TaqMan Fast Universal Master Mix (Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands), optimized concentrations of primers and probes and 2.0 µl isolated DNA was prepared. The temperature profile for the amplification consisted of 20 seconds at 95ºC and 45 cycles of 1 second at 95ºC and 20 seconds at 60ºC (ABI 7900 HT Fast; Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands). The percentages of the different bacteria were subsequently calculated as described by Liu *et al*.(208;209)

Statistical analysis

FISH results with microbial numbers below the detection limit (8.3×10^5/gram) were excluded from statistical analysis. Nonparametric FISH microbiota data were compared using Mann-Whitney tests or Kruskal-Wallis test for subgroup analysis. Independent samples t-test was used to compare differences in Real time PCR microbiota data between IBS patients and healthy subjects. One-way ANOVA with Bonferroni correction was used for analysis of microbiota in the IBS subgroups.

P-values less than 0.05 were considered statistically significant. All statistical analysis was performed using commercially available software (SPSS 12.0.1 for Microsoft Windows).

Results

Characterization of the faecal microbiota of IBS patients and healthy subjects by FISH analysis

The mean percentages of all bacterial groups measured are presented in **Table 2**. The results show that *F. prausnitzii*, *E. rectale* / *C. coccoides* and Bifidobacteria are the most abundant groups in both IBS patients and healthy subjects. The levels of Bifidobacteria were significantly lower ($P<0.05$) in IBS patients (4.2 ± 1.3%) than in healthy subjects (8.3 ± 1.9%). No significant differences were observed between IBS-D, IBS-C and IBS-A subgroups. The *C. lituseburense*

group was detected in significantly lower levels (P<0.01) in IBS patients compared to healthy subjects; however, *C. lituseburense* reached the detection limit only in 14 healthy subjects and 18 IBS patients. The proportions of *Lactobacillus spp*, *C. coccoides*, *C. histolyticum*, *C. difficile*, *Bacteroides* and *F. prausnitzii* showed no differences between IBS patients and healthy subjects. This set of probes covered 44% and 32% of the total faecal microbiota in the healthy subjects and IBS patients, respectively. The low coverage is predominantly due to low counts in *Bacteroides*.

Table 2 *FISH analysis of the composition of the faecal microbiota of healthy subjects, IBS patients and IBS subgroups. Results expressed as a mean percentage (SEM) of the total cell counts as determined by Eub338 probe.*

Probe	HS	IBS	IBS-A	IBS-D	IBS-C
Fprau 645	12.0 (2.1)	9.2 (0.80)	10.6(1.6)	8.2(1.4)	8.6(1.2)
Erec 482	16.6 (5.4)	11.7 (2.5)	6.4(1.3)	7.1(1.2)	20.5(5.8)
Bif 164	8.3 (1.9)	4.2 (1.3)*	1.7(0.63)	7.9(5.2)	4.5(0.94)
Lab 158	4.7 (0.88)	4.0 (0.78)	2.0(0.38)	4.0(1.7)	6.0(1.5)
Chis 150	2.4 (0.43)	2.1 (0.57)	2.0(0.61)	1.6(0.37)	2.4(1.4)
Bac 303	1.5 (0.89)	3.6 (1.5)	0.41(0.21)	4.4(3.3)	5.7(2.8)
Cld73	0.88 (0.28)	0.43 (0.09)	0.61(0.16)	0.32(0.15)	0.21(0.08)
Clit 135	0.39 (0.10)	0.09 (0.03)*	0.06(0.03)	0.09(0.04)	0.11(0.06)
Sum	44.4 (6.85)	32.1 (3.27)	22.7 (2.30)	28.3 (5.90)	43.5 (5.89)

HS: healthy subjects, IBS-A: alternating IBS, IBS-D: diarrhea predominant IBS, IBS-C: constipation predominant IBS
*P<0.05

Characterization of the faecal microbiota of IBS patients and healthy subjects by real-time PCR analysis

In healthy subjects, the proportion of Bifidobacteria identified as analysis *Bifidobacterium catenulatum* (19% ± 2.5) was significantly (P<0.001) higher compared to IBS patients (6% ± 0.6). The low proportion of *B. catenulatum* was consistent in all IBS subgroups (**Table 3**, **Figure 1**). The proportions of the other species (*Bifidobacterium adolescentis*, *Bifidobacterium bifidum* and *Bifidobacterium longum*) were not significantly different between healthy

subjects, IBS patients and IBS subgroups. Low levels of *B. bifidum* were detected in faecal samples of all subjects as compared to the other *Bifidobacterium* species and as compared to the *B. bifidum* level in duodenal samples. (**Table 3**, **Table 4**) The bifidobacterial species covered by these Q-PCR assays were only 43% and 29.5% of the total bifidobacteria population for healthy subjects and IBS patients, respectively.

Table 3 *Real time PCR analysis of faecal bifidobacteria in healthy subjects, IBS patients and IBS subgroups. Results are expressed as mean percentage (± SEM) of the total bifidobacterial load.*

	HS	**IBS**	**IBS-A**	**IBS-D**	**IBS-C**
B. catenulatum	19.31(2.5)	6.24(0.6)*	6.57(1.1)*	5.67(0.8)*	6.49(1.2)*
B. adolescentis	17.05(2.5)	15.96(1.6)	16.86(3.4)	16.73(1.8)	14.06(3.05)
B. bifidum	$2.1x10^{-4}$ ($1.4x10^{-4}$)	$9.1x10^{-4}$ ($6.3x10^{-4}$)	$3.3x10^{-4}$ ($2.2x10^{-4}$)	$1.9x10^{-3}$ ($1.8x10^{-3}$)	$4.5x10^{-4}$ ($3.1x10^{-4}$)
B. longum	7.11(1.4)	7.30(0.8)	6.68(1.4)	8.71(1.7)	6.45(1.33)

HS: healthy subjects, IBS-A: alternating IBS, IBS-D: diarrhea predominant IBS, IBS-C: constipation predominant IBS
*P<0.001

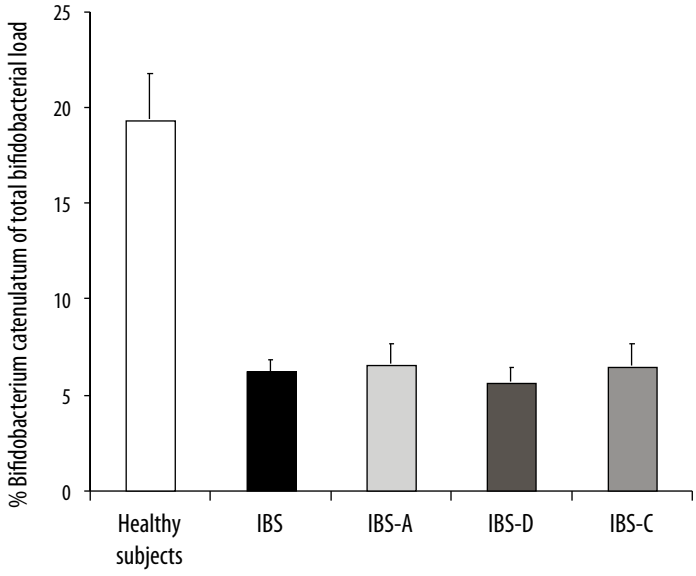

Figure 1 *Percentage of Bifidobacterium catenulatum as percentage of total bifidobacterial load in fecal samples of healthy subjects, IBS patients and IBS subgroups. Results expressed as mean ± SEM*

Table 4 *Duodenal mucosa-associated bifidobacteria in healthy subjects, IBS patients and IBS subgroups. Results are expressed as mean percentage (± SEM) of the total bifidobacterial load.*

	HS	IBS	IBS-A	IBS-D	IBS-C
B. catenulatum	17.04(2.3)	4.85(0.5)*	5.26(0.9)*	4.26(0.7)*	5.04(1.0)*
B. adolescentis	15.68(2.3)	13.90(1.4)	14.66(2.7)	14.58(1.7)	12.21(3.0)
B. bifidum	6.09(0.9)	5.13(0.8)	5.36(1.7)	4.43(1.1)	5.68(1.3)
B. longum	6.88(1.3)	7.27(0.8)	5.84(1.3)	9.01(1.6)	6.88(1.40)

HS: healthy subjects, IBS-A: alternating IBS, IBS-D: diarrhea predominant IBS, IBS-C: constipation predominant IBS
*P<0.001

Characterization of the duodenal microbiota of IBS patients and healthy subjects by real-time PCR analysis

In healthy subjects, *B. catenulatum* level as percentage of total bifidobacterial load (17.04 ± 2.3%) was significantly (P<0.001) higher when compared to IBS patients (4.85 ± 0.5%). The significantly lower proportion of *B. catenulatum* was observed in all IBS subgroups (**Table 4**, **Figure 2**).
The levels of *B. adolescentis*, *B. bifidum* and *B. longum* as percentage of total bifidobacterial load were comparable between healthy subjects, IBS patients and IBS subgroups (**Table 4**). With the set of probes used, the total percentage of bifidobacteria of the bifidobacterial load which could be detected is 46% for healthy subjects and 31% for IBS patients.

Characterization of B. catenulatum in age matched IBS patients and healthy subjects by real-time PCR

Since the patients and healthy subjects were not matched, the age difference between the healthy subjects and IBS patients may be a confounding factor. In a subset of the subjects, 19 IBS patients (33 ± 2.8) matched for age with 19 healthy subjects (33 ± 2.7)) decreased levels of *B. catenulatum* were also shown in duodenal as well as in faecal samples of the IBS patients. The mean percentage of *B. catenulatum* of total bifidobacterial load in duodenal samples was significantly (P<0.001) lower in IBS patients (5.48 ± 0.60%) compared to healthy subjects (17.19 ± 2.43%). Percentage of *B. catenulatum* of total bifidobacterial load in the faecal samples was significantly (P<0.001) lower in IBS patients (6.98 ± 0.69%) compared to healthy subjects (19.50 ± 2.67%).

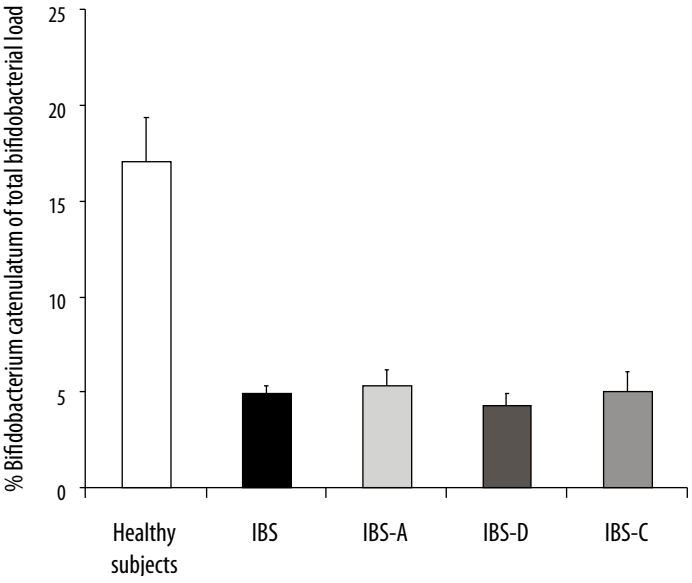

Figure 2 *Percentage of Bifidobacterium catenulatum as percentage of total bifidobacterial load in duodenal samples of healthy subjects, IBS patients and IBS subgroups. Results expressed as mean ± SEM*

Discussion

Composition of gastrointestinal microbiota is known to be relatively stable and composed of permanent, the so called core phyla, and transient species which contribute to gastrointestinal health and disease.(77;210;211) The presence of the beneficial microbes in the intestine prevents colonization by potential pathogenic microbes, referred to as colonization resistance.(212;213) Imbalances in the microbiota are characterized by a decrease in beneficial anaerobic bacteria and increases in aerobic bacteria, fungi and harmful anaerobic bacteria.(213)

In this study, we showed, using FISH analysis, that IBS patients have significantly lower faecal levels of Bifidobacteria but no differences in the other major bacterial groups. Previous studies have also shown microbial alteration in faecal samples of IBS patients both using culturing and molecular-based techniques.(24;202) Using culturing techniques, Balsari *et al*. already showed in

1982 a decrease in bifidobacteria, coliforms and lactobacilli in IBS patients.(202) Using molecular-based techniques decreased *B. catenulatum*, *C. coccoides*, *Lactobacillus* and *Colinsella* counts in the faecal samples of IBS patients were found.(24;26) These studies were limited to the faecal flora. We broadened the study by examining Bifidobacteria levels in duodenal mucosa-associated samples.

Differences in microbiota composition between luminal and mucosa-associated bacteria have been shown.(136;141;159;214;215) The different micro-environment of the epithelium compared to the lumen might lead to a different microbiota composition.(141;216) Bacteria that attach to the mucosa may exert greater influence on innate immune processes in the intestine.(214;217) In addition, the adhesion of non-pathogenic bacteria to the epithelial surface may contribute to the barrier that effects host resistance to pathogenic bacteria.(214)

In this study we showed that the percentages of *B. bifidum* of the total bifidobacterial counts was lower in the faecal samples than in the duodenal mucosa-associated samples in both IBS patients and controls. This might be due to high hydrophobicity of *B. bifidum* which is related to the ability to adhere to surfaces.(218;219) Furthermore, *B. catenulatum* counts were decreased in duodenal mucosa-associated samples as well as in faecal samples of IBS patients compared to controls. The effect of *B. catenulatum* on the health of the host is unknown. However as a group, Bifidobacteria are considered beneficial for the host, as they produce lactic and acetic acids that decrease pH and inhibit the growth of potential pathogenic bacteria.(220-222) Moreover, *Bifidobacterium* spp prevent diarrhea and intestinal infections, alleviate constipation and stimulate the immune system.(222) The lower levels of bifidobacteria might be epiphenomenal or develop as a consequence of altered gastrointestinal motility or genetic makeup of IBS patients rather than the cause of IBS symptoms.(222)

Since the patients and healthy subjects were not matched, the age difference between the healthy subjects and IBS patients might have been a confounding factor. It was reported that elderly (>65 years old) have lower faecal levels of *B. catenulatum*.(60;223) The elderly were not included in our study. The effect of the age difference between healthy subjects (mean age 32 years) and IBS patients (mean age 42 years) on *B. catenulatum* levels is not known. However, in age matched IBS patients and healthy subjects statistically significant decreased levels of *B. catenulatum* were also seen in duodenal as well as in faecal samples of the IBS patients.

An imbalanced microbiota composition may lead to a different fermentation pattern, especially with an increased hydrogen production resulting in bloating.(22;155) Both antibiotics and probiotics have shown to reduce IBS symptoms, which further suggests that microbial imbalance may underlie symptom generation in IBS patients.(17;21;23;224;225) Previously a therapeutic trial suggested that particular *B. infantis* species were efficacious in the treatment of IBS symptoms.(20) No effects of *B. infantis* on stool consistency or frequency could be observed which implies that this therapeutical approach may be applicable to all IBS patients irrespective of their stool pattern.(20) *B. breve* in combination with *L. plantarum* has been shown to decrease pain and the severity of symptoms in IBS patients.(226) Prebiotics, oligofructose and inulin, might reduce symptoms in IBS-C patients as they selectively stimulate bifidobacteria which results in increased stool frequency.(227)
In conclusion, lower bifidobacteria levels were found both in duodenal mucosa-associated samples as in faecal samples of IBS patients when compared to healthy subjects. Specifically *B. catenulatum* was found to be reduced in duodenal mucosa-associated bacteria as well as in the faeces of IBS patients. The relevance of specific *Bifidobacterium* spp in relation to IBS symptoms is unknown however modulation of the gut microbiota by means of prebiotics or bifidobacteria containing probiotics to restore a balanced microbiota composition may have a therapeutic role.

Chapter 5

Molecular analysis of faecal and duodenal samples reveals significantly higher prevalence and numbers of *Pseudomonas aeruginosa* in IBS

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Submitted

Abstract

Introduction
Intestinal microbiota may play a role in the pathophysiology of Irritable Bowel Syndrome (IBS). In this case-control study, mucosa-associated small intestinal and faecal microbiota of IBS patients and healthy subjects (HS) were analysed using molecular based methods.

Material and methods
Duodenal mucosal brush and faecal samples were collected from 37 IBS patients and 20 HS. Bacterial 16SrRNA gene was amplified and analysed using PCR denaturing gradient gel electrophoresis (DGGE). Pooled average DGGE profile of all IBS patients and all HS of both sampling sites were generated and fingerprints of both groups were compared. The DGGE band fragments, which were confined to one group, were further characterized by sequence analysis. Quantitative real time PCR (q-PCR) was used to quantify the disease-associated microbiota.

Results
Averaged DGGE profiles of both groups were identical for 78.2% in the small intestinal samples and for 86.25% in the faecal samples. Cloning and sequencing of the specific bands isolated from small intestinal and faecal DGGE patterns of IBS patients showed that 45.8% of the clones belonged to the genera *Pseudomonas* of which *Pseudomonas aeruginosa* was the predominant species. Q-PCR analysis revealed higher levels ($P<0.001$) of *P. aeruginosa* in the small intestine of IBS patients ($8.3 \pm 0.950\%$) than in HS ($0.1 \pm 0.069\%$). In faeces of IBS patients *P. aeruginosa* was also significantly ($P<0.001$) abundant ($2.34 \pm 0.31\%$) than in HS ($0.003 \pm 0.0027\%$).

Conclusions
This study shows that *P. aeruginosa* is detected more frequently and in higher levels in IBS patients than in HS suggesting its potential role in the pathophysiology of IBS.

Introduction

Irritable bowel syndrome (IBS) is a common gastrointestinal disorder of unknown etiology characterized by abdominal pain and change in bowel habit. Recent evidence is beginning to suggest the potential role of gastrointestinal microbiota in the pathogenesis of IBS. Alteration in faecal microbiota composition, abnormal colonic fermentation, the change in IBS symptoms after using antibiotics and probiotics, the existence of post-infectious variant of the disease and the recognition of an upregulated host immune system response imply that the gastrointestinal microbiota may play a role in the pathogenesis of IBS.(234;235) Indeed, in 7-30% of IBS patients acute gastrointestinal infection has been proposed as trigger of the onset of IBS symptoms, and the inflammatory response to the infection may cause persistent sensory-motor dysfunction.(236-239) Moreover antibiotic therapy and probiotic supplementation have been shown to reduce IBS symptoms in a subset of IBS patients, either by eradication of small intestinal bacterial overgrowth or modulation of the composition of microbiota.(20;23;224;225) Antibiotic therapy may, besides reducing IBS symptoms, also be a risk factor for developing IBS symptoms. This may be due to changes in bowel flora and colonization of pathogenic bacteria such as *Clostridium difficile*.(16) It is well recognized that the microbial community is significantly altered in IBS.(22;184) Conventional culturing methods showed that faeces of IBS patients contain higher numbers of facultative anaerobic bacteria and *Enterobacteriaceae* and lower numbers of lactobacilli and bifidobacteria compared to healthy subjects.(24;202;236;240)

However, detection and identification of gut bacteria by culturing is hampered by the limitations of culture conditions and a large number of intestinal bacteria cannot be cultured.(136;241;242) The advantage of molecular techniques based on 16S ribosomal (rRNA) gene analysis is allowing a more comprehensive assessment of this complex microbial ecosystem.(243) Using PCR based techniques, a significant difference in the faecal predominant groups such as *Clostridium coccoides*, *Bifidobacterium catenulatum*, *Lactobacillus* and *Collinsella* was reported in IBS patients.(24;26) Studies investigating the gut microbiota in IBS using molecular based methods are limited to the faecal flora and did not assess mucosa-associated microbiota in the small intestine which might also be different in composition and abundancy. The mucosa-associated bacteria are thought to have a stronger interaction with the host than luminal bacteria.(57) The composition of luminal

and mucosa-associated bacteria is not the same since the micro-environments are very different at the surface of the intestinal epithelium compared to the lumen.(141;203)
The aim of our research was to determine differences in mucosa-associated small intestinal and luminal faecal microbiota between IBS patients and healthy subjects using a molecular approach based on the sequence variability of 16S rRNA gene.

Material and methods

Subjects
Thirty seven IBS patients who fulfilled Rome II criteria for IBS were included in this study. The IBS patients group consisted of 13 alternating IBS patients, 13 diarrhea predominant IBS patients and 11 constipation predominant IBS patients. Twenty healthy subjects (control group) had neither intestinal complaints nor a history of bowel resection. All subjects had to stop antibiotics at least a month before endoscopy. Use of probiotics, proton pump inhibitors and antisecretory medication had to be stopped at least 3 weeks prior to endoscopy. The Human Ethics Committee of the University Medical Center Utrecht approved the study and all subjects gave written informed consent.

Sampling, preparation and storage
A sterile cytology brush (Uno-Brush, Prince Médical, Ercuis, France) sheathed in a sterile catheter was placed through the endoscope biopsy channel and advanced under direct vision out beyond the endoscope tip.(107) The duodenal mucosa was brushed three times to obtain mucosa-associated bacteria. Brush samples of each subject were obtained from descending and horizontal part of the duodenum. After brushing, the brush was pulled back into the sheath of the catheter, which was removed and the brush was immediately cut off the catheter and placed into a sterile tube in liquid nitrogen and stored at -80^{o}C until analysis.
Faecal samples were collected and were stored at -80^{o}C until analysis. Both faecal and brush samples were shipped in dry ice to the microbiological laboratory for analysis.

DNA extraction and PCR amplification

Faecal samples were thawed and DNA extraction was performed using the Fast DNA Spin kit, (Qbiogene, Irvine,USA) from approximately 0.1 g faecal material. The frozen brush samples were thawed and suspended in 180 µl ATL buffer (Qiagen, Venlo, the Netherlands) and vigorously vortexed to extract the attached bacteria. Subsequently, DNA was isolated from the mucosa-associated bacteria using tissue DNAeasy Blood & Tissue kit (Qiagen, Venlo, The Netherlands) according to the manufacture's instructions. The isolated DNA solution of both faeces and brushes were stored at -20°C.

The extracted DNA was used as a template to amplify the V6 to V8 regions of 16S rRNA with primers U968-GC-f (5'-CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG GAA CGC GAA GAA CCT TAC-3'), which contains at its 5' end a 40-base GC clamp, and U1401-r.(244;245) PCRs were performed using a Taq DNA polymerase kit from Invitrogen (Invitrogen, Paisley,UK). The reaction mixture consisted of 1x PCR buffer, 3 mM $MgCl_2$, 50 µM of each deoxynucleotide triphosphate, 1.25 U of Taq polymerase, 10 pmol of each primer, and 1 µl of appropriately diluted template DNA in a final volume of 50 µl. Samples were amplified in a PTC-200 PCR system (MJ-Research, Waltham,USA) with the following thermocycling program: 94°C for 5 minutes; 10 cycles of denaturation at 94°C for 1 minute, annealing temperature of 65-56°C for 1 minute (reduction of 1°C for each cycle), extension at 68°C for 3 minutes; 33 cycles for faecal and 43 cycles for brush samples of 94°C for 1 minute, 56°C for 1 minute, and 68°C for 3 minutes. Aliquots of 5 µl PCR product were analyzed by electrophoresis on a 1.5% (wt/vol) agarose gel containing ethidium bromide (0.044‰ vol/vol).

DGGE analysis of PCR amplicons

PCR amplicons were separated by DGGE based on the protocol of Muyzer *et al*. using the DGGE Decode system (Bio-Rad Laboratories, Hercules, California, USA) with the following modifications.(245) Polyacrylamide gels consisted of 8% (vol/vol) polyacrylamide (ratio of acrylamide-bisacrylamide; 37.5:1) and 0.5 x Tris-acetate-EDTA (pH 8.0) (TAE) buffer. Denaturing acrylamide of 100% was defined as 7 M urea and 40% formamide. The polyacrylamide gels were made with denaturing gradient ranging from 32.5 to 72.5%. The gels were poured from the top using a gradient maker and a pump (Econo gradient pump; Bio-Rad Laboratories, Hercules, California, USA) set at a speed of 3 ml/min. Prior to the polymerization of the denaturing gel (gradient volume, 28 ml), a 7.5 ml stacking gel without denaturing chemicals was added, and the

appropriate comb was subsequently inserted. Electrophoresis was performed first for 5 min at 200V and then at 80V for 17 hours in 0.5X TAE buffer at a constant temperature of 60°C. The gels were stained with $AgNO_3$ as described by Sanguinetti *et al.* and dried overnight at 50°C.(246)

Analysis of the DGGE profiles and generation of pooled profiles

The average curve feature of GelCompar software (Applied Maths, Kortrijk, Belgium) was used, to generate pooled DGGE profiles generated from IBS patients and healthy subjects, which resulted in an average IBS fingerprint and an average healthy subject fingerprint respectively, based on band intensity and position in the gel. The numeric values of the band intensity (with 5% background substraction) give rise to overlapping band positions for healthy subjects and IBS patients. The overlapping band positions are removed generating a graph with unique bands for healthy subjects and unique bands for IBS patients. Band differences in these average profiles were determined and the bands which were unique for either group were localized in the individual DGGE profiles. The most intense bands in the individual profiles were cut from the original gels. The DNA of these bands was used for a second DGGE analysis using a 46-51.6% gradient. Bands of interest were subjected to sequencing as described below.

DGGE band sequence analysis

In order to identify the bacteria, a small piece from the middle of the selected band was cut from the DGGE gel with a sterile scalpel and then incubated in 50 µl sterile Milli-Q for 24 hours at 4°C to allow diffusion. The eluent containing DNA fragments was used for PCR reamplification with the same primers used earlier. To check whether the DNA of interest on the first DGGE gel and the reamplified DNA migrated to the same position, we carried out a second DGGE in order to compare the two samples. When the two bands comigrated, DNA fragments were purified with the GenElute PCR DNA Purification Kit (Sigma, Zwijndrecht, The Netherlands) and thereafter ligated into the pCR®2.1-TOPO®vector and transformed into *E.coli* One Shot®TOP10 competent cells (Invitrogen, Paisley, UK). Plasmids from colonies of kanamycin resistant transformants were extracted with the Qiagen Plasmid Midi Purification Kit (Qiagen, Venlo, The Netherlands). The extracted plasmids were screened for inserts of the correct size by performing a PCR with the M13 forward and reverse primers as well as with the 968f and 1401r primers. Insert PCR amplicons of selected transformants were purified and were subjected to DNA

sequence analysis. (Baseclear, Leiden, The Netherlands). Sequence similarities of the clones were checked with the Basic Local Alignment Search Tool (BLAST) at the NCBI database http://www.ncbi.nlm.nih.gov/BLAST).(247)

Real time q-PCR analysis

Quantitative Real Time PCR (q-PCR) was performed to determine the percentages of *P. aeruginosa* in brush and faecal samples. Measurement of *P. aeruginosa* was performed as described by Pirnay *et al.* with some slight modifications.(248) The fluorescent labels were changed from LC Red 640 to 6FAM and from fluorescein to TAMRA while the other ingredients and conditions for the described q-PCR stayed the same. The fluorescence signal was measured in the annealing phase on the ABI 7900HT Fast (Applied Biosystems, Nieuwerkerk aan de IJssel, The Netherlands). The total bacterial load was determined as described by Nadkarni *et al*.(249) The relative percentage of *P. aeruginosa* was subsequently calculated according to Liu *et al*.(208;209) The efficiency of each amplification curve was calculated separately and used to determine the initial amount of DNA. Finally, the obtained ratios between the initial amounts of DNA were normalized against a monoculture of the same species, which was set at 100%.

Statistical analysis

DGGE data were analyzed by GelCompar II software (Applied Maths, Belgium). Cluster analysis and calculation of the similarity indices between the different banding patterns were performed using Pearson product-moment correlation and the unweighted-pair group method using arithmetic averages (UPGMA). *P. aeruginosa* levels of IBS patients and healthy subjects were analyzed using independent samples t-test. *P. aeruginosa* levels of IBS subgroups were analyzed using one-way ANOVA with Bonferroni correction. Data are expressed as mean ± SEM. SPSS 12.0.1 for Windows was used for analysis.

Results

Study population

Healthy subjects (5 men, 15 women) were significant (P=0.005) younger (32 ± 2.6 years) than the IBS patients (11 men, 26 women, 42 ± 2.3 years). No differences between both groups in gender distribution were observed.

Bacterial diversity of the dominant microbiota as assessed by DGGE
DGGE analysis of the PCR amplified fragments of the V6-V8 regions of both faecal and duodenal mucosa-associated bacteria 16S rRNA gene of IBS and healthy subjects was performed. Comparing the fingerprints of faecal and small intestinal samples for all subjects, a clear clustering was observed independently from the disease state (data not shown) indicating that the mucosa-associated microbiota is significantly different from that of faecal microbiota as also observed in a previous study.(141) Based on the DGGE profiles no clear difference between the IBS patients and healthy subjects could be detected for the most abundant microbiota at any sampling location. Moreover, statistical analysis of the DGGE profiles from both sampling sites (duodenal brush and faecal samples) did not reveal any specific core microbiota that could be distinguished between healthy subjects, IBS patients or IBS subgroup patients. However some subclusters could be identified suggesting overlap of specific microbial components. To identify possible microbial

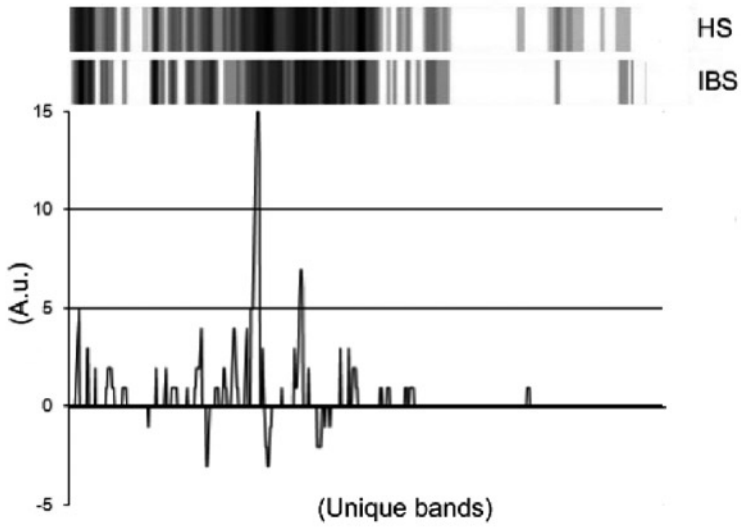

Figure 1 *Average DGGE profiles of all fingerprints of IBS patients (n=34) and all fingerprints of healthy subjects (n=20) in duodenal samples constructed using GelCompar II software. IBS represents the average of 34 IBS patients and HS represents the average profile of 20 healthy subjects. The graph shows a numerical presentation of the average profile with the unique bands present in the healthy subjects on top and the unique bands for the IBS group on the bottom of the graph.*

components specifically associated with healthy subjects or with IBS patients, the DGGE profiles generated from all IBS patients and the DGGE profiles from healthy subjects were pooled in average profiles (**Figure 1 and 2**).
Comparing the generated average duodenal fingerprints of healthy subjects and IBS patients, 14 (78.2%) of the bands were identical for the both groups and 6 bands were specific for IBS patients (10% of all IBS bands) while 27 bands were specific for healthy subjects (34% of all healthy subject bands) (see **Figure 1**). The band location and intensity in the average profiles were used to generate **Figure 1**.
Figure 2 shows the average faecal fingerprints of both groups in which 21 (86.25%) of the bands were identical and 33 bands were confined to healthy subjects (21% of all healthy subjects bands) and 17 bands were confined to IBS patients (10% of all IBS bands). The DGGE band fragments that were of interest were further characterized by sequence analysis.

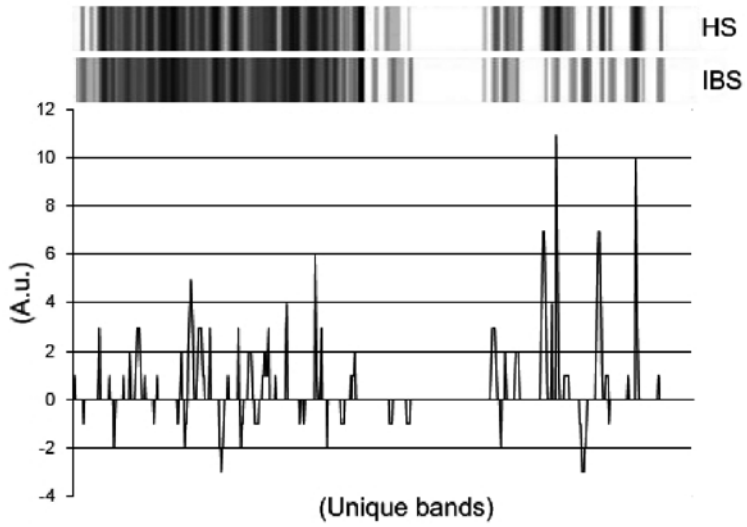

Figure 2 *Average DGGE profiles of all fingerprints of IBS patients (n=34) and all fingerprints of healthy subjects (n=20) in faecal samples constructed using GelCompar II software. IBS represents the average of 34 IBS patients and HS represents the average profile of 20 healthy subjects. The graph shows a numerical presentation of the average profile with the unique bands present in the healthy subjects on top and the unique bands for the IBS group on the bottom of the graph*

Sequence analysis of the dominant microbiota

The 16SrRNA genes from the isolated bands from the DGGE profiles of the healthy subjects and IBS patients of faecal and duodenal samples were amplified and cloned in *E. coli*. The V6 to V8 region of the cloned 16S rRNA gene fragments from the cell lysates of transformants was amplified. In this way, 51 clones were generated and the plasmid DNA from the corresponding clones was purified and, subsequently the nucleotide sequence inserts was determined and compared to the 16S rRNA databases using the Blast tool.(250) From the duodenal samples a total of 19 clones, comprising 15 clones recovered from the IBS patients and 4 clones from the healthy subjects, were generated. A total of 32 clones retrieved from faecal samples, comprising 24 clones of IBS patients and 8 clones of healthy subjects were obtained. Among the 51 sequenced clones retrieved from both groups, 35 shared 95% or less 16S rRNA sequence identity with their nearest relatives based on a blast search. This indicated that the majority of the sequences were derived from new, as yet undescribed phylotypes. Most cloned sequences retrieved from the IBS patients could be assigned to the gamma-proteobacterium phylum mainly to *Pseudomonas* species and other clones retrieved essentially from the faecal samples were assigned to *Clostridium nexile*. However, in the healthy subjects the cloned sequenced were allocated to the 2 major phylotypes commonly encountered in human faecal clone libraries, namely *Clostridium* cluster XIVa and *Bacteroidetes*.

Brush samples:

Out of the 15 clones retrieved from the brush samples of IBS patients, 7 cloned sequences were affiliated with different *Pseudomonas* species with a sequence similarity ranging from 94 to 99%. Remarkably, one cloned sequence was almost identical to *Pseudomonas aeruginosa* (99% sequence similarity). Three clones were closely related to *Klebsiella pneumoniae* with 96 to 97% sequence similarity, one clone almost identical to *Oribacterium sinus* (99% sequence similarity), and 2 new phylotypes (90 to 96% sequence similarity to an uncultured bacterium clone rc2-18; isolated from rat faeces (251)). In addition 1 clone showed a 96% sequence similarity with an uncultured *Neisseria* sp clone isolated from *P. aeruginosa*-colonized patients (252) and shared only 91% similarity to the closest cultured relative that is *Kingella kingae* strain ATCC 23330, another clone was related to an uncultured *Neisseria* sp. clone 101C07 isolated from oral cavity and showed only 91% sequence similarity.

The 4 cloned sequences obtained from the healthy subjects were identified as *Serratia sp*., *Acinetobacter sp.*, *Pantoea sp.* and as uncultured *Clostridiales* bacterium with 99, 98, 91 and 95% sequence similarity, respectively.

Faecal samples:
Out of the 24 clones retrieved from faecal samples of IBS patients, 11 cloned sequences were affiliated with different *Pseudomonas* species with a sequence similarity ranging from 85 to 100%. Noticeably, one cloned sequence showed a perfect match with *Pseudomonas aeruginosa* and 3 other sequences shared 88 to 95% similarity with the same species. Furthermore 8 clones were affiliated with different *Clostridium* species, among those 6 clones were closely related to *Clostridium nexile* with sequence similarity varying from 93-97%, and 2 clones were identified as *Clostridium* sp with sequence similarity varying from 92% to 96%. One clone was identified as *Desulfovibrio vulgaris* (98% similarity), and 1 clone was related to an uncultured faecal bacterium clone isolated by Wang *et al.* with 94% sequence similarity.(253) In addition, 2 clones shared only 92% and 91% sequence similarity with *Burkholderia mallei* and finally one cloned sequence was related with *Alcaligenes faecalis* with 92% sequence similarity.
Out of the 8 clones of the faecal samples of healthy subjects, 5 cloned sequences were affiliated with different *Bacteroides* species with a sequence similarity ranging from 95 to 99% with the closest culturable species being *B.ovatus* (97% sequence similarity), *B.vulgatus* (96% sequence similarity) and *B.coprophilus* (99% sequence similarity). Furthermore one clone was almost identical to *Prevotella corporis* (99% sequence similarity), one clone shared 95% sequence similarity with *Prevotella oulorum* and one clone shared 97% sequence similarity with *Clostridium butyricum*.

Quantification of Pseudomonas aeruginosa using real time PCR
Since the majority of IBS unique bands belonged to the *Pseudomonas* genus from which *Pseudomanas aeruginosa* was the most frequently identified species, real time PCR was used to quantity *P. aeruginosa* in the brush and faecal samples.
The data of **Figure 3A** shows the relative abundance of *P. aeruginosa* (% of total bacterial load) was significantly ($P<0.001$) higher in IBS patients (8.3% ± 0.950) compared to healthy subjects.(0.1% ± 0.069) Moreover, the prevalence of *P. aeruginosa* in the duodenum samples was 97.3% in IBS patients and only 40% in healthy subjects.

The results illustrated in **Figure 3B** reveal that the percentage of *P. aeruginosa* in faecal samples which was also significantly higher (P<0.001) in IBS patients (2.34% ± 0.31) compared to healthy subjects.(0.003% ± 0.0027) We also found that *P. aeruginosa* was detected in 97.2% of the faecal samples of IBS patients whereas only 15.8% of the healthy subjects were positive carriers of *P. aeruginosa*. No differences related to the abundance and prevalence of *P. aeruginosa* between IBS subgroups were detected in both sampling sites.

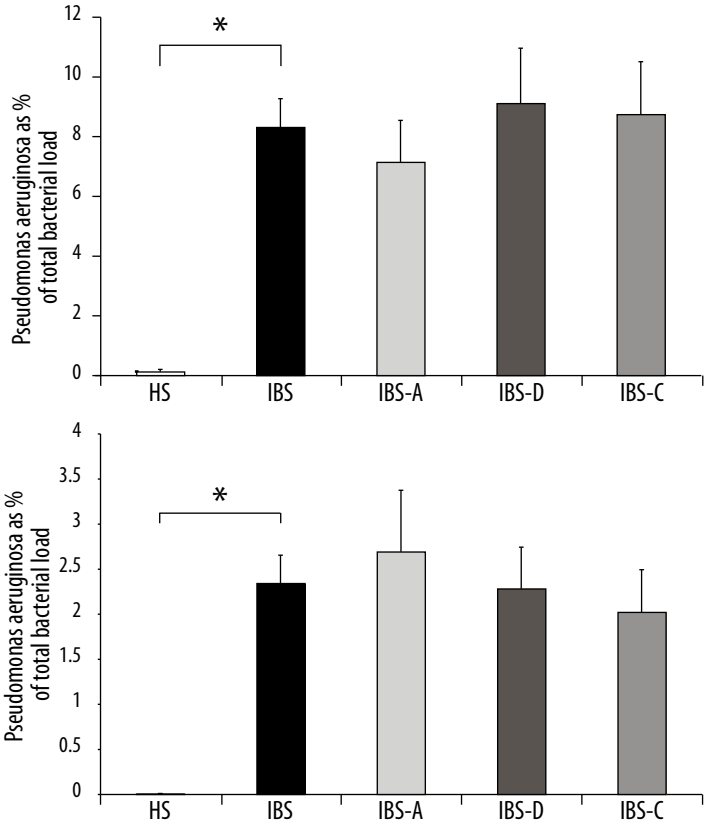

Figure 3 *Duodenal Pseudomonas aeruginosa as percentage of total bacterial load of healthy subjects (HS), IBS patients (IBS) and the IBS subgroups; alternating IBS patients (IBS-A), diarrhea predominant IBS patients (IBS-D) and constipation predominant IBS patients (IBS-C) in duodenal samples (**A**) or faecal samples (**B**).*
Values are represented as means ± SEM.
**P<0.001*

Discussion

This study shows clearly that, analysing the entire faecal and small intestinal microbiota population using DGGE, there are specific DGGE bands for healthy subjects and specific bands for IBS patients. Focussing on the specific bands shows that most clones belonged to the genera *Pseudomonas* of which *Pseudomonas aeruginosa* was the predominant species. The most important finding of this study is the higher prevalence and levels of *Pseudomonas aeruginosa* in small intestinal and faecal samples of IBS patients compared to healthy subjects using Q-PCR analysis.

Previously Kassinen *et al*. also showed distinct microbiota based on their %G+C fractions in faecal samples between IBS subtypes and controls using molecular tools.(26) After faecal microbial genomes of IBS subtypes and controls were pooled differences in the phyla Firmicutes, Bacteroidetes, Actinobacteria and Proteobacteria showed.(26) Our sequence analysis data showed that members of the Proteobacteria and Firmicutes phyla were specific for IBS patients. *Pseudomonas sp* was the most prevalent and Q-PCR analysis showed higher prevalence and levels of *P. aeruginosa* in small intestinal mucosa-associated and faecal samples of IBS patients compared to healthy subjects.

P. aeruginosa, a Gram-negative aerobic rod, is known to predominately infect immunosuppressed and cystic fibrosis patients.(254-256) Prevalence of *P. aeruginosa* in the faecal samples of healthy subjects of 15.8% in this study is within ranges from 0 to 24 %.(257-261) Higher prevalence (97%) and levels of *P. aeruginosa* in IBS patients has not been reported in literature. However, in functional dyspepsia patients, *Pseudomonas* was cultured as predominant bacterium in the small intestine.(107) Previous studies using molecular based methods to determine the quantity of faecal microbiota in IBS patients and healthy subjects showed differences in microbiota composition.(24;26) However, *P. aeruginosa* levels were not tested in these studies.

The age differences between the healthy subject and IBS patients in our study might be a confounding factor. Faecal microbiota composition is only changing in subjects older than 65 years of age.(60;223;262) However elderly (>65 years) were excluded from the study. The effect of the age difference between healthy subjects (mean age 32 years) and IBS patients (mean age 42 years) on bacterial DNA load, prevalence and quantity of *P. aeruginosa* is not known.

The question arises if differences in prevalence and quantity of *P. aeruginosa* might be epiphenomenal or be the cause of IBS symptoms. First of all, it is known that *P. aeruginosa* may occur as sole potential pathogen in patients with diarrhea and *P. aeruginosa* induces signs and symptoms of enteritis in antibiotic treated rats.(263) Besides, a causative relationship between *P. aeruginosa* and diarrhea has been found in immunocompromised individuals.(263) However, in our study *P. aeruginosa* was not only increased in diarrhea predominant IBS patients but also in constipation predominant and alternating IBS patients.

Secondly, proteases of *P. aeruginosa* are known to disable Protease-activated receptor-2 (PAR-2) in the respiratory tract.(264) The effect of *P. aeruginosa* proteases on PAR-2 in the gastrointestinal tract is unknown. PAR-2 activation in the gastrointestinal tract has been shown to modify motility patterns, inflammatory mediator release, intestinal barrier integrity, ion transport and nociceptive functions, all functions which are part of gut physiology involved in generation of IBS symptoms.(265)

Indication that increased *P. aeruginosa* might be epiphenomenal is reported in a recent study which shows high levels of *Pseudomonas* species in pouchitis patients in remission. If the normal microbiota of the gastrointestinal tract is altered, potential pathogens can colonize or resident pathogens can multiply. The remission of the pouchitis patients was induced by antibiotic therapy.(266) It has been shown that antibiotic treatment of mice increased the colonization potential of *Pseudomonas* spp.(267) *P. aeruginosa* is naturally resistant to many antibiotics due to permeability barrier by its outer membrane lipopolysaccharide, tendency to colonize surfaces in a biofilm form and maintenance of antibiotic resistance plasmids.(268-271) Antibiotics and probiotics used more than a month before the start of the study are unlikely to have an effect on the *P. aeruginosa* colonization since stabilization of the intestinal microbiota within one to two weeks after antibiotic therapy has been reported.(139;223;272-274)

In conclusion, our data show no apparent difference in the diversity of predominant microbiota profiles between IBS patients and healthy subjects. However, using the pooled average profiles of the PCR-DGGE fingerprints allowed us to isolate and sequence specific bands of IBS patients and healthy subjects. IBS specific bands were predominantly members of *Pseudomonas* genera. Q-PCR analysis confirmed that *P. aeruginosa* is found in higher prevalence and in higher levels in IBS patients than in healthy subjects.

Therefore, we propose that *P. aeruginosa* may be involved in the pathophysiology of IBS.

Chapter 6

Intestinal permeability in Irritable Bowel Syndrome patients: effects of NSAID

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Under Revision Dig Dis Sci

Abstract

Introduction
Intestinal permeability is suggested to play a role in the pathophysiology of Irritable Bowel Syndrome (IBS). Whether or not IBS patients have altered intestinal permeability is still controversial. IBS is associated with the use of analgesics. Non-steroidal anti-inflammatory drugs (NSAIDs) are known to increase intestinal permeability. We investigated intestinal permeability and the effect of NSAID on permeability in IBS patients and healthy subjects.

Material and methods
Intestinal permeability was investigated in 15 healthy subjects and 14 IBS patients, before and after two days NSAID ingestion. Indicators for permeability were urinary recovery of orally administered polyethylene glycols (PEGs) of molecular masses 400, 1500, 4000, and 10,000, and a lactulose mannitol test. PEG excretion was recorded time-dependently over 24 hours.

Results
There were no significant differences in 24-hours recoveries of PEGs 400, 1500 and 4000 between healthy subjects and IBS patients before or after NSAID ingestion. LM ratios in healthy subjects and IBS patients were not significantly different. Only time-dependent monitoring of PEG excretion showed that two days administration of NSAIDs enhanced intestinal permeability for PEG 4000 in healthy subjects ($p=0.050$) and for PEGs 400, 1500, and 4000 in IBS patients ($p=0.015$, $p=0.041$, and $p= 0.012$, respectively).

Conclusions
Intestinal permeability in IBS patients is not different from that in healthy subjects; NSAIDs compromise intestinal permeability to a greater extent in IBS patients than in healthy subjects. This suggests that IBS is associated with an altered response to noxious agents in the physiology of the intestinal barrier.

Introduction

Irritable Bowel Syndrome (IBS) is characterized by abdominal pain and altered bowel habits. Changes in intestinal microbiota, inflammatory cells, and permeability have been suggested to be involved in the pathophysiology of IBS.(10;237;275) An increase in intestinal permeability will expose the subject to intraluminal antigens, microbiota, and bacterial toxins.

Whether or not permeability is increased in IBS patients is still a matter of controversy in the literature. Intestinal permeability was reported enhanced in 16-50% of post-infectious IBS patients.(31;33) Post-infectious IBS patients make up 16% of the IBS population.(276;277) In post-infectious and non-post-infectious diarrhea-predominant IBS patients permeability of the (proximal) small intestine was increased in comparison with healthy subjects and with constipation-predominant IBS patients.(32) On the other hand, in some studies containing relatively large groups of IBS patients, no indication of enhanced intestinal permeability was found.(34;35) A decrease of intestinal permeability in IBS patients was also reported.(278)

Visceral hypersensitivity is thought to play a pivotal role in IBS symptom generation.(201) Non-steroidal anti-inflammatory drugs (NSAIDs) tend to decrease the initial perception threshold and reduce the basal nerve discharge rate causing visceral hypersensitivity.(279;280) NSAIDs are known to increase intestinal permeability.(281;282) Imbalance in the interaction between luminal aggressive factors and mucosal defence may lead to low grade inflammation.(281;283) Low grade inflammation is known in (post-infectious) IBS patients and may contribute to visceral hypersensitivity.(10;33;284) IBS is significantly associated with the use of analgesics.(285;286) The majority of the IBS patients use NSAIDs without experiencing apparent exacerbation of their symptoms. Nevertheless, IBS patients who use NSAIDs, are likely to have persistent IBS symptoms.(286) We hypothesize that the use of NSAIDs by IBS patients sustains a condition of low-grade inflammation which is mediated by increased intestinal permeability. To test this hypothesis we studied the effect of limited administration of NSAIDs on intestinal permeability in IBS patients. Healthy subjects received the same treatment as reference.

Commonly used tests to assess intestinal permeability such as the sugar absorption test are based on the selective urinary excretion of orally administered probes, usually a combination of a relatively small permeant compound and a larger compound with restricted permeability. Relating recovery of the larger to that of the smaller probe minimizes the influence of factors like

gastric emptying, intestinal motility, and renal function which should affect both probes equally.(287) Parlesak *et al.* introduced a mixture of polyethylene glycols (PEG) with relative molecular masses M_r 400, 1500, 4000, and 10,000 which might offer the possibility to assess size-dependent intestinal permeability.(288;289) The molecular size of PEG 10,000 represents the size of substances of interest such as allergens and bacterial products like lipopolysaccharides.(289;290) Intestinal permeability in IBS has been studied by using sugar absorption tests and by testing the permeability for ^{51} Cr-EDTA.(31-35;278) We decided to test intestinal permeability in IBS by using a polyethylene glycol mixture similar to that described by Parlesak *et al*.(288) and, moreover, by monitoring the kinetics of urinary excretion of the various polyethylene glycols anticipating that kinetic measurements might provide additional information on intestinal permeability. PEG excretion was studied before and two days after administration of NSAIDs in IBS patients and healthy subjects to analyze if permeability would be more affected by limited administration of NSAIDs in IBS patients than in healthy subjects. Enhanced intestinal permeability may also result from intestinal ischemia and enterocyte death. This was evaluated from the release of intestinal fatty acid-binding protein (I-FABP) into urine.(291-293)

Materials and methods

Subjects

The PEG permeability test and analysis were evaluated in 15 healthy persons. Healthy subjects had neither intestinal complaints nor a history of bowel resection, and were not receiving any treatment known to be associated with alterations in gastrointestinal function or with gastrointestinal side effects. The IBS study group consisted of 14 Caucasian IBS patients who were diagnosed according to the Rome II criteria for IBS.(5) Subjects were not allowed to take NSAIDs or acetylsalicylic acid three days prior to the tests.(281;283) The study was approved by the ethics committee of the University Medical Center Utrecht. Written informed consent was obtained from all subjects.

Study protocol

Intestinal permeability was measured in IBS patients and healthy subjects using a PEG and a lactulose mannitol (L/M) test. The L/M test was meant as an alternative and well established indicator of intestinal permeability. The PEG

solution contained 5 g PEG 400, 1.5 g PEG 1500, 5 g PEG 4000, and 10 g PEG 10,000 dissolved in 100 mL water.(288) Sorbate (0.1 %) was added as preservative. PEGs with M_r 400, M_r 1500, and M_r 4000 were obtained from Bufa Chemical Company, Uitgeest, The Netherlands, and PEG with M_r 10,000 from Sigma Chemical Company, St Louis, MO. The L/M solution contained 2 g mannitol, 5 g lactulose and 40 g sucrose in 100 mL water.(294) The PEG and L/M solutions were quality- and purity-controlled by the Department of Pharmacy of the UMC Utrecht. PEG and L/M tests were performed in random order, at least one week apart. In healthy subjects, the tests were performed twice to check the reproducibility of test procedure and assay (test 1 and test 2). At least one week after the PEG and L/M reproducibility tests healthy subjects and IBS patients ingested NSAID at 10 pm for two days and intestinal permeability was measured using the PEG test starting at 8 am the next morning. The NSAID used was 750 mg (250 mg and 500 mg tablets) Naproxen (Centrafarm, Etten-Leur, The Netherlands).
After voiding and discarding overnight urine, the fasting subjects drank a PEG or L/M solution. A further six hours fast followed during which subjects were allowed to drink water according to one's needs.
During the PEG test, before or after NSAID ingestion, subjects collected urine 8 times at 2-hours intervals plus all the urine until the next morning. Aliquots of 30 mL of each urine portion were transferred to separate plastic tubes and the remaining parts were stored together in a container. Volumes of urine were recorded so that PEG excretion over a 24-hours period could be calculated. During the L/M test, subjects collected urine over a single 6-hours period. Urine samples were stored at –20°C until further analysis.

Analysis of urine samples
Urine samples were homogenized and 25 mL were centrifuged at 1000 x g for 10 minutes. Two mL of clear supernatant were desalted by treatment with an ion-exchange resin (Bio-Rad RG 501-X8, Hercules, CA, USA). The resin was removed by centrifugation and 50 µL supernatant were analyzed by high performance liquid chromatography (HPLC).
Parlesak *et al*. analyzed polyethylene glycols by two HPLC systems using differential refraction index detection.(288) These authors purified PEGs 1500, 4000, and 10,000 from urine by extraction with chloroform and analyzed the extracts by one HPLC configuration. PEG 400 was analyzed in the post-extraction residue and analyzed by a different HPLC set-up. We applied HPLC and implemented the novel technique of evaporative light-scattering detection.

(295;296) PEGs were analyzed by reversed-phase HPLC (Shimadzu SCL-10A VP, Shimadzu Benelux, 's-Hertogenbosch, The Netherlands) using a 25 cm 5 µm Lichrospher 100-RP 18E column equipped with a 1.5 cm similar guard column (Li Chro Cart 2504 mm, Merck KgaA, Darmstadt, Germany), and evaporative light-scattering detection (Alltech 500, Grace Alltech Applied Science, Breda, The Netherlands). The mobile phase consisted of a gradient of 40-80% methanol in water allowing analysis and quantification of all four polyethylene glycols in a single run.

Figure 1 shows a chromatogram of PEGs 400, 1500, 4000, and 10,000. The compounds are mixtures of oligomers with molecular masses around the indicated means. PEG 400 was resolved into its various oligomers, the larger PEG compounds showed as a single peak each. Detection limits were 0.05 mg/mL for PEG 400 and 0.005 mg/mL for PEGs 1500, 4000, and 10,000. Recovery of all four PEGs was 100 ± 4 %, reproducibility 97 ± 1 %. The detection limit of the analysis procedure of PEGs 1500, 4000, and 10,000 could be improved by extracting PEGs from urine and concentrating the extract.(288) For this purpose, 8 mL desalted urine supernatant were extracted with 2 mL chloroform. One mL of the chloroform extract was dried under a mild stream of nitrogen, the residue taken up in 200 µL HPLC-mobile phase solvent and 50 µL analyzed.

Urine lactulose, mannitol and creatinine concentrations were measured by routine clinical chemical analysis in the Central Diagnostic Laboratory of the UMC Utrecht with a Synchron CX4 random-access multi-analyzer (Beckman Instruments Inc., Brea, CA, USA). I-FABP was determined in urine using a human I-FABP sandwich ELISA (HyCult Biotechnology B.V., Uden, The Netherlands).

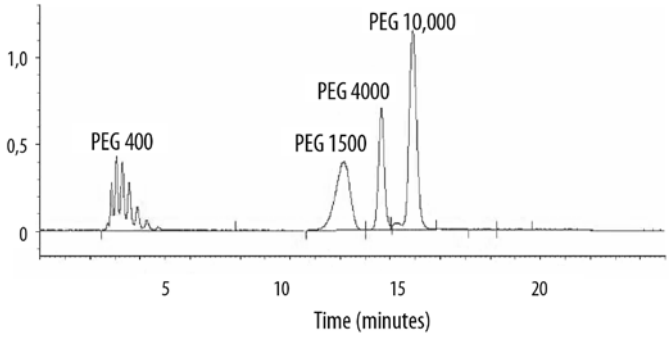

Figure 1 *Chromatogram of a solution containing PEGs 400, 1500, 4000, and 10,000. PEG 400 resolved into various oligomers, PEGs 1500, 4000, and 10,000 were eluted as a single peak each.*

Calculations and data analysis

Intestinal permeability was evaluated from the area under the PEG-excretion time-curves, and from the percentage PEG recovered from the ingested dose. An L/M ratio over 0.03 was considered an indication of increased intestinal permeability.(294;297;298)

Statistical analysis

The significance of differences in recovery of PEGs between IBS patients and healthy subjects before and after NSAID ingestion was evaluated using Mann Whitney U tests. Proportions of subjects with PEG 10,000 recovery before and after NSAID ingestion, and between groups, were compared using the Chi square test. Differences in areas under the curves of PEGs before and after NSAID ingestion were determined using a Wilcoxon signed rank test. Potential differences between the results of L/M test in healthy subjects and IBS patients were evaluated by the Chi square test. Statistical significance was defined as a two-tailed probability < 0.05. Statistical analysis was performed with SPSS version 12.0.1 for Windows.

Results

Subjects

The characteristics of the two groups of subjects are listed in **Table 1**. The mean age of IBS patients was not significantly different from that of healthy subjects. Healthy subjects passed equal volumes of urine in 24 hours before and after NSAID consumption (means ± SEM , respectively: 2.06 ± 0.15 L and 2.11 ± 0.14 L). IBS patients passed 2.80 ± 0.32 L urine in 24 hours before NSAID consumption which is significantly more ($p=0.013$) than after NSAID consumption (2.10 ± 0.30 L), and more ($p=0.050$) than healthy subjects.

PEG excretion test in healthy subjects (Figure 2)

The results of the PEG-excretion reproducibility tests 1 and 2 in healthy subjects are presented in **Figure 2**. It shows the concentrations of PEGs 400, 1500, and 4000 in urine at the various time points over 24 hours. PEG 10,000 could be detected in one healthy subject only and therefore data from PEG 10,000 are not included in this figure. Urinary concentrations of PEGs 400, 1500, and 4000 after NSAID consumption are also presented in **Figure 2**.

Table 1 *Subject characteristics*

	Healthy subjects	IBS patients
Number	15	14
Mean age in years (range)	31 (21-55)	41 (21-63)
Female/Male	8/7	10/4
Rome II:		
IBS-D		8
IBS-C		3
IBS-A		3
Creatinine in urine (mean [mmol/L] (SEM))	7.5 (1.1)	6.2 (1.3)

Subjects with diarrhea-predominant IBS (IBS-D) experienced more than 3 bowel movements per day, loose stools, or urgency while never experiencing less than 3 bowel movements per week, hard stools or straining during a bowel movement. Subjects with constipation-predominant IBS (IBS-C) experienced less than 3 bowel movements per week, hard stools, or straining during a bowel movement while never experiencing more than 3 bowel movements per day, loose stools or urgency. Subjects with alternating IBS (IBS-A) experienced symptoms belonging to both IBS-D and IBS-C criteria.

Excretion of PEGs 400 and 1500 was essentially complete within 12 hours, and excretion of PEG 4000 after 24 hours, independent of NSAID consumption. Peak concentrations of PEGs 400 and 1500 were reached within 2 hours and the peak concentration of PEG 4000 after 4 hours, reflecting a relatively reduced rate of intestinal permeation by the larger compound.
The areas under the concentration curves from tests 1 and 2 were identical (median (range), respectively: PEG 400: 13.3 (7.8-51.8) *vs*. 13.8 (3.8-32.5); PEG 1500: 0.26 (0.08-1.52) *vs*. 0.25 (0.04-0.60); PEG 4000: 0.013 (0.001-0.087) *vs*. 0.012 (0-0.052) $mg.h.mL^{-1}$). **Figure 2** clearly illustrates that similar concentrations of PEGs 400, 1500, and 4000 were found in tests 1 and 2 indicating reliable reproducibility of test procedure and analysis. The means of data from tests 1 and 2 were used to represent data from healthy subjects before NSAID treatment.

PEG excretion in healthy subjects after NSAID ingestion

The 24-hours recoveries of PEGs 400 and 1500 as percentages of the ingested doses were not different before or after NSAID consumption in healthy subjects (**Table 2**). Likewise, the 24-hours recovery of PEG 4000 was not significantly different after NSAID intake suggesting that intestinal

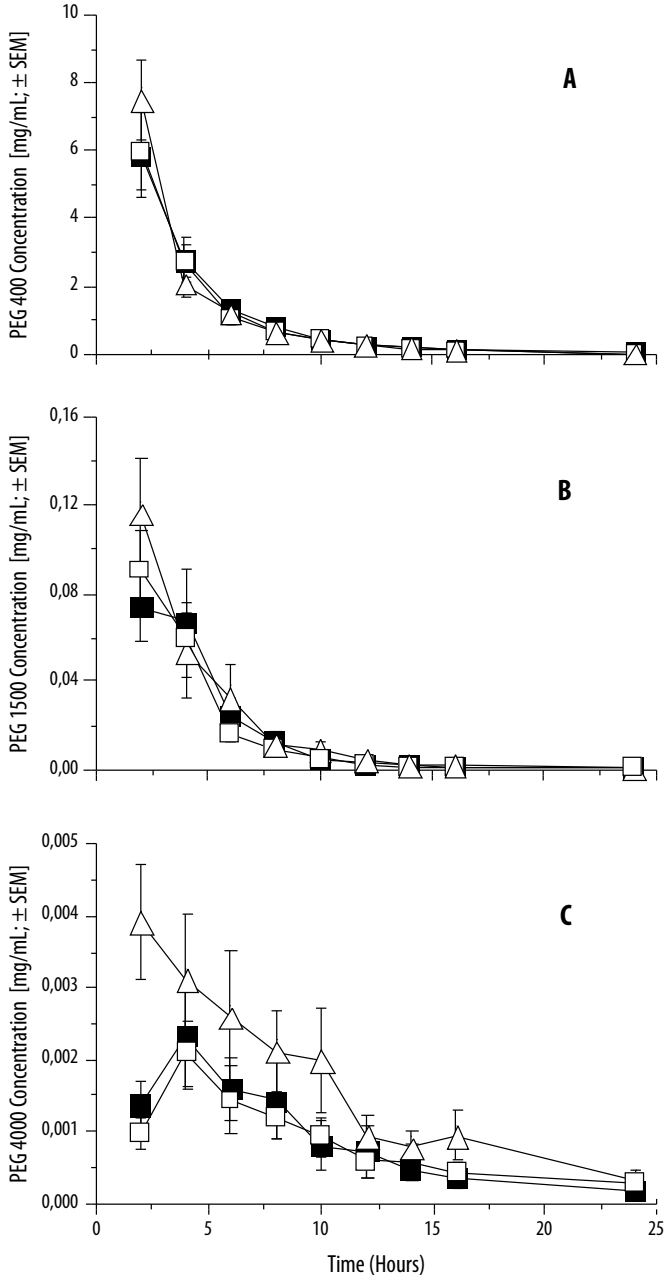

Figure 2 *Concentrations of PEGs 400 (A), 1500 (B), and 4000 (C) in urine of 15 healthy subjects, in mg/mL, at various times after consumption of PEGs . Test 1 (□) and 2 (■) were performed with a one week interval. One week later the test was performed after two-days consumption of NSAID (△).Values represent means ± SEM.*

permeability was not affected by two days NSAID ingestion (**Table 2**). However, the area under the curve of PEG 4000 after NSAID (0.019, range 0.002-0.100 mg.h.mL^{-1}, **Figure 2**) was significantly (p=0.050) enhanced compared to before NSAID ingestion (mean of test 1 and 2) which might be interpreted to indicate increased intestinal permeability after NSAID. The peak urinary excretion of PEG 4000 after NSAID ingestion was reached within 2 hours after administration and the concentration of PEG 4000 at 2 hours was significantly (p=0.002) higher after than before NSAID intake, indicative of accelerated excretion of PEG 4000.

PEG excretion in IBS patients (Figure 3)

Figure 3 shows the recovery of PEGs 400, 1500, and 4000 in mgs in the urine from IBS patients before and after NSAID ingestion. PEG 10,000 was detected in a limited number of subjects only and is therefore not included in this figure. Excretion of PEGs 400 and 1500 before and after NSAID ingestion, and of PEG 4000 before NSAID ingestion were essentially complete within 12 hours. After NSAID intake, complete excretion of PEG 4000 took 24 hours. Peak excretion of PEGs 400, 1500, and 4000 were reached within 2 hours, peak

Table 2 *PEG recovery in healthy subjects and IBS patients before and after NSAID ingestion.*

	Healthy subjects Before NSAID	**Healthy subjects After NSAID**	**IBS patients Before NSAID**	**IBS patients After NSAID**
Recovery PEG 400 (% of administered dose)	27.9 (22.2-38.8)	32.2 (16.0-38.4)	26.0 (13.8-32.1)	30.7 (13.5-45.8)
Recovery PEG 1500 (% of administered dose)	1.29 (0.56-2.85)	1.57 (0.70-4.16)	1.00 (0.67-2.74)	1.62 (0.45-7.50)
Recovery PEG 4000 (% of administered dose)	0.016 (0.008-0.065)	0.040 (0.004-0.124)	0 (0-0.061)	0.020 (0-0.249)
Recovery PEG 10,000	1/15	5/15	1/14	2/14

Values are expressed as median (range). Recovery of PEG 10,000 is expressed as proportion of subjects in which PEG 10,000 was detected. Values before vs. after NSAID, and IBS patients vs. healthy subjects are not statistically significantly different.

excretion of PEG 4000 after 4 hours, independent of NSAID ingestion. IBS patients passed significantly more urine in 24 hours before than after NSAID consumption. Urinary flow and volume will contribute to PEG excretion. However, the rate-limiting step in permeability will be the passage of the

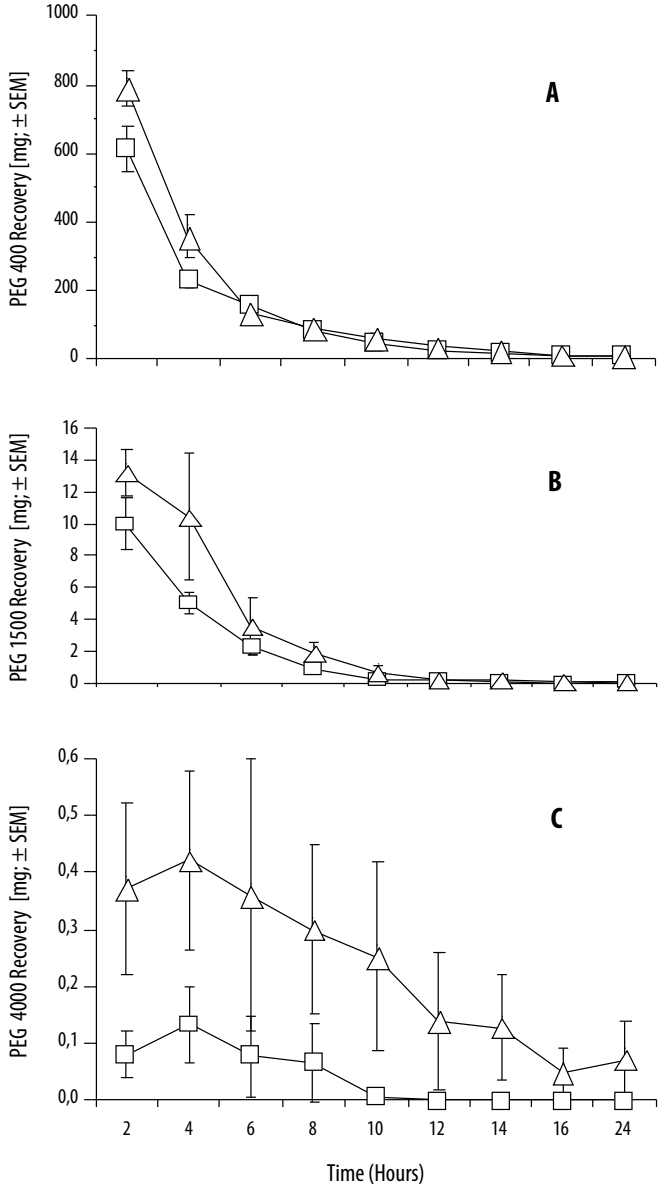

Figure 3 *Recovery of PEGs 400 (A), 1500 (B), and 4000 (C) in urine of 14 IBS patients, expressed in mgs per 24 hours, at various times after consumption of PEGs before (□) and after (△) two-days consumption of NSAID. The tests were performed with an interval of one week. Values represent means ± SEM.*

intestinal mucosal barrier. Therefore, urinary recovery of PEGs will be mostly an indicator of passage of PEGs through the mucosal barrier. By expressing recovery as percentage of the administered quantity (**Table 2**) or as mgs per 24 hours (**Figure 3**), differences in urine volumes will have been accounted for.

PEG excretion after NSAID ingestion in IBS patients

Table 2 shows that 24-hours recoveries of PEGs 400, 1500, and 4000 in IBS patients after NSAID ingestion were not different from before NSAID intake suggesting that two days NSAID consumption did not enhance intestinal permeability in IBS patients. However, the areas under the recovery curves of PEGs 400, 1500, 4000 in **Figure 3** are significantly larger after NSAID ingestion than before NSAID (median (range): PEG 400: 1862 (1266-2314) *vs*. 2162 (921-3988), p=0.012; PEG 1500: 23.7 (13.1-67.3) *vs*. 36.7 (8.11-203.5), p=0.041; PEG 4000: 0 (0-0.564) *vs*. 1.65 (0-23.7), p=0.012, [mg.h]) which again might be interpreted to indicate increased intestinal permeability after NSAID. Figures 3, as well as **Figure 2**, show that time-dependent registration of PEG excretion provides additional information on possible differences in intestinal permeability.

PEG excretion in IBS patients and healthy subjects

There were no significant differences in percentages recovered from the administered doses of PEGs 400, 1500, and 4000 between IBS patients and healthy subjects (**Table 2**) which indicates that intestinal permeability in IBS patients is not different from that in healthy subjects.

L/M excretion in healthy subjects and IBS patients

The median L/M-ratio from IBS patients was 0.013 (range: 0.005 – 0.040) which was similar to that from healthy subjects (0.011; range: 0.008 – 0.030). Both median L/M-ratios are below the cut-off value of 0.03 indicative for enhanced intestinal permeability; three IBS patients had L/M-ratio's above 0.03.

I-FABP

I-FABP results showed no differences between healthy subjects and IBS-patients neither before (mean (range) respectively: 3.4 (0 – 34) *vs*. 6.6 (0 – 48) pg/mL) nor after NSAID consumption (respectively: 9.1 (0 – 55) *vs*. 3.9 (0 – 38) pg/mL) indicating absence of (micro) damage to the intestinal epithelium affecting intestinal permeability as a result of IBS or NSAID consumption.

Discussion

Reports on intestinal permeability in patients with IBS have been contradictory and can be subject to debate.(31-35) Marshall *et al*. found no significant difference in permeability between IBS patients and healthy subjects based on lactulose mannitol excretion using the conventional cut-off point of 0.03.(31) However, when a cut-off value of 0.02 was used, an increase in intestinal permeability reached statistical significance.(31) According to these authors, lowering the threshold might open the possibility to distinguish more subtle disorders like post-infectious IBS. Other studies have shown an increase in intestinal permeability when comparing IBS patients to healthy subjects.(32;33) Using ^{51}Cr-EDTA as permeability marker, Dunlop *et al*. found increased intestinal permeability in post-infectious and diarrhea-predominant IBS patients, but only in the proximal small intestine and not in constipation-predominant IBS patients.(32) Spiller *et al*. reported increased intestinal permeability based on lactulose mannitol recovery in patients following Campylobacter enteritis.(33) These patients were studied acutely after contracting gastroenteritis and 8-48 months following the initial episode of enteritis. The Walkerton study showed that approximately two years after acute gastroenteritis intestinal permeability can be within normal limits.(31) On the other hand, using the L/M test, Lundin *et al*. reported decreased permeability in the proximal gastrointestinal tract of IBS patients without any relation to the predominant bowel habit of these patients.(278) The question remains whether results from studies on post-infectious IBS patients may be extrapolated to the IBS patient in general.

Enhanced intestinal permeability in post-infectious IBS patients may reflect a lack of recovery of tight junction function that had become compromised during acute infection.(28;299) Mast cells are key players in maintaining intestinal permeability integrity and may play a role in this lack of recovery.(29;300;301) The number of mast cells that infiltrate inflamed tissue, is

increased in large intestinal mucosa of IBS patients.(10;302-304) Also tryptase activity is enhanced in IBS mucosa.(10;302-304) Tryptase activates PAR-2 which affects the tight junctions and increases intestinal permeability.(305) However, the effect of infection on permeability can not be generalized since acute infections with Giardia lamblia and rotavirus cause a decrease in intestinal permeability of PEG 400.(306) It is clear that the pathophysiology of altered intestinal permeability in IBS patients in general remains to be clarified.
Intestinal permeability reflects the barrier function of gut mucosa. Disorders of the barrier function may be assessed from changes in permeability markers. In the present approach, intestinal permeability in IBS patients and sensitivity to factors affecting intestinal permeability were studied by 1) using a polyethylene glycol test mixture containing PEGs from M_r 400 to M_r 10,000, 2) monitoring time-dependent urinary excretion of the various PEGs, and 3) testing the effect of two days NSAID administration. Test and analytical procedures showed excellent reproducibility upon repeating the study in healthy subjects (**Figure 2**).
The concomitant administration of a combination of markers is regarded as a useful technique to avoid interference from variances in gastric emptying and intestinal transit (premucosal factors) or urinary excretion (postmucosal factor) since, in those cases, excretion of the various markers should be equally affected. Therefore, the time-dependently increased intestinal permeability for PEG 4000 after the two days ingestion of NSAID in healthy subjects and in particular in IBS patients should be ascribed to NSAID-induced changes in mucosal factors such as putative changes in tight junctions, or an altered composition of the mucous surface layer.(281) NSAIDs can reduce the surface hydrophobicity of the gastrointestinal mucosal barrier facilitating exogenous water-dissolved agents to permeate the mucous barrier and damage the underlying epithelium.(307;308) NSAIDs also intracellularly uncouple mitochondrial oxidative phosphorylation which will contribute to disruption of the integrity of the tight junctions.(309;310)
Our results show the added value of time-dependent registration of PEG excretion. Total 24-hour recoveries of PEGs 400, 1500, and 4000 did not show differences between healthy subjects and IBS patients, neither before nor after two days NSAID ingestion (**Table 2**). Time-dependent monitoring of PEG excretion showed that NSAIDs enhanced intestinal permeability for PEG 4000 in healthy subjects, and for PEGs 400, 1500, and 4000 in IBS patients (**Figures 2** and **3**). The time-dependent results also indicate that a 10-12 hours period to

collect urine would be sufficient to get a valid indication of the extent of PEG recovery, and therefore intestinal permeability.
In conclusion, the important result from this study is that intestinal permeability in IBS patients was found not to be different from that in healthy subjects. Yet, the capacity of the intestinal barrier to cope with luminal aggression is more limited in IBS. Time-dependent monitoring of PEG excretion showed that two days administration of NSAIDs enhanced intestinal permeability for PEG 4000 in healthy subjects, and for PEGs 400, 1500, and 4000 in IBS patients. Therefore, NSAIDs compromise intestinal permeability to a greater extent in IBS patients than in healthy subjects. Since intestinal permeability reflects the functional status of the intestinal barrier, the present findings support the idea that IBS is associated with as yet unknown changes in the physiology of the intestinal barrier, particularly expressed when triggered by action of noxious agents.

Chapter 7

Trypsinogen IV, serotonin transporter transcript levels and serotonin content are increased in small intestine of Irritable Bowel Syndrome patients

Angèle PM Kerckhoffs, José JM ter Linde, Louis MA Akkermans, Melvin Samsom

Neurogastroenterol Motil, 2008, 20, 900-907

Abstract

Introduction
Colorectal and small intestinal visceral hypersensitivity has been demonstrated in Irritable Bowel Syndrome (IBS). Serine protease signalling via protease-activated receptor (PAR)-2 promotes hyperalgesia to mechanical distension. Furthermore, serotonergic pathways are involved in gastrointestinal visceral sensitivity. Abnormalities of serine protease and serotonergic signalling components have been identified in IBS colorectal mucosal biopsies.
We determined the role of altered mucosal serine protease and serotonergic signalling in small intestine of IBS patients.

Material and methods
Duodenal mucosal biopsies of 34 IBS patients (10 constipation-,11 diarrhea-predominant and 13 alternating) and 20 healthy subjects (HS) were collected. Gene transcripts of PAR-2, trypsinogen IV, TPH-1, SERT and 5-HT_3 subunits were quantified using real-time PCR and 5-HT content was measured by ELISA.

Results
IBS patients showed 1.5-fold higher trypsinogen IV mRNA level compared to HS (P=0.016). SERT expression was 1.8-fold higher in IBS compared to HS (P=0.007). Mucosal 5-HT content was 1.7-fold higher in IBS compared to HS (P=0.015). The increase was 2.1-fold in IBS-C relative to HS (P=0.018). Transcript levels of PAR-2, TPH-1 and 5-HT_3 receptor subunits did not differ between IBS and HS.

Conclusions
Enhanced trypsinogen IV expression in IBS may cause increased PAR-2 activation. Increased SERT expression and mucosal 5-HT content in IBS suggest higher 5-HT availability. Both may contribute to small intestinal visceral hypersensitivity in IBS patients.

Introduction

Irritable Bowel Syndrome (IBS) is a multifactorial functional gastrointestinal disorder without a structural or biochemical explanation for the symptoms. The symptoms that characterize IBS are abdominal pain, bloating and altered bowel habits, which may be either diarrhea or constipation, or alternating diarrhea and constipation. Visceral hypersensitivity is thought to play a pivotal role in IBS symptom generation.(201) A range of 75% - 90% of IBS patients are hypersensitive to mechanical distension in the colorectal region.(311;312) Visceral hypersensitivity in IBS patients is not restricted to the colorectal region, since it has also been demonstrated in the oesophagus and small intestine.(313-315) The mechanisms involved in small intestinal visceral hypersensitivity in IBS are unknown. Several studies indicate that abnormalities in serine protease and serotonergic signalling pathways play a role in altered colorectal sensitivity of IBS patients.

IBS patients have an increased serine protease activity in colonic mucosa. Moreover, the mucosal supernatant activates murine extrinsic primary afferent neurons in culture and induces hyperalgesia and allodynia in mice upon colorectal distension. These responses are diminished after pre-incubation with serine protease inhibitor.(42) Furthermore, faecal serine protease activity appeared to be significantly higher in patients with diarrhea-predominant IBS than in both controls and IBS patients with either constipation or alternating bowel habits.(43)

It has been shown in mice that luminal serine proteases increase paracellular permeability, which provokes activation of the local immune system.(44) The subsequent stimulation of extrinsic primary afferents by released immune mediators may induce visceral hyperalgesia and allodynia. Recently, it has been found that the serine protease trypsin IV, produced by intestinal epithelial cells, can activate PAR-2.(41) Increased PAR-2 expression and/or activation induces hyperalgesia to mechanical distension.(38-40) Expression of trypsinogens, zymogens of active trypsins, is elevated in colonic mucosa from IBS patients.(42) Besides, the pronociceptive effects of colonic mucosal supernatant and faecal samples are PAR-2 mediated.(42;44)

Serotonin (5-HT) also modulates visceral sensitivity of the gastrointestinal tract.(316;317) Therefore, alterations in the components of serotonergic signalling that affect 5-HT availability, such as its biosynthesis, release or uptake may lead to changes in visceral sensitivity. 5-HT is synthesized in enterochromaffin (EC) cells by the rate-limiting enzyme tryptophan

hydroxylase (TPH)-1. Release of 5-HT is regulated by 5-HT receptors on EC cells. Activation of the 5-HT_3 autoreceptors triggers a positive feedback mechanism.(318-320) Uptake of 5-HT is mediated by SERT, a serotonin-selective transport protein. Altered mucosal 5-HT content and expression of TPH-1, and SERT in mucosal rectal biopsies of IBS patients have been identified.(47)
Abnormalities in serine protease and serotonergic signalling pathways may be underlying visceral hypersensitivity in the small intestine. We investigated this by comparing mRNA expression levels of PAR-2, trypsinogen IV, TPH-1, SERT and 5-HT_3 receptor subunits and 5-HT content in mucosal biopsies of the duodenum from IBS patients and healthy subjects.

Methods

Subjects

Thirty-four patients with IBS according to Rome II criteria, and 20 healthy subjects , were included.(**Table 1**) Using the Rome II criteria the IBS patients were divided in three subgroups, constipation-predominant IBS (IBS-C) patients, diarrhea- predominant IBS (IBS-D) patients and IBS patients with alternating bowel habits (IBS-A).(5) Subjects with IBS-D experienced > 3 bowel movements per day, loose stools, or urgency while never experiencing < 3 bowel movements per week, hard stools or straining during a bowel movement. Subjects with IBS-C experienced < 3 bowel movements per week, hard stools, or straining during a bowel movement while never experiencing > 3 bowel movements per day, loose stools or urgency. Subjects with IBS-A experienced symptoms belonging to both IBS-D and IBS-C criteria over time. Organic gastrointestinal disorders were ruled out by colonoscopy or sigmoidoscopy. Exclusion criteria also included celiac disease, diabetes mellitus, major abdominal surgery, endocrine, CNS or severe psychiatric disorders as assessed by history taking, physical examination, laboratory tests and when considered appropriate duodenal biopsy, lactose tolerance test, faecal culturing and abdominal imaging. A series of questions about the medical history and the Rome II criteria served to check the health status of the healthy subjects. During endoscopy no signs of abnormality including inflammation in the small intestine were seen in any of the subjects. The study was approved by the medical ethics committee of the University Medical Center Utrecht and written informed consent was obtained from all participants.

Table 1 *Subject characteristics*

	Healthy subjects	IBS	IBS-A	IBS-D	IBS-C
Number	20	34	13	11	10
Age years (± SEM)	31 ± 2.6	41 ± 2.5*	40 ± 4.9	48 ± 3.7*	35 ± 3.6
Gender (Female/male)	15/5	25/9	12/1	7/4	6/4
Medication:					
SSRI	1	4	1	2	1
5-HT_4 agonist	0	1	1	0	0

* $P \leq 0.01$ compared to healthy subjects

Study protocol

After an overnight fast the subjects underwent an upper GI endoscopy. Two mucosal biopsy specimens of the pars descendens of the duodenum per subject were obtained. Biopsies were immediately snap frozen in liquid nitrogen and subsequently stored at -80°C.

mRNA expression analysis

Total RNA isolation from biopsies was performed using the RNeasy micro kit (Qiagen, Hilden, Germany). Spectrophotometric quantification of total RNA was performed and A260/A280 ratios were within normal range. Subsequently, the integrity of total RNA was checked by denaturing agarose gel electrophoresis. First strand cDNA was synthesized from 1 µg of total RNA using the iScript cDNA synthesis kit (BioRad, Hercules, CA, USA) in a volume of 20 µL. Expression analysis was performed by quantitative real time RT-PCR, using the iCycler iQ system (BioRad, Hercules, CA, USA).

Using SYBR green based detection mRNA levels of PAR-2, trypsinogen IV, TPH-1, SERT and 5-HT_3 receptor subunits were monitored. Prior to real-time PCR analysis, cDNA samples were diluted 1:10, except for trypsinogen IV analysis in which case the cDNA samples were diluted 1:25, with RNAse free water. The PCR reactions were set up in a volume of 25 µL, containing 5 µL of the diluted cDNA, 12.5 µL of 2x iQ SYBR Green Supermix (BioRad, Hercules, CA, USA) and 300 nM of the forward and reverse primer each. Specific primers are listed in **Table 2**. The primers were mRNA/cDNA specific (designed on intron/exon boundaries or flanking an intron) to prevent signal formation from

contaminating genomic DNA. All protocols consisted of a 3 min 95°C initial denaturation and enzyme-activating step. The reaction conditions for amplification are listed in **Table 2**. The amplification was followed by a melting curve analysis; performed by increasing the temperature by 0.5 °C increments from 55°C to 95°C and measuring fluorescence at each temperature for a period of 10 sec. All cDNA samples were analyzed in duplicate.

Table 2 Oligonucleotides (5'-3') and thermal cycling conditions for mRNA expression analysis

Gene	Forward primer Reverse primer	PCR product	Amplification
PAR-2	gcaccatccaaggaaccaatag cagtggtcagttttccagtgag	156 bp	15 sec 30 sec 30 sec 95°C 58°C 72°C
Trypsinogen IV	acgcacttggcgagcg aaaggggacagcaactgtgc	67 bp	15 sec 60 sec 95°C 63°C
SERT	tggttctatggcatcactcagttc gttgtggcgggctcatcag	148 bp	15 sec 30 sec 30 sec 95°C 60°C 72°C
TPH-1	tgcaaaggagaagatgagagaattac ctggttatgctcttggtgtctttc	114 bp	15 sec 30 sec 30 sec 95°C 60°C 72°C
5-HT_{3C}	acacttctgctgggctacaac tgaccaccatcagggacagg	115 bp	10 sec 30 sec 95°C 60°C
5-HT_{3E}	aacgctcctgctgggctac agggcgaagtagacaccgatg	93 bp	10 sec 30 sec 95°C 60°C

bp: base pairs

For normalization purposes three endogenous reference genes were measured; porphobilinogen deaminase (PBGD), β-actin (ACTB) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Quantification of PBGD, ACTB and GAPDH was carried out using 5 µL of diluted cDNA, 12.5 µL 2x iQ Supermix (BioRad, Hercules, CA, USA), and 1.25 µL 20x Assays-on-demand gene expression assay mix Hs00609297(PBGD),4333762F(ACTB) and 4333764F(GAPDH) (Applied Biosystems, Foster City, CA, USA). $MgCl_2$ was added to obtain a final concentration of 4 mM in a total volume of 25 µL. Thermal cycling conditions consisted of a 3 min 95°C initial denaturation step, followed by 40 cycles of 15 sec denaturation at 95°C and 1 min annealing and extension at 60°C.

In every run, a relative standard curve was included. The standard curve allows comparison of the expression levels across runs and takes differences in PCR efficiency for the mRNAs analysed into account. cDNA synthesised from total RNA extracted from full thickness jejunum resection material was used to generate the relative standard curve for PAR-2, PBGD, ACTB and GAPDH. We used purified PCR product to generate the relative standard curve for trypsinogen IV, 5-HT_3 receptor subunits, SERT and TPH1.
Expression levels in the various biopsy specimens were quantified by calculating initial target concentrations using the obtained threshold cycle values and the relative standard curve. Subsequently, for each sample the level of a gene of interest was divided by that of the geometric mean of the three reference genes to obtain the normalized mRNA expression.(321)

Measurement of 5-HT content of mucosal biopsies
The biopsy specimens were homogenized in 2.5 mL of 1N formic acid/acetone (15/85, v/v) according to the method of Smith et al.(322) The supernatants were washed by shaking for 10 minutes with 7.5 mL of heptane/chloroform (8/1, v/v). The organic phase and lipid interface were aspirated, and the samples were dried under vacuum. The 5-HT content was quantified using an enzymatic immunoassay kit according to manufacturer's instructions (IBL, Hamburg, Germany) after reconstituting the samples in the supplied enzyme immunoassay buffer.(323) The 5-HT content is expressed as mean nanogram serotonin/ml per milligram of duodenal biopsy (± SEM).

Statistics
An independent samples t test for evaluation of differences between healthy subjects and IBS patients was performed. A $P<0.05$ was considered significant. Differences between healthy subjects and subgroups of IBS were evaluated by applying an univariate analysis of variance (1-way ANOVA) and a post-hoc test (Bonferroni correction). In order to obtain normally distributed data, the normalized mRNA levels were first transformed by taking the natural logarithm. All statistical analysis was performed using commercially available software (SPSS 12.0.1 for Microsoft windows).

Results

No significant differences in gender distribution were present in IBS patients versus healthy subjects and between IBS subgroups. Healthy subjects were significantly (P=0.01) younger than IBS patients. Particularly, the healthy subjects were significantly younger than the IBS-D patients (P=0.005) (**Table 1**).

mRNA expression

For all SYBR Green-based assays, amplification yielded a single product which size was equivalent to that predicted from the relevant sequence. The level of mRNA expression of the reference genes (PBGD, ACTB and GAPDH) was comparable between healthy subjects, IBS patients and IBS subgroups.
The mRNA expression results for PAR-2, TPH-1 and the 5-HT_3 receptor subunits, are shown in **Table 3**.

Table 3 *mRNA expression of PAR-2, TPH-1 and 5-HT_3 receptor subunits, normalized against geometric mean of reference genes.*

	Healthy subjects	**IBS**	**IBS-A**	**IBS-D**	**IBS-C**
PAR-2	245 (20)	273 (23)	275 (46)	276 (42)	266 (32)
TPH-1	6.4 (1.35)	9.4 (3.2)	7.5 (2.3)	9.4 (5.6)	11.8 (8.7)
5-HT_{3C}	6.9 (1.3)	9.1 (2.7)	6.2 (1.1)	4.6 (0.6)	17.2 (8.3)
5-HT_{3E}	4.8 (0.73)	5.5 (1.5)	4.2 (0.88)	3.5 (0.49)	9.5 (4.9)

All values represent mean of normalized mRNA (SEM). The mRNA expression can not be compared between the different genes, since different standard curves were used.

PAR-2: No differences were observed in PAR-2 expression normalized against geometric mean of the three reference genes between healthy subjects on the one hand and IBS patients or subgroups on the other.
TPH-1: No differences were observed in TPH-1 expression normalized against geometric mean of the three reference genes between healthy subjects on the one hand and IBS patients or subgroups on the other.
5-HT_3 receptor subunits: Since we found very low expression of 5-HT_{3A} and 5-HT_{3B} in the duodenum at the mucosal level (unpublished data), and 5-HT_{3D} was reported not to be expressed in the small intestine, we have focused on the 5-HT_{3C} and 5-HT_{3E} receptor subunits.(324) The expression of 5-HT_{3C} and

5-HT_{3E} subunits normalized against geometric mean of the three reference genes did not show significant differences between healthy subjects and IBS patients or between the IBS subgroups and healthy subjects.
Figure 1 shows the mRNA expression results obtained for trypsinogen IV normalized against geometric mean of PBGD, ACTB and GAPDH.

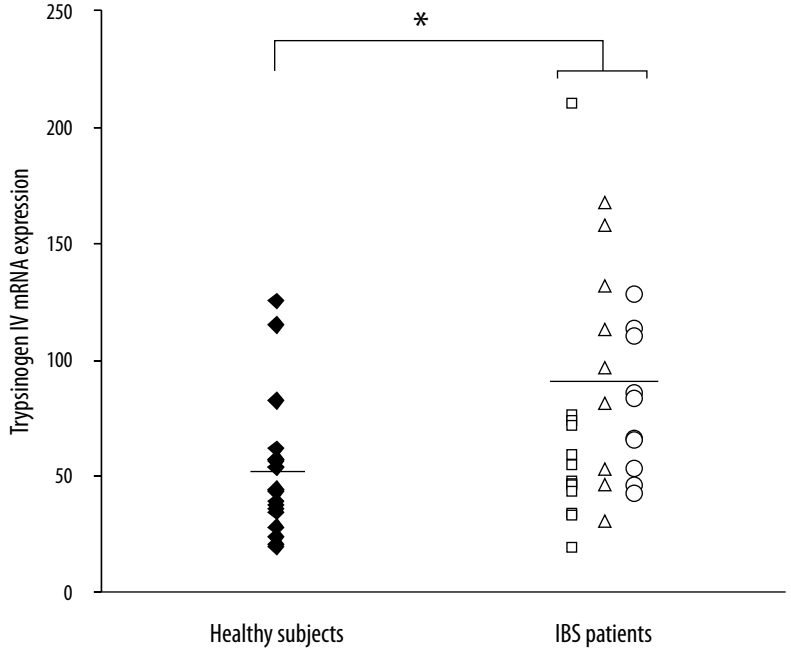

Figure 1 *Trypsinogen IV mRNA expression is increased in IBS patients(□=IBS-A, △ =IBS-D, ○=IBS-C) compared to healthy subjects(◆). The horizontal bars indicate the mean of trypsinogen IV mRNA expression normalized against the geometric mean of the various reference genes. * P =0.016*

In IBS patients, the expression of trypsinogen IV (87.3 ± 13.4) was 1.5-fold higher compared to healthy subjects (51.8 ± 6.28) (P=0.016).
The mRNA expression results obtained for SERT are depicted in **Figure 2**.
In IBS patients, the expression of SERT normalized against geometric mean of the three reference genes (168 ± 22.5) was 1.8-fold higher compared to healthy subjects (86 ± 10.4) (P=0.007). In IBS-C patients expression levels of SERT (225 ± 54.4; mean ± SEM) were 2.3-fold higher when compared to healthy subjects (86

± 10.4; mean ± SEM) (P=0.043). Excluding subjects using serotonin reuptake inhibitors or tegaserod did not effect the SERT mRNA expression results.

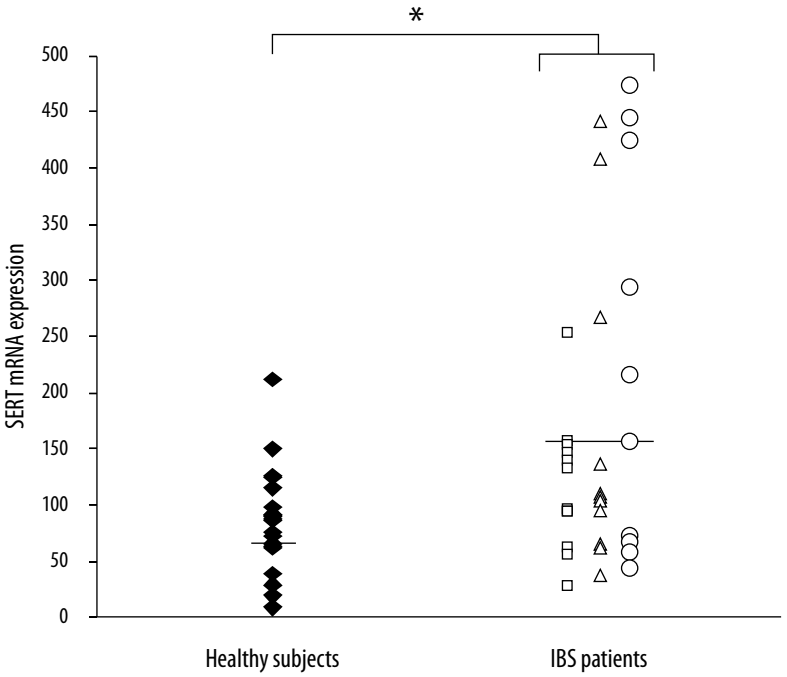

Figure 2 *SERT mRNA expression is increased in IBS patients(□=IBS-A, △ =IBS-D, ○=IBS-C) compared to healthy subjects(◆).*
*The horizontal bars indicate the mean of SERT mRNA expression normalized against the geometric mean of the various reference genes. * P= 0.007*

5-HT content

Duodenal mucosal 5-HT concentrations in IBS patients (300.8 ± 28.7) were significantly higher (P=0.015) than in healthy subjects (181.4 ± 36.1)(**Figure 3**). Particularly, in IBS-C patients duodenal mucosal 5-HT content (373.3 ± 53.9) was significantly higher (P=0.018) than in healthy subjects. Excluding subjects using serotonin reuptake inhibitors or tegaserod did not effect 5-HT content results.

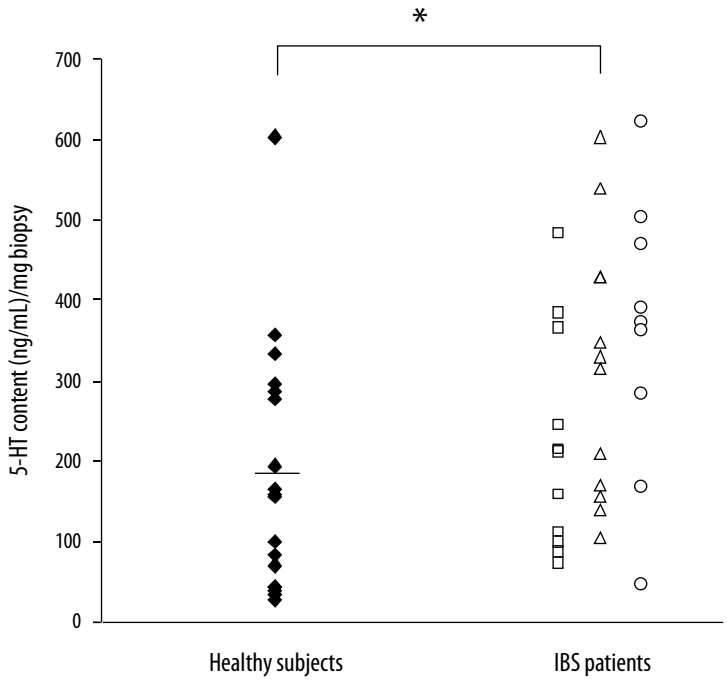

Figure 3 *Small intestinal mucosal 5-HT content is increased in IBS patients compared to healthy subjects.*
*All values represent mean (± SEM) of serotonin content. *P<0.05*

Discussion

This study reveals enhanced mRNA expression of trypsinogen IV and SERT and a higher 5-HT content in the small intestine of IBS patients compared to healthy subjects. These abnormalities in the serine protease and 5-HT signalling systems may contribute to small intestinal visceral hypersensitivity in IBS patients.
Trypsinogen IV is the inactive zymogen form of the serine protease trypsin IV, which can activate PAR-2.(41) Therefore, higher levels of trypsinogen IV in the small intestine may lead to increased PAR-2 activation in IBS patients compared to healthy subjects and enhance visceral sensitivity. PAR-2, present on peripheral terminals of extrinsic primary afferent neurons, acts in a direct manner on the transmission of nociceptive signals.(325) In addition, activation of PAR-2 on these nerve terminals can provoke the release of neuropeptides

such as substance P, thereby inducing neurogenic inflammation.(326) For instance, substance P can trigger the degranulation of mast cells thereby releasing inflammatory mediators, which may stimulate primary afferents.(10;239;327;328) Activation of PAR-2 on enterocytes increases paracellular permeability which induces rectal hypersensitivity.(38-40;305;329;330)
It has been shown in mice that trypsin IV causes PAR-2 dependent mechanical allodynia and hyperalgesia.(331) The mechanism by which trypsin IV contributes to visceral hypersensitivity remains to be determined. Trypsin IV released from enterocytes into the submucosa may act directly on extrinsic primary afferent nerve terminals. So far it has been demonstrated that by activating PAR-2 trypsin IV is capable of signalling to rat dorsal root ganglion neurons in culture that responded to capsaicin and which thus mediate neurogenic inflammation and nociception.(331) In addition or alternatively the pronociceptive effect may result from activation of PAR-2 on enterocytes to promote increased paracellular permeability and subsequent stimulation of the local immune system. Moreover, trypsin IV could potentially cause afferent sensitivity indirectly by acting on PAR-2 located on mast cells, which may promote degranulation and release of preformed mediators from cytoplasmic granules or de novo synthesis of mediators. Released immune mediators may in turn sensitize the extrinsic primary afferents. It is unknown whether enterocytes release trypsin IV into the lumen or the submucosa. It has been suggested that PAR-2 expression on enterocytes is regulated only by luminal serine proteases.(44;332) Considering the absence of a significant difference in PAR-2 mRNA expression despite increased trypsinogen IV expression in our study, one may conclude that enterocytes do not release trypsin IV into the lumen. Thus, bacterial proteases and digestive enzymes are the source of luminal serine protease activity. However, trypsin IV released from enterocytes into the mucosa may increase paracellular permeability by activation of PAR-2 receptors located at the basolateral site of enterocytes. It has been demonstrated that tryptase released from mast cells affects paracellular permeability in this manner.(305) Luminal serine proteases most likely interact with PAR-2 located at the apical site of enterocytes.
In the current study mucosal biopsy specimens were analyzed implying that our data give insight into PAR-2 expressed on enterocytes. The expression of PAR-2 receptors present on peripheral terminals of extrinsic primary afferent neurons in the lamina propria could not be studied, since mRNA for these receptors is situated in the neuronal cell bodies. Although PAR-2 expression on

enterocytes appeared not to be altered, differential expression of PAR-2 on peripheral nerve terminals may play a role in the aetiology of IBS symptoms. We found increased mucosal 5-HT content and SERT expression in the small intestine of IBS patients compared to healthy subjects. Healthy subjects were significantly younger than IBS patients. The effect of age on SERT expression in the gastrointestinal tract is unknown. However, in vivo studies in human healthy subjects have demonstrated an age-related decline in the level of brain SERT.(333) Considering the latter finding, we conclude that the increased SERT expression in IBS cannot be attributed to the difference in age between healthy subjects and IBS patients in our study.

The mucosal 5-HT content reflects 5-HT synthesized and stored in EC cells and the 5-HT released by EC cells and not yet taken up by enterocytes. Once 5-HT is taken up it is rapidly degraded. Our results suggest that higher mucosal 5-HT content does not result from augmented 5-HT synthesis, since TPH-1 mRNA levels were not altered. Therefore, a higher EC cell density or enhanced release may cause the higher mucosal 5-HT content. The increased SERT expression supports the hypothesis of an enhanced release, as the functional role of SERT is to prevent excessive accumulation of free and active 5-HT which would result in potentiation of serotonergic signalling and eventually receptor desensitization. A positive correlation between SERT mRNA expression level and the amount of 5-HT released is supported by studies in rat brain in the serotonin depleted state.(334) More insight into the underlying causes of increased mucosal 5-HT content in the duodenum of IBS patients may be gained by measuring EC cell density, mucosal 5-HIAA content and SERT activity. Although the net effect of the abnormalities in the serotonergic signalling identified may be that 5-HT availability to the 5-HT receptors on peripheral terminals of primary afferents is higher. Visceral sensitivity may be increased by stimulation of extrinsic afferents via a direct effect on perception of gastrointestinal sensation and by activation of intrinsic afferents through modulation of visceral tone or motility.

In rectal mucosal biopsy specimens from patients with IBS mucosal 5-HT content and mRNA levels of TPH-1 and SERT were found to be reduced compared to healthy subjects.(47) In contrast, Camilleri *et al.* observed no difference in mRNA expression of SERT in sigmoid and rectal mucosal biopsy specimens of IBS patients versus healthy controls.(335)The differences in the reported SERT expression may be caused by region-specific effects and by the poor reproducibility of SERT mRNA expression results in rectal mucosal biopsy specimens over time. Like the sigmoid colon, which displayed good

reproducibility, the duodenal region is not heterogeneous with respect to the proportions of the various cell types. As IBS is a heterogeneous disorder, it would be valuable in future studies if upper and lower GI tract samples are compared in the same subjects. Furthermore, it is known that SERT expression, 5-HT content and number of EC cells are altered in inflammatory states.(13;47;336) Moreover, EC cell hyperplasia has been found in rectal biopsies from postinfectious IBS patients, suggesting that inflammatory processes precede changes in 5-HT signalling. Although an effect of inflammation at the microscopic level cannot be ruled out, to explain the increased SERT mRNA levels in IBS patients versus healthy controls as a consequence of low-grade inflammation, the healthy controls should be affected. Although low-grade inflammation has been reported for IBS patients in the colorectal region, it is much less likely to be present in the duodenum of healthy controls.(9) Analyzing the IBS patients for postinfectious origin of IBS resulted in 8 patients equally divided over the IBS subgroups (4 IBS-D, 2 IBS-A, 2 IBS-C). Even with exclusion of the postinfectious IBS group, the IBS patients showed significantly increased 5-HT content and SERT expression compared to healthy subjects. This suggests that increased mucosal 5-HT content and SERT expression are characteristics of IBS and not specific to a postinfectious origin.

The abnormalities in components of serine protease and serotonergic signalling reported in this study are likely to contribute to visceral hypersensitivity in IBS. Moreover, the current findings indicate that changes at the molecular level are not restricted to the colorectal region, but are also observed in the small intestine of patients with IBS.

Chapter 8

Irrespective of visceral sensitivity state in large intestine SERT and TPH-1 mRNA expression are reduced in Irritable Bowel Syndrome patients

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Under Revision Neurogastroenterol Motil

Abstract

Introduction
Colorectal visceral hypersensitivity has been demonstrated in a subset of Irritable Bowel Syndrome (IBS) patients. Serine protease and serotonergic signalling modulate gastrointestinal visceral sensitivity. We evaluated whether altered mucosal serine protease and serotonergic pathway components are related to rectal visceral hypersensitivity in IBS patients.

Material and methods
Colorectal mucosal biopsies of 23 IBS patients and 15 controls were collected. Gene transcripts of PAR-2, trypsinogen IV, TPH-1 and SERT were quantified using real-time PCR. Substance P, 5-HT and 5-HIAA contents were measured by ELISA. The number of enterochromaffin cells, mast cells and intraepithelial lymphocytes was determined using immunohistochemistry. Rectal visceral sensitivity was determined in IBS patients using barostat programmed for phasic ascending distension.

Results
Rectal hypersensitivity (+) and (-) IBS patients showed lower TPH-1 and SERT mRNA levels in the descending colon and rectum compared to controls ($P \leq 0.05$). Rectal hypersensitivity (+) IBS patients ($n=12$) showed lower TPH-1 mRNA level in sigmoid compared to controls ($P=0.015$). Mucosal 5-HT content in descending colon was higher in rectal hypersensitivity (+) IBS compared to controls ($P=0.02$). Rectal substance P content was increased in IBS patients compared to controls ($P=0.045$). No significant differences were found in transcript levels, cell counts and substance P, 5-HT and 5-HIAA contents between rectal hypersensitivity (+) and (-) IBS patients.

Conclusion
Irrespective of visceral hypersensitivity state, several serotonergic signalling components are altered in IBS patients.

Introduction

Visceral hypersensitivity is thought to play a role in IBS symptom generation.(201) A subset of IBS patients is hypersensitive to colorectal distension, indicating that physiologic stimuli are perceived with increased intensity and even cause pain.(311;312;337) This may result from altered expression of receptors or transporters and enzymes determining the availability of their agonists increasing sensitivity of peripheral afferent nerve endings. Serine protease and serotonergic signalling modulate colorectal sensitivity.(42-44;316;317;338-341)

IBS patients have increased serine protease activity in colonic mucosa which activates murine extrinsic primary afferent neurones in culture and induces hyperalgesia and allodynia in mice upon colorectal distension.(42;43) These responses are diminished after pre-incubation with serine protease inhibitor. Mast cell tryptase and nonpancreatic trypsin might be responsible for increased serine protease activity in colonic mucosa. Expression of mast cell tryptase and trypsinogens, zymogens of active trypsins, are elevated in colonic mucosa from IBS patients.(42) Origin of enhanced trypsinogen expression has not been elucidated. Trypsin IV, produced by enterocytes, is a likely candidate. The nociceptive effects of colonic mucosal supernatant are protease-activated receptor (PAR)-2 dependent.(42) Both mast cell tryptase and trypsin IV can activate PAR-2.(42;342)

Activation of PAR-2 on epithelial cells leads to increased paracellular permeability which might increase passage of luminal antigens and toxins with consequent mucosal immune activation contributing to visceral hypersensitivity.(44;340;343) Activation of PAR-2 on nerve terminals can provoke the release of neuropeptides such as substance P.(326) Substance P triggers the degranulation of mast cells thereby releasing inflammatory mediators, which may stimulate primary afferents.(10;239;327;328)

Serotonin (5-HT) also modulates visceral sensitivity of the gastrointestinal tract.(316;317) Alterations in serotonergic signalling components that affect 5-HT availability, such as its biosynthesis, release or uptake may lead to changes in visceral sensitivity. 5-HT is synthesized in enterochromaffin (EC) cells by the rate-limiting enzyme tryptophan hydroxylase (TPH)-1. Uptake of 5-HT into enterocytes is mediated by SERT, a serotonin-selective transport protein. 5-HIAA is the primary metabolite of 5-HT after it has been taken up by enterocytes. The 5-HIAA/5-HT ratio reflects the mucosal 5-HT release and turnover.(12) Altered 5-HIAA/5-HT ratio in mucosal rectal biopsies of IBS

patients has been observed.(12) Contradictory results on EC cell counts, 5-HT content and expression of TPH-1, and SERT in mucosal rectal biopsies of IBS patients have been reported.(12;33;47;49;335) Furthermore, SERT expression, 5-HT content and EC cell counts are altered in inflammatory states.(13;47;336) Low-grade inflammation has been shown in IBS patients.(33)
Since only a subgroup of IBS patients is visceral hypersensitive to colorectal distension, it is conceivable that differences in the portion of IBS patients exhibiting colorectal hypersensitivity underlie discrepancies in serotonergic signalling pathway components in IBS patients. Also for serine protease signalling supporting evidence that alterations in pathway components correlate with visceral hypersensitivity to colorectal distension is lacking. In this study we determined whether or not altered colorectal mucosal serine protease and serotonergic signalling pathways components are related to rectal visceral hypersensitivity of IBS patients.

Methods

Subjects

Twenty-three patients with IBS according to Rome II criteria participated in the study. Using the Rome II criteria 4 patients were classified as constipation-predominant IBS (IBS-C), 11 diarrhea-predominant IBS (IBS-D) patients and 8 as IBS patients with alternating bowel habits. Organic gastrointestinal disorders were ruled out by colonoscopy or sigmoidoscopy. Exclusion criteria also included celiac disease, diabetes mellitus, major abdominal surgery, endocrine, CNS or severe psychiatric disorders as assessed by history taking, physical examination, laboratory tests and when considered appropriate duodenal biopsy, lactose tolerance test, fecal culturing and abdominal imaging. Fifteen subjects who underwent a negative screening colonoscopy or sigmoidoscopy because of family history of colon cancer, unexplained anaemia, haemorrhoids, or previous colonic polyps and who had no gastrointestinal symptoms were used as control subjects. A series of questions about the medical history and the Rome II criteria served to check the health status of the controls. During endoscopy no signs of abnormality including inflammation in the descending colon, sigmoid or rectum were seen in any of the subjects. All participants were not permitted to use erythromycine, octreotide, serotonin antagonists or agonists, antidepressives and prokinetics one week before the endoscopy and rectal barostat test. Furthermore laxatives,

antidiarrheals, antacids containing magnesium or aluminium salts, antispasmodic agents, calcium antagonists or nitrates and opioids or other narcotic analgesics were not permitted 48 hours before the endoscopy and rectal barostat test. The study was approved by the medical ethics committee of the University Medical Center Utrecht and written informed consent was obtained from all participants.

Study protocol

After an overnight fast the subjects underwent a sigmoidoscopy or colonoscopy. Bowel preparation for the simoidoscopy consisted of sennoside containing laxatives (Prunacolon) and phosphosoda enema (Colex) at the morning of the sigmoidoscopy. Bowel preparation for colonoscopy consisted of macrogol containing laxatives (Colofort). Four mucosal biopsy specimens of the rectum, sigmoid and descending colon per subject were obtained. Biopsies for mRNA expression analysis and ELISA measurement were immediately snap frozen in liquid nitrogen and subsequently stored at -80°C. Biopsy for histologic examination was stored in paraformaldehyde 2% at 4°C. Within a week from the endoscopy sensitivity to rectal distension was measured in IBS patients.

mRNA expression analysis

Total RNA isolation, cDNA synthesis and monitoring of mRNA levels of PAR-2, trypsinogen IV and TPH-1 were performed as described previously.(344) Specific primers and reaction conditions for amplification were described previously.(344)

TaqMan MGB probe (oligonucleotides 5'-3': ccagcagatcctccagaa) based detection was used for the quantification of SERT mRNA levels. PCR of SERT was carried out using 5 µl of diluted cDNA, 12.5 µl 2x iQ Supermix (BioRad, Hercules, CA, USA), and 300 nM of the forward and reverse primer each, and 100 nM TaqMan MGB probe 5'-labeled with FAM and a nonfluorescent quencher at the 3'-end. $MgCl_2$ was added to obtain a final concentration of 5 mM in a total volume of 25 µl. Thermal cycling conditions consisted of a 3 min 95°C initial denaturation step, followed by 50 cycles of 15 sec denaturation at 95°C and 1 min annealing and extension at 60°C.

For normalization purposes three endogenous reference genes were measured; porphobilinogen deaminase (PBGD), β-actin (ACTB) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and the geometric mean was obtained, as previously described. Expression levels in the various biopsy

specimens were quantified by calculating initial target concentrations using the obtained threshold cycle values and the relative standard curve.(344) Subsequently, for each sample the level of a gene of interest was divided by that of the geometric mean of the three reference genes to obtain the normalized mRNA expression.(321;344)

Measurement of 5-HT, 5-HIAA and substance P content of mucosal biopsies
The biopsy specimens were homogenized in 1 ml ice-cold buffer (1x PBS, 40 mM Tris-HCl (pH 7.5), 1 mM EDTA, 2 µg/ml aprotinin, 2 µg/ml leupeptin, 2 µg/ml pepstatin-A, 1 mM PMSF) using the Omni µH homogenizer. Suspensions were centrifuged at 25,000 rpm for 20 minutes at 4ºC. In the supernatants the 5-HT content (RE 59121, IBL, Hamburg, Germany), 5-hydroxyindoleacetic acid (5-HIAA) content (RE59131, IBL, Hamburg, Germany), substance P content (583751, Cayman Chemical, Ann Arbor, MI, USA) and protein content (QuantiPro BCA assay, Sigma, Saint Louis, Missouri, USA) were quantified using an enzymatic immunoassay kit according to manufacturer's instructions. C18-SPE extraction of the supernatants prior to substance P content measurement was performed.(345) After the extraction the samples were reconstituted in enzyme immunoassay buffer. Substance P content values out of the linear range of the standard curve (below 20%) were assigned the content value of 20% for statistical purposes. The contents of 5-HT, 5-HIAA and substance P were expressed per milligram of protein (± SEM).

Histopathology and immunohistochemistry
Fixed biopsies were embedded in paraffin wax with orientation optimized using a dissecting microscope to ensure that sections (5 mu) were perpendicular to the mucosa. The sections were stained with haematoxylin-eosin (HE) and immunohistochemistry staining with monoclonal antibodies for CD_3 (detects T lymphocytes, DAKO, Glostrup, Danmark, 1/100), mast cell tryptase (DAKO, Glostrup, Danmark, 1/800), KI-67 monoclonal antibody (MIB-1, DAKO, Glostrup, Danmark, 1/100) and 5-HT (Eurodiagnostica, Malmö, Sweden, rabbit anti-human, PSE 1/200). A single expert pathologist did a conventional histologic assessment on H&E stained sections of the biopsies. The mean numbers of mast cells were expressed per mm^2 of the total mucosal area. The mean number of intraepithelial T lymfocytes were expressed per 100 enterocytes. The mean number of 5-HT- immunoreactive cells were evaluated per colonic gland. Acceptable crypts were defined as being midaxial, with U-shaped sections extending from the muscularis to the lumen with an intact structure.

Number of proliferating cells was expressed as the mean number of Ki-67 positive cells per 100 enterocytes. Crypt height, the distance from base to lumen, was also analyzed as an outcome variable.

Rectal barostat test

A Barostat (Distender Series II Barostat, Protocol Plus, G&J Electronics Inc., Willowdale, Ontario, Canada) was used to inflate a disposable 500 ml polyethylene bag tightly wrapped on the distal end of a polyvinyl tube in the rectum for physiological measurements.(346) The barostat allows continuous recording of rectal volume at a fixed pressure level, which is an indirect measure of rectal tone. In addition, by inflating the intrarectal barostat bag, sensitivity to rectal distension can be assessed in a controlled fashion. The rectum was evacuated with an enema of tap water. The tightly folded bag was inserted into the rectum. During the measurement the patient was in prone position to reduce the gravitational effects of the abdominal organs. After an adaptation period of 10 minutes, minimal distending pressure (MDP) was determined. MDP was defined as the minimum pressure required to overcome mechanical forces and at which the intrabag volume was > 30 mL. To assess basal rectal tone, operating pressure was set at MDP + 2 mmHg and intrabag volume was measured for 15 minutes. Rectal distensions were performed according to a rapid phasic, isobaric distension protocol. The pressure increment was 4 mmHg above MDP, each step lasting 1 min and separated by 1-min intervals at MDP level. The inflation rate was set at 32 mLs^{-1}. After 45 seconds distension, distension evoked sensations of urgency, discomfort and pain were graded from none to an extreme amount on a visual analogue scale. At the threshold for urgency, discomfort or pain or when the maximum pressure of 50 mmHg was reached, the bag was deflated and this completed the distension session.

The discomfort and pain thresholds were defined as the amount of pressure above MDP at which the subject reported a score higher than 80% on the VAS. The threshold for discomfort or pain lower than MDP + 32 mmHg defines visceral hypersensitivity.(311;347) If the subject requested to stop the distensions before 80% on any of the VAS scores was reached the thresholds were not determined. Wall tension was calculated assuming a cylindrical shape of the balloon in the rectum. Laplace's law was applied to a cylinder for calculation of wall tension.

Statistics

In order to obtain normally distributed data, the normalized mRNA levels were first transformed by taking the natural logarithm. The relative differences between the groups are expressed as a fold change. An independent samples t test for evaluation of difference in mRNA expression, 5-HT and substance P content and EC cell, IEL and mast cell counts between controls and IBS patients was performed. Regional differences of mRNA expression, 5-HT and substance P content and EC cell, IEL and mast cell counts between descending colon, sigmoid and rectum were evaluated by applying an univariate analysis of variance (1-way ANOVA) and a post-hoc test (Bonferroni correction).
Mann Whitney U test was performed for evaluation of difference in mRNA expression, 5-HT and substance P content and EC cell, IEL and mast cell counts between controls and rectal hypersensitive (+) and (-) IBS patients.
A $P<0.05$ was considered significant. All statistical analysis was performed using commercially available software (SPSS 12.0.1, SPSS Science, Inc, Chicago, Illinoiis, USA for Microsoft windows).

Results

Subjects

Twenty-three IBS patients (14 female) and 15 controls (6 female) were included in the study. The mean age (range) of the IBS patients was 39.5 (22-65) years versus 42.1 (22-62) years of the controls. There were no statistical differences between patients and controls with regard to gender distribution and age.

Rectal Barostat test

Rectal sensitivity was measured in 19 IBS patients. (**Table 1**) The threshold for discomfort or pain was ≤MDP + 32 mmHg in 12 IBS patients, indicating rectal hypersensitivity.(311) There were no statistical differences between rectal hypersensitivity (+) and (-) IBS patients with regard to gender distribution and age. No statistical difference was found in MDP, rectal tone and rectal wall tension between the rectal hypersensitivity (+) and (-) IBS patients as reported earlier.(348) Perception of rectal distension determined by rectal wall tension correlated with the threshold for discomfort ($P=0.004$, $R=-0.663$) and the threshold for pain ($P=0.034$, $R=-0.501$). Rectal hypersensitivity (+) was found in 63% of IBS patients which confirms previous studies showing that a subgroup of IBS patients is hypersensitive to colorectal distension.(311;312;349)

Table 1 *Rectal Barostat parameters in the IBS patients, divided into a hypersensitivity (+) group on the basis of a discomfort/pain threshold ≤ MDP+ 32 mmHg and hypersensitivity (-) group.*

	Hyper (-) N=7	**Hyper (+) N=12**	**P-value**
MDP (mmHg)	8.0 ± 1.1	9.5 ± 0.92	P=0.316
Discomfort (mmHg)	38.0 ± 1.7	21.3 ± 1.9	**P=0.001**
Pain (mmHg)	38.9 ± 1.1	26.7 ± 2.2	**P=0.002**
Rectal tone (mL)	90.1 ± 15.6	128.1 ± 9.7	P=0.063
Rectal wall tension (mmHg cm^{-1})	26.9 ± 3.3	37.2 ± 4.3	P=0.094

Values expressed as mean ± SEM .

mRNA expression

For all SYBR Green-based assays, amplification yielded a single product which size was equivalent to that predicted from the relevant sequence. In sigmoid and rectum the level of mRNA expression of the reference genes (ACTB, GAPDH and PBGD) was comparable between controls and IBS patients. In the descending colon mRNA expression of the reference genes (ACTB, GAPDH and PBGD) was significantly (P=0.005, P=0.003 and P=0.003 respectively) higher in IBS patients (1.8-, 2.6- and 1.7-fold respectively) than in controls. Between hypersensitivity (+) and (-) IBS patients the level of mRNA expression of the reference genes was comparable in descending colon, sigmoid and rectum. The mRNA expression results for PAR-2, trypsinogen IV, SERT and TPH-1 are shown in **Table 2**.

PAR-2: In descending colon, sigmoid and rectum no differences were observed in PAR-2 expression normalized against the geometric mean of the three reference genes between controls and IBS patients.

Trypsinogen IV: In descending colon of IBS patients, the expression of trypsinogen IV normalized against the geometric mean of the three reference genes was 1.8-fold lower compared to controls (P=0.001). No significant difference in trypsinogen IV expression between rectal hypersensitivity (+) and (-) IBS patients was observed.

In sigmoid and rectum no differences in trypsinogen IV expression between controls and IBS patients were observed.

Notably in descending colon, the expression of trypsinogen IV was markedly lower compared to sigmoid and rectum (P<0.001). The relative differences of

Table 2 *mRNA expression of PAR-2, trypsinogen IV, SERT and TPH-1 normalized against geometric mean of reference genes.*

		Descending colon	Sigmoid	Rectum
PAR-2	IBS	3.2 (0.16)	2.2 (0.13)	4.6 (0.48)
	Controls	3.5 (0.19)	2.3 (0.11)	4.3 (0.38)
Trypsinogen IV	IBS	0.03 (0.004)*	0.51 (0.06)‡	0.94 (0.13)
	Controls	0.06 (0.008)	0.73 (0.11)‡	1.0 (0.21)‡
SERT	IBS	3.6 (1.3)*	5.5 (1.5)	2.6 (1.2)*
	Controls	10.4 (1.8)	14.3 (5.9)	2.8 (0.54)
TPH-1	IBS	1.0 (0.11)*	0.63 (0.13)	1.5 (0.23)*
	Controls	1.9 (0.32)	0.90 (0.14)	3.0 (0.59)

All values represent mean of normalized mRNA level (SEM). The mRNA expression can not be compared between the different genes, since different standard curves were used. * $P<0.05$ compared to controls, ‡ $P<0.001$ compared to descending colon

trypsinogen IV expression in controls were 11.5-fold (descending colon versus sigmoid) and 15.3-fold (descending colon versus rectum).

TPH-1: In IBS patients, the expression of TPH-1 normalized against the geometric mean of the three reference genes was lower (P=0.005) in both descending colon and rectum compared to controls. The relative decrease of TPH-1 expression was 1.8 in the descending colon and 2.2 in the rectum. In respectively the descending colon, sigmoid and rectum of the rectal hypersensitivity (+) IBS patients, TPH-1 expression was 2.2-, 1.8- and 2.2-fold lower (P=0.003, P=0.015 and P=0.051) compared to controls. (**Figure 1a, b**) In rectum of the rectal hypersensitivity (-) IBS patients, TPH-1 expression was 2.4-fold lower (P=0.012) compared to controls. In all three regions no significant difference in TPH-1 expression between rectal hypersensitivity (+) and (-) IBS patients was observed.

SERT: In IBS patients, the expression of SERT normalized against the geometric mean of the three reference genes was 6.7-fold and 2.4-fold lower in respectively descending colon and rectum compared to controls (P=0.001, P=0.013). The mRNA expression of SERT was not significantly different in sigmoid mucosa of controls and IBS patients. However, in rectal hypersensitivity (+) patients SERT transcript level was 2.1-fold lower in the rectum compared to controls (P=0.028). SERT transcript level was not significantly different in

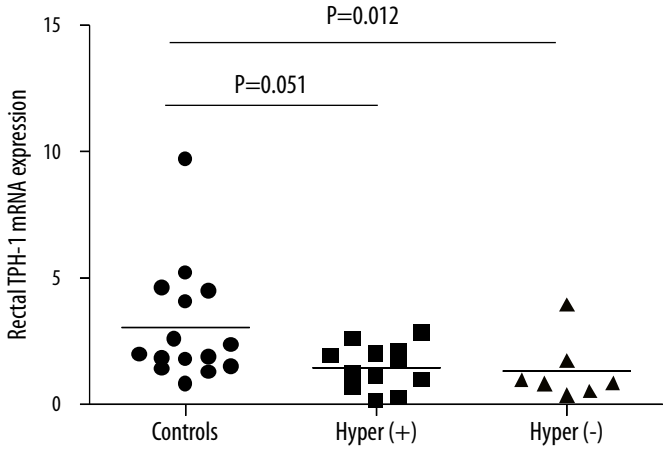

Figure 1a *mRNA expression of TPH-1 in the rectum (normalized against the geometric mean of reference genes). Both rectal hypersensitivity (+) and (-) IBS patients exhibited a lower TPH-1 transcript level compared to controls (P=0.051 and P=0.012 respectively)*

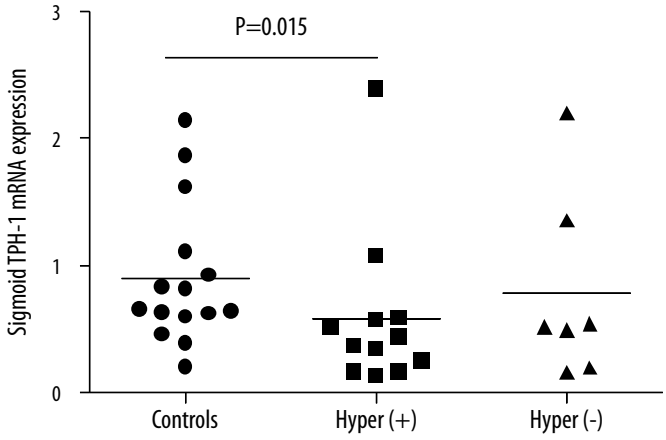

Figure 1b *mRNA expression of TPH-1 in the sigmoid (normalized against the geometric mean of reference genes). TPH-1 transcript level was decreased in rectal hypersensitivity (+) IBS patients relative to controls (P=0.015).*

hypersensitivity (+) and (-) patients. (**Figure 1c**) No significant difference in SERT mRNA expression in sigmoid mucosa of controls and hypersensitivity (+) or (-) IBS patients was observed. Finally, in descending colon and sigmoid no significant difference in SERT expression between rectal hypersensitivity (+) and (-) IBS patients was observed.

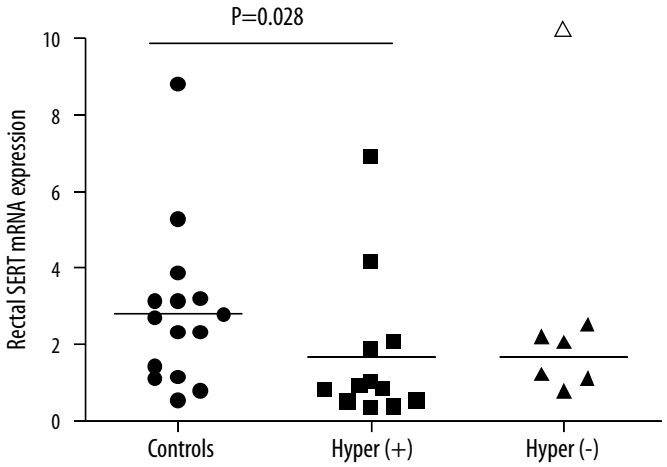

Figure 1c *mRNA expression of SERT in the rectum (normalized against the geometric mean of reference genes). Rectal hypersensitivity (+) IBS patients showed a lower SERT level compared to controls (P=0.028).*

Mucosal 5-HT, 5-HIAA and substance P content

No significant differences in mucosal serotonin content of IBS patients and controls in descending colon (50.0 ± 7.1 versus 36.7 ± 7.6 pg ml^{-1}/mg protein), sigmoid (56.1 ± 7.4 versus 48.7 ± 7.7pg ml^{-1}/mg protein) and rectum (75.9 ± 8.5 versus 61.9 ± 11.4 pg ml-1/mg protein) were observed. In descending colon, mucosal 5-HIAA content was not significantly different between IBS patients (13.0 ± 1.5 µg l^{-1}/mg protein) and controls (12.5 ± 1.4 µg l^{-1}/mg protein). The 5-HIAA/5-HT ratio in descending colon also showed no significant difference between IBS patients and controls. In sigmoid and rectum mucosal 5-HIAA content was not determined. In rectal hypersensitivity (+) patients the serotonin content in descending colon was 1.7-fold higher (P=0.02) compared to controls (63.8 ± 11.7 versus 36.7 ± 7.6 pg ml^{-1}/mg protein) (**Figure 1d**). No

significant differences in mucosal 5-HIAA content and 5-HIAA/5-HT ratio of rectal hypersensitivity (+) and (-) IBS patients and controls were observed.

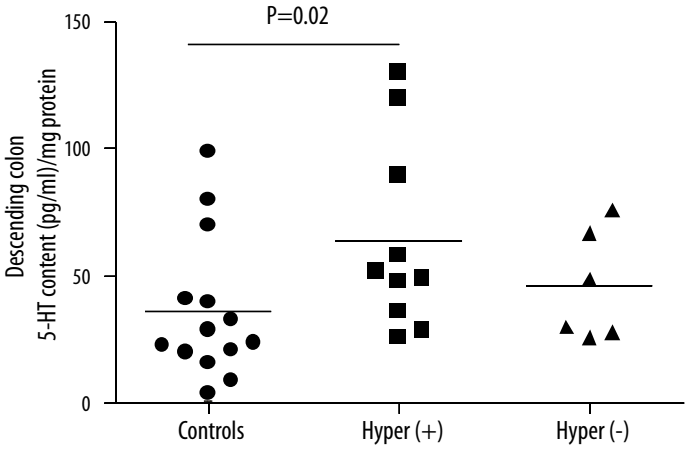

Figure 1d *Rectal hypersensitivity (+) IBS patients showed increased mucosal 5-HT content in descending colon compared to controls (P=0.02).*

Rectal substance P content was higher in IBS patients compared to controls (5.31 ± 0.61 versus 3.40 ± 0.49 pg ml^{-1}/mg protein;P=0.045). No significant differences in mucosal substance P content between IBS patients and controls in descending colon (4.83 ± 0.72 versus 4.51 ± 0.74 pg ml^{-1}/mg protein) and sigmoid (5.38 ± 0.55 versus 3.99 ± 0.54 pg ml^{-1}/mg protein) were observed. In all three regions no significant differences in substance P content between rectal hypersensitivity (+) and (-) IBS patients were observed.

EC, IEL and mast cell counts

In **Table 3**, EC cell, IEL and mast cell counts in IBS patients and controls are presented. The number of EC cells was comparable between patients and controls (P>0.05). No significant difference in the number of EC cells was observed between hypersensitivity (+) and (-) patients. The number of EC cells was larger in rectum compared to the descending colon in IBS patients and controls (P<0.05 and P<0.001 respectively) and compared to the sigmoid in controls (P≤0.001).

The number of mast cells per mm^2 in descending colon, sigmoid and rectum was comparable between IBS patients and controls (P>0.05). No significant

Table 3 *EC cell, intraepithelial lymphocyte (IEL) and mast cell counts in IBS patients and controls*

		Descending colon	Sigmoid	Rectum
EC cell	IBS	1.6 (0.20)	2.2 (0.31)	2.8 (0.41)
	Controls	1.3 (0.24)	1.8 (0.25)	3.8 (0.55)
IEL	IBS	0.03 (0.003)	0.03 (0.005)	0.02 (0.003)
	Controls	0.04 (0.007)	0.03 (0.004)	0.02 (0.003)
Mast cell	IBS	116.8 (8.5)	111.9 (8.5)	97.1 (8.2)
	Controls	112.5 (7.4)	94.8 (7.2)	84.6 (5.0)

All values represent mean (SEM). Mast cells are expressed per mm^2, IEL per 100 enterocytes and EC cells per colonic gland.

difference in the number of mast cells was observed between hypersensitivity (+) and (-) patients. In controls the number of mast cells was larger in descending colon compared to rectum (P=0.015).
No significant differences were found in the number of intraepithelial lymphocytes (IEL) per 100 enterocytes in descending colon, sigmoid and rectum of IBS patients and controls. Furthermore, no significant difference in IEL number was observed between hypersensitivity (+) and (-) patients was observed. Regional differences in IEL number were also not observed in either IBS patients or controls.

Discussion

This study reveals reduced mRNA expression of TPH-1 and SERT in rectum of both rectal hypersensitivity (+) and hypersensitivity (-) IBS patients compared to controls. In rectal hypersensitivity (-) patients the reduction in rectal SERT mRNA did not reach statistical significance due to an outlier. In rectal hypersensitivity (+) IBS patients TPH-1 mRNA expression was also significantly reduced in sigmoid. In rectal hypersensitivity (-) patients no significant difference in sigmoidal TPH-1 transcript level was observed probably due to a smaller difference in expression level and/or the smaller sample size.
Decreased mRNA expression of TPH-1 and SERT in rectal mucosa from IBS patients was found previously by Coates *et al*.(47) In contrast, Camilleri *et al*. observed no difference in SERT mRNA expression in rectal biopsies of IBS

patients versus controls. Similar to the study of Camilleri *et al*. we found no differences in SERT expression in sigmoid mucosal biopsy specimens when comparing the IBS patients with controls.(335) Furthermore, increased 5-HT content normalized for protein concentration in rectal hypersensitivity (+) IBS patients was observed. Previously, 5-HT content was reported to be reduced in IBS patients compared to controls.(47) This contradictory result might be explained by differences in data expression, in which normalization for protein concentration increases data accuracy, and the detection methods employed. Previously EC cell density was shown to be 1.5-fold higher in rectal hypersensitivity (+) IBS-D patients compared to rectal hypersensitivity (-) IBS-D patients.(348) We observed no differences in the absolute number of EC cells expressed per colonic gland between the rectal hypersensitivity (+) and (-) IBS patients. Our results show that several serotonergic pathway components are altered in IBS patients irrespective of visceral sensitivity state and that there exist no significant difference between hypersensitivity (+) and (-) patients. Therefore, we conclude that differences in the portion of IBS patients exhibiting colorectal hypersensitivity are unlikely to explain discrepancies in studies on serotonergic pathway components in IBS patients.

It is known that SERT expression, 5-HT content and number of EC cells are altered in inflammatory states.(13;47;336) Low-grade inflammation has been reported for IBS patients in the colorectal region.(9;302;303) Possibly the contradiction in literature on serotonergic signalling components might be due to differences in the portion of patients exhibiting low-grade inflammation. In our study no changes in intraepithelial lymphocytes or mast cells were present neither in the complete group of IBS patients or the rectal hypersensitivity (+) IBS patients compared to the controls.

The interpretation of the mRNA expression results in the descending colon is complicated due to increased reference gene expression in the descending colon of IBS patients compared to controls. This may be explained by different bowel preparation for colonoscopy and sigmoidoscopy. As preparation for colonoscopy macrogol containing laxatives were used, whereas sennoside containing laxatives and sodiumphosphate enemas for sigmoidoscopy. It has been reported that a single dose of sennoside containing laxatives increases the proliferation rate of colonic epithelial cells and reduces crypt height.(350) During cell proliferation mRNA expression of housekeeping genes, such as GAPDH and ACTB, is enhanced.(351) Sennoside containing laxatives were significantly (P=0.002) more used in the IBS patients (17/23) than in controls (3/15). Therefore, we hypothesized that the elevated reference gene expression

in the descending colon of the IBS patients compared to controls is caused by enhanced proliferation in response to sennoside containing laxatives. Determination of the number of proliferating cells and crypt height supported this hypothesis. The proliferation rate was significantly higher (P=0.038) and crypt height significantly reduced (P=0.028) in descending colon of IBS patients who underwent a sigmoidoscopy compared to controls who underwent a colonoscopy. Decrease in trypsinogen IV and TPH-1 expression in descending colon can be attributed the enhanced reference gene expression. Since the change in SERT expression is clearly larger than the differential reference gene expression, we conclude that SERT expression is reduced in the descending colon of IBS patients.

Furthermore, rectal substance P content was higher in IBS patients compared to controls. However, no significant differences in substance P content between rectal hypersensitivity (+) and (-) IBS patients were observed. Substance P is released from extrinsic primary afferents upon activation and subsequently triggers the degranulation of mast cells, of which the contents may in turn stimulate the extrinsic primary afferents. Therefore it is conceivable that elevated substance P correlates with visceral hypersensitivity. Evidence for a clear-cut relationship with visceral sensitivity is also not supported by a recent study showing substance P immunoreactive fibers to be increased in recto-sigmoid mucosa of IBS patients compared to controls although not related to abdominal pain.(352) Besides from nerve endings substance P is also secreted by inflammatory cells.(353) However, no significant differences in mast cells and IEL were observed in the colorectal mucosal biopsies between IBS patients and controls in our study.

In conclusion the abnormalities in colorectal components of serine protease and serotonergic signalling showed no differences between rectal hypersensitivity (+) and (-) IBS patients. Irrespective of visceral hypersensitivity state, the serotonergic signalling components are altered in IBS patients and may play a role in IBS symptom generation.

Chapter 9

Mast cells in close proximity of Protease Activated Receptor-2 positive neurons in both Irritable Bowel Syndrome patients and controls small intestine

Angèle PM Kerckhoffs, Louis MA Akkermans, Melvin Samsom, Marguerite El Schipper, Greger Lindberg, Bela Veress, Hans Tornblom

Abstract

Introduction
Intestinal visceral hypersensitivity in IBS patients might be due to sensitization of sensory neural endings by low-grade inflammation. Numbers of mast cells and mast cell activation is associated with IBS disease activity. In IBS patients more mast cells are in close proximity to mucosal (lamina propria) nerve fibers than in controls. Mast cell tryptase leads to long-lasting neuronal hyperexcitability due to activation of protease activated receptor (PAR)-2 on visceral afferents. We assessed the spatial interaction between submucosal immunoreactive PAR-2 neurons and mast cells in both IBS patients and controls using immunohistochemistry.

Material and methods
PAR-2 positive neurons and mast cells in the submucosa of full-thickness jejunal biopsy specimens of 16 IBS patients and 9 controls were counted. Furthermore the number of mast cells in vicinity of neurons was measured at different distances up to 30 µm from the neuron.

Results
Counts of submucosal neurons, PAR-2 positive neurons and mast cells were not significantly different between IBS patients and controls. Mast cells were observed in the vicinity of the submucosal plexus in both controls and IBS patients. PAR-2 positivity of neurons was strongly correlated with the number of mast cells in the vicinity of neurons ($P<0.001$, $R=0.94$). Significantly more mast cells were found in the vicinity of PAR-2 positive neurons than PAR-2 negative neurons ($P=0.005$).

Conclusion
Mast cells are in close vicinity to submucosal neurons which are predominantly PAR-2 positive neurons. Although no differences between IBS patients and controls, the cross talk between mast cells and PAR-2 positive neurons might be important in modulating visceral sensitivity.

Introduction

Irritable Bowel Syndrome (IBS) is characterized by abdominal pain and alterations in bowel habits. Visceral hypersensitivity has been identified in the pathogenesis of IBS. In a subset of IBS patients visceral hypersensitivity to mechanical distension in the colon, rectum as well as the small intestine has been shown.(311-315) Visceral hypersensitivity in IBS patients might be due to the sensitization of sensory neural endings in the intestine by mast cells and lymphocytes.(14;284) In a subset of IBS patients increased numbers of mast cells and/or lymphocytes has been observed.(9) In human intestine, mast cells lie in close proximity to mucosal nerve endings.(354) Moreover in IBS it has been reported that a higher number of mast cells are in close proximity to mucosal nerve fibres compared to controls, and most importantly, that this is associated with abdominal pain and discomfort.(10)
Mast cell tryptase induces activation of protease activated receptor (PAR)-2 located on the myenteric and the submucosal plexus and on visceral afferents causing long-lasting neuronal hyperexcitability.(40;342;355-358) Increased PAR-2 expression and activation induces neurogenic inflammation and increased intestinal permeability to macromolecules which leads to hyperalgesia.(38;329) Besides interaction with barrier function and sensory functions, PAR-2 activation interacts with many more aspects of gut physiology such as motility, transports, innate immune response and even proliferation.(359) Since mast cells and tryptase release in close proximity to mucosal nerve endings in IBS patients is correlated with abdominal pain it is interesting to know if PAR-2 receptors are involved. The aim of this study is to assess the spatial interaction between immunoreactive PAR-2 neurons and mast cells to determine differences between IBS patients and controls. Since many nerve fibers, are located in the submucosal area we used full thickness jejunum biopsies since endoscopic biopsies reach only a small part of the submucosal area.

Material and methods

Subjects

The inclusion criteria for IBS patients were fulfilment of the Rome II criteria for the diagnosis of IBS, need for referral to a specialist in gastroenterology and no contraindications for anaesthesia or laparoscopic surgery. All patients were

given oral and written information before acceptance into the study. Patients gave informed consent. They were all aware of the fact that the operative procedure most probably would not change their treatment options. Patients scheduled for an open gastric by-pass operation for morbid obesity were asked to participate in the control group if they had no gastrointestinal symptoms compatible with a functional gastrointestinal disorder. This was checked in the medical history and by asking the patient to fill in a validated Rome II symptom questionnaire. The study was approved by the local ethics committee at Karolinska University Hospital, Huddinge, Sweden.

Biopsy technique IBS patients

A full-thickness biopsy specimen was taken from the proximal jejunum using a laparoscopy-assisted procedure. This technique is performed as follows: two 2-to 4-cm incisions are made. One is situated below the umbilicus and one in the left fossa. A bowel loop of the proximal jejunum is identified laparoscopically and is then exteriorized through the incision below the umbilicus. Without interruption of the blood supply, the bowel loop is held with a surgical forceps while taking the full-thickness biopsy specimen with a surgical knife. Most often, the tissue specimen is at least 10 to 10 mm. The bowel loop is then closed with 2 layers of absorbable suture and repositioned into the abdomen. Visual inspection of the sutured bowel loop is performed laparoscopically before closing the incision in the abdominal wall. The patient is then observed for possible complications for 24 hours and allowed to eat and drink before leaving the hospital.

Biopsy technique controls

All morbid obesity patients had a traditional gastric by-pass procedure.(360) During this procedure, the jejunum is divided approximately 50 cm below the ligament of Treitz in order to create the Roux-en-Y by-pass limb. Anastomosis to the gastric pouch by stapling technique results in a remaining circular fragment of jejunum that normally is destructed, but in this study was saved for examination.

Biopsy specimen preparation and staining

The full-thickness intestinal biopsy specimens were oriented on a cork plate with the mucosa downwards, and fixed in 4% buffered formalin for no longer than 72 hours. Following fixation, the specimens were embedded in paraffin. Serial transversal sections allowed evaluation of all layers with respectively

H&E, mast cell tryptase and PAR-2. The paraffin sections were stained with H&E for general histologic examination, quality of the biopsy and morphological evaluation. Immunohistology was performed with the following antibodies: SAM-11 for PAR-2 (SAM-11, Santa Cruz Biotechnology, Santa Cruz, CA) dilution 1:400 and mast cell tryptase to detect mast cells (DAKO, Glostrup, Danmark, 1/800). Controls for the PAR-2 immunohistochemistry consisted of leaving out the PAR-2 antibodies.

Quantification

Quantification of mast cells was performed on sections immunostained for mast cells with a Olympus BX 50 microscope and Olympus DP 70 camera in blind fashion using Olympus DP-SOFT, a computer-assisted analysis system. Microscopic fields were digitized and the total submucosal area was computed using the Olympus DP-SOFT. All mast cells in the submucosal area of one sections were counted and expressed as mean ± standard error of the mean numbers of cells per square millimetre of submucosal area. Quantification of the neurons was performed in blind fashion on the H&E stained slides by an expert pathologist (MS). Neurons were expressed per square millimetre of submucosal area. Quantification of positive and negative immunoreactive PAR-2 submucosal neurons was performed on sections immunostained with PAR-2 antibodies in concentration of 1:400. Since tryptase released by mast cells is more likely to affect neural function if it is in close proximity to neurons we investigated the spatial relationship between mast cells and neurons. All slides were scanned with the Aperio Scan Scope XT (Aperio Technologies, Inc., Bristol, England) and Aperio Image scope was used to measure the distances between mast cells and positive or negative immunoreactive PAR-2 neurons in the submucosa. In the submucosa the numbers of mast cells located within 10, 15, 20, 25 and 30 μm from both the positive and negative immunoreactive PAR-2 neurons were recorded.

Statistical analysis

The numbers of neurons, mast cells, PAR-2 positive stained neurons and mast cells in vicinity of neurons were compared between IBS patients and controls using the non-parametric Mann Whitney U test. The number of mast cells within a certain distance of the neuron (10, 15, 20, 25 or 30 μm) was compared between PAR-2 positive and negative neurons using the Wilcoxon signed rank test. Pearson correlation coefficients were computed for linear correlation analyses of the number of mast cells within 10 μm of a neuron and the number

of PAR-2 positive neurons of these neurons with mast cells in their close vicinity.

Results

Subjects

No statistically significant difference was found in age between the 16 IBS patients of which 4 IBS-A, 7 IBS-D and 5 IBS-C (44.56 ± 3 years) and 9 controls (47.3 ± 2.97 years). Furthermore no statistically significant difference was found in gender distribution between IBS patients (2 men, 14 women) and controls (2 men, 7 women).

Immunolocalization

Antibodies to PAR-2 in human jejunum showed intense immunoreactivity in the mucosa to the enterocytes, brush border on the luminal side of enterocytes. Moreover smooth muscle of muscularis mucosae was PAR-2 positive stained. In the submucosal layer vascular smooth muscle cells of arterial blood vessels were intense immunostained, peripheral nerve branches and submucosal neurons were less intense immunostained. The submucosal neurons showed immunostaining of variable intensity. The smooth muscle of muscularis externa showed intense immunoreactivity to PAR-2 antibodies. The myenteric neurons were also PAR-2 positive stained.

Histopathology

The number of submucosal neurons per square millimetre of the total submucosal area in the H&E is not significantly different between controls (11.4 ± 1.8) and IBS patients (16.5 ± 3.2). The numbers of PAR-2 immunoreactive positive and negative neurons per square millimetre are also not significantly different between controls and IBS patients. (**Table 1**) Moreover, no differences in submucosal mast cell counts between controls and IBS patients were observed.

Mast cells were observed in the vicinity of the submucosal neurons in both controls and IBS patients. Of the neurons with mast cells in the vicinity (<30µm) 68.44 ± 8.95% were PAR-2 positive in IBS patients and 51.06 ± 14.84% were PAR-2 positive in controls. (**Table 1**) Mast cells in the vicinity of the submucosal neurons are correlated ($P<0.001$, $R=0.940$) with the neuron being PAR-2 positive. The number of mast cells near a PAR-2 positive neuron is

significantly (P=0.005) higher than near a PAR-2 negative neuron. This significantly higher number of mast cells near a PAR-2 positive neuron is not only observed at a distance of 10 µm, but also at 15 µm (P=0.025), 20 µm (P=0.001), 25µm (P=0.003) and 30 µm (P<0.001). (**Figure 1**)

Table 1 *Spatial relationship and counts of submucosal immunoreactive PAR-2 positive and negative neurons and mast cells in IBS patients and controls.*

	IBS	**controls**	**P-value**
PAR-2 positive stained neurons/mm^2	7.2 ± 1.7	4.2 ± 0.9	P=0.141
PAR-2 negative stained neurons/mm^2	3.5 ± 0.8	1.6 ± 0.6	P=0.101
Mast cells/mm^2	67.9 ± 7.8	49.66 ± 10.47	P=0.141
Mast cells in close vicinity(<10µm) of neurons/mm^2	0.75 ± 0.17	0.51 ± 0.18	P=0.646
% Mast cells in close vicinity of PAR-2 positive neurons of total mast cells in close vicinity of neurons	68.4 ± 8.9	51.1 ± 14.8	P=0.269

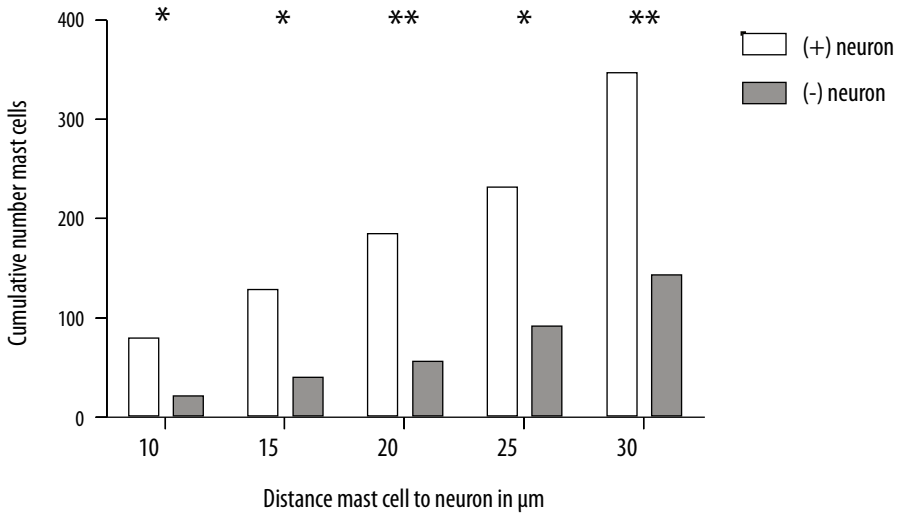

Figure 1 *Number of mast cells near PAR-2 positive and negative neurons at a distance of 10, 15, 20, 25 and 30 µm from the neuron.*P<0.05, **P≤0.001*

Discussion

In this study, using immunohistochemistry, we have provided evidence for the presence of mast cells in close vicinity to submucosal neurons which are predominantly PAR-2 immunostained neurons in human jejunum biopsies. No statistical significant difference in the number of mast cells and in the number of mast cells in the vicinity of submucosal neurons between IBS patients and controls was found.

Previously, increased and unchanged numbers of intestinal mucosal mast cells in IBS patients compared to controls have been found.(9;13;302;303) Discrepancies among studies regarding mast cell numbers in IBS could be due to differences in counting methods, the regions of the gut that were sampled, and the detection methods employed.

In contrast to our study the number of mast cells in close proximity to nerve fibers in mucosa was reported to be higher in IBS patients compared to controls by Barbara *et al*.(10) This might be due to several reasons. First of all, in our study mast cells were investigated in the submucosal layer compared to mucosal area in other studies. Secondly, in contrast to other studies the mast cell numbers were not increased in IBS patients compared to controls in this study. This may suggest that a critical relationship between nerves and mast cells only exists in the setting of a significant increase in mast cell numbers. Thirdly the problem of finding a control group for this study is of major concern. To obtain full thickness jejunum biopsies in healthy volunteers is unethical. Postmortal changes due to autolysis of autopsy material might reduce the ability to detect changes since immunohistochemistry is negatively influenced by post mortal changes. Although obese subjects might be second best as control group we consider them useful because they all lacked gastro-intestinal symptoms. The main disadvantage is that obesity in itself has an impact on the immune system.(361) In the gut, fat tissue may have an active proinflammatory role exemplified by its intriguing association with inflamed areas of bowel in Crohns disease.(362) There are also a few studies reporting of an association between obesity and gastrointestinal symptoms of functional character, but not specifically IBS.(363) If this proinflammatory action also involves mast cells is still not known but may be a potential confounder to our results. At last, in the study of Barbara *et al*. mast cell count was performed by measuring the mucosal area occupied by mast cells as in our study the number of mast cells was counted.

The most important finding in this study is the strong spatial correlation between mast cells in the vicinity of neurons and that these neurons were PAR-2 positive in both controls and IBS patients. To our knowledge this is the first human study to show this interaction between mast cells and PAR-2 positive neurons in the intestine. Close proximity between mast cells and enteric nerve fibers might lead to bidirectional communication.(364) A relevant aspect is the ability of mast cells to evoke activation of nociceptive neural pathways which correlates with abdominal pain, at least in patients with IBS.(10;365) Tryptase, activating PAR-2, may be a physiological agonist in cells that are in close proximity to mast cells and which could be exposed to high concentrations of this enzyme, such as sensory nerves.(326;342;356;358) PAR-2 activation increases signalling at the spinal cord level which leads to amplification and persistence of pain sensation.(366)
In conclusion, in human jejunum mast cells are in close vicinity to submucosal neurons that are predominantly PAR-2 immunostained neurons. This cross talk might be important in modulating visceral sensitivity in both IBS patients and controls.

Chapter 10

General discussion and summary

Algemene discussie en samenvatting

References

Dankwoord

Curriculum Vitae

General discussion and summary

Irritable Bowel Syndrome (IBS) is a functional bowel disease in which abdominal pain or discomfort is associated with disordered defecation. Many pathophysiological explanations have been suggested for IBS; however no unique mechanism has been identified. It is most likely a multifactorial disease. In this thesis we focus on several peripheral factors influencing visceral hypersensitivity in IBS. In the introduction (**chapter 1**), the role of intestinal microbiota, intestinal permeability and serine protease and serotonergic signalling on the pathophysiology of visceral hypersensitivity in Irritable Bowel Syndrome are reviewed. Furthermore in the introduction the aims of this thesis are presented, which have been laid out in the following chapters.

In **chapter 2 to 5**, sampling microbiota in the gastrointestinal tract and diagnosing alterations in the composition of intestinal microbiota are described.

The sampling and research of the gastrointestinal tract microbiota, especially in the case of humans, is often restricted to faecal material. In **chapter 2** a range of invasive sampling techniques to access the entire human gastrointestinal tract are reviewed, as well as non-invasive sampling methods that are used in medical practice. This is an important issue since faeces represent only the luminal microbiota of the terminal colon and will provide insufficient information about other, more proximal, parts of the intestinal tract. Furthermore, mucosa-associated bacteria are thought to have a stronger interaction with the host than luminal bacteria.(57)

Both invasive and non-invasive sampling methods are used to diagnose clinical small bowel bacterial overgrowth syndrome (SBBO). Steatorrhea, diarrhea, flatulence, abdominal pain and bloating characterize SBBO. SBBO is a condition associated with the proliferation of colonic-type bacteria within the small intestine. In **chapter 3** diagnosing bacterial overgrowth using experimental and standard breath tests (BT) and culture and molecular based methods were evaluated. Direct culture of jejunal aspirates is considered the

gold standard but is impractical and leaves cases with isolated distal bacterial overgrowth undiagnosed.(155) Therefore, non-invasive tests such as breath tests are used. A major drawback of lactulose BT is false positive testing due to rapid lactulose transit to the colon.(170) To avoid this rapid transit we limited lactulose to the small intestine using an occlusive balloon during experimental breath testing. We analyzed 11 controls and 15 SBBO predisposed subjects for bacterial overgrowth. The group of SBBO predisposed subjects consisted of 10 IBS patients and 5 patients with the clinical suspicion of SBBO syndrome and conditions favouring SBBO. Jejunal fluid was analysed using culture and quantitative PCR. Using culture based methods, bacterial overgrowth was diagnosed, on the basis of more than 10^3CFU/ml excluding lactobacilli and streptococci, in 0 out of 9 controls and 4 out of 12 SBBO subjects. Bacterial overgrowth, using culture based methods, was also diagnosed by means of all previously published definitions for SBBO and also this revealed no significant differences between controls and SBBO subjects. Furthermore, total bacterial counts showed no significant differences between controls and SBBO subjects using both culture and molecular based methods. Using standard breath tests, bacterial overgrowth was diagnosed, on the basis of 20 ppm hydrogen or methane increase above baseline within 90 minutes, in 3 out of 11 controls and 8 out of 15 SBBO subjects. No changes in start of increase of hydrogen excretion in both experimental and standard BT between controls and SBBO subjects are shown. These findings indicate that in this small group of subjects the experimental BT does not improve the ability of lactulose BT to diagnose bacterial overgrowth. Interestingly, culturing shows less positive diagnoses of bacterial overgrowth in controls compared to the standard BT. Remarkably, current diagnostic criteria do not seem to be accurate in discriminating between SBBO subjects and controls. Recently Simren *et al*. and Khoshini *et al*. also showed in reviews that definitions of normal and abnormal results of breath tests and culturing to prove small bowel bacterial overgrowth are variable.(178;367) Furthermore the use of lactulose breath tests cannot be recommended in the evaluation of patients with functional gastrointestinal disorders.(178) Schiller *et al*. also conclude that the perfect test for small bowel bacterial overgrowth is still to be devised.(368) Khoshini *et al*. recommend a combination of testing, treatment with antibiotics and symptomatic outcome techniques since accurately determining SBBO is limited.(367) In conclusion, a gold standard for diagnosing bacterial overgrowth is currently missing which makes it very unreliable to obtain this clinical diagnosis in an individual

patient. In future studies new techniques such as molecular-based methods should be evaluated.

Similar to diagnosing SBBO, research on gut microbiota composition in healthy subjects and IBS patients has been limited to culture-based methods. Although most of the indigenous bacteria cannot be isolated in pure culture owing to difficulties mimicking in vitro their niche within the enteric microenvironment. This has recently been circumvented with the advent of molecular-based techniques. In **chapter 4** we determined the composition of the total microbiota and especially bifidobacteria of both faecal and duodenal mucosa-associated microbiota in 41 IBS patients and 26 healthy subjects using fluorescent in situ hybridization (FISH) and real time PCR. The FISH analysis of faecal samples reveals a 2-fold decrease in the level of bifidobacteria in IBS patients compared to healthy subjects whereas no major differences in other bacterial groups were observed. At the species level, *Bifidobacterium catenulatum* was significantly lower in the IBS patients in both faecal and duodenal brush samples than in the healthy subjects. The alterations in the microbiota composition might play a key role in the IBS pathophysiology since microbiota influence the structure and function of the gastrointestinal tract.(57) A major obstacle in relating microbial composition with function is the fact that each individual is inhabited by a specific microbial community shaped by host and environmental factors.(159) Therefore in future studies it is necessary to determine if the decrease in bifidobacteria and especially *Bifidobacteria catenulatum* is causative by evaluating the symptoms before and after restoring the total number of bifidobacteria and especially the number of *Bifidobacteria catenulatum*.

In **chapter 5** we determined mucosa-associated small intestinal and faecal microbiota of 37 IBS patients and 20 healthy subjects by PCR denaturing gradient gel electrophoresis (DGGE) and Q-PCR analysis. Bacterial 16SrRNA gene is amplified and analysed and an average DGGE profile of all IBS patients and of all healthy subjects is constructed using GelCompar II software and fingerprints of both groups are compared. IBS patients and healthy subjects have identical averaged DGGE profiles for 78.2% in the small intestinal samples and for 86.25% in the faecal samples. Bands, which are confined to one group (IBS or healthy subjects), are cut out of the gel and sequenced. Clones of these small intestinal and faecal bands confined to IBS are mainly identified as *Pseudomonas* species of which *Pseudomonas aeruginosa* is the predominant

species. Q-PCR analysis, used to quantify the percentages *P. aeruginosa* of total bacterial load in the mucosa-associated small intestinal and faecal samples showed significantly higher percentages in IBS patients than in healthy subjects. The role of *P. aeruginosa* in the pathophysiology of visceral hypersensitivity is unknown. However it is known that *P. aeruginosa* influence protease-activated receptor (PAR)-2 in the respiratory tract.(264) Future studies should evaluate the effect of the proteases of *P. aeruginosa* on PAR-2 in the gastrointestinal tract . Complementary to chapter 4, we showed alterations in microbiota composition in both faecal and small intestinal mucosa associated samples which might play a role in the pathophysiology of IBS. Kassinen *et al*. and Malinen *et al*. also showed that the microbial community in faecal samples in IBS is significantly altered.(24;26) However the question remains from our and their studies if the change in the microbial composition is causal, consequential or merely the result of constipation and diarrhea. Studies evaluating the effect on IBS symptoms using antibiotics or probiotics to eliminate *P. aeruginosa* are warranted with, besides healthy controls, clinical controls having diarrhea or constipation in the absence of IBS. Moreover the stability of the change in microbiota needs to be demonstrated because it is known that subsets of IBS patients undergo transition from being diarrhea predominant to constipation predominant and vice versa over time.(369)

Besides microbiota, increased intestinal permeability may play an important role in the pathophysiology of IBS because it exposes the mucosa to an abnormal challenge of luminal antigens of dietary and bacterial origin promoting and maintaining mucosal activation.(370) In **chapter 6** we investigated intestinal permeability and the effect of a relatively low dose of a NSAID (naproxen 750 mg/day for two days) on permeability in 14 IBS patients and 15 healthy subjects. NSAIDs are known to increase intestinal permeability which may lead to low-grade inflammation which contributes to visceral hypersensitivity.(281;282) Intestinal permeability is investigated before and after two days NSAID ingestion. Indicators for permeability are urinary recovery of orally administered polyethylene glycols (PEGs) of molecular masses 400, 1500, 4000, and 10,000, and a lactulose mannitol test. There are no significant differences in 24-hours recoveries of PEGs 400, 1500 and 4000 between healthy subjects and IBS patients before or after NSAID ingestion. Lactulose mannitol ratios in healthy subjects and IBS patients are also not significantly different. However PEG excretion, recorded at 2-hours intervals and permeability evaluated from the area under the PEG excretion time curve,

shows that two days administration of NSAIDs enhanced intestinal permeability for PEG 4000 in healthy subjects and for PEGs 400, 1500, and 4000 in IBS patients. These results suggest that IBS is associated with an altered response to noxious agents in the physiology of the intestinal barrier. Whether or not altered microbiota composition in IBS, as discussed in chapters 3 to 5, play a role in this altered response and the interactions between luminal bacteria with the epithelial receptors regulating mucosal integrity in IBS should be examined. However it is known that bacteria, such as *Clostridia perfringens* and *Vibrio cholerae*, can increase intestinal permeability.(371) Furthermore, post-infective IBS patients are known to have increased intestinal permeability.(33) Using probiotics in patients with atopic dermatitis reduced the skin disease but also decreased elevated small intestinal permeability.(372) In future studies the ability to interfere therapeutically with pathways that regulate intestinal permeability, such as restoring the microbiota composition, will allow us to establish whether increased permeability is involved in IBS symptom generation.

In **chapter 7**, **chapter 8** and **chapter 9** the role of serine protease and serotonergic signalling components in IBS patients were studied.
Colorectal and small intestinal visceral hypersensitivity has been demonstrated in IBS. Serine protease signalling via protease-activated receptor (PAR)-2 promotes hyperalgesia to mechanical distension. Furthermore, serotonergic pathways are involved in gastrointestinal visceral sensitivity.
Abnormalities of serine protease and serotonergic signalling components have been identified in IBS colorectal mucosal biopsies.(43;47;373) In **chapter 7** we aimed to determine the role of altered mucosal serine protease and serotonergic signalling in small intestine of IBS patients. Gene transcripts of PAR-2, trypsinogen IV, TPH-1, SERT and 5-HT_3 subunits were quantified using real-time PCR and 5-HT content was measured by ELISA in duodenal mucosal biopsies of 34 IBS patients (10 constipation-, 11 diarrhea-predominant and 13 alternating) and 20 healthy subjects (HS). IBS patients show 1.5-fold higher trypsinogen IV mRNA level compared to HS. SERT expression is 1.8-fold higher in IBS compared to HS. Mucosal 5-HT content is 1.7-fold higher in IBS compared to HS. The mucosal 5-HT content increase was 2.1-fold in IBS-C relative to HS. Transcript levels of PAR-2, TPH-1 and 5-HT_3 receptor subunits did not differ between IBS and HS. Enhanced trypsinogen IV expression in IBS may cause increased PAR-2 activation. In future studies it has to be evaluated if the enhanced trypsinogen IV expression results in a sufficient protease

concentration to activate PAR-2. Furthermore development of PAR antagonist or trypsinogen IV inhibitors for human use could be an excellent opportunity to clarify the importance of PARs in IBS pathophysiology and symptom generation. Increased SERT expression and mucosal 5-HT content in IBS suggest higher 5-HT availability. Both increased trypsinogen IV and SERT expression may contribute to small intestinal visceral hypersensitivity in IBS patients. We aimed to answer the remaining question whether or not altered mucosal serine protease and serotonergic signalling pathway components are related to rectal visceral hypersensitivity in IBS patients in **chapter 8**. In order to study this we collected colorectal mucosal biopsies of 23 IBS patients and 15 controls. Gene transcripts of PAR-2, trypsinogen IV, TPH-1 and SERT were quantified using real-time PCR. Substance P, 5-HT and 5-HIAA contents were measured by ELISA. EC cells, mast cells and intraepithelial lymphocytes were immunohistochemically studied. Rectal visceral hypersensitivity was determined in 19 IBS patients using barostat programmed for phasic ascending distension. Hypersensitive IBS patients were defined as having a threshold for discomfort and pain lower than minimal distending pressure + 32 mmHg. (311;348) IBS patients showed lower TPH-1 and SERT mRNA levels in the descending colon and rectum compared to controls. The twelve rectal hypersensitive IBS patients showed lower TPH-1 mRNA level in the colorectal region compared to controls. In addition we found that SERT expression was lower in descending colon and rectum of rectal hypersensitive IBS patients compared to controls. Mucosal 5-HT content was higher in rectal hypersensitive IBS patients compared to controls. Furthermore, rectal substance P content was increased in IBS patients compared to controls. No differences were found in PAR-2, trypsinogen IV, TPH-1 and SERT transcript levels, EC cells, mast cells and intraepithelial lymphocytes cell counts and substance P, 5-HT and 5-HIAA content measurements between rectal hypersensitive and normosensitive IBS patients.

This study indicates that the abnormalities in colorectal components of serine protease and serotonergic signalling show no differences between rectal hypersensitive and normosensitive IBS patients. However, irrespective of visceral hypersensitivity state, the serotonergic signalling components are altered in IBS patients and may play a role in the IBS symptom generation. Conflicting results in the serotonergic signalling components between chapter 8 and chapter 7 could be due to the inclusion of a different group of IBS patients. Theoretically increased SERT levels in the duodenum (chapter 7) in contrast to decreased SERT levels in the colorectal region (chapter 8) might

be due to different numbers of patients included with visceral hypersensitivity or low grade inflammation. However as shown in this study the differences found in serotonergic signalling were irrespective of the visceral hypersensitivity state and furthermore we couldn't show differences in low grade inflammation between IBS patients and controls. As previously suggested in chapters 4 and 5 other factors such as microbiota composition might play a role in the pathogenesis of IBS. Differences in serotonin signalling components have been shown in post-infectious IBS patients however data on serotonin components and microbiota composition in non-post-infectious IBS patients are lacking.(374)

As mentioned previously, visceral hypersensitivity in IBS patients might be due to the sensitization of sensory neural endings in the intestine by low-grade inflammation. Increased numbers of mast cells and mast cell activation are associated with disease activity in IBS patients.(10) In IBS patients more mast cells are in close proximity to mucosal nerve fibers than in controls. Mast cell tryptase leads to long-lasting neuronal hyperexcitability due to protease activated receptor (PAR)-2 activation on visceral afferents by mast cell tryptase. In **chapter 9** we investigated in a preliminary study the role of PAR-2 in the mast cell-nerve interactions in IBS patients and controls using immunohistochemistry. In order to study this, PAR-2 positive neurons and mast cells in the submucosa of full-thickness jejunal biopsy specimens of 16 IBS patients and 9 controls were counted. Furthermore the number of mast cells in vicinity of neurons was measured at different distances up to 30 μm from the neuron. This study indicates that counts of submucosal neurons, PAR-2 positive neurons and mast cells are not significantly different between IBS patients and controls. Mast cells are observed in the vicinity of the submucosal neurons in both controls and IBS patients. Positive immunostained neurons are strongly correlated with mast cells in close vicinity of the neuron. Significantly more mast cells are seen in the vicinity of PAR-2 positive neurons than PAR-2 negative neurons. This cross talk might be important in generating visceral sensitivity in both IBS patients and controls. Close proximity between mast cells and enteric nerve fibers might lead to bidirectional communication.(364) A relevant aspect is the ability of mast cells to evoke activation of nociceptive neural pathways which correlates with abdominal pain, at least in patients with IBS.(10;375) Tryptase, activating PAR-2, may be a physiological agonist in cells that are in close proximity to mast cells and which could be exposed to high concentrations of this enzyme, such as sensory nerves.(326;342;356;358)

PAR-2 activation increases signalling at the spinal cord level which leads to amplification and persistence of pain sensation.(366) However the strong spatial correlation between mast cells and PAR-2 positive neurons is shown in both controls and IBS patients which suggest other pathophysiological mechanisms for visceral hypersensitivity in IBS patients.

This thesis showed that the pathophysiology of visceral hypersensitivity of Irritable Bowel Syndrome is multi-factorial including microbiota, intestinal permeability and serotonin and serine protease signalling. In conclusion much work has yet to be done to define whether microbiota composition, intestinal permeability, serine protease and serotonergic signalling yield pathogenetic, diagnostic and therapeutic value for IBS patients.

Answers to questions addressed in this thesis

(1) What are the current views on invasive and non-invasive sampling techniques to collect information about microbiota in the small intestine?

Investigating the microbiota composition of the small intestine is still challenging. Using upper gastrointestinal endoscopy the sampling area is limited to the duodenum and upper part of the jejunum. Important during sampling of the small intestine is to reduce the risk of contamination with microbiota from the proximal habitats. Furthermore during the endoscopy insufflation with nitrogen is necessary if the analysis of the microbiota on biopsies and brush samples is done using culturing techniques. Besides mucosa-associated bacteria, which can be investigated using brush and biopsy samples, luminal microbiota are also sampled to investigate the microbiota composition. The luminal microbiota are investigated in aspirates which can be gained using endoscopy or intestinal intubation. Techniques such as preoperative needle aspiration, self opening capsules and string tests are not used routinely to access the small intestine. Besides invasive sampling techniques non-invasive breath testing techniques are used to determine small bowel bacterial overgrowth. However the use of non-invasive methods seems to be limited in clinical practice.

(2) Are lactulose breath test, culture and molecular based methods useful in diagnosing bacterial overgrowth?

The lactulose breath test has been used as "gold standard" in a number of studies. In this thesis it has been shown that the lactulose breath test is unreliable in detecting small bowel bacterial overgrowth. A major drawback of the lactulose breath test might be rapid transit of lactulose to the colon. Limiting lactulose to the small intestine using an occlusive balloon did not improve the ability of the lactulose breath test to diagnose bacterial overgrowth. Ideally, the diagnosis of the bacterial overgrowth syndrome is the demonstration of increased levels of bacteria in jejunal aspirate. Culturing of jejunal aspirates seems to be more specific in diagnosing small bowel bacterial overgrowth than lactulose breath test since less false positive results in controls has been observed. The use of molecular based methods should be further analysed but did not show differences in our study.

(3) What is the difference in composition of both faecal and duodenal mucosa-associated microbiota in IBS patients and healthy subjects?

Composition of gastrointestinal microbiota is known to be relatively stable. The presence of the beneficial microbes in the intestine prevents colonization by potential pathogenic microbes, referred to as colonization resistance. Imbalances in the microbiota are characterized by a decrease in beneficial anaerobic bacteria and increases in aerobic bacteria, fungi and harmful anaerobic bacteria. Both faecal and duodenal mucosa-associated microbiota of IBS patients show a decrease in beneficial *Bifidobacteria catenulatum* and an increase in potentially harmful *Pseudomonas aeruginosa*.

(4) Do NSAIDs increase intestinal permeability in IBS patients more than in healthy subjects?

The capacity of the intestinal barrier to cope with luminal aggression of NSAIDs is more limited in IBS than in healthy subjects. Since intestinal permeability reflects the functional status of the intestinal barrier, the present findings support the idea that IBS is associated with as yet unknown changes in the physiology of the intestinal barrier, particularly expressed when triggered by action of noxious agents.

(5) What is the role of altered mucosal serine protease and serotonergic signalling in small and large intestine of IBS patients and are they related to rectal visceral hypersensitivity?

In the small intestine enhanced mRNA expression of trypsinogen IV and SERT and a higher 5-HT content of IBS patients compared to healthy subjects have been revealed. Higher levels of trypsinogen IV in the small intestine may lead to increased PAR-2 activation in IBS patients compared to healthy subjects and enhance visceral sensitivity. We hypothesized that the higher mucosal 5-HT content may result from a higher EC cell density or enhanced release. The increased SERT expression supports the hypothesis of an enhanced release, as the functional role of SERT is to prevent excessive accumulation of free and active 5-HT which would result in potentiation of serotonergic signalling and eventually receptor desensitization. These abnormalities in the serine protease and 5-HT signalling systems may contribute to small intestinal visceral hypersensitivity in IBS patients.
In the large intestine mRNA expression of TPH-1 and SERT in rectum of both rectal hypersensitive and normosensitive IBS patients is reduced compared to controls and may play a role in IBS symptom generation. The abnormalities in colorectal components of serine protease and serotonergic signalling showed no differences between rectal hypersensitive and normosensitive IBS patients.

(6) What is the role of PAR-2 in the submucosal mast cell-nerve interactions in the small intestine of IBS patients?

Strong spatial correlation between mast cells in the vicinity of neurons that were PAR-2 positive in both controls and IBS patients has been found. Close proximity between mast cells and enteric nerve fibers might lead to bidirectional communication. This cross talk might be important in modulating visceral sensitivity in both IBS patients and controls since mast cells are able to evoke activation of nociceptive neural pathways which correlates with abdominal pain. Tryptase, activating PAR-2, may be a physiological agonist in cells that are in close proximity to mast cells and which could be exposed to high concentrations of this enzyme, such as sensory nerves.(326;342;356;358) PAR-2 activation increases signalling at the spinal cord level which leads to amplification and persistence of pain sensation.(366)

Algemene discussie en samenvatting

Het prikkelbare darmsyndroom (PDS) is een functionele ziekte waarbij pijn in de buik of een onbehaaglijk gevoel in de buik wordt geassocieerd met een veranderd ontlastingspatroon. Vele pathofysiologische verklaringen zijn voorgesteld voor PDS; echter geen uniek mechanisme is geïdentificeerd. Het is waarschijnlijk een multifactoriële ziekte. In dit proefschrift hebben we ons gericht op verschillende perifere factoren die viscerale overgevoeligheid in PDS beïnvloeden. In de inleiding (**hoofdstuk 1**) wordt de rol van de intestinale microbiota, de darmdoorlaatbaarheid, serine protease en serotonerge signaal transductie op de pathofysiologie van viscerale overgevoeligheid in PDS beschreven. Verder worden in de inleiding de doelstellingen van dit proefschrift gepresenteerd, die hebben geleid tot de volgende hoofdstukken.

In **hoofdstuk 2 tot 5** worden het verzamelen van de microbiota in het maagdarmkanaal en het vaststellen van veranderingen in de samenstelling van de microbiota in de darm beschreven.

Het monsters nemen en onderzoeken van de gastrointestinale microbiota, vooral in het geval van mensen, is vaak beperkt tot fecaal materiaal. In **hoofdstuk 2** wordt een verscheidenheid van invasieve verzameltechnieken om tot het volledige menselijke maagdarmkanaal toegang te hebben besproken, evenals niet-invasieve verzameltechnieken die in de medische praktijk worden gebruikt. Dit is van belang aangezien feces slechts de luminale microbiota van het terminale colon vertegenwoordigen en daarom ontoereikende informatie over andere, meer proximaal gelegen delen van het darmkanaal zullen verstrekken. Voorts worden de mucosa-geassocieerde bacteriën verondersteld een sterkere interactie met de gastheer te hebben dan luminale bacteriën.(57)
Zowel invasieve als niet-invasieve verzameltechnieken worden gebruikt om het klinische dunne darm bacteriële overgroei (BO) syndroom te diagnosticeren. Steatorrhea, diarree, flatulentie, buikpijn en een opgeblazen gevoel kenmerken BO. BO wordt geassocieerd met de aanwezigheid van colon bacteriën in de dunne darm. In **hoofdstuk 3** wordt het stellen van de diagnose BO met behulp van experimentele en standaard ademtesten (AT) en op

kweek- en moleculaire technieken gebaseerde methoden geëvalueerd. Het kweken van jejunum aspiraat wordt beschouwd als de gouden standaard, maar is onpraktisch en diagnosticeert niet de individuen met geïsoleerde distale BO.(155) Daarom worden niet-invasieve testen zoals ademtesten (AT) gebruikt. Een belangrijk nadeel van de lactulose AT is het vals positief testen door snelle passage van de lactulose naar het colon.(170) Om deze snelle passage te voorkomen hebben we tijdens de experimentele AT met behulp van een occlusieve ballon de passage van de lactulose beperkt tot de dunne darm. We analyseerden 11 controles en 15 personen waarbij de diagnose BO erg waarschijnlijk was. De groep BO proefpersonen bestond uit 10 PDS patiënten en 5 patiënten die klinisch verdacht waren voor BO. Jejunale vloeistof werd geanalyseerd met behulp van kweek en kwantitatieve PCR. Gebruikmakend van conventionele kweekmethoden werd op basis van meer dan 10^3 CFU/ml met exclusie van de lactobacillen en streptococcen, in 0 van de 9 controles en in 4 van de 12 personen met BO, BO gediagnosticeerd. Bacteriële overgroei, gebruikmakend van de kweektechnieken, werd ook gediagnosticeerd op basis van alle eerder gepubliceerde definities en ook dit toonde geen significante verschillen aan tussen controles en BO personen. Voorts toonde het totale aantal bacteriën in het aspitaat geen significante verschillen aan tussen controles en BO, gebruikmakend van zowel kweek- als moleculaire technieken. Met behulp van de standaard ademtest werd bacteriële overgroei gediagnosticeerd op basis van een toename van meer dan 20 ppm waterstof of methaan boven de uitgangswaarde binnen 90 minuten, in 3 van de 11 controles en 8 van de 15 BO personen. Er werd geen verandering aangetoond in het begin van de toename van waterstof excretie in zowel de experimentele als de standaard AT tussen controles en BO. Deze bevindingen wijzen erop dat in deze kleine onderzochte groep de experimentele AT niet de gevoeligheid van de lactulose AT om BO te diagnosticeren verbetert. Interessant is dat kweken minder positieve diagnoses van BO tonen in de controles in vergelijking met de standaard AT. Opmerkelijk is dat de huidige diagnostische criteria niet nauwkeurig genoeg zijn om BO patiënten van controles te kunnen onderscheiden. Onlangs toonden Simren *et al*. en Khoshini *et al*. ook aan dat de definities van normale en abnormale resultaten van ademtesten en kweken om BO aan te tonen variabel zijn.(178;367) Verder is het gebruiken van lactulose ademtesten niet aan te bevelen in de evaluatie van patiënten met functionele maagdarmziekten.(178) Schiller *et al*. concluderen dan ook dat de perfecte test voor BO nog steeds ontworpen moet worden.(368) Khoshini *et al*. adviseren een combinatie van testen voor BO (zoals

behandelen met antibiotica en symptomatisch het resultaat beoordelen) aangezien het nauwkeurig bepalen van BO beperkt is.(367) Concluderend, een gouden standaard voor het diagnosticeren van BO ontbreekt, hetgeen het stellen van een klinische diagnose in een individuele patiënt zeer onbetrouwbaar maakt. In toekomstige studies zullen nieuwe technieken zoals de moleculaire technieken geëvalueerd moeten worden.

Net zoals het diagnosticeren van BO is het onderzoek naar de samenstelling van de intestinale microbiota beperkt tot conventionele kweekmethoden. De meeste bacteriën kunnen niet geïsoleerd worden door middel van kweektechnieken omdat het in vitro moeilijk is om hun niches in de darm na te bootsen. Dit is recent omzeild met de komst van moleculair-gebaseerde technieken. In **hoofdstuk 4** bepaalden wij de samenstelling van de totale microbiota, met name die van de bifidobacteriën in zowel fecale als duodenale mucosa-geassocieerde samples van 41 PDS patiënten en 26 gezonde controles met hulp van fluorescent in situ hybridization (FISH) en real time PCR. De FISH analyse van de fecale monsters toonde een halvering aan in het aantal bifidobacteriën in PDS patiënten ten opzichte van gezonde controles terwijl geen belangrijke verschillen in andere bacterie groepen werden waargenomen. Op het species niveau was *Bifidobacterium catenulatum* beduidend lager in PDS patiënten ten opzichte van de gezonde controles, zowel in fecale als duodenale brush samples. De veranderingen in de microbiota samenstelling kunnen een belangrijke rol spelen in de PDS pathofysiologie aangezien microbiota de structuur en functie van het maagdarmkanaal beïnvloeden.(57) Een belangrijk obstakel bij het in verband brengen van de microbiële samenstelling met de functie van de microbiële samenstelling is het feit dat ieder individu zijn eigen specifieke microbiële samenstelling heeft die gevormd en in stand gehouden wordt door zowel de gastheer als omgevingsfactoren.(159) Daarom is het in toekomstige studies noodzakelijk te bepalen of de afname van bifidobacteriën en met name de *Bifidobacterium catenulatum* een oorzakelijk verband heeft met de PDS symptomen. Door middel van het evalueren van de symptomen voor en na het herstellen van het totaal aantal bifidobacteriën en met name de *Bifidobacterium catenulatum* valt dit verband te bestuderen.

In **hoofdstuk 5** bepaalden we de mucosa-geassocieerde dunne darm en fecale microbiota van 37 PDS patiënten en 20 gezonde controles door middel van PCR denaturing gradient gel electrophoresis (DGGE) en Q-PCR analyse. Bacterieel 16SrRNA werd geamplificeerd en geanalyseerd en een gemiddeld

DGGE profiel van alle PDS patiënten en alle gezonde controles werd geconstrueerd, gebruikmakend van GelCompar II software, waarna de fingerprints van beide groepen werden vergeleken. PDS patiënten en gezonde controles hebben voor 78.2% van de dunne darm samples en 86.25% van de fecale samples identieke DGGE profielen. DGGE banden, die tot één groep beperkt zijn (PDS of gezonde controle), werden uit de gel gesneden en daarvan werd een sequence analyse gemaakt. De klonen van de PDS dunne darm en fecale banden werden hoofdzakelijk geïdentificeerd als *Pseudomonas* species, waarbij *Pseudomonas aeruginosa* de meest voorkomende species was. Q-PCR analyse werd gebruikt om de percentages van *P. aeruginosa* ten opzichte van de totale bacteriële hoeveelheid te kwantificeren in de dunne darm mucosa-geassocieerde samples en fecale samples. Dit toonde beduidend hogere *P. aeruginosa* percentages in PDS patiënten ten opzichte van de gezonde controles. De rol van *P. aeruginosa* in de pathofysiologie van viscerale hypersensitiviteit is onbekend. Het is echter bekend dat *P. aeruginosa* de protease geactiveerde receptor (PAR)-2 in de luchtwegen beïnvloedt.(264) Toekomstige studies zouden het effect van de *P. aeruginosa* proteases in het maagdarmkanaal moeten evalueren. Aanvullend aan hoofdstuk 4 toonden we veranderingen aan in de bacteriële samenstelling in zowel de fecale als dunne darm mucosa geassocieerde samples die een rol kunnen spelen in de pathofysiologie van PDS. Kassinen *et al*. en Malinen *et al*. toonden ook aan dat de microbiële samenstelling in fecale samples van PDS patiënten significant veranderd is.(24;26) Nochtans resteert de vraag uit onze en hun studies of de verandering in bacteriële samenstelling de oorzaak dan wel het gevolg zijn van obstipatie of diarree. Studies die het effect op PDS symptomen evalueren waarbij antibiotica of probiotica gebruikt worden om *P. aeruginosa* te elimineren zijn noodzakelijk. Verder moeten naast gezonde controles ook klinische controles van patiënten met diarree of obstipatie zonder PDS worden gebruikt. Bovendien moet de stabiliteit van de bacteriële verandering aangetoond worden omdat sommige PDS patiënten in de loop van de tijd kunnen veranderen van diarree predominant naar obstipatie predominant en vice versa.(369)

Naast bacteriën kan een verhoogde darmdoorlaatbaarheid een belangrijke rol spelen in de pathofysiologie van PDS, omdat het de mucosa blootstelt aan luminale antigenen afkomstig van voedingsstoffen of bacteriën die de mucosale immuniteit kunnen activeren.(370) In **hoofdstuk 6** onderzochten we de darmdoorlaatbaarheid en het effect van een relatieve lage dosering NSAIDs

(naproxen 750 mg/dag gedurende twee dagen) op de darmdoorlaatbaarheid in 14 PDS patiënten en 15 gezonde controles. NSAIDs verhogen de darmdoorlaatbaarheid wat kan leiden tot laaggradige ontstekingen hetgeen weer bij kan dragen aan een toename in viscerale hypersensitiviteit.(281;282) De darmdoorlaatbaarheid werd onderzocht voor en na 2 dagen NSAID inname. Indicatoren voor de doorlaatbaarheid zijn oraal toegediende en in urine gemeten polyethyleenglycolen (PEGs) met een moleculaire massa van 400, 1500, 4000 en 10000 en de lactulose mannitol test. Er zijn geen significante verschillen gevonden in de 24 uurs terugwinning van PEGs 400, 1500 en 4000 tussen gezonde controles en PDS patiënten voor en na de NSAID inname. Lactulose mannitol ratios toonden ook geen significante verschillen tussen PDS patiënten en gezonde controles. Echter de PEG excretie, vastgesteld in 2-uurs intervallen, en de doorlaatbaarheid geëvalueerd als oppervlakte onder de PEG excretie-tijd-curve, tonen dat 2 dagen NSAID toediening de darmdoorlaatbaarheid van PEG 4000 in gezonde controles verhoogde alsook de doorlaatbaarheid van PEGs 400, 1500 en 4000 in de PDS patiënten. Deze resultaten suggereren dat PDS een toegenomen reactie op schadelijke stoffen in de fysiologie van de darmbarrière vertoont. Of de bacteriële samenstelling, besproken in hoofdstukken 3 tot en met 5, al dan niet een rol speelt in deze veranderde reactie en de interactie tussen luminale bacteriën en epitheliale receptoren die mucosale integriteit beïnvloeden in PDS patiënten moet onderzocht worden. Het is al bekend dat bacteriën, zoals *Clostridia perfringens* en *Vibrio cholerae*, de darmdoorlaatbaarheid kunnen vergroten.(371) Verder hebben postinfectieuze PDS patiënten een verhoogde darmdoorlaatbaarheid.(33) Het gebruik van probiotica in patiënten met allergische dermatitis vermindert de huidziekte maar vermindert ook de toegenomen darmdoorlaatbaarheid.(372) Toekomstige therapeutische mogelijkheden om de darmdoorlaatbaarheidsmechanismen te beïnvloeden, zoals het herstellen van de bacteriële samenstelling, kunnen ons in staat stellen om te bepalen of toegenomen darmdoorlaatbaarheid betrokken is bij het ontstaan van PDS symptomen.

In **hoofdstuk 7**, **hoofdstuk 8** en **hoofdstuk 9** worden de rol van serine protease en serotonerge signaaltransductie componenten in PDS patiënten bestudeerd.
Colorectale en dunne darm viscerale hypersensitiviteit is aangetoond in PDS. Serine protease signalering via protease geactiveerde receptor (PAR)-2 bevordert hyperalgesie door mechanische distensie. Verder zijn ook

serotonerge componenten betrokken in de gastrointestinale viscerale gevoeligheid. Veranderingen in serine protease en serotonerge signaaltransductie componenten zijn geïdentificeerd in mucosale colorectale biopten van PDS patiënten.(43;47;373) In **hoofdstuk 7** hadden we als doelstelling de rol van de veranderde mucosale serine protease en serotonerge signaaltransductie componenten in de dunne darm van PDS patiënten te bepalen. De mRNA expressie van PAR-2, trypsinogen IV, TPH-1, SERT en 5-HT_3 subunits werd gekwantificeerd door middel van real time PCR en de hoeveelheid serotonine werd gemeten door middel van ELISA in mucosale duodenum biopten van 34 PDS patiënten (10 obstipatie predominante, 11 diarree predominante en 13 alternerende) en 20 gezonde controles. De PDS patiënten toonden een 1,5 maal hoger trypsinogen IV mRNA niveau ten opzichte van de gezonde controles. SERT expressie was 1,8 maal hoger in PDS patiënten ten opzichte van de gezonde controles. De mucosale serotonine hoeveelheid was 2,1 maal hoger in obstipatie predominante PDS patiënten ten opzichte van de gezonde controles. Transcriptie levels van PAR-2, TPH-1 en 5-HT_3 receptor subunits verschilden niet tussen PDS patiënten en gezonde controles. Verhoogde trypsinogen IV expressie in PDS kan een toegenomen PAR-2 activiteit veroorzaken. In toekomstige studies moet onderzocht worden of de verhoogde trypsinogen IV expressiewaardes kunnen resulteren in protease concentraties die PAR-2 kunnen activeren. Verder zal de ontwikkeling van een PAR-2 antagonist of trypsinogen IV inhibitor voor humaan gebruik het belang van PAR in de PDS pathofysiologie en symptoom ontwikkeling kunnen verhelderen. De toegenomen SERT expressie en mucosale serotonine inhoud in PDS suggereren een verhoogde serotonine beschikbaarheid. Zowel de verhoogde trypsinogen IV en SERT expressie kunnen bijdragen aan de dunne darm viscerale overgevoeligheid in PDS patiënten. In **hoofdstuk 8** stelden wij ons ten doel de vraag te beantwoorden of de veranderde mucosale serine protease en serotonerge signaaltransductie componenten al dan niet gerelateerd zijn aan rectale viscerale hypersensitiviteit in PDS patiënten. Om dit te bestuderen verzamelden we colorectale mucosale biopten van 23 PDS patiënten en 15 controles. De mRNA expressie van PAR-2, trypsinogen IV, TPH-1 en SERT werd gekwantificeerd met behulp van real-time PCR. De hoeveelheid substance P, 5-HT en 5-HIAA werd gemeten met behulp van ELISA. Enterochromafin cellen (EC), mestcellen en intraepitheliale lymfocyten werden immunohistochemisch bestudeerd. Rectale viscerale hypersensitiviteit werd gemeten in 19 PDS patiënten met behulp van een barostat, geprogrammeerd voor geleidelijk toenemende distensies. Hypersensitieve PDS patiënten

werden gedefinieerd als het hebben van een barostat intraluminale drukwaarde voor ongemak of pijn lager dan de minimale distensie druk plus 32 mmHg. (311;348) De PDS patiënten toonden lagere TPH-1 en SERT mRNA niveaus in het colon descendens en het rectum in vergelijking met de controles. De 12 rectale hypersensitieve PDS patiënten toonden lagere TPH-1 mRNA niveaus in de colorectale regio ten opzichte van de controles. Daarnaast vonden wij dat de expressie van SERT lager was in het colon descendens en het rectum van rectale hypersensitieve PDS patiënten ten opzichte van controles. De mucosale 5-HT hoeveelheid was hoger in de rectale hypersensitieve PDS patiënten ten opzichte van de controles. Voorts was de rectale substance P hoeveelheid toegenomen in PDS patiënten ten opzichte van de controles. Er werden geen verschillen in de PAR-2, trypsinogen IV, TPH-1 en SERT transcriptie niveaus, EC cellen, mestcellen en intraepitheliale lymfocyten celgetallen en de substance P, 5-HT en 5-HIAA gehaltes aangetoond tussen rectale hypersensitieve en normosensitieve PDS patiënten. Deze studie wijst erop dat afwijkingen in de colorectale componenten van de serine protease en serotonerge signalering geen verschillen aantonen tussen rectale hypersensitieve en normosensitieve PDS patiënten. Echter, onafhankelijk van het aanwezig zijn van viscerale hypersensitiviteit, zijn de serotonerge signaleringscomponenten in de PDS patiënten veranderd en kunnen een rol spelen in het ontstaan van de PDS symptomen.
De strijdige resultaten in de serotonerge signaleringscomponenten tussen hoofdstuk 8 en hoofdstuk 7 zou kunnen worden toegeschreven aan een verschil in de inclusie van de PDS patiënten. Theoretisch kunnen de toegenomen SERT niveaus in het duodenum (hoofdstuk 7) in vergelijking tot de afgenomen SERT niveaus in de colorectale regio (hoofdstuk 8) veroorzaakt worden door inclusie van een groter aantal patiënten met viscerale hypersensitiviteit of met laaggradige ontsteking. Echter, zoals aangetoond in deze studie waren de serotonerge signaleringsverschillen onafhankelijk van de viscerale hypersensitiviteitstoestand en voorts konden we geen verschillen aantonen in laaggradige ontsteking tussen PDS patiënten en controles. Zoals eerder voorgesteld in hoofdstuk 4 en 5 kunnen andere factoren, zoals bacteriën, een rol spelen in de pathogenese van PDS. Verschillen in de serotonerge signaleringscomponenten zijn aangetoond in postinfectieuze PDS patiënten maar gegevens over serotonine componenten en bacteriële samenstelling in niet postinfectieuze PDS patiënten ontbreken.(374)

Zoals eerder vermeld kan viscerale hypersensitiviteit in PDS patiënten het gevolg zijn van het sensibiliseren van sensorische zenuwuiteinden in de darm door laaggradige ontstekingen. Toegenomen mestcel aantallen en toegenomen mestcel activering zijn geassocieerd met de ernst van de klachten van de PDS patiënten.(10) Bij PDS patiënten liggen meer mestcellen in de nabijheid van mucosale zenuwvezels dan bij controles. Mestcel tryptase leidt tot een langdurige lagere prikkeldrempel van de zenuw door activering van de protease activated receptor 2 op viscerale afferente zenuwen. In **hoofdstuk 9** onderzochten we de rol van protease activated receptor (PAR)-2 bij de mestcel-zenuw interactie in PDS patiënten en controles, gebruikmakend van immunohistochemie. Om dit te bestuderen werden de PAR-2 positieve neuronen en mestcellen in de submucosa van full thickness jejunum biopten van 16 PDS patiënten en 9 controles geteld. Voorts werd het aantal mestcellen in de nabijheid van neuronen geteld op verschillende afstanden tot een afstand van 30 µm van het neuron. Deze studie toont aan dat het aantal submucosale neuronen, PAR-2 positieve neuronen en mestcellen niet significant verschilt tussen PDS patiënten en controles. Mestcellen werden geobserveerd in de nabijheid van de submucosale neuronen in zowel controles als PDS patiënten. PAR-2 positieve neuronen zijn sterk gecorreleerd met mestcellen in de nabijheid van een neuron. Significant meer mestcellen werden gezien in de nabijheid van PAR-2 positieve neuronen ten opzichte van PAR-2 negatieve neuronen. Dit kan belangrijk zijn in het ontstaan van viscerale hypersensitiviteit in zowel PDS patiënten als controles. Nabijheid van mestcellen bij zenuwvezels en neuronen van de darm kan leiden tot communicatie van mestcel naar zenuwvezel en vice versa.(364) Relevant is de mogelijkheid van mestcellen om pijntrajecten te activeren wat correleert, in ieder geval bij PDS patiënten, met buikpijn.(10;375) Tryptase, dat PAR-2 activeert, kan een fysiologische agonist zijn voor cellen, zoals sensorische zenuwen die in de nabijheid van mestcellen liggen en die blootgesteld kunnen worden aan hoge concentraties van dit enzym.(326;342;356;358) Activering van PAR-2 laat de signalering op ruggenmerg niveau toenemen, hetgeen leidt tot toename en aanhouden van de pijnsensatie.(366) Echter de sterke ruimtelijke correlatie tussen mestcellen en PAR-2 positieve neuronen werd aangetoond in zowel controles als PDS patiënten hetgeen andere pathofysiologische mechanismen voor viscerale hypersensitiviteit in PDS suggereert.

Dit proefschrift toont aan dat de pathofysiologie van viscerale hypersensitiviteit in PDS multifactorieel is waarbij bacteriën, darmdoorlaatbaarheid en serotonerge en serine protease signalering een rol spelen. Wij concluderen dat er veel meer werk verricht moet worden om aan te tonen of bacteriële samenstelling, darmdoorlaatbaarheid, serine protease en serotonerge signalering leiden tot pathogenese, diagnostische en therapeutische waarde voor PDS patiënten.

Antwoorden op de vragen die in de introductie van dit proefschrift gesteld werden:

(1) Wat zijn de huidige meningen over de invasieve en niet-invasieve verzameltechnieken om informatie te verkrijgen over de bacteriële samenstelling van de dunne darm?

Het onderzoeken van de bacteriële samenstelling van de dunne darm is nog steeds uitdagend. Gebruikmakend van gastroduodenoscopie blijft het te onderzoeken gebied beperkt tot het duodenum en het bovenste deel van het jejunum. Het is van belang om tijdens het nemen van monsters in de dunne darm het risico op vervuiling met bacteriën van meer proximaal gelegen gebieden te verminderen. Voorts is tijdens endoscopie het opblazen van de darm met stikstof noodzakelijk, indien de analyse van de bacteriën op biopten en in borstelmonsters met behulp van kweektechnieken wordt gedaan. Naast mucosa-geassocieerde bacteriën, onderzocht met borstel- en bioptmonsters, worden ook luminale bacteriën onderzocht om de bacteriële samenstelling te bepalen. De luminale bacteriën worden onderzocht in aspiraten die verkregen worden met behulp van endoscopie of intestinale intubatie. Technieken zoals peroperatieve aspiratie, zelfopenende capsules en touwtesten worden niet routinematig gebruikt om de dunne darm te onderzoeken. Naast invasieve monstermethodes worden niet-invasieve verzamelmethodes, zoals ademtesten, gebruikt om dunne darm bacteriële overgroei aan te tonen. Echter het gebruik van niet invasieve methodes lijkt klinisch beperkt te zijn.

(2) Zijn lactulose ademtesten, kweek- en moleculair gebaseerde methodes bruikbaar in het diagnosticeren van bacteriële overgroei?

De lactulose ademtest is gebruikt als " gouden standaard" in een aantal studies. In dit proefschrift is aangetoond dat de lactulose ademtest onbetrouwbaar is om dunne darm bacteriële overgroei aan te tonen. Een groot nadeel van deze lactulose ademtest kan het snelle transport van lactulose naar het colon zijn. Lactulose beperken tot de dunne darm met behulp van een afsluitende ballon verbeterde de mogelijkheid van de lactulose ademtest om bacteriële overgroei aan te tonen niet. De meest optimale methode om bacteriële overgroei te diagnosticeren is het aantonen van toegenomen aantallen bacteriën in het jejunum aspiraat. Het kweken van jejunum aspiraat lijkt meer specifiek in het aantonen van dunne darm bacteriële overgroei dan de lactulose ademtest aangezien er minder vals-positieve resultaten in de controles werden gevonden. Het gebruik van moleculair gebaseerde technieken moet nog verder worden onderzocht, maar toonde in onze studie geen verschillen.

(3) Wat is het verschil in de samenstelling van fecale en duodenale mucosa-geassocieerde bacteriën van PDS patiënten en gezonden?

De samenstelling van de maagdarm bacteriën is relatief stabiel. De aanwezigheid van goedaardige bacteriën in de darm voorkomt kolonisatie door potentiële ziekteverwekkende bacteriën. Dysbalans in de bacteriële samenstelling wordt gekenmerkt door een afname van goedaardige anaërobe bacteriën en toename van aërobe bacteriën, schimmels en schadelijke anaërobe bacteriën. Zowel fecale als duodenale mucosa-geassocieerde bacteriën van PDS patiënten tonen een afname aan van goedaardige *Bifidobacterium catenulatum* en een toename van potentieel schadelijke *Pseudomonas aeruginosa* bacteriën.

(4) Verhogen NSAIDs darmdoorlaatbaarheid meer in PDS patiënten dan in gezonden?

De capaciteit van de intestinale barrière om het hoofd te bieden aan de luminale invloed van NSAIDs is beperkter bij PDS dan in gezonden. Aangezien darmdoorlaatbaarheid de functionele status van de intestinale barrière reflecteert, steunen de huidige bevindingen het idee dat PDS wordt

geassocieerd met tot nu toe nog onbekende veranderingen in de fysiologie van de intestinale barrière, voornamelijk tot uitdrukking gebracht door de verhoogde gevoeligheid voor schadelijke stoffen zoals NSAIDs.

(5) Wat is de rol van de veranderde serine protease en serotonerge signalering op dunne en dikke darm van PDS patiënten en zijn ze gerelateerd aan rectale viscerale hypersensitiviteit?

In de dunne darm toonden we een toegenomen mRNA expressie van trypsinogen IV en SERT en hogere 5-HT waardes in PDS patiënten ten opzichte van controles aan. Meer trypsinogen IV in de dunne darm kan leiden tot toegenomen PAR-2 activering in PDS patiënten ten opzichte van controles en tot toegenomen viscerale sensitiviteit. We stelden een hypothese op dat hogere mucosale 5-HT niveaus mogelijk het resultaat zijn van een hogere EC cel dichtheid of van toegenomen afgifte van 5-HT. De toegenomen expressie van SERT steunt de hypothese van een toegenomen afgifte, aangezien de functionele rol van SERT het voorkomen van een overvloedige ophoping van vrij en actief 5-HT is, hetgeen zou leiden tot versterking van de serotonine signalering en het ongevoelig worden van de receptor. Deze afwijkingen in het serine protease en serotonerge signaleringssysteem kunnen bijdragen aan dunne darm viscerale hypersensitiviteit in PDS patiënten.
In de dikke darm is de mRNA expressie van TPH-1 en SERT in het rectum van zowel rectale hypersensitieve als normosensitieve PDS patiënten lager ten opzichte van controles en speelt mogelijk een rol in het ontstaan van PDS symptomen. De afwijkingen in de colorectale componenten van serine protease en serotonerge signalering toonden geen verschillen tussen rectale hypersensitieve en normosensitieve PDS patiënten.

(6) Wat is de rol van PAR-2 in de submucosale mestcel-zenuw interactie in de dunne darm van PDS patiënten?

Sterke ruimtelijke correlatie tussen mestcellen en PAR-2 positieve neuronen werd aangetoond in zowel controles als PDS patiënten. Nabijheid van mestcellen bij darmzenuwvezels kan leiden tot communicatie in beide richtingen tussen mestcellen en zenuwvezels. Deze communicatie kan van belang zijn in het moduleren van viscerale sensitiviteit in zowel PDS patiënten en controles aangezien mestcellen pijnpaden kunnen activeren correlerend met buikpijn. Tryptase dat PAR-2 activeert kan een fysiologische agonist zijn

voor cellen, zoals zenuwcellen, die in de nabijheid liggen van mestcellen waardoor ze blootgesteld kunnen worden aan hoge concentraties van dit enzym.(326;342;356;358) PAR-2 activering doet de signalering op ruggenmerg-niveau toenemen, hetgeen leidt tot versterking en aanhouden van de pijnsensatie.(366)

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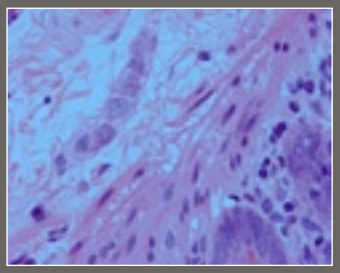
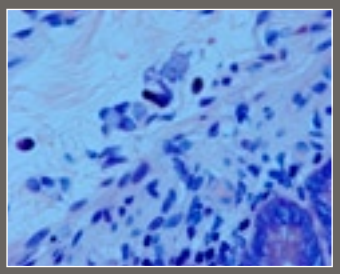
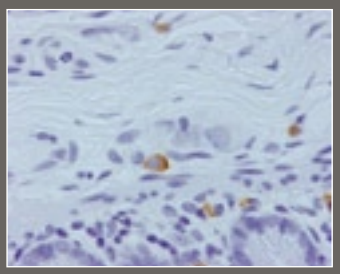
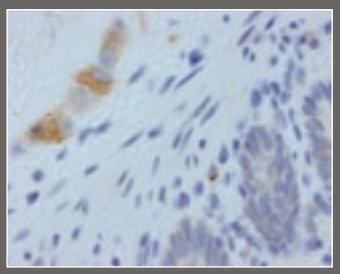
Curriculum vitae

Angeline Pauline Marcelle (Angèle) Kerckhoffs werd geboren op 24 oktober 1976 in Geleen. Na het behalen van het VWO diploma aan het Bisschoppelijke College te Sittard heeft zij 2 jaar geneeskunde gestudeerd aan de Katholieke Universiteit te Leuven, België alvorens zij begon aan de studie geneeskunde aan de Universiteit Maastricht in 1998. Zij behaalde het basisartsexamen in 2001. Tijdens de laatste jaren van haar studie verrichte zij onderzoek naar "hyperlipidemie na levertransplantatie" en in het kader van een wetenschapsstage " PPAR γ: a modifier gene in familial combined hyperlipidemia" in het laboratorium Moleculaire Metabolisme en Endocrinologie van de Universiteit Maastricht onder begeleiding van Prof. T.W.A. de Bruin. In 2003 behaalde zij haar artsexamen aan de Universiteit Maastricht. Aansluitend werd zij aangesteld als arts-onderzoeker op het onderzoeksproject "Innovatieve diagnostiek en behandeling van patiënten met prikkelbaar darmsyndroom" in het Universitair Medisch Centrum te Utrecht onder begeleiding van Prof. L.M.A. Akkermans, Prof. G.P. van Berge Henegouwen en Prof M. Samsom. De resultaten heeft u kunnen lezen in dit proefschrift. Op 1 maart 2008 is zij gestart met de opleiding interne geneeskunde in het Twee Steden Ziekenhuis te Tilburg (opleider Dr H. Goey)









Additional full color figures for chapter 9 of serial sections of a mast cell near a neuron stained with respectively H&E, Giemsa and Mast cell tryptase for mast cells and Sam-11 for PAR-2.

2009