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TOWARDS A BACTERIAL ORIGIN OF IRRITABLE BOWEL SYNDROME

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ABSTRACT

Background: The irritable bowel syndrome (IBS) is one of the most prevalent disorders and affects about 15% of the Swedish population. Patients with IBS suffer from abdominal pain and disturbed bowel function. Despite being so common, little is known about causality in IBS. Patients with IBS have been found to exhibit low-grade inflammation of nerve plexuses in the gut. The driving force for observed immune activation is yet unknown.
Aim of the thesis: To assess if bacteria may have a role in the pathogenesis of IBS. This was addressed using different models. The first was to investigate if *Chlamydia* antigens were present in the small bowel of patients with severe IBS. Then the interaction between *Chlamydia* and enteroendocrine cells (EEC) *in vitro* using enteroendocrine cell lines was studied and finally the composition of small bowel mucosa-associated microbiota was explored in patients with IBS and healthy controls using pyrotag sequencing.
Material and methods: In Study I full-thickness jejunum biopsies and mucosa biopsies from the duodenum and the jejunum from patients with severe IBS and healthy controls were investigated with immunofluorescence for chlamydial antigens. In studies II and III two different human enteroendocrine cell lines were studied: LCC-18 from a neuroendocrine colonic tumour and CNDT-2 from a small intestinal carcinoid. Cell lines were infected with *C. trachomatis* serovar LGV II strain 434. Penicillin G was used for inducing persistent infection. The ultrastructure of infected cells was studied using transmission electron microscopy and immunofluorescence and we used RT-PCR analysis and microarray analysis (Affymetrix GeneChip®) for studying changes in gene expression at different stages of infection. In Study IV capsule biopsies from the jejunum of patients with IBS and healthy volunteers were studied using barcoded 454-pyrosequencing to determine the composition of microbial communities in the upper small bowel.
Results: *Chlamydia* LPS was detected in enteroendocrine cells of the mucosa or subepithelial macrophages in 89% of patients with IBS, but in only 14% of healthy controls ($p < 0.001$) and 79% of LPS-positive biopsies were also positive for *C. trachomatis* major outer membrane protein. The cell line experiments showed that both cell lines could be infected with *C. trachomatis* yielding productive infection and persistence could be induced using penicillin G. The cellular distribution of serotonin and chromogranin A was altered by infection from a cytoplasmatic distribution to a location mostly in chlamydial inclusions. Significant differences in the gene transcription levels between persistently infected and non-infected cells were found in 10 genes coding for different solute carrier transporters (SLC) and 5 genes related to endocrine function (GABARAPL1, GRIP1, DRD2, SYT5 and SYT7). Study IV showed no major difference in small bowel mucosa-associated microbiota between patients with IBS and healthy controls.

Conclusions: Study I introduced the novel concept that infection with *C. trachomatis* might be involved in the pathogenesis of IBS. *In vitro* studies confirmed that such an infection affects enteroendocrine cell function. The luminal flora of the small bowel was not identified as a host factor for developing IBS.

Key-words: Chlamydia, irritable bowel syndrome, enteroendocrine cells, immunofluorescence, microarray, pyrotag sequencing, small bowel microbiota

LIST OF PUBLICATIONS

I. **Dlugosz A**, Törnblom H, Mohammadian G, Morgan G, Veress B, Edvinsson B, Sandström G, Lindberg G. *Chlamydia trachomatis* antigens in enteroendocrine cells and macrophages of the small bowel in patients with severe irritable bowel syndrome. BMC Gastroenterol. 2010; 10: 19.

II. **Dlugosz A**, Zakikhany K, Muschiol S, Hultenby K, Lindberg G. Infection of human enteroendocrine cells with *Chlamydia trachomatis*: a possible model for pathogenesis in irritable bowel syndrome. Neurogastroenterol Motil. 2011; 23: 928-34.

III. **Dlugosz A**, Muschiol S, Zakikhany K, D'Amato M, Lindberg G. Gene expression analysis of *Chlamydia trachomatis* infected human enteroendocrine cells. (Submitted for publication)

IV. **Dlugosz A**, Lundin E, Zakikhany K, Sandström G, Engstrand L, Lindberg G. Small bowel microbiota in patients with irritable bowel syndrome and healthy controls – a pyrosequencing study. (Submitted for publication)

Related publication

Zucchelli M, Camilleri M, Nixon Andreasson A, Bresso F, **Dlugosz A**, Halfvarson J, Törkvist L, Schmidt PT, Karling P, Ohlsson B, Duerr RH, Simrén M, Lindberg G, Agréus L, Carlson P, Zinsmeister AR, D'Amato M. Association of *TNFSF15* polymorphism with irritable bowel syndrome. Gut. 2011 Jun 2 doi:10.1136/gut.2011.241877

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LIST OF ABBREVIATIONS

A-IBS	alternating stool pattern irritable bowel syndrome
ANOVA	analysis of variance
ASS1	argininosuccinate synthetase 1
BIRC3	baculoviral IAP repeat-containing 3
BMI	body mass index
C-IBS	constipation-predominated irritable bowel syndrome
C. trachomatis	*Chlamydia trachomatis*
CFU	colony-forming units
CgA	chromogranin A
CHGA	chromogranin A gene
CI	confidence interval
CNDT-2	CNDT-2 cell line
D-IBS	diarrhoea-predominated irritable bowel syndrome
DAVID	Database for Annotation, Visualization and Integrated Discovery
DN	degenerative neuropathy
DNA	deoxiribonucleic acid
DRD2	D2 subtype of the dopamine receptor
EB	elementary body
EEC	enteroendocrine cells
emPCR	emulsion polymerase chain reaction
ESTs	expressed sequence tags
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
GABA	gamma-aminobutyric acid
GAPARAPL1	gamma-aminobutyric acid A receptor-associated protein like 1
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
gDNA	genomic deoxiribonucleic acid
GI	gastrointestinal
GRIP 1	glutamate receptor interacting protein 1
HBD-2	human β-defensin-2
HC	healthy controls
HSPB1	heat shock protein 1
5-HT	5-hydroxytryptamine

IBD	inflammatory bowel disease
IBS	irritable bowel syndrome
IBS-C	irritable bowel syndrome with constipation
IBS-D	irritable bowel syndrome with diarrhoea
IBS-M	mixed irritable bowel syndrome
IELs	intraepithelial lymphocytes
IF	immunofluorescence
IgG	immunoglobulin G
IL6	interleukin 6
IL8	interleukin 8
LCC-18	LCC-18 cell line
LDCV	large dense-core secretory vesicles
LEG	lymphocytic epithelio-ganglionitis
LG	lymphocytic ganglionitis
LMPC	Laser Microdissection Pressure Catapulting
LPS	lipopolysaccharide
MEV	MultiExperiment Viewer
mGluR4	glutamate receptor metabotropic 4
M-IBS	mixed stool pattern irritable bowel syndrome
MOI	multiplicity of infection
MOMP	major outer membrane protein
OTU	Operational Taxonomic Units
PCoA	Principal Coordinates Analysis
PCR	polymerase chain reaction
penG	penicillin G
PI-IBS	post-infectious irritable bowel syndrome
RB	reticulate body
RDP	Ribosomal Database Project
RMA	robust multi-array average
RNA	ribonucleic acid
SD	standard deviation
SIBO	small intestinal bacterial overgrowth
SLC	solute carrier transporters
SYT5	synaptotagmin 5
SYT7	synaptotagmin 7
TEM	transmission electron microscopy

TFCP2	transcription factor CP2
TLR4	toll-like receptor 4
TPH1	tryptophan hydroxylase 1
TRPA1	transient receptor potential cation channel
VMAT1	vesicular monoamine transporter

1 INTRODUCTION

Disorders of gut function are among the most prevalent problems presented to gastroenterologists of the Western world and irritable bowel syndrome (IBS) is the most common functional gastrointestinal disorder (1). Although IBS is not a life-threatening disease, the chronic nature of IBS tends to interfere with the normal daily life and IBS can have a strong impact on the patient's quality of life (2, 3). Patients with IBS and co-morbid somatic disorders report more severe IBS symptoms and low health-related quality of life. IBS has been associated with fibromyalgia, chronic fatigue syndrome, temporo-mandibular joint disorder and chronic pelvic pain (4). In addition to individual patient discomfort, the costs related to IBS are substantial. In the United States, the total costs associated with IBS include $10 billion in direct medical costs and $20 billon in indirect costs, such as absence from work and loss of productivity (5).

1.1 THE ROME CRITERIA FOR FUNCTIONAL GASTROINTESTINAL DISORDERS

IBS is a syndrome, i.e. defined by the presence of certain symptoms. Thus defined, IBS seems to be a rather heterogeneous disorder with multiple presentations, possibly of different origins (6). In order to improve diagnosis and management, patients with IBS were classified into different groups according to their predominant symptoms (7). The development of symptom criteria for IBS has been an iterative process over the last 20 years.

1.1.1 Rome-I criteria for IBS

At least 3 months of continuous or recurrent symptoms of:
1. abdominal pain or discomfort which is
a. relieved with defecation
b. and/or associated with a change in frequency of stools
c. and/or associated with a change in the consistency of stools
and
2. Two or more of the following are present at least 25 percent of occasions or days:
a. altered stool frequency
b. altered stool form (lumpy/hard or loose/watery stools)
c. altered stool passage (e.g. sensations of incomplete evacuation, straining, or urgency)
d. passage of mucus
e. bloating or feeling of abdominal distention (8)

1.1.2 Rome-II criteria for IBS

At least 12 weeks, which need not be consecutive, in the preceding 12 months of abdominal discomfort or pain that has 2 out of 3 features:
1. Relieved by defecation

2. Onset associated with a change in frequency of stool
3. Onset associated with a change in form (appearance) of stool (9).

1.1.3 Rome-III criteria for IBS

Recurrent abdominal pain or discomfort at least 3 days per month associated with 2 or more of the following:
1. Improvement with defecation.
2. Onset associated with a change in frequency of stool
3. Onset associated with a change in the appearance of stool.
Start of symptoms at least 6 months previously (10).

1.2 IBS – DEFINITION

All studies concerning patients utilized the Rome-II criteria for IBS. Patients were diagnosed by experienced specialists in gastroenterology. The Rome-II diagnostic criteria define IBS by the presence of recurrent abdominal pain or discomfort for at least 12 weeks, that need not be consecutive, during the last 12 months associated with two or more of the following: improvement with defecation, onset associated with a change in frequency of stool, or onset associated with a change in form (appearance) of stool. Additional symptoms that are not part of the diagnostic criteria cumulatively support the diagnosis of IBS: abnormal stool frequency; abnormal stool form; abnormal stool passage; passage of mucus; and bloating or feeling of abdominal distension (9).

1.3 IBS – SUBTYPES

The latest Rome III criteria subtype patients exclusively according to their stool form because it better reflects transit time:
1. IBS with constipation (IBS-C): hard or lumpy stools more than 25% of the time and loose/watery stools appearing in fewer then 25% of bowel movements.
2. IBS with diarrhoea (IBS-D): loose/watery stools more than 25% of the time and hard or lumpy stool appearing in fewer than 25% of bowel movements.
3. Mixed IBS (IBS-M): hard or lumpy stools more than 25% of the time and loose/watery stools in more than 25% of bowel movements.
4. Unsubtyped IBS: insufficient abnormality of stool consistency to meet criteria for IBS-C, IBS-D, or IBS-M. IBS-C, -D and -M subtypes are believed to have a similar distribution, each accounting for approximately 30% of cases (11, 12).

Rome-II criteria identify diarrhoea-predominant IBS (D-IBS) and constipation-predominant IBS (C-IBS) (9). Patients who fulfil the symptom criteria for IBS but not for any of the two subtypes are sometimes referred to as A-IBS (alternating stool pattern) or M-IBS (mixed stool pattern IBS).

1.4 IBS – PREVALENCE

Estimates of the prevalence of IBS vary across studies between 5-20% of the population of most countries with peaks in the third and fourth decades (13, 14). IBS is more common in women. In the community, the ratio of women to men with IBS is about 2:1, and the difference is even greater in the health care-seeking population, with women leading men by up to 4:1 (15). Female patients report greater overall IBS symptom severity, intensity of abdominal pain and bloating, and impact of symptoms on daily life, and lower health-related quality of life than male patients. The reason for this is not fully understood, but women also report more extraintestinal symptoms, such as nausea and urinary urgency. IBS symptoms in women vary according to the menstrual cycle, with increased symptoms just before and during the menses (15).

1.5 IBS – PATHOPHYSIOLOGY

The pathophysiology of IBS is still not well understood. Several mechanisms have been proposed, including psychological disturbances, abnormal gastrointestinal motility, visceral hyperalgesia, altered central perception of visceral events and hypothalamic-pituitary axis dysfunction (16). Recent studies have focused on low-grade inflammation and dysregulation of intestinal microflora (17-19).

1.5.1 Post-infectious IBS

Studies have repeatedly found that IBS can arise after an acute gastroenteritis. This phenomenon, known as post-infectious IBS (PI-IBS) denotes the persistence of abdominal discomfort, bloating and diarrhoea that continues despite clearance of the inciting pathogen (20). Probably the first consistent report on PI-IBS dates back to the beginning of the century when Sir Arthur Hurst described a condition defined as ‘‘postdysenteric colonic irritability’’ (21). This condition was quite frequent in British troops returning from war campaigns in areas endemic for bacillary or amoebic gastroenteritis. Over the past decade, this concept has been revitalized and PI-IBS has been reported to develop in 3% to 30% of individuals after bacterial gastroenteritis (22). A recent systematic review and meta-analysis identified that the pooled incidence for IBS development after infectious gastroenteritis was 10% (23). Salmonella, Shigella, and Campylobacter are among the most frequently isolated infectious agents (16), but viral infection has also been documented as a trigger of IBS (24, 25). Connell (26) postulated that in the United States organisms such as *Shigella* or *Salmonella* are being replaced in frequency by *Campylobacter*, *Chlamydia* and *Giardia*.

The underlying mechanism of post-infectious IBS has not been established but ongoing inflammation appears to play a role, with an increase in enteroendocrine cells, lymphocytes, mast cells, and proinflammatory cytokines (27). Intestinal dysbiosis induced by infection also has been proposed to produce low-grade inflammation and chronic gut dysfunction (16).

1.5.2 IBS and inflammation

An immune activation and inflammatory response at the mucosal level may play a role in generating and perpetuating symptoms in patients with IBS. It is well known that patients with inflammatory bowel disease frequently develop symptoms overlapping those of IBS patients (28, 29). Several studies revealed the presence of intestinal inflammation in IBS (30). Both innate and adaptive immune responses seem to be involved in IBS pathogenesis (31). Histopathologic data demonstrated low-grade inflammation in intestinal mucosa and ganglia in a subset of patients with IBS (17). Increased numbers of immunocytes, mainly lamina propria T cells, intraepithelial lymphocytes (IELs), and mucosal mast cells, have been observed in intestinal mucosa biopsies from IBS patients when compared with specimens from asymptomatic control subjects (32, 33).

Some studies have shown that patients with IBS have increased plasma levels of interleukins 6 and 8 (IL6 and IL8) (34, 35), which are proinflammatory cytokines primarily produced by monocytes and macrophages. Spiller et al. (36) found 50% reduction in the number of resident mucosal macrophages in rectum but increased levels of activated macrophages identified by calprotectin expression in patients with PI-IBS. Mast cells and resident macrophages are key players in the early immune response to microorganisms that affect the mucosal tissue. The secretion of immune mediators by these cell populations is crucial for recruitment of phagocytic cells, macrophages and neutrophils to the damaged tissue (31).

1.6 ENTEROENDOCRINE CELLS

The gastrointestinal tract contains the largest endocrine organ in the body. Enteroendocrine cells (EEC) are present in the mucosa of the stomach, small intestine, colon, and rectum and are highly specialized cells that produce hormones and other signalling substances that are vital to the normal function of the gut. Due to their diffuse localisation EEC are difficult to study and so far, their role in the pathogenesis of bowel disorders has been only little explored. A well characterised subset of EEC are the enterochromaffin cells, which are the main source of the biogenic amine serotonin (5-hydroxytryptamine, 5-HT) in the GI tract (37). The aminoacid tryptophan serves as a precursor for the production of serotonin, which is transported from the cytosol to large dense-core secretory vesicles (LDCV) by the vesicular monoamine transporter (VMAT1), a glycoprotein located in the membrane of LDCV (38).

Serotonin influences the intestinal homeostasis by altering gut physiology (motility and secretory function) and has been implicated in the pathophysiology of various GI disorders, including inflammatory bowel disease (IBD) and IBS (39). The mechanisms regulating serotonin homeostasis in EEC during infection and inflammation processes are yet unclear. Chromogranin A (CgA) is a well known protein constituent of LDCVs and has been identified as a “common denominator” peptide for EEC (40). CgA is released by regulated secretion in response to extracellular stimulation of the cells (41)

and can furthermore act as an on/off switch for secretory granule biogenesis (42). Elevated serum levels of CgA have been reported in both IBD (43) and IBS (44).

EEC play a pivotal role in sensory signalling for the enteric reflex regulating intestinal propulsive motility and secretion under basal and stress-sensitive manifestations of functional bowel disorders (45). Alteration of endocrine cell function, particularly in the context of serotonin (5-HT), has been shown to be associated with a number of GI diseases including inflammatory bowel disease (IBD), coeliac disease, and IBS (46-48). The association between alteration in the production of gut hormones from enteric endocrine cells and various GI diseases emphasizes the significance of these cells in intestinal homeostasis (45).

In addition, EEC seems to be an important contributor to inflammatory processes occurring in the gut in response to pathogens (49) and an increased number of serotonin positive EEC has been observed in post-infectious IBS (36). Alterations in the number of EEC have been observed in different bacterial, viral and parasitic infections of the GI tract (50). EEC can be activated by bacterial ligands like lipopolysaccharide (LPS) and may play a role in immune activation and in the regulation of gut inflammation (51). Altered endocrine function of the gut accompanies the inflammatory immune response (52).

1.7 IBS AND MICROBIOTA

Abnormalities in the gut flora may be of importance in the pathogenesis of IBS as the gut flora can modulate the immune response. The human microbiota consist of about 100 trillion microbial cells that outnumber our cells by 10 to 1 (53). The human gastrointestinal microbiota have been extensively studied but is not yet fully described. In the healthy host, enteric bacteria colonize the alimentary tract soon after birth, and the composition of the intestinal microflora is believed to remain relatively constant throughout life (54). Maternally acquired bacteria during birth are followed by hundreds of environmentally acquired species, which differ between individuals but mainly belong to two bacterial phylotypes, *Firmicutes spp*. and *Bacteroidetes spp*. (55). In a recently published metagenomic study Arumugam *et al*. (56) identified three major enterotypes in the humen gut microbiome; these were dominated by *Bacteroides*, *Prevotella*, and *Ruminococcus*, respectively.

The adult human intestine is home to between 1000 and 1150 prevalent bacterial species (57), which normally remain confined to the distal gut (colon) where the concentration of organisms is approximately 10^{11} organisms per gram of content (58). Because of peristalsis and the antimicrobial effects of gastric acidity, the stomach and proximal small intestine contain small numbers of bacteria in healthy individuals. The bacterial counts of coliforms rarely exceed 10^3 colony-forming units (CFU)/mL in jejunal juice (54). The intestinal epithelium provides an essential barrier between host and microbe. However, there is constant cross-talk across this barrier between the

immune system and microbiota that is equally important for homeostasis in the gut (59).

The relationships between microbiota and different diseases in the gut have been studied regarding colonic microbiota and some associations have been observed for inflammatory bowel diseases (IBD) (57, 60), metabolic syndrome (61), and obesity (62). A contribution of the gastrointestinal microbiota has been suggested in IBS (63). Intestinal dysbiosis induced by infection also has been proposed to produce low-grade inflammation and chronic gut dysfunction in IBS (16).

The potential differences of the intestinal microbiota between IBS patients and healthy controls (HC) have mostly been studied using fecal material, as this is the most accessible source of the GI microbiota (63, 64), but fecal samples reflect mostly colonic flora. Small bowel microflora is not fully described, partly because small bowel samples are relatively difficult to access. Kerckhoffs *et al*. (65) found decreased *Bifidobacteria* levels in both fecal and duodenal brush samples of IBS patients compared to healthy subjects. Small intestinal bacterial overgrowth (SIBO) has been proposed to be common in IBS (66). Bacterial overgrowth is a condition caused by an abnormal number of bacteria in the small intestine, exceeding 10^5 organisms/ml (5 log colony-forming unit (CFU)/ml) owing to different predisposing conditions, such as impaired motility or failure of the gastric-acid barrier (67, 68). The direct aspiration and culture of jejunal fluid, with results expressed as CFU/mL of jejunal fluid, although invasive, has been regarded by many investigators as the gold standard for the diagnosis SIBO but molecular techniques such as genomics and metabolomics suggest that as much as 60% of the normal flora is not identified by culture-based methods (54).

1.8 IBS AND *CHLAMYDIA*

Connell (26) postulated that in the United States infections with organisms such as *Shigella* or *Salmonella* prior to PI-IBS are being replaced in frequency by *Campylobacter*, *Chlamydia* and *Giardia*. Törnblom *et al*. (25) showed that the actual agent causing gastroenteritis was not a predictor of risk for IBS. Consequently, a host factor, such as a pre-existing chronic infection with a different microbe than the agent causing gastroenteritis, might explain the development of IBS. Such a candidate agent should be compatible with an asymptomatic carrier-ship, have a preference for female gender, and have the ability to become persistent and to live in bowel epithelium. There are some observations to support the idea that a persistent infection with *Chlamydia trachomatis* might constitute such a host factor.

Bacteria of the genus *Chlamydia* are gram-negative, obligate intracellular pathogens, which multiply and infect a wide range of eukaryotic cells causing both chronic and acute diseases in humans and animals (69). The growth of *Chlamydia* is characterised by a unique developmental cycle with morphologically distinct infectious (elementary body (EB)) and reproductive forms (reticulate body (RB)). Upon infection, the EB differentiates into the metabolically active RB, which is able to replicate within the host

inside a membrane-bound compartment, termed inclusion. In association with chronic diseases, alterations of the life cycle such as a persistent state leading to asymptomatic infections have been discussed for *C. trachomatis* (69). Bacteria in a persistent state down-regulate their metabolism and may exhibit atypical morphology. They may therefore be difficult to detect for the immune system and they are relatively inaccessible for antibiotic therapy (70). Trachoma-related blindness is 2-4 times more likely to affect females compared to males (71). It is known that IBS occurs in 35%-50% of females with chronic pelvic pain syndrome, which is believed to often be caused by chronic infection with *C. trachomatis* (4, 72, 73). A previous attempt to link *C. trachomatis* to IBS using serum IgG antibodies failed (74), but IgG antibody patterns may be insufficient to rule out persistence of *Chlamydia* due a dominating cellular immune response to infection (75, 76).

2 AIM OF STUDY

The general aim of this thesis was to assess the role of bacteria in the pathogenesis of IBS.

The following research questions were central to the studies included in this thesis:

- The aim of study I was to find out if Chlamydia antigens were present in the small bowel in patients with severe IBS.
- The aim of study II was to characterise the interaction between Chlamydia and EEC in vitro using enteroendocrine cell lines from both small bowel and large bowel.
- The aim of study III was to evaluate the response pattern of enteroendocrine cells to intracellular bacteria in vitro using microarray technology.
- The primary aim of study IV was to deeply explore the composition of small bowel mucosa-associated microbiota using pyrotag sequencing. The secondary aim of study IV was to study differences in small bowel mucosa associated microbiota between IBS patients and healthy controls.

3 MATERIAL AND METHODS

The patients and controls included in study I and IV received oral and written information before consenting to participation in the studies. In study I we reanalyzed archived biopsy material. The local Ethics Committee at Karolinska University Hospital, Huddinge, Stockholm, Sweden (HS 144/98, HS 517/98, HS 518/98, HS 39/02) and the Regional Board of Research Ethics, Stockholm (2006/398-31/2, 2006/1328-31/1, 2007/1151-31/2) approved all human studies.

3.1 SUBJECTS

3.1.1 Study I

All patients fulfilled Rome-II criteria for IBS. A total of 65 patients (61 females and 4 males) with a median age of 48 (range 22-67) years and a median duration of IBS symptoms of 6.5 years (range 0.6-33.2 years) were investigated. Diarrhoea predominant IBS was present in 21 patients (32%), 22 patients (34%) had constipation predominant IBS and 22 patients (34%) had IBS with alternating bowel habits. All patients had severe symptoms of IBS and 26 patients also exhibited abnormalities on small bowel manometry, thus qualifying for a pathophysiological diagnosis of enteric dysmotility. Full thickness jejunum biopsies had been taken in 60 of our patients. Previous histopathological analysis had revealed neuropathic changes in 58 patients. Neuropathy was associated with low-grade inflammation (LG = lymphocytic ganglionitis) in 46 patients and 20 of these also exhibited increased numbers of intraepithelial lymphocytes (LEG = lymphocytic epithelio-ganglionitis), whereas 12 patients had degenerative neuropathy (DN) without inflammation. Deficient staining for alpha-actin without neuronal damage was observed in 2 patients.

The control group comprised 42 persons (29 females) in whom IBS and all other functional bowel disorders had been excluded by medical interview and a validated questionnaire for the Rome-II symptom criteria. Ten controls (7 females) were obese but otherwise healthy (BMI mean = 42.8, SD = 4.3). The rest of the control group (32 persons, 22 females) consisted of healthy volunteers with normal weights. The median age of the controls was 36 (range 19-60) years.

3.1.2 Studies II and III

Studies II and III were *in vitro* studies on human enteroendocrine cell lines.

3.1.2.1 Cell lines

We used two different human enteroendocrine cell lines: LCC-18, derived from a neuroendocrine colonic tumour and CNDT-2, derived from a small intestinal carcinoid. LCC-18 cells (a kind gift from K. Öberg, Uppsala University Hospital, Uppsala, Sweden) were maintained in DMEM/F-12 medium (Invitrogen, UK) supplemented with 5% fetal bovine serum (FBS) transferrin, β-estradiol, insulin, hydrocortisone,

sodium selenite (Sigma-Aldrich, Sweden) and HEPES buffer (Invitrogen). CNDT-2 cells (a kind gift from LM. Ellis, University of Texas, Houston, USA) were cultured in DMEM/F12 medium (Invitrogen), supplemented with 10 % FBS, vitamin solution, L-glutamine and HEPES (Invitrogen). Both cell lines were incubated at 37 °C in a 5 % CO_2 humidified environment.

3.1.2.2 Chlamydial strains and infection

Chlamydia trachomatis L2 strain 434 (ATCC) was propagated in HeLa cells as described by Boleti *et al*. (77) For infection experiments, LCC-18 and CNDT-2 cells were inoculated with *Chlamydia* at a multiplicity of infection (MOI) of 0.5-1 for 1 h at 37°C. Monolayers of infected CNDT-2 cells were washed with PBS (phosphate buffered saline) and incubated in fresh media. Infected LCC-18 cells were spun at 500 x g for 5 min to pellet the cells. The inoculum was removed and fresh media containing 8 µg/ml gentamycin (Invitrogen) to kill off extracellular bacteria was added for the indicated times. To induce persistent infection, LCC-18 and CNDT-2 cells were infected and incubated in medium containing 100 U/ml penicillin G (Sigma-Aldrich) for 24 h at 37°C.

3.1.3 Study IV

All patients fulfilled Rome-II criteria for IBS. A total of 35 patients (26 females) with a median age of 36 (range 18-50) were investigated. Diarrhoea predominant IBS (D-IBS) was present in 13 patients (37%), 9 patients (26%) had constipation predominant IBS (C-IBS) and 13 patients (37%) had IBS but did not fulfil criteria for D-IBS or C-IBS (M-IBS).

The control group comprised 16 healthy volunteers (11 females) in whom IBS and all other functional bowel disorders had been excluded by medical interview and a validated questionnaire for the Rome-II symptom criteria. The median age of the controls was 32 (range 20-48) years. Obesity was excluded in both patients and controls.

3.2 METHODS

3.2.1 Full thickness jejunum biopsy (I)

Previously obtained full-thickness biopsy specimens were available in 60/65 patients. The biopsies had been taken from the proximal jejunum using a laparoscopy assisted procedure described by Törnblom *et al*. (17). Ten obese controls underwent full thickness biopsy of the jejunum at the time of gastric by-pass surgery.

3.2.2 Mucosa biopsy (I, IV)

Mucosa specimens from the proximal jejunum were taken with a Watson capsule in 32 controls and 41 patients (6 patients in study I and 35 patients in study IV). The capsule was swallowed by the subject and brought by peristalsis to a position distal to the

ligament of Treitz as determined by fluoroscopy. In study I we analyzed also archived endoscopic mucosa biopsies from the duodenum of 20 patients and in 15 of these full thickness biopsies were also available. In 2 patients we analyzed mucosa biopsies from both the jejunum and the duodenum. The presence of villous atrophy or inflammation was excluded in all biopsies.

3.2.3 Immunofluorescence (I, II)

The following antibodies were used for immunofluorescence and immunohistochemistry:

Target	Primary antibody	Secondary antibody
Chlamydia lipopolysaccharide (LPS)	Mouse monoclonal - FITC conjugated with Evans blue	-
C. trachomatis major outer membrane protein (MOMP)	Mouse monoclonal	Rabbit polyclonal anti-mouse -FITC conjugated
C. pneumoniae MOMP	Mouse monoclonal	Rabbit polyclonal anti-mouse - FITC conjugated
C. trachomatis LPS	Rabbit polyclonal	Goat anti-rabbit Streptavidin-biotin complex
Chromogranin A	Rabbit polyclonal	Goat anti-rabbit conjugated with Alexa Fluor 568
Serotonin	Rabbit polyclonal	Goat anti-rabbit conjugated with Alexa Fluor 568
Mast cells CD117	Rabbit polyclonal	Goat anti-rabbit conjugated with Alexa Fluor 568
Macrophages CD68	Rabbit polyclonal	Goat anti-rabbit conjugated with Alexa Fluor 350
Dendritic cells CD11c	Rabbit polyclonal	Goat anti-rabbit conjugated with Alexa Fluor 350

Stained sections were examined using a Universal Laser Scanning Confocal Microscope System Leica TCS and a Fluorescent Microscope System Leica DMRXA (Leica Microsystems, Wetzlar, Germany).

In study I two independent investigators (AD and GM), who were unaware of clinical data, made the final assessment of slides. The slides were reviewed by a third investigator (BV). The immunofluorescence stainings were considered positive if more than one cell showed fluorescence. If only one positive cell was found, the staining-procedure was repeated. The case was regarded positive if the colour signal was again present, otherwise the case was recorded as negative. Double immunofluorescence

stainings were also performed for the identification of LPS positive cells (LPS with chromogranin, CD68, CD117, or CD11c, respectively).

3.2.4 Western blotting

Snap-frozen biopsies from 4 patients that were positive for *Chlamydia* LPS staining, were examined by Western blotting. HeLa cells infected with *C. trachomatis* served as positive control and we used non-infected HeLa cells as negative control. Equivalent amounts of protein from each specimen were loaded onto a sodium dodecyl sulphate-polyacrylamide gel. After electrophoresis, samples were transferred to nitrocellulose membranes. We used a mouse monoclonal antibody to *C. trachomatis* LPS (AbD Serotec, Oxford, UK) as primary antibody and goat anti-mouse antibody conjugated to horseradish peroxidase (BioRad, Herculaes, CA, USA) as secondary antibody. For MOMP we used the same primary antibody as for immunofluorescence.

3.2.5 Microlaser system (I)

We used Laser Microdissection Pressure Catapulting (LMPC) for laser based non-contact extraction of tissue areas in paraffin embedded biopsies from 6 patients. Regions of interest were manually delineated using fluorescence microscopy and the LMPC software. Tissue collection was achieved by laser cutting along the delineation lines to separate tissue of interest from surrounding regions, and secondly the laser catapulted the tissue of interest up into the lid of an Eppendorf cap containing sterile water. DNA was extracted using the QIAamp DNA mini kit, according to the instructions of the manufacturer, and analyzed by PCR.

3.2.6 Transmission electron microscopy (I, II, III)

In study I biopsies from the distal duodenum of 4 patients that were positive for *Chlamydia* LPS staining were fixed according to a procedure described by Muschiol *et al*. (78). Semi thin sections were cut and stained with toluidin blue and used for light microscopic analysis. Ultra-thin sections were contrasted with uranyl acetate followed by lead citrate and examined in a Tecnai 10 transmission electron microscope at 80 kV. Digital images were taken by using a MegaView III digital camera (Soft Imaging System, Münster, Germany).

In studies II and III cells were grown in 6-well plates and infected with *Chlamydia trachomatis* L2 in presence or absence of penicillin G as described. At 24 h post infection the cells were harvested, fixed in 0.1 M sodium cacodylate buffer (pH 7.4) containing 2 % glutaraldehyde, 0.5 % paraformaldehyde, 0.1 M sucrose and 3 mM $CaCl_2$ and prepared for transmission electron microscopy as previously described and analysed in a BioTWIN (Fei, The Netherlands). Digital images were obtained by a Veleta digital camera (Soft Imaging System, Germany).

3.2.7 Real-time PCR (I, II)

In study I we used the real-time PCR assay developed by Everett *et al*. (79), which amplifies 23S ribosomal DNA and detects all members of the family *Chlamydiaceae*. An internal amplification control was included to monitor possible inhibition of the PCR. DNA from frozen biopsies, taken from 4 patients previously positive for *Chlamydia* LPS staining, was extracted using a Qiagen minikit according to the tissue protocol (Qiagen, Solna, Sweden). Extracted DNA was quantified and quality controlled using a Nanodrop spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA) before being subjected to PCR.

In study II total RNA from non-infected, infected and persistently infected cells was extracted using the RNeasyMini RNA extraction kit (Qiagen, Germany) including an on-column DNase treatment procedure, following the manufacturer's instructions. PCR amplification of selected target genes: chromogranin A (CHGA), vesicular monoamine transporter (VMAT1), glutamate receptor metabotropic 4 (mGluR4), tryptophan hydroxylase 1 (TPH1), transient receptor potential cation channel (TRPA1), transcription factor CP2 (TFCP2), heat shock protein 1 (HSPB1) and toll-like receptor 4 (TLR4) was performed under the following conditions: 1 cycle at 50 °C for 2 min; 1 cycle at 95 °C for 10 min, 45 cycles at 95 °C for 15 s; and 60 °C for 60 s, with fluorescence acquisition at 60 °C. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) served as internal normalization control and relative expression values were assessed from triplicates per real-time PCR assay and five technical replicates relative to a calibrator value (non-infected cells).

The following primers were used:

Target gene	Sense	Antisense
CHGA	GCATCGTTGAGGTCATCT	ACCGCTGTGTTTCTTCTG
VMAT1	ACACATACACAGAATCCTCAG	TCAACTTGCCAGCTTATCC
mGluR4	CCAGGCTACCACAAGAAC	CCAACAGCACAGACAGAG
TPH1	ATATACCATACAAGCCACTGAC	CATAATACTCCTCAAGTTATTCTAAGC
TRPA1	TGACGATATGGACACCTT	AAGACCTTCATCACCTCAT
TFCP2	TGGATTTGGTTCTAAGCCGTG	CCCAGGTCTCGCTCTCTC
HSPB1	CTCAAACACCGCCTGCTA	GCTGACTCTGCTCTGGAC
TLR4	TTCAAGGTCTGGCTGGTT	CGAGGTAGTAGTCTAAGTATGCTA
GAPDH	GGTCGGAGTCAACGGATT	ATCGCCCCACTTGATTTTG

3.2.8 Microarray (III)

In study III total RNA was isolated from 1 x 10^6 LCC-18 and CNDT-2 cells infected with *Chlamydia trachomatis* L2 (MOI 1) in presence or absence of penicillin G for 24 h. RNA samples were prepared using the RNeasy Mini kit (Qiagen) including an on-column DNase treatment step according to the manufacturer´s instructions. RNA concentrations were measured with a ND-1000 spectrophotometer (NanoDrop Technologies, USA) and RNA quality was assesed using the Agilent 2100 Bioanalyzer system (Agilent Technologies Inc, USA). 250 nanograms of total RNA from each sample were used to generate amplified and biotinylated sense-strand cDNA according to the Ambion WT Expression Kit (P/N 4425209 Rev B 05/2009) and Affymetrix GeneChip® WT Terminal Labeling and Hybridization User Manual (P/N 702808 Rev. 1, Affymetrix Inc., USA). GeneChip® ST Arrays (GeneChip® Mouse Gene 1.0 ST Array) were hybridized for 16 hours in a 45 °C incubator, rotated at 60 rpm. According to the GeneChip® Expression Wash, Stain and Scan Manual (PN 702731 Rev 2, Affymetrix Inc., USA) the arrays were then washed and stained using the Fluidics Station 450 and finally scanned using the GeneChip® Scanner 3000 7G.

3.2.9 DNA extraction (IV)

Extraction of total genomic DNA (gDNA) from frozen biopsies was performed with the DNeasy Blood & Tissue Kit (Qiagen, Germany). Biopsy samples where homogenized with a pestle in 1.5 ml tubes containing 200 µl freezing buffer. 100 µl of the homogenate was added to 200 µl lyses buffer (180 µl ATL buffer, 20 µl Proteinase K) and incubated overnight at 56°C in a shaking incubator. A negative extraction control was included for each batch of DNA extraction. After extraction of gDNA following the manufacturer's instruction the gDNA was eluted in 100 µl Buffer AE and stored at -20°C until further usage.

3.2.10 PCR and template preparates for 454-sequencing (IV)

The protocol for barcoded 454-pyrosequencing has been adapted from Andersson et al. (80). In order to create barcoded sequencing templates, PCR was performed using barcoded reverse primer 805r (1-55) targeting the 16S rRNA gene. For each sample, a PCR mix was prepared (triplicate) containing 1x PCR buffer, 10mM dNTP´s (Finnzymes, Finland), 1 U Phusion High-Fidelity DNA Polymerase (Finnzymes, Finland), 0.4 µM of each primer and 1-5 µl template DNA. For the PCR reaction the following conditions were applied: after an initial denaturation step (95°C, 5 min), 30 cycles of 95°C for 40 sec, 55°C for 40 sec and 72°C for 60 sec were followed by an final elongation step with 72°C for 10 min. Following agarose gel electrophoresis (1% agarose in TBE buffer containing gel red (Sigma Aldrich, Sweden), PCR products with the right size (approx. 500 bp) were excised and purified using the QIAquick gel extraction kit (Qiagen, Germany). The DNA concentration was assessed using the Qubit Fluorometer (Invitrogen, USA). After an initial concentration determination, the PCR products were diluted to a concentration between 3 and 5 ng/µl and then pooled for the 454 sequencing run. The final concentration of the DNA pool was measured for

further dilutions for the emulsion PCR (emPCR) in the 454 workflow. Pyrosequencing was performed according to the Genome Sequencer FLX System Methods Manual.

3.2.11 Data analysis (IV)

3.2.11.1 Sequence processing

The raw sequences from the 454 pyrosequencing run were processed to eventually assign the sequences a taxon. All sequences that i) contained ambiguous nucleotides ii) were shorter than 200 bases (primer sequence and barcode tag excluded) or iii) did not perfectly match the barcode or primer, were removed. The remaining sequences were trimmed down to 200 bases excluding barcode and primer. The RDP Pyrosequencing Pipeline (http://rdp.cme.msu.edu/) was then used for alignment and clustering of the non-redundant sequences into Operational Taxonomic Units (OTU), using complete linkage clustering and a 3% distance threshold. The most prevalent sequence within each OTU was used for taxonomic classification of the OTU.

3.2.11.2 Taxonomic classification

A local BLAST database was created from 16S rRNA gene sequences longer than 1200 bases and with good pintail score, downloaded from the Ribosomal Database Project (RDP) website (release 10, update 15). All OTU representative sequences were BLAST searched against the local database using default parameters. The OTUs were assigned the taxonomic classification of the best scoring RDP hit if the identity similarity to this was at least 95% over at least 180 nucleotides. The OTU was classified as a "no_match" if no such hit was found. If there were several equally good (with the same score) BLAST hits for an OTU, the taxonomy was taken down to the level, which was consistent for all the hits. The normalized values for the number of sequences assigned to each taxon (relative abundance of each taxon) were used for comparison of samples. MultiExperiment Viewer 4_0 (TMEV) (http://www.tm4.org/mev/) was used to visualize the relative abundance of the different OTUs in a heatmap.

3.2.11.3 Sample clustering

The online version of Fast Unifrac (http://bmf2.colorado.edu/fastunifrac/) was used for calculating weighted sample distances using the Greengenes reference sequences and the corresponding tree (downloaded from the Fast Unifrac website). We first clustered our sequences into OTUs using 1% distance threshold and the most prevalent sequence for each OTU was mapped to the best matching Greengenes sequence by using BLAST and an e-value cutoff of 10^{-30} (81). Based on pair-wise sample differences (Unifrac values), a Principal Coordinates Analysis (PCoA) plot was created on the Fast Unifrac website.

3.3 STATISTICAL METHODS

In Study I we used logistic regression with age and gender as covariates for calculation of odds ratios and *p*-values for comparisons of proportions. The size of the study group was determined from the assumption that LPS positivity would be no greater than 20%

among controls. In order to detect a risk factor with an odds ratio of at least 6 at $p<0.01$ with power >90% we needed to include at least 37 patients and 37 controls (two-sided test).

Differences in gene expression between infected and non-infected cells in Study II were analysed using Student's *t*-test. Differences in staining intensity were analysed using analysis of variance (ANOVA). A *p*-value of less than 0.05 was considered statistically significant.

In Study III subsequent analysis of the gene expression data was carried out in the freely available statistical computing language R (http://www.r-project.org) using packages available from the Bioconductor project (http://www.bioconductor.org). The raw data was normalized using the robust multi-array average (RMA) method first suggested by Li and Wong in 2001 (82, 83). In order to search for the differentially expressed genes between acute and persistent infection and the control samples (non-infected) an empirical Bayes moderated t-test was applied (84), using the 'limma' package (85).

To address the problem with multiple testing, p-values were adjusted using the method of Benjamini and Hochberg (86). Genes with an expression ratio ≥2-fold and adjusted $p<0.05$ were regarded as differentially transcribed genes (87). Gene clustering was performed using the Database for Annotation, Visualization and Integrated Discovery (DAVID) (http://david.abcc.ncifcrf.gov).

In Study IV significant differences at phylum, class, genus and OTU level were investigated using the Bioconductor R package (88), comparing all groups to each other (M-IBS with controls, C-IBS with controls, D-IBS with controls). EdgeR returns a non-paired p-value for each taxon taking the actual number of reads of the different samples into account. The probability for false positive significant differences was also calculated to correct for multiple testing (False Discovery Rate). Shannon diversity index, a measure of biodiversity, was calculated for each of the four groups (controls, D-IBS, C-IBS and M-IBS) and also for all IBS cases as one group (89). The Shannon diversity index takes into account the number of species present and also the evenness of these species (90).

4 RESULTS

4.1 THE PRESENCE OF *CHLAMYDIA* IN THE SMALL BOWEL OF PATIENTS WITH SEVERE IBS (I)

4.1.1 Immunofluorescence and Western blot

65 patients (61 females) with severe IBS and 42 (29 females) healthy controls without IBS were included. Immunofluorescence (IF) staining showed that 53/60 patients were positive for *Chlamydia* LPS in full thickness jejunum biopsies and 21/24 (88%) patients in mucosa biopsies. Positive staining occurred in a few cells both within the epithelium and in lamina propria. No *Chlamydia* LPS-positive cells were found in the submucosa and muscularis propria or the enteric nervous system in full thickness jejunum biopsies. In 19 patients biopsies had been taken with a time interval of more than 1 year. *Chlamydia* LPS was present in biopsies with a median time difference of 5.2 (range 1-11) years. Overall, 58/65 (89%) patients were classified as *Chlamydia* LPS positive. Six women and one man with IBS were negative in all biopsies. In 90% of positive biopsies from patients *Chlamydia* LPS was localised to mucosal cells at the level of the crypts. Double staining with antibodies to chromogranin- A showed that *Chlamydia* LPS was present in enteroendocrine cells within the epithelium. *Chlamydia* LPS was found in l. propria cells in 69% of the biopsies from patients. Double staining with antibodies to CD117, CD11c and CD68 revealed that in this location *Chlamydia* LPS was present in macrophages. Seventy-nine percent of *Chlamydia* LPS positive biopsies were also positive to *C. trachomatis* MOMP. Staining for *C. pneumoniae* was negative in all patients. We confirmed the presence of *Chlamydia* LPS antigen in new biopsies taken from duodenum mucosa in 4 LPS-positive patients with Western blotting but the new biopsies were negative for *C. trachomatis* MOMP both in immunofluorescense and Western blot.

Only 6/42 (14%) controls (5 women) were positive for *Chlamydia* LPS (6 in *l. propria* macrophages and 2 in enteroendocrine cells) and 2/6 LPS-positive controls were positive to *C. trachomatis* MOMP. No biopsies from controls were positive for *C. pneumoniae*. The prevalence of *Chlamydia* LPS was much higher in patients with IBS (58/65) than it was among controls (6/42). The odds ratio, corrected for differences in age and gender distributions, for mucosal *Chlamydia* LPS being indicative for presence of IBS was 43.1 (95% CI: 13.2- 140.7). Biopsies were analysed by two independent investigators unaware of clinical data. The agreement between the two investigators regarding individual biopsies was 94%, whereas their agreement regarding patient classification was 100%. We found no correlation between presence of *Chlamydia* antigens and the type of neuropathy in patients with full thickness biopsies. Although the majority of patients had neuropathy with inflammation, *Chlamydia* antigens were common also among those who had neuropathic changes without inflammation (LEG: 19/20 = 95%; LG 23/26 = 88%; DN 9/12 = 75%). We found no difference between IBS

subgroups with regard to positivity for *Chlamydia* antigens and 19/21 with D-IBS, 22/22 with C-IBS, and 17/22 with A-IBS were LPS positive.

4.1.2 Transmission electron microscopy

Electron microscopy of biopsies taken from the duodenum mucosa in 4 LPS-positive patients revealed small oval structures resembling intermediate bodies of *Chlamydia* with characteristic condensed nucleoids of nucleic acid. Mitochondria were observed surrounding the inclusions but did not appear to be in close association with the inclusions.

4.1.3 Real-time PCR

We were unable to confirm the presence of *Chlamydia* DNA in new biopsies taken from the duodenum mucosa in 4 LPS positive patients using 23S ribosomal DNA as target. Results were negative for *Chlamydia* DNA also when we analyzed micro-dissected *Chlamydia* LPS positive cells.

4.2 INTERACTION BETWEEN *CHLAMYDIA* AND HUMAN ENTEROENDOCRINE CELLS *IN VITRO* (II)

4.2.1 Immunofluorescence

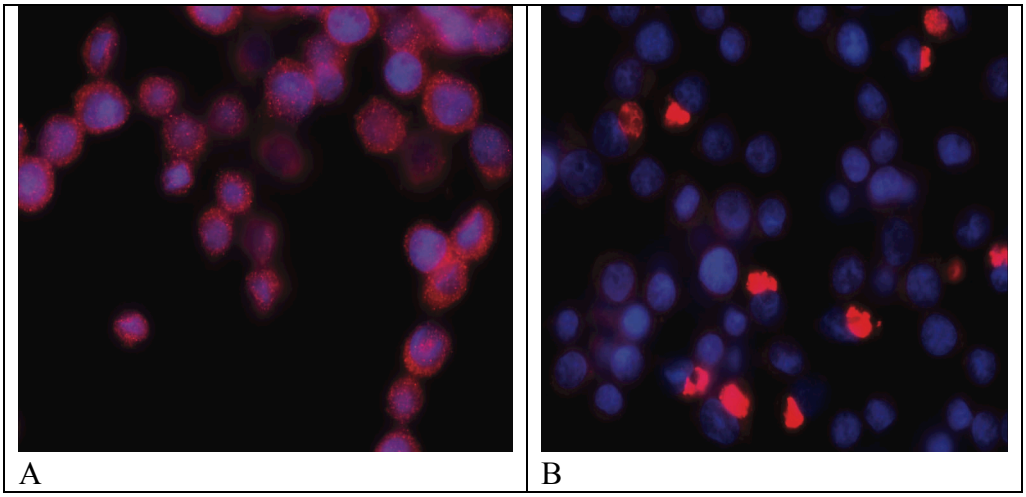

Figure 1. Altered cellular distribution of chromogranin A in EEC after infection with *Chlamydia trachomatis* L2. A: Cytoplasmatic distribution of chromogranin A (CgA) in non-infected LCC-18 cells (rabbit CgA antibody and Alexa 568-red and DAPI nucleus staining (blue), original magnification x63). B: Chromogranin A labelling in *Chlamydia* containing inclusions in infected LCC-18 cells (rabbit CgA antibody and Alexa 568-red and DAPI nucleus staining (blue), original magnification x63).

Immunofluorescence demonstrated differences in the cellular distributions of serotonin and CgA between infected and non-infected cells. In infected cells serotonin and CgA were mainly localized within chlamydial inclusions (Figure 1 B), whereas in non-infected cells these markers predominantly exhibited a cytoplasmatic distribution (Figure 1 A). Mean staining intensity was measured using Nikon NIS Elements.

Differences in staining intensity between inclusions and cytoplasm of both infected and non-infected cells, and between cytoplasm of infected and non-infected cells were highly significant ($p<0.0001$) for both serotonin and CgA staining in both cell lines. No serotonin or CgA was detected in infected or non-infected HeLa cells, which in this instance served as negative control.

4.2.2 Transmission electron microscopy

In order to investigate whether *C. trachomatis* is able to grow and multiply within EEC, human EEC from both the colon (LCC-18) and the small bowel (CNDT-2) were infected with *C. trachomatis* and incubated for 24 h. TEM confirmed the growth of *C. trachomatis,* manifested through inclusions containing both elementary bodies (EB) and reticulate bodies (RB), for both cell lines (Figure 2 A). Similar growth was observed in HeLa cells, which were used as positive control (data not shown). Similar to previously described infection cycles in other cell types, *C. trachomatis* successfully infected and multiplied within the confinements of the inclusion in EEC and yielded productive EBs and this can therefore be considered as an active infection (data not shown). To investigate whether the persistent life-cycle could be induced in the EEC, cells were infected with *C. trachomatis* and incubated in the absence and presence of penicillin G (penG), which has been demonstrated previously to induce persistent growth of *Chlamydia* in HeLa cells (91). When EEC were treated with penG we observed enlarged, aberrant bacteria reminiscent of persistent growth forms suggesting that persistence can be induced in both EEC tested (Figure 2 B).

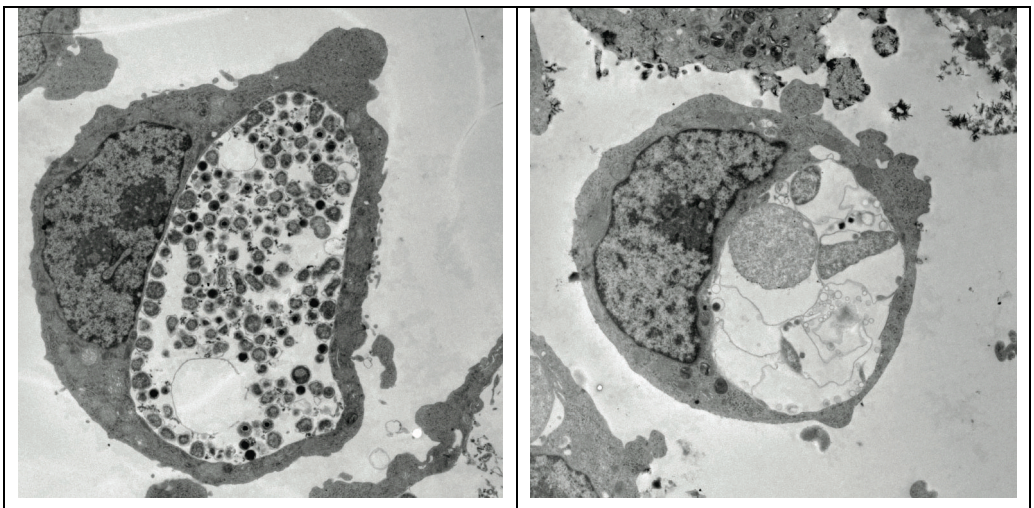

Figure 2. Infection of enteroendocrine cells from cell line LCC-18 with *Chlamydia trachomatis*. A: Active infection. B: Persistent infection.

4.2.3 Real-time PCR

In order to analyze the infection of EEC with *C.trachomatis* at the molecular level, we investigated the expression of selected target genes using real time PCR. Genes coding for EEC protein markers (CHGA, VMAT1, mGluR4, TPH1, TRPA1), house-keeping proteins (TFCP2, GAPDH), enviromental stress marker (HSPB1) as well as TLR4,

which is believed to mediate the main line of response upon *Chlamydia* infection, where subjected to PCR analysis. Transcriptomes of *C. trachomatis* infected EEC (LCC-18 and CNDT-2) were analysed after 24h. The same time-point was investigated after induction of persistence using penicillin G. We found significant down-regulation of VMAT1 expression in persistent infection compared to non-infected cells ($p<0.05$) and up-regulation of TLR4 expression in active and persistent infection ($p<0.05$). Expression of CHGA, the gene coding for CgA, as well as TPH1 and TRPA1, genes coding for proteins associated with serotonin synthesis and release, were not changed upon infection with *Chlamydia*. Gene expression changes were associated with infection and did not appear in cells exposed to heat-treated bacteria.

4.3 RESPONSE PATTERNS OF ENTEROENDOCRINE CELLS TO INTRACELLULAR BACTERIA *IN VITRO* (III)

4.3.1 Transmission electron microscopy

With TEM we confirmed *C. trachomatis* active and persistent infection in enteroendocrine cells both from colon and small bowel. Growth of *C. trachomatis,* manifested through inclusions containing both elementary bodies (EB) and reticulate bodies (RB) at 24h post infection could be observed for both cell lines. When EEC were treated with penG we observed enlarged, aberrant bacteria reminiscent of persistent growth forms.

4.3.2 Microarray

In order to screen host-cell gene regulation in response to *C.trachomatis* infection we used microarrays representing 30 000 human genes. We studied gene changes at the same time point (24 h post infection) in both active and persistent infection. Twenty-four hours after infection represents a period of active metabolism and binary fission of *C. trachomatis* reticulate bodies within the host cell.

4.3.2.1 Cell line specific responses to active and persistent infection

We identified 66 differently transcribed genes or expressed sequence tags (ESTs) (61 up-regulated) in active C*hlamydia* infected LCC-18 cells compared to non-infected and 411 (108 up-regulated) in persistently infected LCC-18 cells compared to non-infected cells. Twelve differently transcribed genes (11 up-regulated) were identical for both active and persistent infection of LCC-18 cells. DAVID Functional Annotation Tool recognized 4 clusters (apoptosis, transcription regulation activity, vesicle-mediated transport, intrinsic to membrane).

Similarly, we identified 68 differently transcribed genes or expressed sequence tags (ESTs) in actively infected CNDT-2 cells (58 up-regulated) and 170 differently transcribed genes or ESTs (120 up-regulated) in persistently infected CNDT-2 cells compared to non infected cells. Thirty-nine differently transcribed genes (all up-regulated) were identical for both active and persistent infection of CNDT-2 cells. Genes were grouped into 8 clusters (amine biosynthesis, apoptosis, intracellular

organelle lumen, plasma membrane, ion-binding, transcription regulator activity, intracellular non-membrane-bounded organelle, amino-acid transporter activity).

4.3.2.2 C. trachomatis active and persistent infection gene signatures

Host response to infection in two different enteroendocrine cell lines in active chlamydial infection seemed to be similar but not identical. Only 8 differently transcribed genes (6 up-regulated) were identical for both CNDT-2 and LCC-18 cell lines but all of differently transcribed genes were grouped in the same clusters. Clusters consisted of genes involved in apoptosis, negative regulation of cell communication, ion binding, cellular protein catabolic process, cell cycle, membrane-enclosed lumen, cytokine activity, and vesicle mediated transport.

Forty-four of differently transcribed genes (18 up-regulated and 26 down-regulated) were identical for both CNDT-2 and LCC-18 cells in persistent infection. Among them were genes involved in apoptosis, cytokine activity, growth factor activity, cell cycle, DNA metabolic process, intracellular organelle lumen, amine biosynthesis, transcription regulation activity, cell-cell signalling, regulation of phosphorylation, ion binding and plasma membrane. Remaining genes were involved in immune response, vesicle lumen, cytoplasmic vesicle, vesicle mediated transport, intrinsic to membrane and regulation of cell motion. Baculoviral IAP repeat-containing 3 (BIRC3) was the only gene that was up-regulated in the two cell lines during both active and persistent infection.

4.3.2.3 Selected genes of interest

Grouping of particular genes into specific functional classes is arbitrary to a certain degree, because some genes may be involved in several cellular functions and pathways. In order to study the cell-type specific response to infection, we decided to specifically look at the genes associated with transport and enteroendocrine function. We found significant differences in the gene transcription levels between persistently infected and non-infected cells in 10 genes coding for different solute carrier transporters (SLC).

Moreover, we found significant differences in expression of genes related to glutamate transport and synthesis. (SLC1A4, SLC7A11, argininosuccinate synthetase 1 (ASS1) and glutamate receptor interacting protein 1 (GRIP 1)) in persistently infected cells compared to non-infected. We identified 5 genes related to EEC function that were differently transcribed in persistent infection compared to non-infected cells: GABARAPL1, GRIP1, DRD2, SYT5 and SYT7.

We found many similarities but also many differences in response to infection probably due to significant differences in basal gene transcriptions (non-infected cells, data not shown) between the two studied lines. For example basal expressions of receptors for gamma-aminobutyric acid: (GABA)A receptor, alpha 1 (GABRA1), (GABA)A receptor, alpha 3 (GABRA3), (GABA)A receptor, beta 2 (GABRB2) and (GABA)B receptor 1 (GABBR1) were significantly lower in non-infected CNDT2 than in non-

infected LCC-18 and in contrast (GABA)A receptor-associated protein like 1 (GAPARAPL1) expression was higher in non-infected LCC-18 cells (data not shown).

4.4 SMALL BOWEL MUCOSA-ASSOCIATED MICROBIOTA IN PATIENTS WITH IBS AND HEALTHY CONTROLS (IV)

4.4.1 454-sequencing of small bowel mucosa-associated microbiota

4.4.1.1 Sequencing run

An average number of 3837 reads per sample was obtained after processing of the raw data from the pyrosequencing run. The total number of reads per sample ranged from 732 to 5870. After classification of the reads by comparison to the 16S database from RDP, an average of 235 reads per sample (approximately 6.3% of the total number of reads per sample) could not be assigned any taxonomic classification. Taxa with an average number of reads in the entire data set of less than 1 were excluded from further processing and visualization.

4.4.1.2 Characterisation of small bowel microbiota

We characterized small bowel microbiota at both phylum and taxon level. We found that 6 phyla (*Firmicutes*, *Proteobacteria*, *Bacteroidetes*, *Actinobacteria*, *Fusobacteria* and Candidate division TM7) dominated in the jejunum of both controls and patients with IBS (Figure 3).

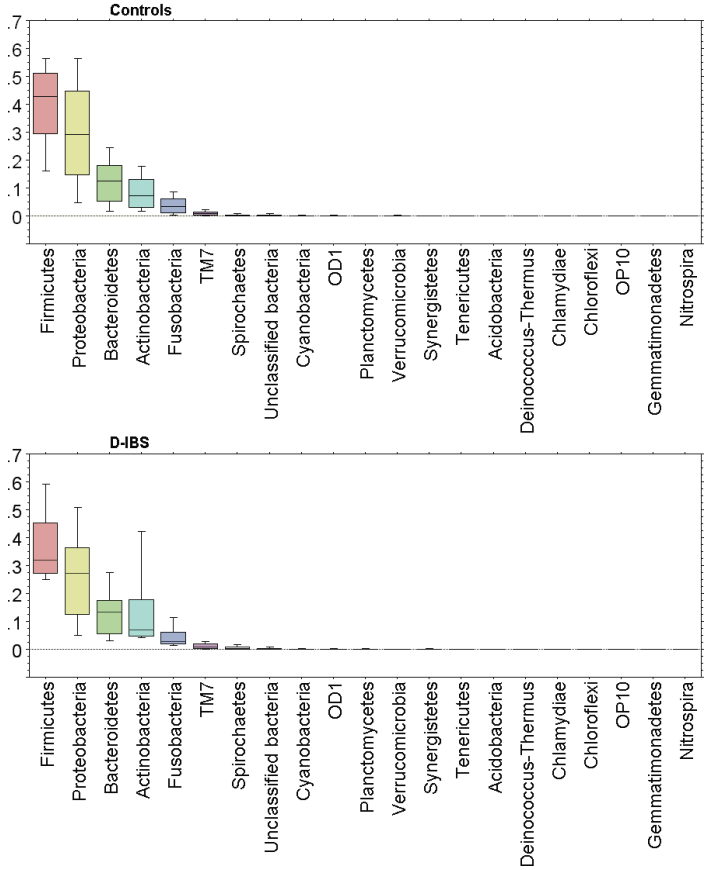

Figure 3 A. Prevalence of sequences belonging to different phyla among healthy controls and patients with diarrhoea-predominated irritable bowel syndrome (D-IBS).

4.4.1.3 Principal coordinates analysis

Principal Coordinates Analysis (PCoA) of all samples was performed in UniFrac (81). Clustering in UniFrac showed a rather tight cluster that contained all samples which indicating that samples were similar to one another. Controls and D-IBS cases were more dispersed, whereas C-IBS and M-IBS showed tendencies to forming their own two clusters in the middle of the main cluster. In conclusion, samples appeared to have a similar bacterial composition and did not form any distinct clusters and thus, do not indicate any clear differences between the four groups.

4.4.1.4 Investigation of significant differences

The three subgroups of IBS and all IBS cases were compared with the control group using the statistical tool "R" in search for significant differences between the controls and the IBS cases. No significant difference was found at either phylum or taxon level when comparing the controls to the other four groups. At genus level, several genera turned out to differ significantly between the different IBS groups and the controls. *Ochrobactrum* and *Escherichia/Shigella* were two of the genera differing significantly ($p < 0.01$) between the combined IBS group and controls but also between M-IBS and controls. However, the prevalences were very low for these genera (below 1%) and when plotting the abundance of them for each individual tested, it became clear that there was no consistent pattern of higher or lower prevalence in the different groups.

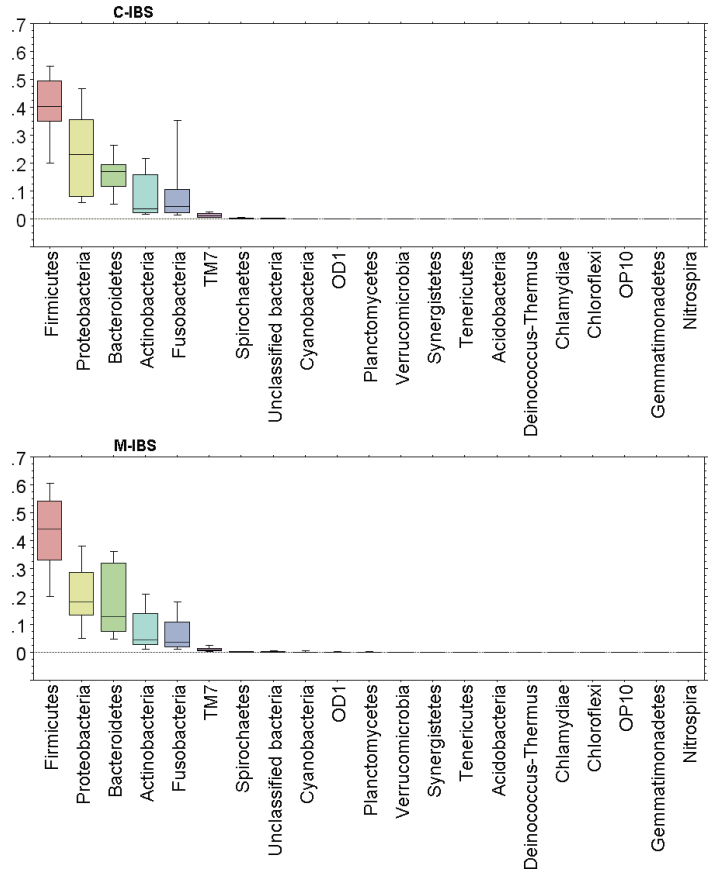

Figure 3 B. Prevalence of sequences belonging to different phyla among patients with constipation-predominated (C-IBS) and mixed-type (M-IBS) irritable bowel syndrome.

4.4.1.5 Bacterial prevalence patterns

MultiExperiment Viewer (MeV) is a tool for visualization of prevalence patterns and among other things also for clustering. The prevalence patterns for the characterized samples are all similar and a clustering of samples based on phyla abundance results in a random clustering with no distinct clusters based on the different groups. The clustering of samples based on taxa abundance indicated lower prevalence of unclassified *Enterobacteriaceae sp*. among IBS patients (C-IBS and M-IBS) than among controls, however the differences were not statistically significant.

4.4.1.6 Shannon diversity index

Shannon diversity indices were calculated for each of the studied groups using R. The variance was larger for the M-IBS group than for the other groups, showing a particularly a lower diversity index for some of the samples. However, the M-IBS group showed a larger variance for the diversity than the other groups where the sample with the lowest diversity index was much less diverse than the mean values of all other groups but was still not considered as an outlier (compared to the outlier in the control group). A lower number of pyrosequencing reads for these samples could be the cause of them showing low diversity indices (92).

5 DISCUSSION

The central part of this thesis was the investigation of the presence of *Chlamydia* antigens in small bowel biopsies in patients with a diagnosis of IBS. This has never been done before. The novel parts of immunofluorescence findings will be discussed briefly with the possible influence of *Chlamydia* infection on enteroendorine cells investigated *in vitro* in studies II and III followed by major findings in study IV.

5.1 *CHLAMYDIA TRACHOMATIS* ANTIGENS IN ENTEROENDOCRINE CELLS AND MACROPHAGES OF THE SMALL BOWEL IN PATIENTS WITH SEVERE IRRITABLE BOWEL SYNDROME

We report the novel finding of chlamydial antigens in enteroendocrine cells and macrophages of the small bowel mucosa in patients with severe IBS. LPS and MOMP antigens were detected in mucosa and l. propria of the small bowel in a large majority of patients with IBS, but rarely so in healthy controls. The odds ratio for mucosal *Chlamydia* LPS being indicative for presence of IBS is much higher than any previously described pathogenetic marker in IBS (31). These findings raise several questions:

5.1.1 Do observed immunofluorescence findings represent presence of bacterial antigens?

The specificity of the LPS antibody is important in this respect. There is a risk that antibodies may give rise to unspecific binding but such binding should not differ between patients and controls. We therefore think that unspecific binding is an unlikely explanation for our findings. It has previously been shown that this monoclonal anti-LPS antibody does not bind to environmental *Chlamydiae* (93, 94). The genus-specific LPS epitope is not shared by other known gram-negative bacteria and monoclonal antibodies do not bind LPS of those organisms (95). Thus, positive staining of LPS can be considered as a marker of past or present *Chlamydia* infection.

We used different techniques to visualize *Chlamydia* LPS. In addition to immunofluorescence with a FITC-conjugated monoclonal antibody, we used a polyclonal antibody to *Chlamydia* LPS and the standard streptavidin-biotin technique for light microscopy. The latter technique also showed presence of antigen in the same cell types. We used Western blot on new biopsies from a limited number of patients and confirmed that *Chlamydia* LPS was present in the tissue samples from these patients. These findings further support the hypothesis that observed antigens have a bacterial origin.

5.1.2 What is the identity of the species involved?

We used a genus-specific antibody to *Chlamydia* LPS and species-specific antibodies to *C. pneumoniae* and *C. trachomatis* MOMP. Since we found positive staining for *C.*

trachomatis MOMP in 79% of *Chlamydia* LPS-positive biopsies and none of them was positive for *C. pneumoniae* we hypothesized that the origin of these antigens was a past or present infection with *C. trachomatis*. Also, since we found positivity for *C. trachomatis* antigens using two different monoclonal antibodies, one for LPS and one for MOMP, cross-reaction with another, similar species, is unlikely but not fully excluded.

5.1.3 Do observed antigens represent an ongoing infection or not?

We investigated archived biopsy material from the small bowel and this puts certain limits on our ability to ascertain the underlying cause for observed findings as well as their specificity. In order to confirm and strengthen our immunohistochemical findings we used antibodies to different antigens and also different methods of visualisation. In a limited number of cases we had the opportunity to take new biopsies for more advanced analyses using molecular biology techniques, proteomics and electron microscopy. Whereas electron microscopy showed presence of *Chlamydia*-like organisms in the cytoplasm of enteroendocrine cells, similar to those described in persistent *C. trachomatis* infection (96), we were unable to detect chlamydial DNA in a small number of patients using standard extraction and amplification protocols. It is possible that the standard methods we used for nucleic acid retrieval were inadequate for the detection of a persistent infection (97). Another explanation for the contradictory results could be that chlamydial antigens were remainders of a past, but no longer present infection (98). The latter seems unlikely in light of the long-term presence of *C. trachomatis* antigens observed in several of our patients. Long-term presence of antigens is more likely to be attributable to replicating *Chlamydiae* residing in the diseased tissue (99). At present, however, we cannot determine if patients with severe IBS have an ongoing intestinal infection with *C. trachomatis* or not. Neither can we explain the presence of inflammation in myenteric ganglia or neuropathy by the finding of chlamydial antigens in enteroendocrine cells.

5.2 IN VITRO INTERACTION BETWEEN EEC AND *CHLAMYDIA TRACHOMATIS*

Enteroendocrine cells play a pivotal role in the control of gut motility and secretion and increased numbers of enteroendocrine cells have been detected in patients that developed IBS after an acute gastroenteritis (100). It is unknown if infection alters the function of enteroendocrine cells in man but animal experiments using different models of enteric infection have shown pronounced changes in both numbers of enteroendocrine cells and their function (101, 102). Serotonin-producing enteroendocrine cells may present an ideal location for *Chlamydia* due to their abundance of tryptophan. Tryptophan is required for normal development in *Chlamydia* species and tryptophan metabolism has been implicated in *Chlamydia* persistence and tissue tropism (103).

Studies II and III showed that it is possible to infect EEC from both small bowel (CNDT-2 cell line) and large bowel (LCC-18 cell line) with *C. trachomatis*, which are able to grow and multiply within EEC. Furthermore, in both cell lines a state of persistent infection with *C. trachomatis* could be induced. We think the latter is more important since it may mimic the situation in patients with IBS.

Study II is the first to show that the presence of *C. trachomatis* alters the cellular distributions of serotonin and CgA in vitro. Both serotonin and CgA are important for immune activation and gut inflammation *in vivo* and several serotonergic receptors have been characterized in lymphocytes, monocytes, macrophages and dendritic cells (104). Serotonin has been shown to activate immune cells, which are responsible for the production of proinflammatory mediators. Consequently, a manipulation of the serotonin system could possibly modulate responses to gut inflammation (105). CgA on the other hand has antimicrobial activity (106) and exhibits both proinflammatory and anti-inflammatory functions (105).

Altered protein distributions together with down-regulation of the vesicular monoamine transporter VMAT1 suggest bacterial influence on vesicular transport since the expressions of genes associated with serotonin synthesis (TPH1) and release (TRPA1) were not impaired. At this stage, it is not clear whether the altered serotonin and CgA distribution in *C. trachomatis* infected EEC is induced by the bacteria themselves or is part of the innate immunity response via TLR4 up-regulation. Kidd et al. (107) reported increased LPS-induced serotonin secretion in EEC derived from patients with Crohn's disease. TLR4 stimulation with its agonist LPS also caused the release of human β-defensin-2 (HBD-2) from EEC (51) and elevated HBD-2 levels have recently been found in patients with IBS (108).

Study III is the first to describe *Chlamydia*-induced transcriptome changes in enteroendocrine cells. Studying cell-type specific response to infection we found significant differences in the gene transcription levels between persistently infected and non-infected cells in 10 genes coding for different solute carrier transporters (SLC) and we identified 5 genes related to endocrine function that were differently transcribed in persistent infection compared to non- infected cells: GABAA receptor-associated protein like 1 (GABARAPL1), glutamate receptor interacting protein 1 (GRIP1), D2 subtype of the dopamine receptor (DRD2), synaptotagmin 5 (SYT5) and synaptotagmin 7 (SYT7). Transporters are the gatekeepers for all cells and organelles, controlling uptake and efflux of crucial compounds such as sugars, amino acids, nucleotides, inorganic ions and drugs. The SLC family include genes encoding passive transporters, ion coupled transporters and exchangers (109).

Although the majority of investigators in this area have focused on alterations to 5-HT signalling during GI inflammation study III shows the impact of *Chlamydia* infection on genes encoded proteins associated with neurotransmitters (GABARAPL1, GRIP, DRD2) and genes encoding synaptotagmins (proteins responsible for neurotransmitters exocytosis from vesicles) (110). EEC, being mucosal sensory transducers, are the part

of enteric nervous system (ENS) together with afferent neurons, interneurons and motor neurons (52). Obtained results support the thesis that inflammatory effects on the ENS contribute to altered function during GI inflammation. It appears that changes in enteroendocrine cells and the effectors of their neuroactive substances are adaptive responses to inflammatory insults. Studies II and III reveal potentially important *Chlamydia*-induced alterations in EEC signalling.

5.3 SMALL BOWEL MICROBIOTA

Several studies have indicated that colonic microbial flora may exhibit important differences between patients with certain subtypes of IBS and healthy controls. Little is known about the microbial flora of the small bowel. In Study IV we used pyrotag sequencing in order to deeply explore and compare the composition of small bowel mucosa-associated microbiota between patients with IBS and healthy controls, something that has never been done before. We detected representatives of several genera with a predominance of Firmicutes. On the taxon level *Streptococcus spp*., *Veillonella spp.*, *Prevotella spp*., unclassified *Pasteurellaceae spp*., *Rothia spp*., *Haemophilus spp*. and unclassified *Enterobacteriaceae spp*. were the dominating species. 454-barcoded pyrosequencing is a very powerful method to explore the diversity within human gut ecosystems but it also has some limitations. One of them is the inability to distinguish between live and dead bacteria. Using jejunal biopsies instead of jejunal fluid we analyzed mucosa associated bacteria, thus diminishing the possibility of sequencing dead bacteria passing gastrointestinal tract. The mucosa-associated bacteria are of particular interest since they, generally speaking, are more likely to have a direct effect on the host through the mucosal layer than the bacteria only passing through the intestinal tract.

We cannot exclude the contamination from oral or esophageal flora although the probability is very low. We rinsed Watson capsules in sterile NaCl-solution before opening them and our method for DNA extraction was designed to extract only mucosa-associated flora. However, we found similarities at both phylum and genus levels with previously described microbiota from the distal esophagus (111). The same 6 phyla (*Firmicutes, Bacteroidetes, Actinobacteria, Proteobacteria, Fusobacteria,* and Candidate division TM7) although differing in relative proportions were represented in both the distal esophagus and in our biopsies from the jejunum. *Streptococcus*, *Prevotella* and *Veillonella* were the most prevalent genera in both esophagus and jejunum.

We found bacteria belonging to the genus *Chlamydia* (*unclassified Parachlamydia*) in only 5 patients (data not shown). This is perhaps surprising in view of our previous studies but there are several explanations for a negative result:

i. *Chlamydia* is an obligate intracellular pathogen and in study I *Chlamydia* LPS was localised to EEC at the level of the crypts not at the villous surface of the mucosa;

ii. We used a method for DNA extraction that was designed to extract mucosa-associated flora, not intracellular bacteria; and
iii. Barcoded pyrosequencing is a method to study the diversity of microbiota, not to detect specific bacteria. The amount of *Chlamydia* DNA available for extraction and 454-sequencing may have been below the detection level of the method.

We found no major difference in small bowel microbiota between patients with IBS and healthy controls. Subject-to-subject variation was large among both patients and controls. Our results correlate with some previous studies describing faecal microbiota in IBS patients and healthy controls (112-114). Our study does not support a role of SIBO in IBS. The relationship between SIBO and IBS is highly inconsistent among studies (115). In a recently published study Yu et al. (116) demonstrated that breath testing detects oro-caecal transit, not small intestinal bacterial overgrowth in patients with IBS.

From an ecological perspective, abnormal variation likely reflects a loss of homeostasis, in which the microbial community is unable to maintain its normal structure. A high degree of variation is typical for disturbed and re-establishing communities undergoing succession, while the loss of variation is usually associated with a loss of diversity and outgrowth of certain taxa. Our results suggest that the luminal flora is probably not a host factor for the development of IBS.

6 CONCLUSIONS

Study I introduced the novel concept of a bacterial aetiology in cases of severe IBS. Our results suggest that *C. trachomatis* may be involved in the pathogenesis in IBS.

The findings from Study II and Study III support the hypothesis that *C. trachomatis* may have a role in IBS by showing *in vitro* that enteroendocrine cells from both the small bowel and the large bowel can be infected by *C. trachomatis*. In both cell lines a state of persistent infection with *C. trachomatis* can be induced.

Studies II and III give new insights into the cellular events that take place during *Chlamydia* infection of enteroendocrine cells. The results of Study II and Study III provide *in vitro* evidence that an infection of enteroendocrine cells *in vivo* can lead to disturbances in the regulation of the gut function.

In Study IV we found no major difference in small bowel microbiota between patients with IBS and healthy controls, and this suggests that the luminal flora is probably not a host factor for the development of IBS.

7 SAMMANFATTNING PÅ SVENSKA

Syftet med de arbeten som redovisas i denna avhandling var att ta reda på om bakterier har någon betydelse för uppkomsten av irritabel tarm (eng. IBS = irritable bowel syndrome). Irritabel tarm är en av våra största folksjukdomar och man räknar med att ungefär 15 % av befolkningen har någon variant av denna sjukdom. Orsaken eller orsakerna till IBS är inte kända men sjukdomen har länge betraktats som psykosomatisk. Modern forskning har emellertid visat att patienter med IBS har störningar i såväl de immunologiska som de enteroendokrina reglersystemen i tarmen. Det immunologiska systemet reglerar vårt försvar mot olika mikroorganismer samtidigt som det tillåter närvaron av stora mängder bakterier, virus och parasiter i vår mag-tarmkanal. Det enteroendokrina systemet utgörs av ett stort antal celler i mag-tarmkanalens slemhinna som känner av vad vi har ätit och som genom utsöndring av särskilda signalsubstanser kan styra tarmens motorik och enzymsekretion så att matsmältning och näringsupptag blir så effektivt som möjligt. Vävnadsprover från tunntarmen undersöktes med hjälp av immunofluorescens avseende förekomsten av klamydia-specifik lipopolysackarid (LPS) hos 65 patienter med IBS och 42 friska försökspersoner. LPS påvisades i enteroendokrina celler och/eller makrofager hos 58/65 (89 %) patienter men endast 6/42 (14 %) kontroller. Ytterligare undersökning visade att 79 % av de LPS-positiva proverna också var positiva i immunfärgning för membranproteinet MOMP från bakterien *Chlamydia trachomatis*. Dessa fynd indikerade att en persistent infektion med *C. trachomatis* i tunntarmen kunde ha betydelse för uppkomsten av IBS. Fyndet av bakteriella antigener i enteroendokrina celler talade för en tidigare icke känd celltropism för *C. trachomatis*. För att validera detta fynd genomfördes en serie *in vitro* experiment med enteroendokrina cellinjer. Två olika cellinjer, LCC-18, från en endokrin colontumör, och CNDT-2 från en tunntarmscarcinoid studerades före och efter infektion med *C. trachomatis*. Båda cellinjerna kunde infekteras med *C. trachomatis* och persistent infektion kunde induceras med hjälp av penicillin G. Infektionen ledde till en tydlig omfördelning intracellulärt av proteinet chromogranin A och signalsubstansen serotonin så att dessa i infekterade celler huvudsakligen återfanns i de bakteriella inklusionerna i stället för i cytoplasman. Genuttrycket för en lång rad gener uppreglerades eller nedreglerades i infekterade celler. Bland celltypsspecifika förändringar av genuttryck märktes signifikant förändring av genuttrycket för 10 transportproteiner inom gruppen SLC (solute carrier transporters) och 5 gener som har med cellernas endokrina funktion att göra (GABARAPL1, GRIP1, DRD2, SYT5 and SYT7). Slutligen studerades den mucosa-associerade bakteriefloran in tunntarmens övre del hos patienter med IBS och friska kontroller. Florans sammansättning analyserades med 454-pyrosekvensiering. Inga betydande skillnader hittades mellan patienter och kontroller. Sammanfattningsvis pekar denna avhandling på möjligheten att IBS orsakas av en persistent klamydia-infektion i tarmen. Studier av cellinjer visar att sådan infektion leder till uttalade förändringar i enteroendokrin funktion som kan tänkas vara en del av förklaringen till många av de symtom patienter med IBS lider av. Den luminala floran däremot förefaller inte vara av betydelse för uppkomsten av IBS.

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