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INVESTIGATIONS INTO THE ROLE OF MAST CELL MEDIATED EFFECTS ON THE INTESTINAL EPITHELIAL BARRIER *IN VITRO*: IMPLICATIONS FOR THE PATHOGENESIS OF IRRITABLE BOWEL SYNDROME

by

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A thesis submitted to the University of Dublin, Trinity College
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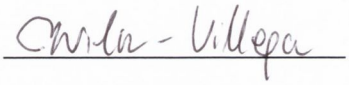

ACKNOWLEDGEMENTS

I would like to sincerely thank my supervisor Professor Maria O'Sullivan for this opportunity to do my PhD in her research group. I am very grateful for her guidance, her constructive suggestions, her enthusiasm and optimism, and her patience throughout my PhD. I am deeply grateful for her help on developing my career, for giving me the opportunity to attend several conferences and workshops, and that she could find time for me even though she was very busy.

I am also extremely grateful to my PI Dr Siobhán McClean for supervising my research at ITT. I am thankful for all her guidance, her constructive suggestions, her friendly approach and her very quick responses to my e-mails.

I would like to thank Professor Joseph H. Butterfield (Mayo Clinic) for the generous gift of the HMC-1 cell line and for his advices on troubleshooting. I would like to thank Lorraine Coleman (ITT) for teaching me methodologies on confocal microscopy; it was a pleasure to work with her. I thank James Reilly (ITT) for his assistance with statistical analysis. I wish to thank Professor Dimitri Scholz and Dr Cormac O 'Connell (UCD) for preparing the transmission electron microscopy images. I thank Professor Stephan C. Bischoff and Dr Axel Lorenz (Hohenheim University) for the great opportunity to visit their laboratory as a part of European COST action programme.

I acknowledge the support of the Irish Higher Education Authority Program for Research in Third Level Institutes (PRTLI) Cycle 4 funding 'Centre of Applied Sciences for Health' CASH consortium'.

I would also like to thank my colleagues for the nice welcome, the sharing of ideas and the invigorating atmosphere in the lab; especially to Ruth Pilkington,

Kevin Keane, Lydia Fabunmi, Agnieszka Foltyn Arfa-Kia, Cormac Walsh, David Hughes, Dariusz Karcz and Tara Raftery.

Special thanks to my family. Thank you to my sister for her support and for her endless faith in me. Thank you to my mother for all the support she gave me, which made this possible. Thank you to my father and my grandmother. Thank you for inspiring me.... Thanks to my aunt and uncle for their encouragement.

I dedicate this thesis to my husband for his support, his never ending patience, for making my days happier and for all the time spent listening about my research, even though it was not so interesting for him. And also thanks for fixing my computer.

God bless you all.

SUMMARY

Irritable bowel syndrome (IBS) is a highly prevalent functional bowel disorder characterised by abdominal pain or discomfort and associated with altered bowel habit, abdominal bloating and disturbed defecation. The aetiology of IBS is multifactorial. Increasing evidence has implicated impaired barrier function and immune activation in this condition.

The aim of this thesis was to investigate mechanisms by which the increased mast cell activity documented in IBS may alter intestinal permeability and junctional proteins *in vitro*, using cell culture models, and to explore translation to IBS.

This thesis investigated the effect of: 1) tryptase on Caco-2 monolayers 2) mast cell stimulation with compound 48/80 in a novel Caco-2/Human Mast Cell-1 (HMC-1) co-culture model 3) mast cell stimulation +/- tryptase inhibition with nafamostat mesilate (NM). Epithelial integrity was assessed by transepithelial resistance (TER), permeability to fluorescein isothiocyanate (FITC)-dextran and transmission electron microscopy (TEM). The expression of tight junction (TJ) proteins zonula occludens (ZO)-1, junctional adhesion molecule (JAM)-A, claudin (CLD)-1, CLD-2, CLD-3, occludin and adherens junction protein E-cadherin was determined by western blotting and immunofluorescence confocal microscopy. Based on the *in vitro* results, the thesis further assessed JAM-A, ZO-1, CLD-1 and E-cadherin protein expression in caecal biopsy tissue from 34 IBS patients comprising both alternating (IBS-A) and diarrhoea predominant (IBS-D) subtypes, 12 controls and 8 inflammatory bowel disease (IBD) patients using immunofluorescence confocal microscopy, and explored associations with IBS symptoms. Protein expression in the surface epithelium was the primary outcome, whereas expression in the crypt epithelium the secondary one.

Tryptase disrupted epithelial integrity in Caco-2 monolayers as shown by a significant decrease in TER, an increase in permeability to FITC-dextran and a decrease in the expression of JAM-A, CLD-1 and ZO-1 proteins within 24 hours. Correspondingly, in the Caco-2/HMC-1 co-culture model mast cell stimulation with compound 48/80 induced a significant decrease in TER, an increase in permeability to FITC-dextran, ultrastructural abnormalities in TJ regions (dilated TJs, TEM) a decrease in JAM-A and CLD-1 expression within 24 hours following stimulation. Tryptase inhibition (NM) significantly reduced the effect of compound 48/80-stimulated mast cells on JAM-A expression, TER and FITC-dextran flux. In IBS, JAM-A, ZO-1 and E-cadherin expression was significantly reduced in the surface epithelium of the caecal mucosa of IBS patients compared to controls, whereas for CLD-1 there was a trend towards reduction. In the crypt epithelium, only JAM-A expression was significantly reduced in IBS patients compared to controls. Significantly lower JAM-A was also found in IBD patients compared to controls. In the IBS subgroup analyses, JAM-A and ZO-1 expression was significantly reduced in both IBS-D and IBS-A patients, whereas E-cadherin was reduced only in IBS-A patients. A trend for reduced levels of E-cadherin and CLD-1 was reported in the IBS-D subgroup. Lower JAM-A expression and E-cadherin was associated with more severe abdominal pain and longer duration of symptoms in IBS patients.

The findings of this thesis are novel - they provide insights to intestinal permeability, junctional proteins and mast cells in IBS. Reduced expression of junctional proteins appeared to underlie mechanisms of altered intestinal epithelial permeability either in response to tryptase or to compound 48/80-stimulated mast cells *in vitro.* In IBS, the expression of junctional proteins was reduced in caecal tissue and was associated with IBS symptoms. This is the first reporting of reduced JAM-A and E-cadherin protein expression in IBS tissue and of a corresponding association with IBS symptoms. These findings may improve understanding of IBS pathogenesis and therapeutic strategies in IBS.

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ABBREVIATIONS

5-HT	5-hydroxytryptamine, serotonin
AJ	Adherens junction
AJC	Apical junctional complex
Anova	Analysis of variance
APS	Ammonium persulfate
BSA	Bovine serum albumin
Caco-2	Colorectal adenocarcinoma cell line
CD34+, 3+, 8+, 4+, 69, 25	Cluster of differentiation
CD	Crohn disease
CDAI	Crohn's Disease Activity Index
CLD	Claudin
CRF	Corticotrophin releasing factor
CRF-R	Corticotrophin releasing factor receptor
CS	Calf serum
CTMC	Connective tissue mast cells
DAPI	4',6-diamidino-2-phenylindole
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DSS	Dextran sulfate sodium
ECACC	European Collection of Animal Cell Culture
EDTA	Ethylenediaminetetraacetic acid
F-actin	Filamentous actin

FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GUK	Guanylase kinase
HADS	Hospital anxiety and depression scale
HBSS	Hank's Balanced Salt Solution
HEPES	4-(2-Hydroxyethyl)piperazine-1-ethanosulfonic acid
HMC-1	Human mast cell line-1
HLA	Human leukocyte antigen
HPA axis	Hypothalamic-pituitary-adrenal axis
HR	Histamine receptor
HRP	Horseradish peroxidase
IBD	Inflammatory bowel disease
IBS	Irritable bowel syndrome
IBS-A	Irritable bowel syndrome-alternating
IBS-C	Irritable bowel syndrome-constipation
IBS-D	Irritable bowel syndrome-diarrhoea
IBS-M	Irritable bowel syndrome-mixed
IBS-SSS	Irritable bowel syndrome Severity Scoring System
IBS-U	Irritable bowel syndrome-unclassified
ICAM	Intercellular adhesion molecule
IFN	Interferon
IL	Interleukin
IP	Intestinal permeability
JAM	Junctional adhesion molecule

J-T test	Jonckheere-Terpstra test
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
MC	Mast cell
MC_C	Mast cell expressing chymase
MC_T	Mast cells expressing tryptase
MC_{TC}	Mast cells expressing tryptase, chymase and carboxypeptidase A
MDCK	Madine-Darby canine kidney cell line
MHC	Major histocompatibility complex
MLC	Myosin light chain
MLCK	Myosin light chain kinase
MMC	Mucosal mast cells
mMCP	Mouse mast cell protease
mTMT	Mouse transmembrane tryptase
NFκB	Nuclear factor κB
NGF	Nerve growth factor
NM	Nafamostat mesilate
NOD	Nucleotide-binding oligomerisation domain
NSF	Soluble N-ethylmaleimide-sensitive factor
Papp	Apparent permeability coefficient
PAR	Proteinase-activated receptor
PBS	Phosphate buffered saline
PDZ	Postsynaptic density -95/Drosophila disc large/ zonula occludens-1
PEG	Polyethylene glycol

PI-IBS	Post-infectious irritable bowel syndrome
PKC	Protein kinase C
PLC	Phospholipase C
RIPA	Radioimmunoprecipitation
RT	Room temperature
SDS	Sodium dodecyl sulfate
SEM	Standard error of the mean
SERT	Serotonin reuptake transporter
SGLT1	Sodium glucose transporter 1
SIBO	Small intestinal bacterial overgrowth
SNARE	Soluble NSF attachment protein receptor
SNP	Single nucleotide polymorphism
TBS	Tris-buffered saline
TEM	Transmission electron microscopy
TEMED	N,N,N′,N′-Tetramethylethylenediamine
TER	Transepithelial resistance
TGF	Transforming growth factor
TJ	Tight junction
TLR	Toll-like receptor
TMT	Transmembrane tryptase
TNF	Tumour necrosis factor
UC	Ulcerative colitis
UTR	3′- untranslated region
ZO	Zonula occludens

CHAPTER 1

INTRODUCTION

1.1. The gastrointestinal barrier: an overview of intestinal permeability, integrity and mast cells

1.1.1. Gastrointestinal barrier physiology and function

The gastrointestinal tract, from the lower oesophageal sphincter to the anus, contains a continuous epithelial cell layer forming a barrier between the outside world and the internal human body. The separation of the body from a variety of environmental agents is important, as they might initiate or perpetuate mucosal inflammation if they cross the epithelial barrier. Thus, the epithelial barrier must act as a selective filter allowing the passage of solutes that are beneficial to the host, such as dietary nutrients and electrolytes, while preventing the passage of harmful entities such as bacterial toxins or pathogens – a concept known as intestinal barrier function (1-3). Several mechanisms have evolved to protect the host against luminal threats including immune (epithelial and immune cells) and non-immune components (mucus layer, water secretion). The continuous interactions between these components maintain gut homeostasis whereas defects in any of them lead to an impaired mucosal barrier (4). The importance of epithelial barrier function was clearly demonstrated in the experiments performed in 1993 by Yamada *et al.* (5) where cell wall products of luminal bacteria were injected into the colonic wall of rats. Placing the bacterial products directly to the colonic wall, thereby bypassing the epithelial barrier, induced an inflammatory condition resembling inflammatory bowel disease (IBD). These experiments showed that abrogating epithelial barrier function may initiate inflammatory disease.

At the same time, the intestinal barrier allows small amounts of antigen to penetrate the intestinal mucosa to interact with the immune system, a

phenomenon that plays a key role in the induction of immune tolerance. The key responsibility for transport of molecules from the intestinal lumen to the lamina propria resides within the plasma membrane of epithelial cells. Since this membrane consists of a phospholipid bilayer, it is permeable to lipid-soluble substances. In contrast, most hydrophilic particles must be transferred in the presence of energy-dependent transporters via the transcellular pathway (2, 6, 7) or passively through the paracellular pathway (<600 Da) governed by multi-protein complexes called apical junctional complexes (AJCs) (Figure 1.1).

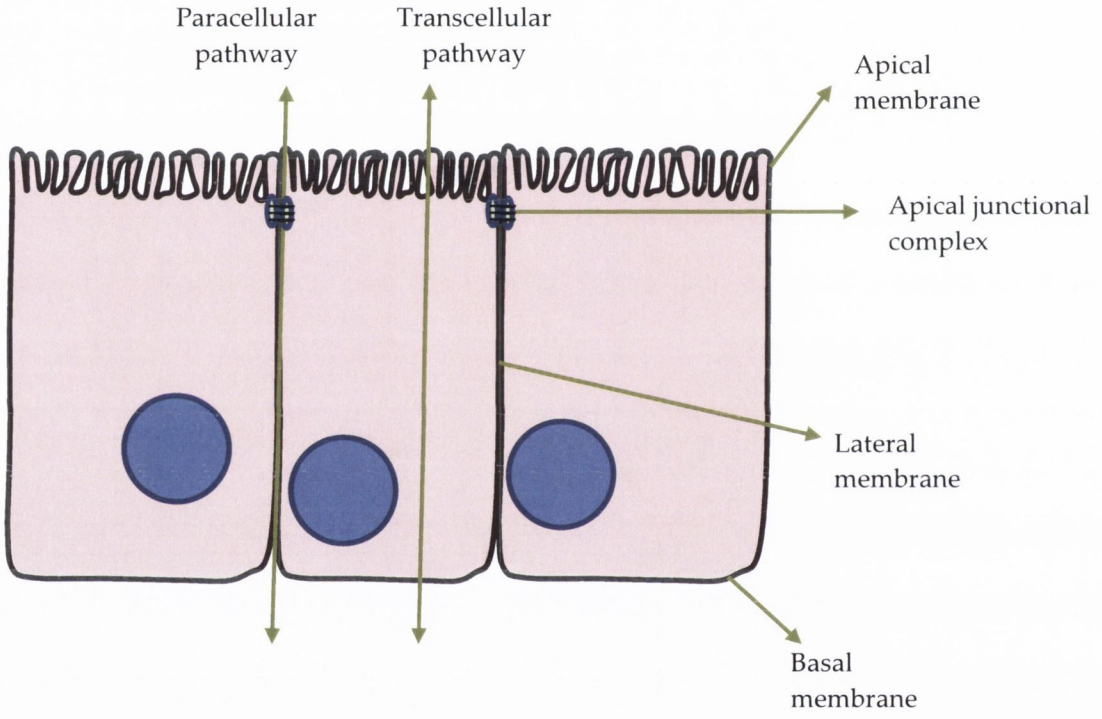


Figure 1.1 Pathways of epithelial permeability.

1.1.1.1. Cellular components of the intestinal barrier

There is an array of cells composing the intestinal barrier. The epithelium consists of absorptive enterocytes, mucus-producing goblet cells, Paneth cells of crypts which secrete antimicrobial peptides, and enteroendocrine cells which produce peptide hormones (8). Beneath the epithelial lining, in the gut lamina propria,

there are endothelial cells, myofibroblasts, the enteric nervous system and endocrine system, and plethora of innate and adaptive immune cells (e.g. mast cells, lymphocytes, macrophages, dendritic cells and eosinophils) that interact to maintain a balanced immune response against intestinal antigens (3, 9). Epithelial cells and immune cells in the lamina propria express a number of receptors that recognise conserved microbial molecules such as intracellular nucleotide-binding oligomerisation domain (NOD)-like receptors and the toll-like receptors (TLRs) (10). In health, TLRs protect the intestinal epithelial barrier and provide commensal tolerance, whereas NODs regulate antimicrobial activities. In the case of injury or infection, the activation of epithelial TLRs and NODs stimulates epithelial cells to release a number of pro-inflammatory mediators. These activate and recruit innate immune cells (11) which generate an antigen specific response and finally resolve to heal wounds and prevent chronic inflammation. However, if the control of the intestinal barrier is impaired it can lead to the excessive entrance of luminal macromolecules and bacteria ("leaky gut") resulting in inflammation and infection or even abscess formation or fibrosis (9, 12). Possibly such barrier dysfunction could lead to inflammatory bowel diseases, coeliac disease, inflammatory reactions such as food allergy, and irritable bowel syndrome (IBS) (13). In addition, more substantial impairments could lead to more severe complications such as systemic inflammatory response syndrome, sepsis or multiple organ dysfunction syndrome (14).

1.1.1.2. Extracellular components of the intestinal barrier

Most intestinal epithelial cells are covered with a layer of mucins and lipids (mucus layer) that limits the exposure to sheer forces and other physical traumas from entities within the lumen. The mucus layer also prevents large particles, including bacteria, from directly contacting epithelial cells and colonising the gut (15). Moreover, this layer contributes to the retention of mucosal secretions rich in

antimicrobial peptides (e.g. defensins) and immunoglobulin (Ig)A to fight invading pathogens (16, 17). Additionally, the mucus layer limits fluid flow and contributes to the development of an unstirred fluid layer at the epithelial cell surface. In the small intestine, this unstirred fluid layer slows the absorption of nutrients by reducing the rate at which nutrients reach microvillus brush border rich in protein transporters and digestive enzymes. Microvilli are covered with an array of glycoproteins and glycolipids, i.e. glycocalyx, that limits pathogen colonisation (Figure 1.2) (18). In addition, intestinal peristalsis, together with ion and water secretion, allows the intestine to wash away noxious substances, preventing their adhesion to the intestinal walls and penetration to the lamina propria (4).

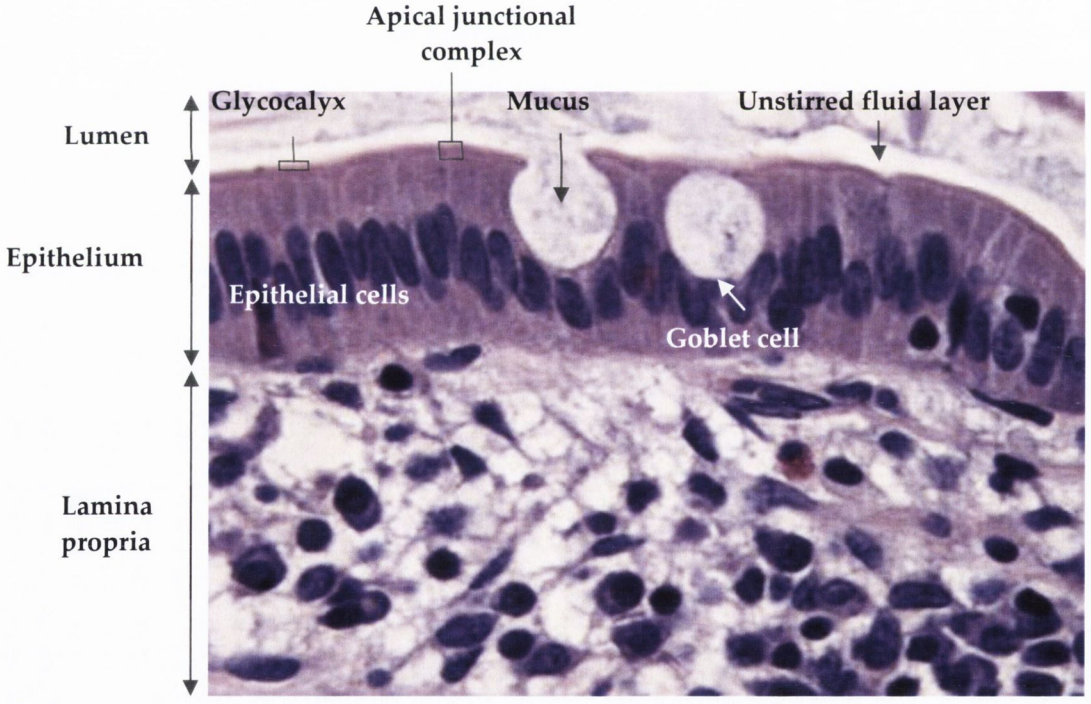


Figure 1.2 Anatomy of the human intestinal mucosa. The image was adapted from Turner (19).

1.1.2. AJC: structural characteristics and regulation of epithelial permeability

AJCs at intercellular contacts maintain the integrity of the intestinal epithelial barrier and regulate solute flux via the paracellular pathway. The paracellular pathway is typically more permeable than the transcellular pathway; thus, it is the rate-limiting step in transepithelial transport and the principal determinant of mucosal permeability. Therefore, recognition of the specific barrier properties of junctional complexes might help to understand mechanism underlying mucosal permeability.

AJCs are composed of tight junctions (TJs), adherens junctions (AJs) and desmosomes. TJs are typically located at the apical tip of the basolateral membrane of epithelial cells, whereas AJs and desmosomes are located below within the basolateral membrane. TJs and AJs are both connected to the actin cytoskeleton encircling the perimeter of the cell and these interactions regulate permeability properties of TJs (20). Desmosomes are linked to intermediate filaments but the role of these interactions in the gastrointestinal tract is unclear (Figure 1.3) (21).

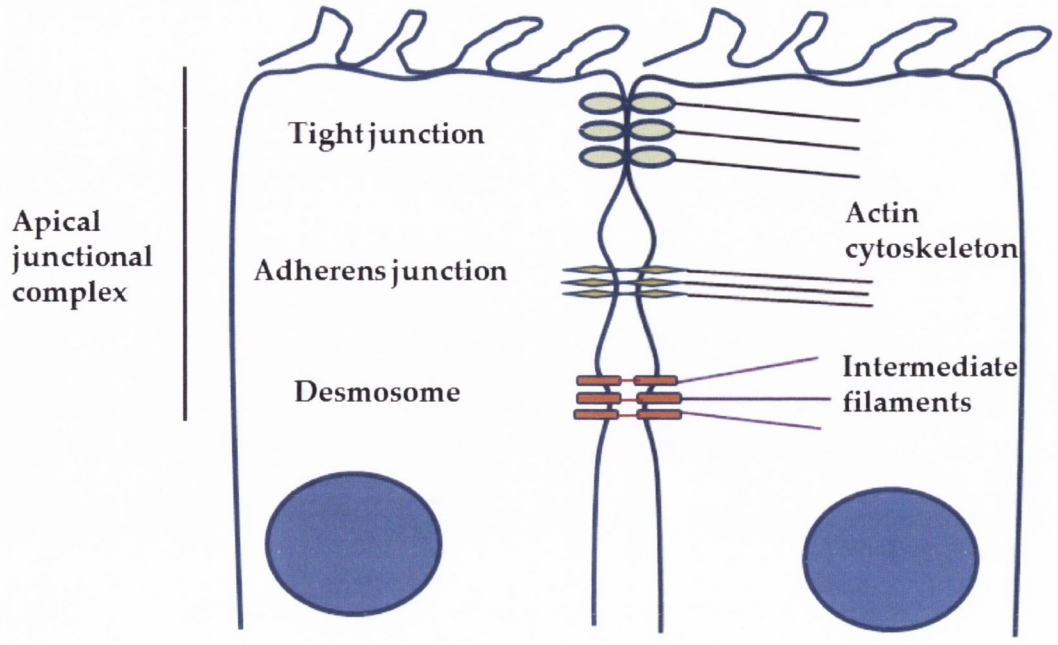


Figure 1.3 Adjacent epithelial cells held together via the apical junctional complex.

Tight junctions, also known as *zonula occludens (ZO)*, are the major constituents of the AJC and more than 50 TJ proteins have been identified to date. They serve as a gate for the paracellular pathway, regulating epithelial transport towards and away from the lumen (gate function), and as a fence that separates the plasma membrane into the apical and basolateral domains (fence function) (22). In addition to these classical gate and fence functions, more recently TJs emerged as dynamic signalling complexes which receive signals from the cell interior (e.g. via transcription factors) to regulate their assembly and function, and they send information back to the cell to regulate gene expression and subsequently proliferation and differentiation (23, 24). TJs also open and close in response to a variety of external stimuli (25) and the mechanisms underlying these will be explained in greater detail in Section 1.1.3.

Electron microscopy demonstrates that TJs appear as a network of fibrils/strands with focal contacts ("kisses") between the plasma membrane of neighbouring cells (26) that appear as 10 nm rows (27). Each fibril within the plasma membrane associates laterally with another fibril in the opposing membrane of the neighbouring cell to form paired TJ fibrils (28). The tightness of TJs is determined by the number of TJ fibrils, the quality of the fibrils and the complexity of the network pattern (29-31). These fibrils consist of multiple transmembrane proteins including occludin (65-85 kDa) (32, 33), members of claudin (CLD) protein family (~23 kDa) (34), members of junctional adhesion molecule (JAM) protein family (~40 kDa) (35) and tricellulin (~60 kDa) (36). Transmembrane proteins are connected to protein complexes called cytoplasmic plaques. These consist of peripheral (scaffolding) proteins including members of zonula occludens proteins (ZO) (130-225 kDa) (37, 38) and cingulin (~150 kDa) (39), and regulatory molecules such as kinases. Transmembrane proteins span the plasma membrane of epithelial cells. Claudins, occludin and tricellulin span the plasma membrane 4 times while members of the JAM protein family span only once. Several transmembrane proteins interact with the cytoplasmic plaque proteins (e.g. ZO-1) via postsynaptic density -95/Drosophila disc large/zonula occludens-1 (PDZ) domains. Cytoplasmic plaque proteins link transmembrane proteins to the cytoskeleton (Figure 1.4).

Occludin was the first transmembrane protein to be discovered in TJs; however, its role in barrier permeability seems to be secondary since occludin knockout mice appeared to have structurally normal TJs and intestinal barrier functions (40). Furthermore, occludin-deficient embryonic stem cells exhibited numbers and morphology of TJ fibrils comparable to controls (41). Possibly, occludin is involved in cell signalling rather than in the regulation of epithelial permeability (42). *Tricellulin*, which shares structural similarities with occludin, is primarily abundantly expressed at tricellular TJs and to a lesser extent at bicellular TJs (43).

It seems that tricellulin might compensate for occludin loss in TJs (44). In occludin knockout Madine-Darby canine kidney (MDCK) epithelial cells, tricellulin appeared to be redistributed to bicellular TJs (44). Another study reported that the loss of tricellulin expression resulted in a lower resistance and increased macromolecular paracellular permeability of mammary epithelial cells (36).

Claudins are thought to be both the main structural components of TJs and the principal regulators of epithelial paracellular permeability. In mammals, 27 members of the claudin family (CLD-1 to CLD-27) have been described to date (45). Claudins appear to determine, in a tissue-specific manner, properties of the epithelial barrier such as electrical resistance and paracellular ionic selectivity. They regulate permeability by forming unique paracellular channels; however, specific characteristics of individual claudins have just begun to be recognised (46, 47). For example, several *in vitro* studies showed that CLD-1 and CLD-3 decrease cation permeability and increase epithelial resistance, CLD-2 and CLD-10 create cation selective channels and a consecutive decrease in resistance, while CLD-11 and CLD-15 can either increase or decrease resistance depending on the presence of other claudins forming TJs (47).

JAM-A has been the most extensively studied member of the JAM protein family in the context of permeability and inflammation. JAM-A is expressed in several cell types, but it is particularly abundant at the intercellular contacts of endothelial and epithelial cells (48). At the endothelial sites JAM-A mediates leukocyte recruitment to sites of inflammation through the paracellular pathway (49-52), whereas at epithelial sites JAM-A is crucial for TJ assembly (35). *In vitro* studies have shown that silencing of JAM-A in intestinal epithelial cells increased paracellular permeability (53) and its inhibition perturbed the intestinal barrier recovery after epithelial injury (35). Furthermore, Laukoetter *et al.* (54) reported that JAM-A$^{-/-}$ mice exhibited increased permeability and decreased transepithelial

resistance (TER) of colonic mucosa. In a more recent study, Vetrano *et al.* (53) confirmed that JAM-A$^{-/-}$ mice had increased mucosal permeability and reported enhanced pro-inflammatory cytokine production in these mice.

The TJ transmembrane proteins occludin, claudins and JAMs bind with peripheral ZO proteins. For example, occludin binds to the region of homology to guanylase kinase (GUK) of ZO, claudins to PDZ1 domain, whereas JAM-A binds to PDZ3 domain. ZO proteins also interact directly with the actin cytoskeleton (55). These interactions are crucial to TJ assembly and the regulation of paracellular permeability. For example, suppression of both ZO-1 and ZO-2 expression has been shown to prevent claudin recruitment to TJs, the subsequent TJ assembly and the development of barrier function of mammary epithelial cells (56). ZO-1, occludin and perijunctional filamentous (F)-actin redistribution from TJs has been associated with decreased TER and increased paracellular permeability of intestinal epithelial cells (57).

Adherens junctions, also known as *zonula adherens*, are protein complexes on the lateral membrane located below TJs where they link adjacent cells. In AJs, plasma membranes of apposing cells are 15-20 nm distant apart (28). The major AJ proteins belong to the cadherins (calcium-dependent adhesion molecule), the catenins (-α, -β, -δ) and the nectins (-1, -2, -3, -4), and serve multiple roles including the regulation of cell-cell adhesion, epithelial migration and proliferation, and maintenance of cell polarity (58, 59) (Figure 1.4).

E-cadherin (120 kDa) is the principal mediator of cell adhesion between intestinal epithelial cells. This protein interacts with cadherins of neighbouring cells to promote cell-cell adhesion and associates to the cytoskeleton through *catenins* (~80-100 kDa) (59-64) containing the F-actin binding site (65). Disruption of E-cadherin in the transgenic/chimeric murine model was shown to weaken cell-cell

adhesion and was associated with impaired cell-cell contacts, followed by intestinal inflammation similar to IBD (66).

Desmosomes, also known as *maculae adherents*, are intercellular junctions located below AJs at the lateral cell surfaces and appear as punctuate "spot welds" which form adhesion between the epithelial cells. Desmosomes consist of components of at least 3 separate protein families: the catherins (e.g. desmoglein, desmocollin), the armadillo proteins (e.g. plakoglobin) and the plakins (e.g. desmoplakin) (Figure 1.4) (21). Desmosomes typically provide strength and resistance to mechanical stress by forming stable cell-cell contacts that are linked to keratin intermediate filaments (21). A recent study, however, reported that desmosome-keratin link was not required for cell-cell adhesion in the intestinal epithelium but appeared to determine proper shape and length of microvilli (67).

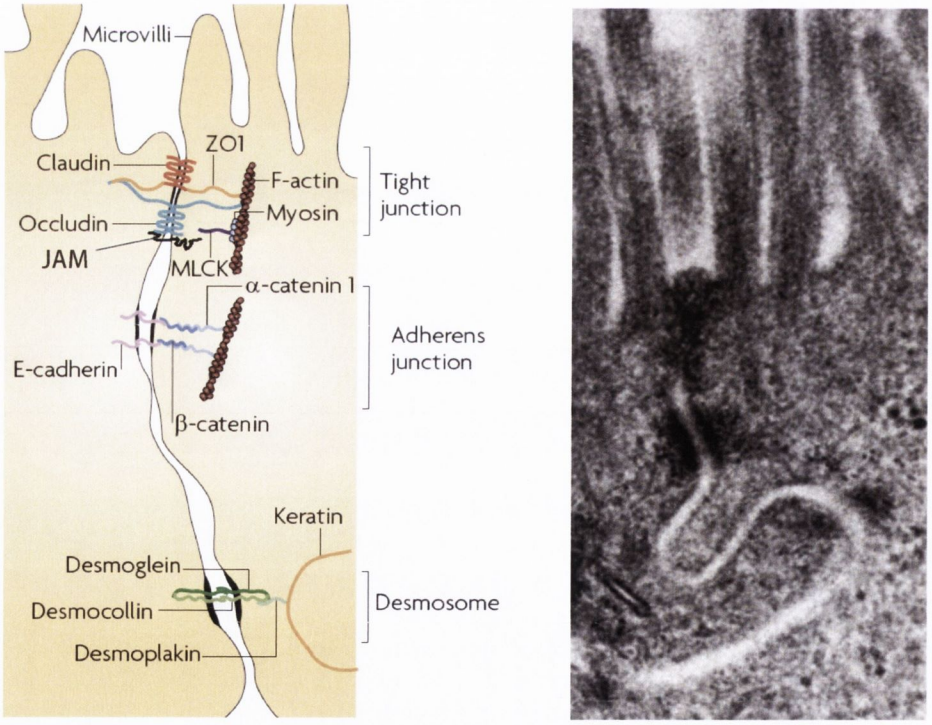


Figure 1.4 Apical junctional complex in epithelial cells. The tight junction and the adherens junction are connected to the actin cytoskeleton, while desmosomes to the keratin cytoskeleton. The image was adapted from Turner (19).

1.1.3. Factors regulating intestinal epithelial permeability

TJs are recognised as dynamic structures whose permeability can be regulated by physiological, pathological and pharmacological stimuli. These include dietary factors, humoral and neuronal signals, inflammatory mediators such as mast cell products, and a variety of cellular pathways that are susceptible of being usurped by microbial or viral pathogens (25). The regulation of intestinal permeability can occur via actomyosin cytoskeleton contractions, achieved via phosphorylation of the myosin light chain (MLC) by myosin light chain kinase (MLCK), and by endocytosis of TJ proteins. However, longstanding permeability disturbances involve transcriptional alterations of TJ proteins, epithelial cell apoptosis and epithelial structural alterations (4).

The best studied physiological example is the modulation of the paracellular pathway by transcellular absorptive processes. In this case, the activation of the apical sodium dependent glucose transporter (SGLT1) results in a reversible increase in intestinal paracellular permeability through actomyosin cytoskeleton contractions and subsequent TJ opening. This mechanism allows for the passive movement of nutrients and water to amplify nutrient absorption, particularly when high luminal nutrient concentrations exceed the SGLT1 capacity (19).

Intestinal epithelial permeability may also be modulated by pathogenic bacteria which found several ways to alter TJs, presumably to improve their growth conditions. An interesting example of this pathway is the so called zonulin pathway usurped by *Vibrio cholerae*. These bacteria produce a "zonula occludens toxin" which binds to an apical membrane receptor on enterocytes, activates the actomyosin cytoskeleton and subsequently increases permeability (68) .

Inflammatory mediators, including pro-inflammatory cytokines tumour necrosis factor (TNF)-α and interferon (IFN)-γ, may also increase intestinal epithelial permeability. The ability of TNF-α and IFN-γ to regulate the TJ barrier was described more than 20 years ago (69) and since then several mechanisms have been proposed to mediate cytokine-induced loss of barrier function, including endocytosis of TJ proteins, TJ protein degradation and cytoskeletal contractions (4). The increased levels of these pro-inflammatory cytokines are found in several diseases associated with the intestinal barrier dysfunction, including IBD and IBS (19, 70, 71).

1.1.4. Cellular mechanisms underlying gastrointestinal barrier: functions of selected immune cells

While epithelial cells are the primary cellular determinant of intestinal epithelial barrier, the properties of intestinal barrier depend on interactions between epithelial cells and immune cells such mast cells, T-cells, macrophages, eosinophils and dendritic cells. Immune cells in the gastrointestinal tract reside both within the epithelium and the lamina propria, and their migration towards gut lumen is regulated to maintain homeostasis and to induce tolerance towards food antigens and normal gut flora (4). However, immune cells have been also implicated in intestinal barrier dysfunction. In IBS, for example, increased numbers of immune cells, predominantly mast cells (described in Section 1.1.5) and T-cells, and their increased activation have been frequently reported (72).

T-cells, in general, comprise 3 populations of cells in the gut. There are T-cells associated with lymphoid tissues, T-cells distributed in the lamina propria, and intraepithelial lymphocytes (IELs) that are localised between intestinal epithelial cells and presumably constitute the first T-cells to encounter pathogens (73).

Mucosal T-cells, as components of gut adaptive immunity, have many functions. These cells activate other cells such as B-lymphocytes and macrophages, secrete cytokines and kill infected host cells (4). IELs also stimulate mucosal healing following acute injury (74). Increased numbers of T-cells have been reported in the gut mucosa of IBS patients compared to controls (4, 72). Furthermore, T-cells products such as pro-inflammatory cytokines (e.g. TNF-α, IFN-γ) and interleukins (e.g. IL-4, IL-13) have been implicated in the disruption of intestinal epithelial barrier function either *in vitro* or *in vivo* (4).

Macrophages and their precursors **monocytes** are cells of the innate immune system; they also help in the modulation of adaptive immunity (75). Upon tissue damage or infection, monocytes migrate to the tissue, where they differentiate into tissue macrophages (76). Gut macrophages are the largest population of mononuclear phagocytes in the body. They are found throughout the intestinal tract of mammals both in the mucosa and submucosa. However, they are found in the highest numbers in the lamina propria where they are located strategically in close proximity to luminal bacteria and antigenic stimuli (77). Macrophages regulate inflammatory responses to bacteria and antigens, trigger an acute inflammatory response to infectious stimuli, and scavenge dead cells and foreign debris (78). They mediate their functions by interacting directly with bacteria or host cells or through secretion of their products such as cytokines (79). Activated macrophages release pro-inflammatory cytokines such as TNF-α, IL-1β and IL-6, which recruit other inflammatory cells and also are involved in the intestinal barrier dysfunction (80-82). Macrophages appear to be involved in the pathophysiology of IBD (79, 83); they exhibit enhanced pro-inflammatory responses to infection (84). In IBS, their role is unclear (4). Macrophage levels were reported to be reduced in the rectal mucosa of IBS after a bout of gastrointestinal infection (85), whereas in the colonic mucosa of IBS their levels were comparable to controls (86).

Eosinophils are pro-inflammatory leukocytes that are implicated in the pathogenesis of numerous inflammatory processes, including infection, asthma and gastrointestinal diseases (4). In health, most of eosinophils reside in the gastrointestinal tract in the lamina propria of the stomach and intestine where they store mediators, but also, when activated, synthesise mediators *de novo*. These mediators include eosinophilic cationic protein, major basic protein, eosinophil protein X, eosinophil derived neuroendotoxin and eosinophil peroxidase (87). Eosinophils play multifunctional roles through these mediators. They cause tissue damage, insert pores into target cells and disrupt epithelial barrier function (4, 87). Interestingly, eotaxin, which is a chemotactic agent for eosinophils, was reported to be up-regulated in the serum of IBD patients compared to controls (88), and elevated eotaxin levels were associated with higher numbers of eosinophils in IBD tissue (89). Also, higher levels of eotaxin were observed in the gut lumen of IBS patients following corticotrophin releasing factor (CRF) injection. However, eosinophil levels in the majority of studies appear to be unaltered in IBS compared to controls (72, 86, 90-92).

Dendritic cells are one of the most important antigen presenting cells in the gut, where they play a pivotal role in the regulation of mucosal immune responses. Dendritic cells are scattered in the lamina propria to sample antigens from periphery and migrate to secondary lymphoid tissue where they promote immune responses by stimulation of T-cells (93, 94). These functions differ depending on location, state of maturation, and state of inflammation (95, 96). Interestingly, phenotypic and functional changes of lamina propria dendritic cells were identified in mice infected with *Trichinella spiralis* and increased numbers of dendritic cells were associated with the severity of intestinal inflammation after infection (97). Furthermore, alterations in the functions and phenotype of intestinal dendritic cells, and changes in cytokine production (e.g. IL-12, IL-6) appear to be involved in the pathophysiology of IBD (98, 99).

1.1.5. Mast cells in the gastrointestinal tract: regulators of mucosal and epithelial barrier functions

Mast cells (Figure 1.5) are tissue cells strategically located at the interface between the host and the external environment and abundantly found adjacent to blood and lymphatic vessels, and nerves (100, 101). Mast cells comprise 2-3% of cells within the lamina propria in the intestinal mucosa whereas in the submucosa their density is lower, accounting for 1% of the total cell population in healthy individuals (100). In the course of intestinal diseases mast cell numbers can be significantly increased up to 10-fold (13, 101). Mast cells have been implicated in the pathophysiology of a number of diseases including IBD, IBS, coeliac disease, systemic mastocytosis, and also food allergy (13, 102).

Human mast cells derive from pluripotential CD34+ hematopoietic progenitor cells in the bone marrow (103). Mast cells leave the bone marrow in an immature state and migrate as committed progenitors to peripheral tissues. In the tissue environment, mast cells can remain as a homeostatic pool or complete their maturation under the influence of growth factors, such as stem cell factor (SCF) and IL-4, which also regulate the development of mast-cell subtypes (13, 104). In humans, 3 types of mast cells (MCs) have been described based on their proteinase content: MC_{TC} (tryptase, chymase and carboxypeptidase A), MC_T (tryptase), MC_C (chymase). Most mast cells in the gastrointestinal mucosa represent the MC_T subtype, whereas the subtype MC_{TC} dominates in the intestinal submucosa (100). In mice, mast cells are divided into the mucosal mast cells (MMC) expressing predominantly chymase, and connective tissue mast cells (CTMC) expressing predominantly chymase but also tryptase and carboxypeptidase A (105). Through a constitutive release of chymase, mast cells appear to play a role in the maintenance of homeostatic intestinal barrier function, epithelial cell migration and architecture in mice (106).

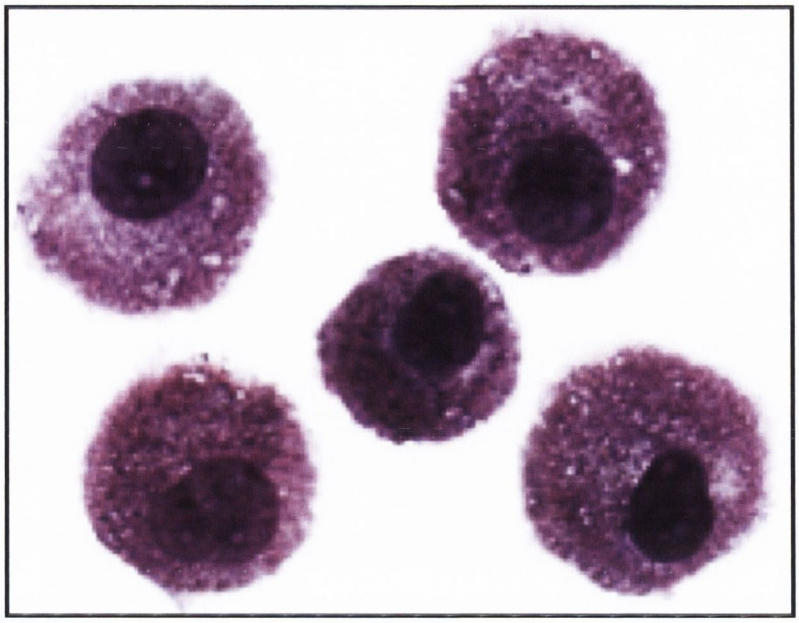


Figure 1.5 Mast cells isolated from human intestinal tissue (13).

A striking morphological feature of mast cells is the presence of numerous cytoplasmic granules with an array of mediators inside. These mediators can be categorised into 3 main groups. The first group comprises preformed mediators stored in secretory granules such as proteases (e.g. tryptase), histamine, proteoglycans, serotonin and cytokines/growth factors such as IL-6, IL-4, TNF-α transforming growth factor (TGF)-β, nerve growth factor (VEGF), and SCF. These mediators are released to the local microenvironment immediately (within minutes) after mast cell activation. Another group consists of lipid derived mediators (e.g. prostaglandin D_2, leukotriene C_4 and platelet-activating factor) which are synthesised simultaneously with the primary response and released immediately after mast cell activation. Finally, pro-inflammatory and immunoregulatory cytokines [e.g. IL-1, IL-3, IL-4, IL-5, IL-6, IL-8, IL-10, IL-13, IL-16, TNF-α, TGF-β and IFN-γ] and chemokines (e.g. macrophage inflammatory protein-1) are produced within hours after mast cell activation. Of note, IL-6, IL-4

and TNF-α are both stored as preformed mediators but are also synthesised *de novo* by activated mast cells (107-110).

Mast cells also contain lysosomal enzymes such as β-hexosaminidase, β-glucuronidase, β-D-galactosidase, arylsulfatase A, and cathepsins (i.e. cathepsin C, B, L, D and E). Out of these β-hexosaminidase appears to be the most well-known. It is present ubiquitously in mast cells of different subtypes and throughout all the species (110).

Mast cells exert their different functions depending on mediators released on activation. Histamine is one of the best known mast cell mediators. It is involved in a multitude of biological activities including immunomodulation, vasodilation, increased capillary permeabilities, smooth muscle construction, excitation of submucosal neurons and stimulation of ion secretion in intestinal epithelium (110, 111). Due to these functions, histamine appears to be implicated in pathophysiological conditions including airway inflammation, systemic anaphylaxis and IBS (110, 112). In patients with IBS and food allergies histamine receptors HR1 and HR2 were reported to be up-regulated within the gastrointestinal tract (113).

Classically, mast cells have been viewed as key effector cells in allergy reactions. Mast cells express a membrane based high-affinity IgE receptor (FcεRI) and bind IgE on their surface (13). Crosslinking of surface-bound IgE by circulating immunoglobulin light-free chains or superallergens is the central mechanisms in type I hypersensitivity reactions, which are fundamental for many pathological conditions including food allergies, bronchial asthma and rhinitis (13). Mast cells release an array of mediators upon IgE crosslinking in a process called degranulation. IgE crosslinking induces an anaphylactic-type degranulation, characterised by coordinated fusion of intergranular membranes and the plasma

membrane, to form a tunnel for mediators to the pericelullar space and an explosive and massive release of mast cells mediators. This pattern has also been observed for experimentally induced activation with compound 48/80 or calcium ionophore (114). A piecemeal-type of degranulation has been also described. In contrast to the anaphylactic type, piecemeal degranulation is a slow and more selective emptying of mast cells granules which occurs without the fusion of intergranular membranes but through microvesicular transport of granule content to the pericelullar space (115, 116). This type of degranulation is typically triggered by cytokines, lymphokines, microbial products and neuropeptide activation (116, 117). Piecemeal degranulation has been identified as a common morphologic change of mast cells in the gut of IBS patients (118).

Mast cell degranulation appears to modulate intestinal chloride ion secretion, under normal and pathological conditions (102). Enhanced water and ion secretion typically occurs in allergy reactions to food antigens. The role of mast cells in this context has been extensively studied using a number of models of intestinal hypersensitivity (119). An *in vitro* study showed that chloride secretion in human colonic epithelial cells (HCA-7) was observed only when these cells were sandwiched in Ussing chambers with rat mast cells stimulated with IgE crosslinking in the presence of antigen ovalbumin, but not when sandwiched with unstimulated mast cells (120). Animal studies reported that no secretory response was identified in sensitised rat jejunal tissues in Ussing chambers that had been pretreated with mast cells stabilisers (121). Furthermore, mast cell activation by IgE crosslinking induced a chloride secretory response of human colonic tissue that involved histamine, eicosanoids, and enteric nerves (122-124).

Based on the emerging evidence, mast cells appear to be implicated in a variety of pathologies beyond allergy. Human mast cells are capable of recognising a number of so-called pathogen-associated molecular patterns and other microbial

products through TLRs. The expression of TLRs varies slightly between mast cells of different origin. Human mast cells were reported to express TLR1-to-TLR10 with the exception of TLR8, though the data for TLR1, TLR4, TLR6 and TLR9 are inconsistent (125-131). In mice, the expression of TLR1-to-TLR4 and TLR6-to-TLR9 has been identified at least at the mRNA level (125, 132-138). Also responsiveness of mast cell to different TLR ligand may differ. For example, mast cells generated from a sterile environment (e.g. bone marrow, peripheral blood) respond well to different ligands such as LPS or peptidoglycan, while mast cells derived from an unsterile environment such as the intestine respond less efficiently to such stimuli (13). These differences may suggest that mast cells localised at sites constantly challenged with bacterial products such as the intestine acquire a kind of tolerance (13). Furthermore, while allergens induce a release of pro-inflammatory mediators such as histamine and eicosanoids, bacterial products primary induce a release of cytokines (e.g. TNF-α, IL-5) but also leukotrienes (e.g. leukotriene B4) and chemokines to initiate innate and adaptive immune responses (13, 139). These responses include recruitment of immune cells such as macrophages and neutrophils to infected/injured tissue to promote defence against pathogens and wound healing (75). Mast cells appear also to be activated by anti-microbial peptides such α- and β-defensins, as evidenced by histamine and prostaglandin D2 release (140-142).

Also, the interaction between mast cells and enteric nerves is part of the intestinal mucosal defence system. In the human gastrointestinal mucosa, a reported 47-78% of mast cells appear to be localised in close proximity to nerve fibres (143). Mast cells detect and signal the presence of antigens to the enteric nervous system, which reads the mast cell signal as a threat and initiates secretory and propulsive motor behaviour to eliminate the threat and protect the individual, at the expense of symptoms such as abdominal pain and diarrhoea (13). Interestingly, the distance between mast cells and enteric nerves has been

reported to be reduced in IBS tissue and even membrane-membrane contacts were occasionally observed (144). This suggests potentially enhanced interaction between the immune and nervous system (144) that might contribute to pain perception in IBS patients (112). Co-culture studies showed that sympathetic neurons formed contacts with mast cells, suggesting bidirectorial interaction between nervous and immune system (145). However, the factors that mediate this association are poorly explored. Potential candidates include nerve growth factor (NGF) (146), which is abundantly expressed by mast cells and it is involved in the survival, differentiation, and sprouting of sympathetic and sensory neurons (147). On the other hand, it is also possible that mast cells are attracted by neuropeptides (108). Enhanced mast cell activity is also associated with diarrhoea which might be induced as part of the mucosal defence system that protects the host against harmful entities (13). Furthermore, mast cells have been implicated in mechanisms underlying increased mucosal permeability evoked by stress and inflammation, as evidenced by a number of studies on animal models (109). Based on the emerging evidence, mast cells appear to be crucial to impaired intestinal barrier function reported in a subset of IBS patients (148) and this might be due to abnormal mast cell protease activity (57).

1.1.6. Mast cell tryptase – a possible link to epithelial permeability regulation in the gut

Mast cells appear to be one of the major sites for stored and secreted proteases with tryptase, chymase and carboxypeptidase A accounting for more than 25% of the total mast cell proteins. Different types of mast cells were also shown to express cathepsins (149) and renin (150). In humans, among all mast cell proteases, tryptase is the most abundant mediator (151). Basophils are the only other cells that are known to express tryptase however they appear to contain less than 1% of the tryptase that is found in mast cells (152, 153). Mast cell tryptase belongs to serine peptidases with trypsin-like target properties, which means that it cleaves peptide and protein substrates at lysine and arginine (154). Tryptase was shown to cleave extracellular substrates such as vasoactive intestinal peptide, calcitonin gene-related peptide, fibronectin and kininogens (110).

Mast cell tryptase is efficiently stored in the secretory granules in a fully processed form. Thus, its release from degranulating mast cells might have a rapid impact on the local environment (105). In mast cell granules low tryptase activity is maintained due to an acidic environment; optimally active tryptase requires neutral pH (155). A unique feature of mast cell tryptase is its tetrameric structure with each monomer having an active site directed to a narrow central pore (Figure 1.6) (156). The tetramer is stabilised by heparin proteoglycans present in mast cells granules and other highly negatively charged polymers (157-159). The active sites of tryptase are largely inaccessible for macromolecular protease inhibitors, making it resistant to any biological inhibitors of serine proteases and allowing it to stay active for a prolonged time. Additionally, this characteristic structure determines its high-selectivity properties (152, 156).

Human mast cells express 2 main enzymatically active tryptases, both tetrameric, α- and β-tryptase with the latter considered as a main form. Their counterparts in mice, mouse mast cell proteases (mMCPs) mMCP-6 and mMCP-7 respectively, are also released as tetrameters during mast cell degranulation. Additionally, both human and murine mast cells express a monomeric transmembrane tryptase TMT (mTMT in mice) (105).

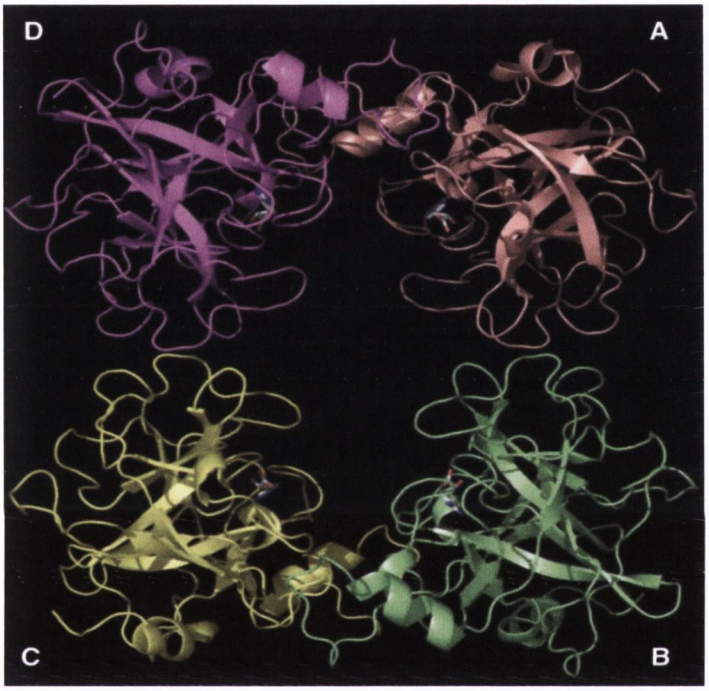


Figure 1.6 Structure of tetrameric human β-tryptase. Monomers (A, B, C, D) are positioned at the corners of a flat rectangular frame. Each monomer has its active site directed to a narrow pore in the middle of the tetramer. The image was adopted from Pereira *et al.* (156).

The role of mast cell tryptase is still not fully explored; however, it appears to be essential for combating a variety of infections. In mice, for example, mMCP-6 was involved in eosinophils recruitment into skeletal muscle infected with *Trichinella spiralis* (160) and in neutrophil recruitment towards *Klebsiella pneumoniae* infection (161). Tryptase may also potentiate allergic inflammation in human asthma (162) and allergic rhinitis (163). In asthma tryptase is typically overexpressed or it is released prematurely from mast cells, which can result in airway inflammation and bronchoconstriction (110). Furthermore, serum or plasma levels of total and mature tryptase are typically increased in systemic anaphylaxis and systemic mastocytosis and measurements of tryptase levels are recommended to be considered for the diagnostic evaluation of these conditions (152).

In inflammation tryptase might be important for the recruitment of inflammatory cells to sites of mast cell activation. Tryptase was shown to stimulate the release of IL-8, which is a chemoattractant for granulocytes, and to up-regulate expression of intercellular adhesion molecule (ICAM)-1 on lung epithelial cells (164). In addition, tryptase was reported to induce an increase in expression of IL-1β mRNA and IL-8 mRNA in endothelial cells (165). Moreover, the release of tryptase from activated mast cells may stimulate neighbouring mast cells, thus amplifying the response of mast cells to stimuli (166). On the other hand, tryptase appears to promote anti-inflammatory reactions by cleavage of pro-inflammatory chemokines and cytokines; for example, human β-tryptase was shown to cleave and markedly reduce the activity of eotaxin (eosinophil chemotaxin) *in vitro* (167).

Mast cell tryptase activates proteinase-activated receptor (PAR)-2, selectively out of 4 PARs belonging to a family of G-protein-coupled receptors (168, 169) (Figure 1.7). PAR-2 is also activated by trypsin, cathepsin G, coagulation factors VIIa and Xa. PAR-2 is expressed on several cell types in the gastrointestinal tract including enterocytes (both on apical and basolateral membranes), smooth muscle cells,

myenteric neurones, fibroblasts, endothelial cells of the lamina propria and immune cells including mast cells, neutrophils, lymphocytes, monocytes and macrophages (169, 170). Therefore, these cells appear to be potential targets for tryptase. It was reported that 63% of rat sensory neurons express PAR-2 receptors (171) and that tryptase caused excitation of sensory neurons, as evidenced by a raise in intracellular calcium. Excited neurons released neuropeptides such as substance P and calcitonin gene related peptide, which are involved in inflammatory processes (171). Furthermore, mast cell tryptase was suggested to activate PAR-2 located on enteric nerves and visceral afferents which resulted in long-lasting hypersensitivity of submucosal neurons in the ileum of guinea pigs (172). In addition, mast cell tryptase was shown to increase permeability and decrease TER of intestinal epithelial cells *in vitro* (57). This study, however, did not investigate the effects of tryptase on the expression on junctional proteins which are key determinants of epithelial permeability. Instead, this study showed that PAR-2 agonist induced a redistribution of TJ proteins (ZO-1, occludin) and perijunctional F-actin paralleled by increased permeability and decreased TER of epithelial cells. This was in line with the studies on animal models where intracolonic administration of PAR-2 agonists induced both increased paracellular permeability of the colon (173) and visceral hypersensitivity (174).

Interestingly, increased tryptase content has been repeatedly reported in IBS tissue and its levels positively correlated with mast cell numbers (144, 175). Moreover, increased intestinal permeability that was identified in rectal biopsy tissue from IBS patients was associated with enhanced tryptase activity (176). Thus, mast cell tryptase may be involved in the pathophysiology of IBS, particularly in disturbed sensory-motor function and impaired barrier function mechanisms.

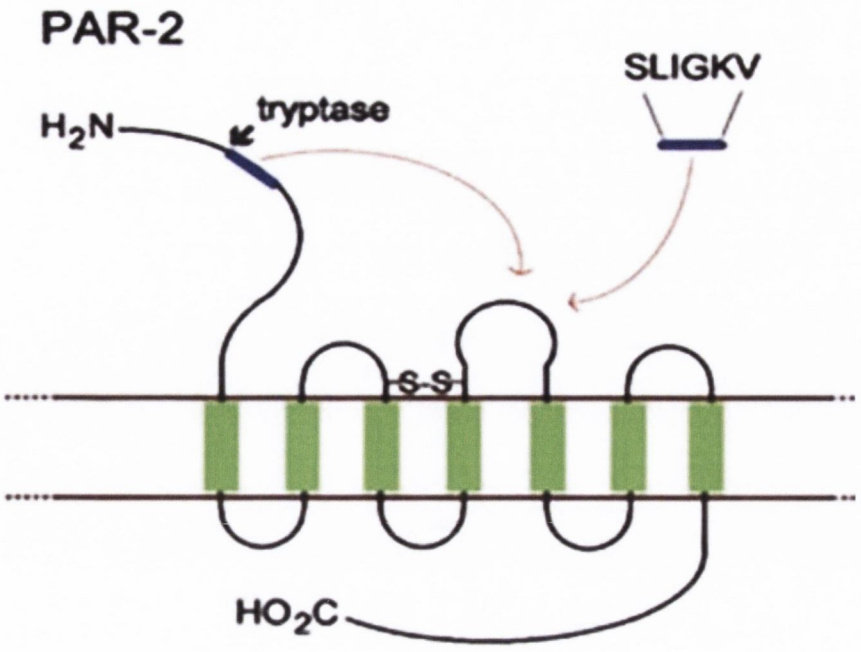


Figure 1.7 General mechanism of PAR-2 activation by tryptase. Tryptase cleaves at specific sites within the extracellular amino terminus of the receptors to expose a new amino terminus that serves as a tethered ligand domain (SLIGKV). SLIGKV binds to conserved regions in the second extracellular loop of the cleaved receptor that activates the receptor (168, 177-180). The image was adapted from Miller and Pemberton (151).

1.2. Intestinal permeability and mast cells in the pathogenesis of IBS: a review

1.2.1. Definition and classification of IBS

IBS is a highly prevalent functional bowel disorder characterised by the presence of abdominal discomfort or pain associated with defecation or altered bowel habit, abdominal bloating and features of disordered defecation (181). Although the aetiology of IBS is unclear, it is thought to result from the dysregulation of the brain-gut axis (182). Among a number of pathophysiological mechanisms that have been postulated to underlie the brain-gut dysfunction, increasing evidence has implicated impaired barrier function (148) and immune activation (72).

First diagnostic criteria for IBS were established in the 1970s, by Manning and colleagues who described abdominal symptoms more likely to be present in patients with IBS than in organic bowel disease (183). The first consensus definition was presented in 1988 in Rome and the guidelines for the first Rome criteria (Rome I) were published a year later (184). Revised guidelines, the Rome II criteria, were published in 1999, and the most recent Rome III criteria were established in 2006 (181) (Table 1.1).

Manning criteria (183) 1. Pain relieved by defecation 2. More frequent stools at onset of pain 3. Looser stools at onset of pain 4. Visible abdominal distension 5. Passage of mucus per rectum 6. Sense of incomplete evacuation
Rome I criteria (184) At least 3 months of continuous or recurrent symptoms of abdominal pain or discomfort relieved upon defecation or associated with change in stool frequency or consistence, plus 2 or more of the following on ≥25% of occasions or days: 1. Altered stool frequency (>3 bowel movements/day or <3 bowel movements/week) 2. Altered stool form (lumpy/hard or loose/watery stools) 3. Altered stool passage (straining, urgency, or feeling of incomplete evacuation) 4. Passage of mucus per rectum 5. Bloating or feeling of abdominal distension
Rome II criteria (185) At least 12 weeks, which need not be consecutive, in the preceding 12 months of abdominal discomfort or pain that has 2 of 3 features: 1. Relieved with defecation; and/or 2. Onset associated with a change in frequency of stool; and /or 3. Onset associated with a change in form (appearance) of stool.
Rome III criteria* (181) Recurrent abdominal pain or discomfort at least 3 days a month in the past 3 months, 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 form (appearance) of stool *Criteria fulfilled for the last 3 months with symptom onset at least 6 months before diagnosis.

Table 1.1 Diagnostic criteria for IBS.

1.2.2. Symptoms and subtypes

The Rome III criteria distinguish 5 subgroups of IBS according to bowel habit based on stool consistency (181):

- Diarrhoea predominant IBS (IBS-D): patients have loose stools more than 25% of the time and hard stools less than 25% of the time;
- Constipation predominant IBS (IBS-C): patients have hard stools more than 25% of the time and loose stools less than 25% of the time;
- Mixed bowel pattern IBS (IBS-M): patients have both hard and loose stools more than 25% of the time;
- IBS-A (alternating bowel pattern): patients whose bowel habit changes from one subtype to another during follow up over months and years. It is estimated that one quarter of IBS patients change their IBS predominant pattern at least once per year (186).
- Unclassified IBS (IBS-U): patients with neither loose nor hard stools more than 25% of the time (Figure 1.8) (186).

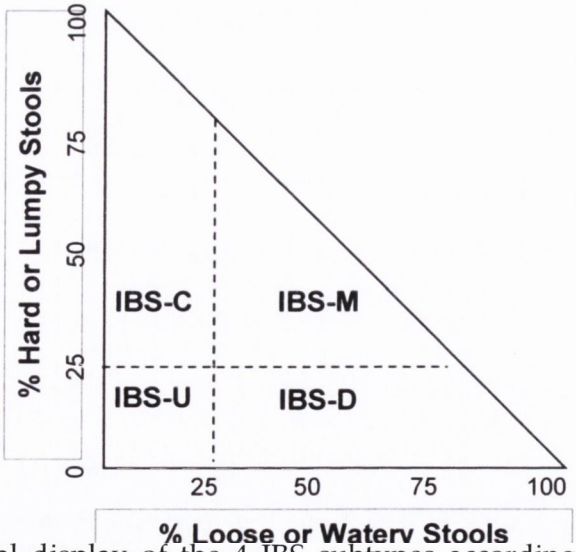


Figure 1.8 2-dimensional display of the 4 IBS subtypes according to bowel form at a particular point in time (181).

particular point in time (181).

30

In addition, there is a subset of IBS where symptom onset is associated with a documented preceding incidence of infectious gastroenteritis and, based on this etiological factor, it may be classified as post-infectious IBS (PI-IBS) subtype (187).

1.2.3. Epidemiology of IBS

Prevalence

IBS is a common condition with a worldwide prevalence of 3-20% (median 8.1%) based on Rome criteria (188, 189).

- The prevalence of IBS in European countries ranges from 3.1% to 15.7% (median 7.4%). The lowest prevalence is reported in France (median 3.1%) while the highest in Greece (15.7%).
- The prevalence in Asian countries appears to be higher and within 5.1-19.8% (median 8.1%), with the lowest prevalence in Hong Kong (median 5.1%) and the highest in Taiwan (19.8%).
- The median prevalence in USA is comparable to European countries and accounts for 7.3%, though in Canada is much higher (median 19%).
- The studies on prevalence in Latin and South America are limited and include a study in Mexico with 16.9% prevalence, and a study in Colombia with 19.9% prevalence.
- The median prevalence in Australia appears to be low in comparison to the prevalence in other continents and is 6.1% (189) (Figure 1.9).

The prevalence of IBS is higher in females in the general population using Rome criteria (males/females ratio=1/1.5). This pattern is consistent in European countries but several studies in Asia showed a lower prevalence in females (e.g. Korea males/females ratio=1/0.85) (189, 190).

Both in Europe and USA, the alternating (mixed) bowel habit is predominant (50% and 44% respectively) while the constipation bowel habit is the least common (24% and 18% respectively). On the contrary, both in Asian countries and in Latin America, constipation predominance is the most common pattern (42% and 39% respectively) while the prevalence of the other IBS subgroups is on a comparable level (189).

The prevalence of IBS appears to be higher in young individuals. In a large survey study recruiting almost 42000 respondents across 8 European countries, the prevalence in the youngest group (18-34 years) reached 12.2%, in the middle age group (35-54 years) 9.9%, while in the group with responders of 55 years or more 7% (188).

The heterogeneity of IBS prevalence might be due to demographic characteristics of the population studied (e.g. access to health care, cultural influences), geographical location, use of different diagnostic criteria and quality of the designed study (191, 192). For example, depending on diagnostic criteria used, a comparative study of Rome II and Rome III criteria reported significantly higher prevalence with Rome III criteria (193).

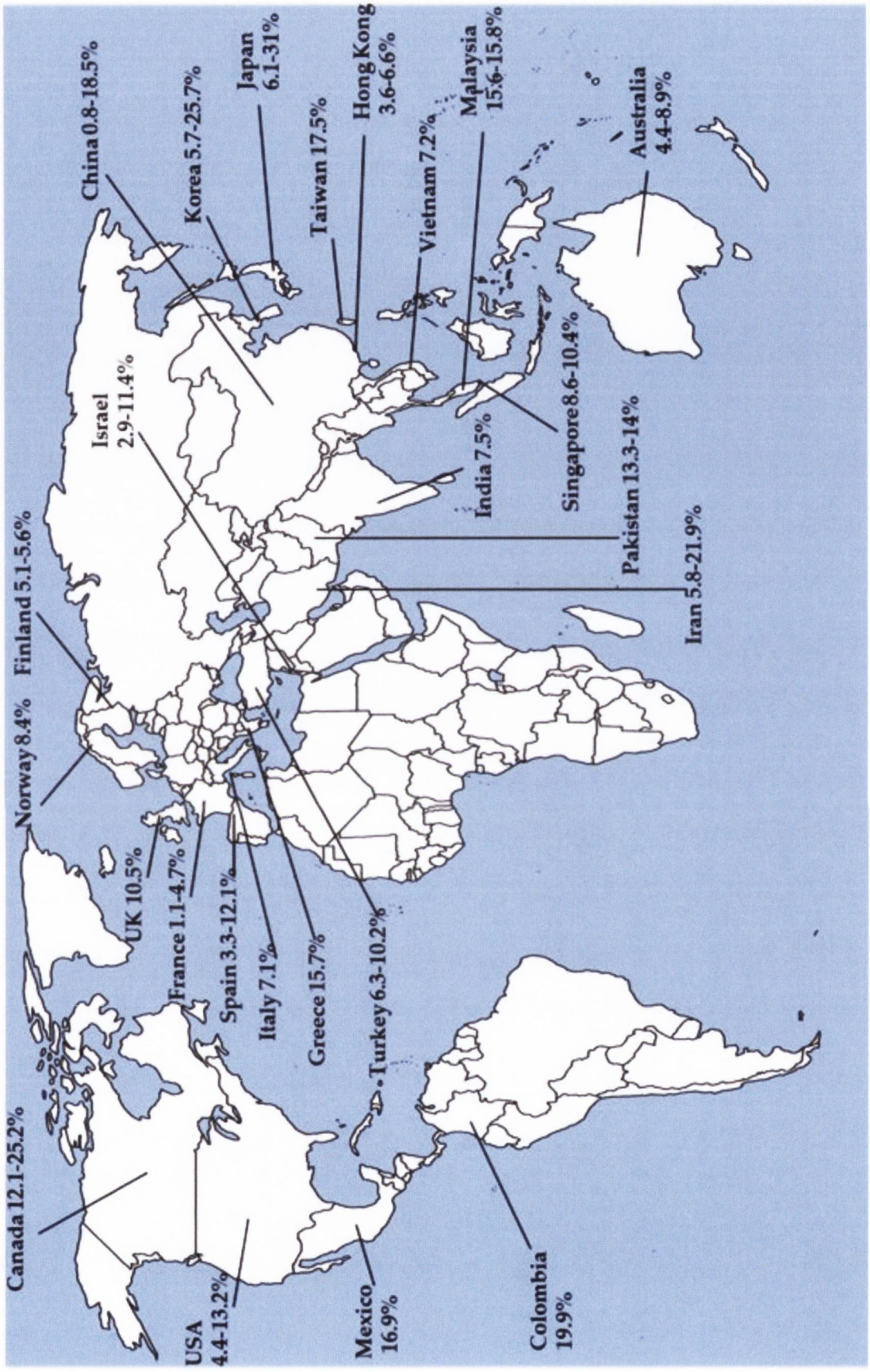


Figure 1.9 World map of IBS prevalence (2000-2010) based on Rome I, II and III criteria. Compiled from Chang *et al.* (189).

Impact of IBS: health care burden and quality of life

IBS is a chronic condition and although not associated with increased mortality it accounts for substantial illness, reduced quality of life and health care costs. IBS patients constitute about 3% of all general practice consultation and up to 40% of all gastrointestinal referrals, which generates enormous health care costs (194). This economic burden includes direct costs due to health services (consultations, diagnostic procedures and medications), as well as indirect costs including increased work absence and reduced work productivity (195). Surveys carried out in US and UK show that the average number of work days lost per year because of IBS varied between 8.5 and 21.6 per patient (196).

Quality of life is decreased in IBS patients as compared to general population (197, 198) and IBS patients have been reported to have more impaired mental and physical functioning than healthy controls (199). However, it appears that 33-90% of sufferers do not consult health care services. The main predictors of health care seeking are symptomatic, including abdominal pain or distension and pain severity. Psychological and social factors are relevant to the decision to seek medical advice (200, 201).

1.2.4. Pathophysiology of IBS

The pathophysiology of IBS is complex and not fully understood; however, it is generally accepted that IBS is caused by the dysregulation of the brain-gut axis, involving abnormal function of the enteric, autonomic and/or central nervous system, or disturbed interactions between these systems (202). These alterations appear to be implicated in the development of visceral hypersensitivity (203), altered gut motility (204) and secretion (205), which in turn contribute to the hallmark IBS symptoms i.e. abdominal pain and/or discomfort, bloating and altered bowel habit (diarrhoea and/or constipation) (75). Putative biological

factors associated with IBS pathophysiology include a bout of gastrointestinal infection (206), stress (207), genetic predisposition (208), food sensitivity (209) and bile acid malabsorption (210). Currently there is a growing interest to better understand the mechanisms underlying these changes and research is focused towards multidisciplinary analysis including genetics (e.g. polymorphism for cytokines) (211), immunology (low-grade inflammation) (70, 72), microbiology (prior incidence of infectious gastroenteritis, abnormal gut microbiota, host microbial interactions) and epithelial biology (defective barrier function, altered intestinal permeability) (212, 213). The complex interplay between these components leads to the generation of IBS symptoms (Figure 1.10).

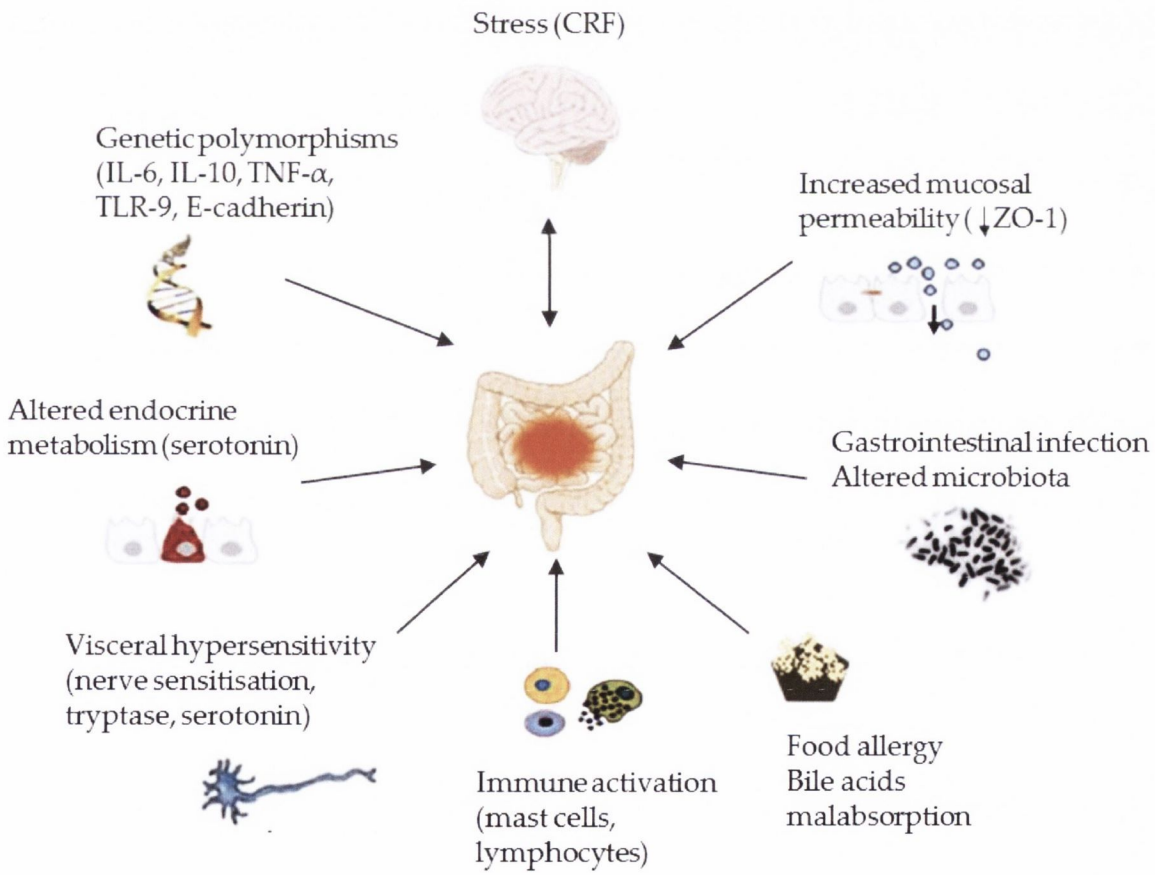


Figure 1.10 Putative biological factors associated with the IBS pathophysiology. CRF, corticotrophin releasing factor. The image was adopted from Barbara *et al.* (71).

1.2.4.1. Visceral hypersensitivity

Visceral hypersensitivity has been described in 20-90% of IBS patients (214). It is characterised by 2 components: hyperalgesia, which is an enhanced response to a painful stimulus, or allodynia, which is described as a painful response to an innocuous stimulus. Several studies demonstrated that IBS patients exhibit lower thresholds to pain induced by balloon distension in the oesophagus (215), stomach (216), small bowel (217) and rectum (218-220) suggesting that visceral hypersensitivity is diffused along the gastrointestinal tract. However, these findings have not been confirmed by other studies (221-223).

Visceral sensation is the result of a complex process consisting of chemical or mechanical nerves stimuli within the gut wall, and transduction of these stimuli into information that is transferred through afferent nerves to the spinal cord and to the brain (214). Lower thresholds to painful stimuli or perception of innocuous stimuli as painful in IBS patients may be due to abnormal processing of sensory information by the central and/or peripheral nervous system (214). During rectal distension, IBS patients appear to abnormally process the sensory input from the periphery as evidenced by greater brain activity, particularly in regions associated with pain modulation and emotional arousal (224). In the gut of IBS patients, immune, neural, endocrine and microbiological abnormalities, and their interactions, are thought to result in impaired peripheral sensitisation of sensory nerve endings within the gut wall and pain transmission to the brain (203).

1.2.4.2. Psychological factors in IBS

The importance of psychological factors in the development of IBS symptoms is well recognised. IBS symptoms are frequently associated with psychological co-morbidities such as anxiety, hypochondriasis and depression (225, 226), as well as an increased level of fatigue (227). Perhaps, the strongest example of stress involvement in the pathophysiology of IBS is described in PI-IBS (206). Approximately half of IBS patients experience more chronic stress compared to healthy volunteers (228). Higher rates of these psychological factors in IBS have a severe impact on the quality of life and are associated with seeking medical care.

Stress is recognised as a risk factor for IBS and stressful life events frequently precede the onset of IBS symptoms (229). Furthermore, stress is associated with symptom onset, severity and persistence in subsets of IBS patients (230, 231). The hypothalamic-pituitary-adrenal (HPA) axis and the sympathetic branch of the autonomic nervous system constitute the core stress system in humans that mediates communication between the brain and the gut immune system. HPA is activated by both physical and psychological stressors and in response releases CRF from hypothalamus. CRF promotes the synthesis of corticotrophin in the pituary gland, which subsequently stimulates the adrenal cortex to release cortisol. Cortisol circulates in the blood and interacts with peripheral tissues. Dinan *et al.* (232) reported that IBS patients, in response to intravenous CRF infusion, exhibited elevated levels of pro-inflammatory cytokines and enhanced release of cortisol, indicating exaggerated activation of HPA axis. Thus, psychological factors interact with, and may trigger, immune activation in the gut. Piche *et al.* (90) showed that depression and fatigue correlate with mast cell numbers in the colonic mucosa of IBS patients compared to controls. This finding suggests that chronic psychological factors were associated with gut inflammation, particularly with mast cell infiltration.

A number of studies have reproduced IBS symptoms in animal models where experimental stressors were shown to alter gastrointestinal motility (233-235), to alter visceral perception (235-239), to disrupt the intestinal epithelial barrier (235, 240, 241), to increase ion secretion (235) and to induce mucosal inflammation (235, 242).

1.2.4.3. Altered serotonin metabolism

Serotonin (5-hydroxytryptamine, 5-HT) is one of the key neurotransmitters in the gut. Approximately 95% of 5-HT is produced in the gastrointestinal tract and is mainly synthesised by enterochromaffin cells (243). 5-HT biological activity is terminated by the serotonin reuptake transporter (SERT) expressed on nerve terminals or on the mucosal enterocytes and vascular endothelial cells (244, 245). The possible contribution of 5-HT in the pathophysiology of IBS is supported by the efficacy of 5-HT_3 receptor antagonists and 5-HT_4 agonist on a number of IBS symptoms including, the modulation of colonic transit (246, 247). Increased postprandial plasma levels of 5-HT have been documented in IBS-D (248-250) whereas lower levels were associated with delayed colonic transit in IBS-C patients (249, 250). In addition, increased 5-HT levels have been reported in rectal (251) and colonic (252) biopsy tissue from IBS patients, though other studies did not confirm these findings (253). Interestingly, in a recent study by Cremon *et al.* (252) increased 5-HT release was associated with enhanced mast cell infiltration of the colonic mucosa of IBS patients, suggesting that immune activation might affect 5-HT metabolism in IBS. Other studies suggested that abnormal 5-HT plasma levels in IBS patients were a consequence of impaired reuptake by SERT (253, 254). Furthermore, Foley *et al.* (254) reported that reduction in SERT mRNA expression was associated with immune activation, as evidenced by increases in both mast cell and lymphocyte numbers and higher tryptase levels in IBS duodenum.

1.2.4.4. Immune activation

The importance of intestinal immune activation in the pathophysiology of IBS is based on at least 2 clinical observations: 1) the development of IBS symptoms after a bout of enteric infection (PI-IBS) and 2) the prevalence of IBS-like symptoms in patients with IBD in remission, or in other organic gastrointestinal diseases in the absence of active inflammation. The causes of immune activation in IBS are not completely understood; however, based on emerging evidence, the proposed mechanisms include previous gastrointestinal infection (255), stress (256), increased intestinal permeability (148), changes in microbiota (257), food allergies (209) and bile acid malabsorption (210).

Immune activation has been documented both in PI-IBS and in IBS patients without a previous incidence of acute gastroenteritis (72). A number of recent studies have reported immune cell infiltration (e.g. mast cell, T-cells) and activation (e.g. release of proteases, cytokines) in the intestinal mucosa of IBS patients (72, 75), abnormal systemic immune response (e.g. circulating T-cells, cytokines) (71), and an increase in antimicrobial agents (e.g. β-defensin-2) (258) along with alterations of cytokine gene expression (e.g. IL-10, TNF-α) (208). Several studies have demonstrated that immune infiltrate is gender-dependent, with increased mast cell numbers (144, 259) but lower CD3+ and CD8+ counts in females than males (259). Perhaps, gonadal hormones that are known to regulate immune activity (260), including mast cell degranulation *in vitro* (261, 262), might determine differences in immune responses in IBS between genders. Equally, differences in psychological factors or other host factors may account for gender differences.

The most consistent finding is the increased number of mast cells which has been shown along the gastrointestinal tract from the duodenum (101) through jejunum

(228), ileum (263), caecum (90) and colon (86, 112, 144, 175, 264-266) to the rectum (259); however, these findings have not been confirmed by other studies (85, 267, 268) (Table 1.2). Several studies have shown a similar degree of mast cell infiltration in intestinal tissue of IBS-D and IBS-C patients (90, 144, 259) whereas other studies have reported higher mast cell counts in IBS-D tissue (91, 228). The higher mast cell numbers have been linked to stress (256), previous infection (206), abnormal microbiota (257) and increased intestinal permeability (212). The presence of increased mast cell numbers could be due to local hyperplasia of resident mast cells, or due to an influx of newly migrated mast cells from the bone marrow, or a combination of both (240). Furthermore, a higher percentage of activated mast cells has been reported in IBS tissue as evidenced by ultrastructural features of activation by transmission electron microscopy (TEM) analysis (118, 144, 175), and/or by the measurement of enhanced spontaneous tryptase release from mucosal biopsy tissue (112, 144, 175, 252, 269), or increased content of luminal tryptase (228) (Table.1.2).

Beyond mast cells, an increase in the number of T-lymphocytes (CD3+, CD4+, CD8+) has been frequently documented in mucosal tissue from IBS patients (85, 91, 228, 259, 264, 270, 271). There is also evidence of increased T-cell activation in IBS. Increased mucosal staining of the CD25 marker (IL-2 receptor) in the colonic mucosa of IBS patients suggested an increased activation of T-lymphocytes (270). In agreement with this study, Öhman *et al.* (272) reported increased frequencies of T-lymphocytes (CD8+ and CD4+) co-expressing human leukocyte antigen(HLA)-DR (major histocompatibility complex (MHC) class II receptor), expressed on activated T-cells, and integrin B7, involved in homing of lymphocytes through the blood to the gut, in blood samples from IBS patients. In addition, elevated counts of circulating T-lymphocytes (CD8+ and CD4+) expressing CD69, a cell surface marker for recent T-cell activation, have been identified in subsets of IBS patients (272). In the case of B-lymphocytes counts in IBS the evidence is less consistent,

with reports of both reduced numbers (273) in mucosal biopsy tissue of IBS patients or comparable numbers to controls (86, 259). Other immune cells whose increased counts have been reported in IBS patients include enterochromaffin cells, and these have been predominantly related to PI-IBS (85, 252, 274) (Section 1.2.4.3). Other studies, however, did not confirm these findings (251, 253).

Furthermore, several studies have reported increased levels of pro-inflammatory cytokines in colonic mucosa (TNF-α) (265), rectal mucosa (IL-1β mRNA) (263, 275), in peripheral blood mononuclear cells (IL-1β, IL-6, TNF-α) (276) and in plasma (IL-6 and IL-8) (232, 277, 278) from IBS patients. In contrast, mucosal concentrations of IL-6 and IFN-γ were decreased in one study (265) whereas other studies demonstrated that the levels of IL-1β, IL-6 and TNF-α in colonic biopsy tissue from IBS patients (70) and IL-1β, IL-6 in serum (279) were comparable to controls. An abnormal IL-10/IL-12 ratio, indicative of an inflammatory response, was shown in blood cell culture of IBS patients (280). In addition, a genotype producing low levels of the anti-inflammatory cytokine IL-10 (281), a genotype producing high levels of TNF-α (282) and a genotype combination of IL-10 low producer and TNF-α high producer (282) have been described more often in IBS patients.

Other studies have reported immune responses to microbial antigens in IBS patients. For example, an increased concentration of antimicrobial protein β-defensin-2, whose expression can be induced by pro-inflammatory cytokines, was shown both in faecal specimens and in the colonic epithelium of IBS patients (258). Furthermore, increased levels of circulating antibodies to luminal antigens, i.e. anti-flagellin antibodies, have been detected in IBS patients, more often in PI-IBS than in non-PI-IBS (283).

Table 1.2 Summary of studies on mast cells (numbers, mast cell tryptase, ultrastructural features of degranulation by TEM) in IBS patients according to intestinal site.

Study	*Cases/ (controls)*	*Location*	*MCs in IBS v controls*	*MC tryptase/MC degranulation by TEM in IBS v controls*
Foley *et al.*, 2011[254]	20 IBS-D/(29)	duodenum	↑	↑tryptase released from biopsy tissue
Walker *et al.*, 2009[91]	41 IBS/(48)	duodenum	↑	NA
Wang *et al.*, 2007[284]	38 IBS/(20)	duodenum	unaltered	NA
Martínez *et al.*, 2012[285]	25 IBS-D/(23)	jejunum	↑	↑tryptase (mRNA & protein) in biopsy tissue
Guilarte *et al.*, 2007[228]	20 IBS-D/(14)	jejunum	↑	↑ luminal tryptase
Wang *et al.*, 2007[284]	38 IBS/(20)	jejunum	unaltered	NA
Wang *et al.*, 2007[284]	38 IBS/(20)	ileum	↑	NA
Park *et al.*, 2006[266]	18 IBS-D/(15)	ileum	↑	NA
Wang *et al.*, 2004[263]	56 IBS/(12)	ileum	↑	NA
Vivinus-Nébot *et al.*, 2012[269]	34 IBS/(15)	caecum	↑	↑tryptase released from biopsy tissue
Piche *et al.*, 2008[90]	50 IBS/(21)	caecum	↑	NA
Park *et al.*, 2003[118]	14IBS-D/14)	caecum	↑	↑ degranulated MC
O'Sullivan *et al.*, 2000[86]	14 IBS/(7)	caecum	↑	NA
Cenac *et al.*, 2007[267]	15 IBS/(7)	AC	unaltered	↑tryptase mRNA in biopsy tissue
Park *et al.*, 2006[266]	18 IBS-D/(15)	AC	↑	NA
O'Sullivan *et al.*, 2000[86]	14 IBS/(7)	AC	unaltered	NA
Cremon *et al.*, 2011[252]	25 IBS/(12)	DC	↑	↑tryptase released from biopsy tissue
Klooker *et al.*, 2010[268]	29 IBS/(15)	DC	unaltered	NA
Coeffier *et al.*, 2010[265]	25 IBS/(18)	DC	↑	NA

Table 1.2 continued.

Study	*Cases/ (controls)*	*Location*	*MCs in IBS v controls*	*MC tryptase/MC degranulation by TEM in IBS v controls*
Buhner *et al.*, 2009[112]	18 IBS/(7)	DC	↑	↑tryptase released from biopsy tissue
Bian *et al.*, 2009[286]	10 IBS-D/(13)	DC	↑	↑tryptase in biopsy tissue
Cremon, *et al.*, 2009[259]	48 IBS/(48)	DC	↑	NA
Barbara *et al.*, 2007[175]	29 IBS/(15)	DC	↑	↑tryptase released from biopsy tissue; ↑ degranulated MC
Barbara *et al.*, 2004[144]	44 IBS/(22)	DC	↑	↑tryptase released from biopsy tissue; ↑degranulated MC
O'Sullivan *et al.*, 2000[86]	14 IBS/(7)	DC	unaltered	NA
Akbar *et al.*, 2008[287]	23 IBS/(22)	RC	↑	NA
Wang *et al.*, 2004[263]	56 IBS/(12)	RC	unaltered	NA
Klooker *et al.*, 2010[268]	58 IBS/(21)	rectum	unaltered	↓tryptase released from biopsy tissue
Lee *et al.*, 2008[264]	42 IBS/(12)	rectum	↑	NA
Cenac *et al.*, 2007[267]	15 IBS/(7)	rectum	unaltered	↑mRNA tryptase in biopsy tissue
Park *et al.*, 2006[266]	18 IBS-D/(15)	rectum	↑	NA
Park *et al.*, 2003[118]	14 IBS-D/(14)	rectum	↑	↑degranulated MC
O'Sullivan *et al.*, 2000[86]	14 IBS/(7)	rectum	unaltered	NA

MC, mast cell; AC, ascending colon; DC, descending colon; RC, rectosigmoid colon. IBS patient cohorts that comprised more than one bowel predominance subtype are denoted as IBS; while IBS-D denote study cohorts with the 'diarrhoea' subtype only. ↑means an increase, ↓means a decrease.

1.2.4.5. Gastrointestinal infection

Between 4-36% of patients with acute gastroenteritis develop PI-IBS (206). Dunlop *et al.* (187) has defined PI-IBS as "the acute onset of new IBS symptoms in an individual, who has not previously met the Rome criteria for IBS, immediately following an acute illness characterised by 2 or more of the following: fever, vomiting, diarrhoea, or a positive bacterial stool culture".

PI-IBS is most common in patients who suffered from bacterial infection with *Salmonella spp.* (288), *Campylobacter jejuni* (274, 289) or *Shigella spp.* (290). The highest incidence was reported in Walkerton town in Canada, where 36% of inhabitants developed PI-IBS due to waterborne infection with *Campylobacter jejuni* and *Escherichia coli* (291). Recent evidence also implicates viral (Norovirus) gastroenteritis in the development of PI-IBS (292). Several risk factors for the development of this subtype of IBS have been established and these include female gender, the severity of gastroenteritis (e.g. weight loss, duration of the episode), smoking, psychological status (depression, anxiety, hypochondriasis) and genetic factors (206). Sustained immune activation may contribute to the development of PI-IBS. Spiller *et al.* (85) suggested that increased numbers of T-cells, macrophages and enteroendocrine cells in the rectal mucosa reported during acute enteritis failed to decline in patients who developed PI-IBS. Another study reported that PI-IBS patients had more anti-flagellin antibodies compared to IBS without a history of acute gastroenteritis (283). This suggests that immune activity to luminal antigens is relevant to the development of PI-IBS.

1.2.4.6. Abnormal microbiota

Changes in gut microbiota have been found in IBS patients (293-295). For example, the faecal microbiota of IBS patients was shown to be less diverse compared to controls (296). Furthermore, alterations in relative numbers of Firmicutes and Bacteroidetes, which are the major beneficial bacterial phyla in gut microbiota, have been reported in faecal samples of IBS patients (297). Of note, the composition of microbiota differs between the gut epithelium and lumen (298). In IBS-D patients, a significantly increased quantity of aerobic bacteria and *Lactobacilli* were reported in faecal samples but not in mucosa-associated microbiota (299).

The normal microbiota is important in the development of host mucosal immune system (300, 301), which appeared to be undeveloped in germ-free animals. For example, these animals appeared to have reduced numbers of T-cells in the gut (302, 303). Alterations in gut microbiota due to, for example, enteric infection or antibiotic therapy, may lead to abnormal immune responses (304). Therefore, abnormalities in gut microbiota might affect immune activity in IBS patients. Commensal bacteria also protect the host against pathogenic bacteria activity and can either have direct bactericidal effects or prevent the adherence of pathogenic bacteria to the gut wall (305). Alterations in gut microflora may influence the ability of microbiota to adhere to intestinal mucosa and thus facilitate the access of pathogenic bacteria to the epithelium and impair intestinal barrier function (306). Gecse *et al.* (307) demonstrated that faecal supernatants of IBS-D patients caused barrier dysfunction *in vitro* and visceral hypersensitivity in mice. These supernatants exhibited increased protease activity, possibly of bacterial origin (307).

A number of studies have reported that an abnormally high bacterial count ($\geq 10^5$ colony-forming units/ml) in the small intestine, termed small intestinal bacterial overgrowth (SIBO) (308), is common among IBS patients (309-312). SIBO was described predominantly in IBS-D patients and was associated with IBS-related symptoms such as abdominal bloating and diarrhoea (311, 312). However, the diagnostic test for SIBO, i.e. lactulose hydrogen breath test, has poor sensitivity and specificity (313, 314) and is not validated to accurately detect SIBO, as considered in a recent report (315).

1.2.4.7. Impaired host-microbial interactions

Host-microbial interactions seem to be relevant to the pathophysiology of IBS. Recent attention has focused on TLRs, which play a central role in the mucosal innate immune response (316). These receptors are activated by several bacterial and viral components (317) and their activation leads to the production of cytokines, chemokines and antimicrobial molecules (318). In a recent study, Brint *et al.* (319) have investigated TLR1-to-TLR10 receptors and reported an up-regulation of mRNA expression of TLR4 and TLR5 and down-regulation of TLR7 and TLR8 mRNA in the colonic mucosa of IBS patients compared to controls. The increased expression of TLR4 and TLR5 was possibly determined by the enhanced levels of their endogenous ligands, i.e. lipopolysaccharide (LPS) (320) and flagellin (283) respectively. Previous studies reported that LPS and flagellin up-regulated the corresponding TLR (283, 320). Both TLR7 and TLR8 respond to single-stranded RNA and their down-regulation in IBS patients suggests that viral infections may contribute to the development of IBS (305). TLR2 mRNA expression appeared to be unchanged in IBS colonic tissue compared to controls as shown by Brint *et al.* (319); however, its increased expression on blood monocytes in IBS has been recently reported (279) indicating differences for microbial engagement between the systemic and mucosal immune system. The

implication of altered host-microbial interactions in the pathogenesis of IBS is further supported by the reports of genetic susceptibility markers in genes involved in these interaction both in PI-IBS (e.g. TLR9) (211) and non-PI-IBS patients (e.g. TL1A) (321, 322) .

Up-regulation of TLRs can contribute to the higher levels of pro-inflammatory cytokines observed in IBS patients. For example, in a recent study the activation of TLR2 was reported to enhance release of TNF-α, while the activation of TLR4 resulted in amplified secretion of IL-1β and TNF-α in whole blood from IBS patients compared with controls (278).

1.2.4.8. Genetic susceptibility to IBS

Genetic predisposition to IBS has been documented in epidemiological twin studies, which are the traditional way to estimate the genetic and environmental contribution to a disease (208). In the majority of these studies concordance rates for monozygotic twins (genetically identical) were higher than concordance in dizygotic twins, suggesting genetic aetiology of IBS (323-325). However, having a parent with IBS appeared to be a stronger predictor of IBS than having a twin with IBS, indicating that social learning (what an individual learns from his or her environment) may have greater influence on the development of IBS than genetics alone (323).

A number of studies have reported associations between IBS symptoms and genetic polymorphism for genes that regulate immune activation, interactions with bacteria, maintenance of the epithelial barrier and motility. Among these studies, genetic associations with IBS were found for genes coding either pro-inflammatory or anti-inflammatory cytokines. The imbalance between pro- and anti-inflammatory cytokines may affect disease susceptibility and clinical

outcomes (70). For instance, the combined presence of a low producer of anti-inflammatory IL-10 (-1082 A/A) and a high producer of pro-inflammatory TNF-α (-308 A/A) genotype was reported to be more prevalent in IBS patients compared with controls (282). Since IL-10 is known to inhibit TNF-α synthesis and initial inflammatory responses, the individuals genetically predisposed to produce both high levels of TNF-α and low levels of IL-10 might be at particular risk to develop low-grade inflammation and IBS-like symptoms. Furthermore, the high producer TNF-α genotype (-308 G/A) was more common in IBS (282), whereas frequencies of IL-10 high producer genotype (-1082 G/G) in IBS patients appear to be reduced compared with controls (281), though this finding was not confirmed by another study (282). Recently, a single nucleotide polymorphism (SNP) in the gene for another pro-inflammatory cytokine, i.e. IL-6, was associated with the risk for IBS (211). The recognised promoter variant of the IL-6 gene (-G174C, rs1800795) was more common in PI-IBS. Previously, the same variant was reported to increase the transcription rate of the IL-6 gene (326). Thus, potentially higher IL-6 levels might promote intestinal inflammation (276) and predispose individuals to the development of PI-IBS.

Further 3 SNPs associated with IBS have been found in genes that regulate defence against pathogens and host-commensal bacteria interactions in the gut. The first 2 SNPs were identified in the TLR9 gene, with one localised in promoter region (-T1237C, rs5743836) and one in coding region (P545P, rs352139), and were associated with risk for PI-IBS (211). The third SNP was found in TNFSF15 coding for TL1A (rs42633839, SNP G/A), a member of TNF (ligand) superfamily (327, 328). A recent study demonstrated that carrying a risk allele "G" in the TL1A gene enhanced transcriptional activity and was associated with a higher risk for IBS (322). These gene variables might play a critical role in recognising the microbial threat and initiating the immune response.

One SNP associated with risk for IBS was also found in a gene involved in the regulation of epithelial barrier function, i.e. CDH1 coding for E-cadherin (211). More specifically, a promoter variant of CDH1 (-C160A, rs16260) carrying the "A" allele instead of "C" was associated with a higher risk for the development of PI-IBS. Previously, this variant was shown to decrease transcriptional activity (329) and may predispose individuals to impaired integrity of the epithelial barrier.

In several studies genes related to 5-HT metabolism have been investigated, including SERT and 5-HT receptors. An association has been reported between "G" allele of gene for SERT, i.e. SLC6A4 (rs25531, A/G), and IBS (330) and between *76G/A polymorphism in 3'-untranslated region (UTR) in subunit of SERT type 3 5-HT_3, i.e. HTR_{3E}, and IBS-D (331). Both polymorphisms seem to affect gene expression and impact sensorimotor function in the gut (330-332).

1.2.5. Intestinal barrier dysfunction: implications for IBS

The intestinal barrier selectively filters substances in the gut lumen allowing for the passage of luminal nutrients, ions and water while preventing the passage of molecules that may be harmful to the host, such as microbial products. The paracellular pathway is the principal determinant of epithelial permeability and is tightly regulated by junctional proteins. Intestinal barrier integrity can be assessed by a number of approaches, including the measurement of permeability, analysis of the expression levels and localisation of epithelial junctional proteins (structural determinants of gut "leakiness") by western blot analysis, immunofluorescence, immunohistochemistry or mRNA quantification.

In vitro epithelial permeability can be assessed, for example, by mounting biopsy tissue in Ussing chambers; with this approach tissue conductance can be monitored as well as the transport of small molecular probes such as horseradish

peroxidase (HRP) or fluorescein isothiocyanate (FITC)-dextran. *In vivo*, a variety of probes have been employed to evaluate permeability of different sites in the gastrointestinal tract. For example, sucrose is used to assess gastroduodenal permeability as it is degraded by digestive enzymes once it leaves the stomach (25). In a similar manner, small sugar probes such as lactulose or mannitol are used to assess small intestinal permeability. These probes are degraded in the large intestine by bacterial enzymes and cannot be used to study colonic permeability. Thus, the lactulose/mannitol test is a tool to assess small intestinal permeability. With this test, urinary excretions are assessed for lactulose and mannitol content after ingestion of a standard oral load. Lactulose is absorbed through the paracellular pathway and lactulose permeation is increased by intestinal damage, while mannitol uptake occurs via the transcellular pathway and correlates with intestinal surface area. The calculated lactulose/mannitol ratio provides a measure of intestinal damage normalised to surface area (68). In addition, polyethylene glycol (PEG)-400 has been used as a marker of small intestinal permeability (333). However, it has been suggested that this test in comparison to the other probes, may not be sufficiently sensitive to detect more subtle changes in intestinal permeability that may occur, for example, in IBS (334). In order to evaluate colonic permeability properties chromium-labelled ethylenediaminetetraacetic acid (^{51}Cr-EDTA) can be used and, as it is stable throughout the gut, it also allows for measurement of small intestinal permeability. The timing of the ^{51}Cr-EDTA urine collection relates to permeability measurement of distinct part of the gut (148); for example, 3 hours for gastroduodenal and a longer duration of 5 hours for distal small bowel permeability (335, 336).

A summary of studies and assessment approaches to intestinal permeability in IBS is compiled in Table 1.3.

1.2.5.1. Evidence of altered intestinal permeability in IBS

Increased intestinal permeability is a common finding in IBS patients (85, 176, 212, 310, 335-339) and has been observed in IBS patients along the gastrointestinal tract including small bowel, colon and rectum (Table 1.3). The lactulose/mannitol test has been the most frequently used method to assess intestinal permeability in IBS; small bowel permeability appeared to be increased from 12% up to 50% of IBS patients with this test (85, 337-340). Increased colonic permeability has been reported in IBS patients as evidenced both by measuring the urine excretion of orally ingested ^{51}Cr-EDTA (335) and the apical-to-basolateral flux of a probe through mucosal biopsy tissue *in vitro* (212) (Table 1.3).

The analysis of intestinal permeability has been predominantly performed in the IBS-D subtype using both oral probe excretion assays and mucosal biopsy tissue *in vitro,* and showed enhanced permeability both in the small bowel and the colon. Recent analyses employing mucosal tissue have also shown that intestinal permeability is independent of IBS subtype and is found across all the subgroups (Table 1.3).

Table 1.3 Summary of studies on intestinal permeability in IBS patients.

Study	*Cases/ (controls)*	*Method*	*Intestinal permeability in IBS v controls*
Vivinus-N. *et al.*, 2012[269]	13 IBS-D, 10 IBS-C, 11 IBS-M/(15)	Biopsy tissue in Ussing chamber: IP to FITC-sulfonic acid	Caecum: ↑in all IBS subtypes
Gecse *et al.*, 2012[336]	18 IBS-D, 12 IBS-C/(10)	^{51}Cr-EDTA excretion	Proximal small bowel: ↓in IBS-C; distal small bowel: no change; colon: ↑in IBS-D
Lee *et al.*, 2010[176]	14 IBS-D/(21)	Biopsy tissue in Ussing chamber: IP to HRP	rectum: ↑
Zhou *et al.*, 2010[338]	19 IBS-D/(10)	L/M ratio (≥0.07)	Small bowel: ↑in 42% IBS
Kerckhoffs *et al.*, 2010[341]	14 IBS/(15)	PEG excretion; L/M ratio (>0.03)	Small bowel: no change
Zhou *et al.*, 2009[339]	54 IBS-D/(22)	L/M ratio (≥0.07)	Small bowel: ↑in 39% IBS
Park *et al.*, 2009[310]	27 IBS-D, 8 IBS-C, 3 IBS-A/(12)	PEG excretion;	Small bowel: ↑in IBS overall and, based on bowel subtype, in IBS-D and IBS-C
Piche *et al.*, 2009[212]	4 IBS-D, 3 IBS-C, 5 IBS-A/(5)	Biopsy tissue in Ussing chamber: IP to FITC-sulfonic acid	Colon: ↑in all IBS subtypes
Dunlop *et al.*, 2006[335]	15 PI IBS-D, 15 IBS-C/(15)	^{51}Cr-EDTA excretion	Proximal small bowel: ↑in PI IBS-D v IBS-C and v controls; distal small bowel: no change; large bowel: no change

Table 1.3 continued.

Study	*Cases/ (controls)*	*Method*	*Intestinal permeability in IBS v controls*
Dunlop *et al.*, 2006[335]	15 PI IBS-D, 15 IBS-D/(12)	^{51}Cr-EDTA excretion	Small bowel: ↑in IBS-D v PI IBS-D and v controls; large bowel: ↑in IBS-D v controls; no change for PI IBS-D
Marshall *et al.*, 2004[337]	27 IBS, 115 PI-IBS/(86)	L/M ratio (>0.02)	Small bowel: ↑in 35.6% IBS v 18.6% controls
Tibble *et al.*, 2002[342]	339 IBS/(263 organic disease)	lactulose/ L-rhamnose ratio (>0.05)	Small bowel: no change
Spiller *et al.*, 2000[85]	10 PI-IBS/(10)	L/M ratio (>0.03)	Small bowel: ↑in 50% PI-IBS
Berstad *et al.*, 2000[343]	18 IBS/(0)	^{51}Cr-EDTA excretion	Distal small bowel: 0.07% in IBS
Dainese *et al.*, 1999[340]	33 IBS/(0)	L/M ratio (>0.025)	12% above normal

Patients were classified to IBS group using Rome criteria, with the exception of Berstad *et al.* (abdominal pain and/or diarrhoea without indication of organic disease) and Dainese *et al.* (not specified). IP, intestinal permeability. L/M ratio, lactulose/mannitol ratio. ↑ means an increase, ↓ means a decrease.

1.2.5.2. Evidence of altered expression of junctional proteins in IBS

Paracellular permeability is regulated by junctional complexes at the intestinal mucosal barrier. Junctional proteins have not been extensively studied in IBS; however, recently there is a growing interest in this area. To date, occludin and members of the ZO and claudin families have been studied in IBS tissue. A summary of these studies is compiled in Table 1.4.

ZO-1 protein expression has been reported by Bertiaux-Vandaële *et al.* (213) to be lower in the colonic mucosa and redistributed from TJs in IBS tissue compared to controls. More recently, Martínez *et al.* (285) have shown reduced ZO-1 protein expression and redistribution in jejunal biopsy tissue of IBS patients. Similarly, lower ZO-1 mRNA expression has been demonstrated in the colonic (212) and jejunal (285) mucosa of IBS patients; however, other authors did not confirm these findings (213) (Table 1.4). Furthermore, Piche *et al.* (212) showed that integrity of Caco-2 cells exposed to colonic supernatants of IBS biopsy tissue was impaired, as evidenced by lower ZO-1 mRNA levels. ZO-2 protein was also reduced in the jejunal mucosa of IBS patients, though mRNA expression was comparable to controls whereas ZO-3 expression was reduced only at mRNA level (285). In addition, Martínez *et al.* (285) reported that reduced ZO protein expression was associated with enhanced tryptase mRNA expression in IBS tissue and suggested that alterations in ZO proteins are related to increased mast cell activity (285).

Occludin appeared to be reduced in the colonic mucosa (213, 265) and redistributed from the membrane to the cytoplasm in the jejunal mucosa (344) of IBS patients compared to controls (Table 1.4). In addition, its phosphorylation was decreased in IBS jejunal tissue compared to controls (344). These findings were paralleled by increased MLCK expression, enhanced myosin phosphorylation and significantly increased paracellular space between adjacent

enterocytes, as shown with ultrastructural analysis. Enhanced mast cell activation was also reported and associated with these molecular and ultrastructural alterations, and hence may be linked to alterations in TJ complex integrity in IBS (344). Coëffier *et al.* (265) implied that the lower occludin expression was due to proteasome-mediated degradation and that proteasome trypsin-like activity was increased in IBS tissue. The expression of proteasome subunits is regulated by inflammatory mediators; IFN-α was shown to up-regulate proteasome subunits in intestinal epithelial cells (345). Mast cell contribution to proteasome-mediated occludin degradation is not well studied. Coëffier *et al.* (265) however, did not show any correlation between mast cell numbers and proteasome activity.

Altered intestinal barrier integrity has been further evidenced by changes in the expression of claudin proteins in IBS tissue as shown in Table 1.4. CLD-1 expression was reduced in the small intestinal (346) and colonic mucosa of IBS-D patients compared to controls (213, 346) and appeared to be redistributed from the membrane to the cytoplasm (213). However, in a more recent study no alterations were observed in IBS tissue compared to controls (344) (Table 1.4). The data on CLD-1 in IBS-C patients are inconsistent, since one study demonstrated its redistribution from the apical membrane to the cytoplasm in the colonic mucosa (213) while other researchers showed that its expression was increased in the colonic and ileal mucosa (346). Similarly, data on CLD-3 and CLD-4 in IBS are inconsistent (344, 346). In a recent study, Martínez *et al.* (344) reported higher levels of CLD-2 in the jejunal mucosa of IBS patients compared to controls.

Taken together, these studies show altered expression of TJ proteins in mucosal tissue of IBS patients and these alterations are found both in the small and large intestine, though the data are inconsistent. The most consistent and strongest finding appears to be reduced ZO-1 expression in IBS tissue. The exact role of TJ proteins in IBS pathogenesis is not clear and remains an area of current research.

Study	*Cases/ (controls)*	*Protein in IBS v controls*	*mRNA in IBS v controls*
Martínez *et al.*, 2012[344]	7 IBS-D/(7)	Jejunum: **CLD-2↑**; **occludin** redistribution,↓in phosphorylation; **CLD-1**, **CLD-3**, **CLD-4** no change	NA
Martínez *et al.*, 2012[285]	*16 IBS-D/(15); **17 IBS-D/(12)	Jejunum: **ZO-1↓** and redistribution; **ZO-2↓**; **ZO-3** no change	Jejunum: **ZO-1, ZO-3↓**; **ZO-2** no change
Bertiaux-Vandaële *et al.*, 2011[213]	19 IBS-D, 15 IBS-A, 14 IBS-C, 2 IBS-U/(31)	Descending colon: **ZO-1, occludin, #CLD1↓** in IBS and redistribution in IBS-D and IBS-C	Descending colon: **ZO-1, occludin, CLD-1** no change
Coëffier *et al.*, 2010[265]	10 IBS/(8)	Descending colon: **occludin↓**	Descending colon: **occludin** no change
Piche *et al.*, 2009[212]	21 IBS/(12)	NA	Colon: **ZO-1↓**; **occludin** no change
Kong *et al.*, 2007[346]	23 IBS-D, 20 IBS-C/(20)	Ascending colon, ileum: **CLD-1**, **CLD-4**: ↓ in IBS-D, ↑ in IBS-C; **CLD-3↑** in IBS-C, no change in IBS-D	NA

Table 1.4 Summary of studies on TJ protein expression/mRNA levels in IBS patients. Patients were classified to IBS group using Rome criteria.* means protein expression, ** means mRNA expression. # means a trend towards decreased expression. ↑ means an increase, ↓ means a decrease.

1.2.6. Intestinal barrier dysfunction in the pathophysiology of IBS

There is growing evidence that alterations in intestinal barrier function are involved in IBS pathogenesis. As previously discussed, increased intestinal permeability and alterations in junctional proteins have been documented in IBS. Impaired barrier function in IBS appears to be associated with several factors, including a previous incidence of acute gastroenteritis, immune activation, visceral hypersensitivity and severity of abdominal pain, abnormal motility, stress, altered gut microbiota, food allergy, and bile acid malabsorption. There are also considerable data on genetic predisposition to structural abnormalities of barrier function in IBS (Figure 1.11). Thus, the breakdown of the intestinal epithelial barrier might play an important role in the pathogenesis in subsets of IBS patients. This section explores the potential mechanisms underlying barrier dysfunction and its involvement in the pathophysiology of IBS.

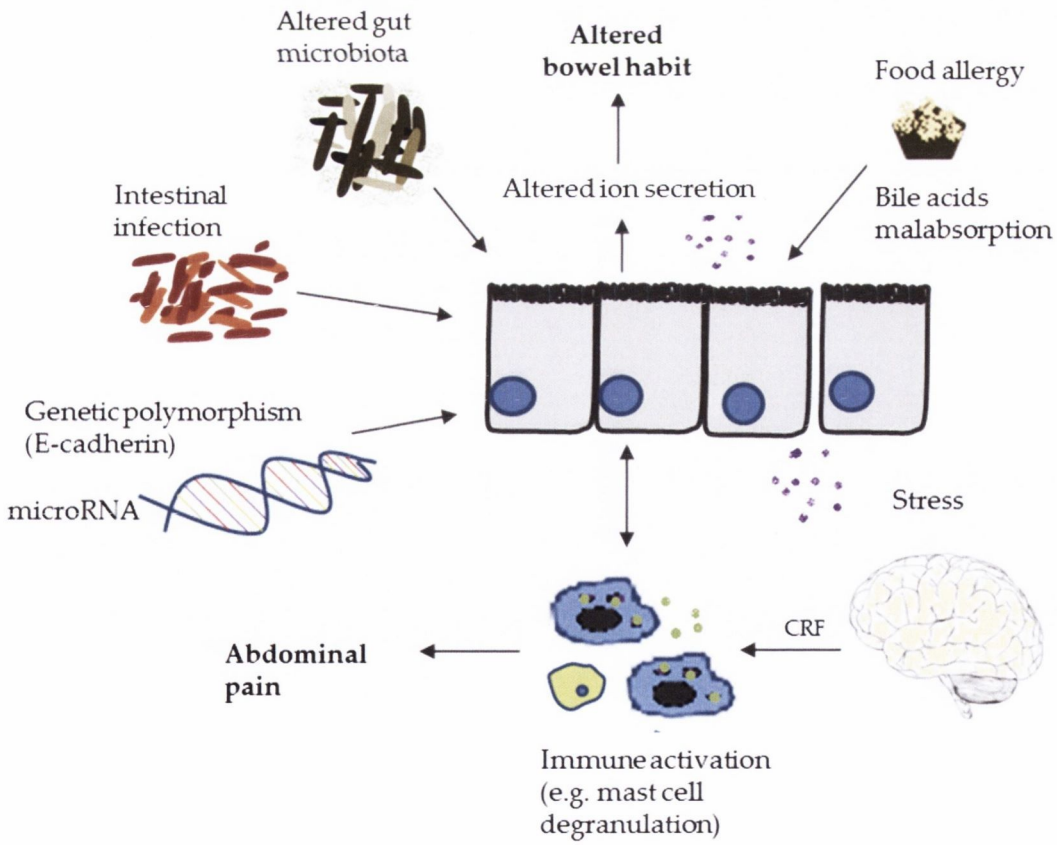


Figure 1.11 Putative role of impaired intestinal barrier in IBS pathophysiology.

1.2.6.1. Intestinal barrier dysfunction following gastrointestinal infection

Increased permeability has been reported in patients with PI-IBS as shown in Table 1.3. It has been suggested that this patient group has a reduced ability to suppress the immune response triggered by previous acute infections with persistence of mildly increased mucosal infiltration of T-cells and increased permeability (85, 335, 337). It is likely that the presence of increased intestinal permeability in PI-IBS reflects the lack of recovery of TJ structure that was disrupted during acute infection. Several enteric pathogens including *Clostridium difficile, Escherichia coli, Bacteroides fragilis, Campylobacter jejuni* and *Vibrio cholera* have abilities to increase intestinal permeability either by direct disruption of TJ proteins (e.g. ZO-1) or through changes in the actin cytoskeleton (347). Interestingly, Dunlop *et al.* (335) demonstrated that small bowel permeability in PI-IBS patients was increased compared to healthy controls but was lower in comparison to IBS patients without the history of acute gastroenteritis, indicating that infection is not an exclusive risk factor for increased permeability in IBS. In line with this, the alterations in TJ proteins outlined in Table 1.4 have been reported in patients without a known history of gastroenteritis.

1.2.6.2. Intestinal barrier dysfunction and immune activation

In general, sustained increased intestinal permeability could lead to uncontrolled passage of luminal antigens and promote local mucosal immune responses that manifest as inflammation (348). Immune activation may also lead to structural alterations in the intestinal epithelial barrier and thus to increased epithelial permeability (57). One component of the immune response that links with barrier dysfunction in IBS is mast cells. In experimental studies, animals exposed to stress exhibited both mast cell activation and increased intestinal permeability. Interestingly, the latter was prevented by mast cell stabilisers (349, 350) indicating

that mast cell activation leads to increased permeability. In IBS, the increased permeability of mucosal biopsy tissue mounted in Ussing chambers significantly correlated with increased mast cell numbers (269). Among mast cell mediators, tryptase appears to be highly relevant since it has been shown to activate PAR-2 on epithelial cells (168) and to increase intestinal permeability of epithelial (351) and mucosal tissue *in vitro* (176). Furthermore, significantly higher tryptase levels have been detected in the colonic (144, 175) and jejunal mucosa (285, 344), and in jejunal fluids (228) of IBS patients compared to controls. Moreover, increased spontaneous release of tryptase from IBS colonic biopsy tissue positively correlated with mucosal mast cell counts in a number of studies (144, 175, 269).

Beyond tryptase, altered levels of cytokines appear to be relevant to barrier dysfunction in IBS pathophysiology. For example, levels of TNF-α, IFN-γ, IL-1β and IL-6 have been shown to be altered in IBS patients (71) (Section 1.2.4.4). Furthermore, there is growing evidence that these are implicated in the regulation of intestinal barrier function *in vitro*. TNF-α and IFN-γ were shown to increase the expression and activity of MLCK, resulting in MLC phosphorylation and a redistribution of TJ proteins (ZO-1, CLD-1 occludin), leading to increased permeability of intestinal epithelial cells (352). IFN-γ appeared to increase paracellular permeability via a redistribution of occludin, JAM-A, CLD-1 and ZO-1 from TJ regions in intestinal epithelial cell culture (353). A reduction of ZO-1 expression and reorganisation of the actin cytoskeleton was also reported (354). This barrier-regulation effect of inflammatory cytokines was further supported by *in vivo* studies where mucosal levels of TNF-α and IFN-γ increased upon T-cell activation and led to MLCK-mediated MLC phosphorylation, ZO-1 reorganisation and enhanced intestinal paracellular permeability (355). Among other cytokines, for example, IL-6 has been recently reported to increase the permeability of intestinal epithelial cells through the up-regulation of CLD-2 protein expression (82). Similarly, IL-β increased the permeability of intestinal

epithelial cells *in vitro*, but through the up-regulation of CLD-1 expression and parallel down-regulation of occludin expression (356).

Immune activation is a reasonably consistent finding reported in IBS patients; however, its exact role and underlying mechanisms in the regulation of the intestinal epithelial barrier in IBS remain under investigation. Intestinal barrier dysfunction in IBS may lead to immune activation or, in contrast, activated immune cells may alter junctional proteins and barrier function. Research is focussing on pro-inflammatory mediators and mast cell activity, particularly mast cell tryptase. While cytokines appear to influence TJ protein expression *in vitro*, the effects of tryptase on the expression of junctional proteins has not yet been explored *in vitro* or in IBS.

1.2.6.3. Intestinal barrier dysfunction and visceral hypersensitivity

Features of barrier dysfunction and visceral hypersensitivity may co-exist in IBS patients. A study by Zhou *et al.* (339) has shown that IBS-D patients with increased intestinal permeability reported increased sensitivity to visceral (rectal distension) and thermal stimuli and higher pain intensity rates compared to IBS-D patients with normal intestinal permeability and to healthy controls. This indicates that enhanced intestinal permeability may precede the development of other IBS symptoms and lead to visceral hypersensitivity in IBS patients (339). Although cause and effect have not been established, alterations in TJ proteins have been reported in IBS patients, as presented in Section 1.2.5.2, and reduced expression of TJ proteins (CLD-1, occludin) was negatively correlated with the severity of abdominal pain (213). A cause and effect relationship was however demonstrated between paracellular permeability and visceral hypersensitivity in an *in vivo* study, where chemical blockage of stress-induced permeability was associated with a decrease in sensitivity to colonic distension in rats (241). The

relationship between impaired intestinal barrier and visceral hypersensitivity might be translated to IBS. Piche *et al.* (212) reported a significant correlation between severity of abdominal pain and paracellular permeability in colonic biopsy tissue of IBS patients. In addition, colonic supernatants from IBS tissue induced increased paracellular permeability of Caco-2 cells that was significantly associated with the severity of abdominal pain. These associations between increased permeability and pain severity might be related to the activity of soluble mediators released from IBS colonic tissue.

There is evidence that mast cell mediators affect both the epithelial barrier, leading to increased permeability, and sensitise nerve endings, leading to visceral hypersensitivity (i.e. tryptase). Interestingly, increased numbers of mucosal mast cells have correlated with visceral hypersensitivity in patients with IBS-D (266). Barbara *et al.* (144) showed that the severity and frequency of perceived abdominal painful sensations in IBS patients correlated with the activated mast cells in close proximity to colonic nerves. This study was later confirmed by Buhner *et al.* (112), who also reported increased levels of histamine and tryptase in IBS mucosa, and showed that the excitation of human submucosal neurons was mediated by histamine, proteases and serotonin. In animals, CRF receptor (CRF-R1) activation has been implicated in both the development of hyperalgesia and increased intestinal permeability (357). Although the exact site of CRF-R1 has not been identified, the study suggested that both central and peripheral CRF-R1 has been involved in these mechanisms.

Furthermore, Gecse *et al.* (307) have suggested that altered microbial activity contributes to both abnormal visceral sensitivity and increased permeability in IBS. In that study, enhanced protease activity identified in faecal supernatants from IBS-D patients was shown to cause visceral hypersensitivity in mice and increased permeability of colonic tissue *in vitro*.

The studies show that mechanisms that alter intestinal permeability and mediate visceral hypersensitivity co-exist in IBS patients. Although these mechanisms remain to be clarified, there is emerging evidence that mast cell activation may link barrier dysfunction and visceral hypersensitivity.

1.2.6.4. Intestinal barrier dysfunction and stress

Stress, either psychological or physical, and either chronic or acute, is a potential threat to intestinal barrier integrity. Healthy female volunteers who had experienced a moderately stressful life event appeared to have an increased macromolecular intestinal permeability in response to experimental acute stress compared to low-stressed volunteers (207). Interestingly, acute stress induced mast cell activation as determined by enhanced release of luminal tryptase in both groups; however, no difference in tryptase concentration was observed between groups. This suggests that mast cell mediators other than tryptase might be involved in stress-induced enhanced permeability; alternatively moderate stress had induced epithelial abnormalities and consequently the defective response to experimental acute stress (207). Recently, Vicario *et al.* (235) have reported that rats exposed to crowding stress exhibited epithelial abnormalities such as increased ion secretion and mucosal intestinal inflammation. However, the same rats did not respond to the exogenous putative stress mediator CRF, while in the control rats increased ionic secretion was observed (235). These chronic stress-induced changes in epithelial physiology, such as the inability to evoke a secretory response to stressful stimuli, might have important clinical manifestations. The epithelium might become vulnerable to pathogenic entities in the gut lumen, thus increasing antigenic uptake and promoting inflammatory responses.

The effect of stress on paracellular permeability appears to involve mast cells. Higher mast cells numbers and their increased degranulation, and increased

intestinal permeability have been reported in rats subjected to water avoidance (240) or crowding (350) stress. However, mast cell-deficient rats exposed to chronic stress appeared to have intestinal permeability comparable to non-stressed controls (240, 350). A higher proportion of degranulated mast cells in stressed rats indicates enhanced release of mast cells pro-inflammatory mediators to the local environment, which might affect epithelial integrity. Among these mediators, tryptase is considered as a potential permeability regulator as described previously in Section 1.2.6.2. In addition, human mucosal mast cells express CRF (358) and respond to it through the activation of cell surface receptors, CRF-R1 and CRF-R2 (359). The activation of these receptors on subepithelial mast cells appears to increase permeability of human colonic mucosa, as evidenced by increased transcellular transport of antigen-sized macromolecules via endosomes (359). In animals, a CRF-R1 agonist increased colonic permeability and altered colonic secretory activities (360). Furthermore, rats exposed to chronic stress appeared to have higher mucosal CRF-R1 expression dependent on the stress level; however, they did not respond to externally administered CRF (235). Therefore, stress might promote enhanced uptake of antigenic molecules, the mechanism associated with mucosal inflammation and possibly implicated in IBS pathogenesis. In line with this, IBS patients appeared to have increased levels of pro-inflammatory cytokines following intravenous CRF infusion (232).

Stress is likely to have a role in initiating and in sustaining barrier dysfunction. The classical study for the role of stress in IBS performed by Qiu *et al.* (361) showed that chronic stress was capable of reactivity of intestinal inflammation in mice. In humans, stress may activate low-grade inflammation and barrier dysfunction after a previous infectious insult.

1.2.6.5. Intestinal barrier dysfunction and microbial activity

Microorganisms adhering to the intestinal wall are thought to influence the host immune system and modulate the epithelial barrier (306). Therefore, alterations in the gut microflora may disrupt epithelial integrity. In IBS-D patients, as shown by Gecse *et al.* (307), enhanced protease activity in faecal supernatants appeared to increase permeability of colonic tissue *in vitro* via activation of PAR-2, redistribution of ZO-1 protein and cytoskeleton contractions. The origin of the elevated protease activity remains to be confirmed, but Gecse *et al.* (307) suggested it could be of microbial origin.

Microorganisms may activate epithelial TLRs and, through this activation, subsequently either strengthen or weaken epithelial barrier integrity (362). TLRs expression appears to be altered in IBS colonic tissue, as described in Section 1.2.4.7, and individuals with polymorphism in genes regulating host-microbial interactions (TLR9) and barrier function (E-cadherin) appear to be more susceptible to develop PI-IBS (211). It is likely that these alterations may affect epithelial barrier properties in subsets of IBS patients. Data from *in vitro* study showed that stimulation of intestinal epithelial TLR2 enhanced barrier resistance through tightening and sealing of TJs, including ZO-1 redistribution to further apical TJ areas (363). In contrast, LPS, which is a ligand for TLR4, increased paracellular permeability of colonic epithelial cells. Interestingly, this effect was ameliorated by TLR2 activation, suggesting a cross-talk between these TLRs in terms of epithelial integrity regulation (364).

Taken together, there are emerging data that gastrointestinal microbiota may alter intestinal barrier function in IBS. This is an important area of research, given the potential of therapeutic intervention directed at the gut flora such as probiotics (315).

1.2.6.6. Intestinal barrier dysfunction and food hypersensitivity

There is a high prevalence of IBS among patients with self-reported food hypersensitivity (365) and IBS patients were reported to be more likely to have a positive skin test against food allergens (269). Furthermore, severity of IBS symptoms was reported to be significantly associated with the presence of an allergic background, including food allergies in IBS patients, possibly mediated by enhanced mast cell activation and paracellular permeability (269). Newer evidence has proposed gluten sensitivity in IBS patients (366). Food allergy has been implicated in IBS pathogenesis and in barrier dysfunction, although this remains controversial (367, 368). 'True food allergy' is rare (13), and would not, on its own, account for the pathogenesis of a common condition such as IBS. It is likely that factors other than allergy (e.g. stress, abnormal gut microbiota) are associated with the development of IBS symptoms. However, there are emerging data that nutrients may play a role in the regulation of intestinal barrier function, for example with ongoing research from my group investigating the role of vitamin D in maintaining intestinal permeability.

1.2.6.7. Emerging areas: intestinal barrier and bile acids

IBS-D patients have been reported to synthesise and excrete higher levels of bile acids compared to controls (369) and up to one-third are thought to have had adult-onset idiopathic bile acid malabsorption (369, 370). Bile acids, such as deoxycholic acid and chenodeoxycholic acid, were shown to induce occludin dephosphorylation and cytoskeletal reorganisation paralleled by increased paracellular permeability of intestinal epithelial cells *in vitro* (371). In animals, deoxycholic acid was shown to increase colonic permeability by activating enteric neurons (372). Whether there is a link between bile acids and intestinal permeability in IBS has yet to be shown.

1.2.6.8. Emerging areas: intestinal barrier and microRNAs

Recently, attention has focused on microRNAs as potential factors involved in the regulation of intestinal permeability. It has been shown that IBS-D patients with increased permeability had elevated expression of miR-29a and reduced expression of glutamine synthetase in the small bowel and the colon, compared with healthy controls and IBS patients with normal permeability. MiR-29a was shown to regulate intestinal permeability through regulation of glutamine synthetase expression *in vitro* (338). These preliminary studies suggest that impaired intestinal permeability observed in IBS-D patients might be due to the altered level of microRNAs. Beyond glutamine synthetase, miR-29a has more than 1000 known estimated target mRNAs. Of note, CLD-1 has been identified as a target (373) and, based on the evidence of its altered expression in IBS colonic tissue (Section 1.2.5.2), is particularly of interest in the context of IBS. However, regulation of CLD-1 by miR-29a has not yet been explored in IBS to date. Another *in vitro* study suggested that ZO-1 expression might be regulated by its corresponding microRNA i.e. miR-212 (374). It has been demonstrated that due to up-regulation of miR-212, the target levels of ZO-1 decreased resulting in enhanced paracellular permeability of intestinal epithelial cells (374). However, whether junctional proteins are targets of microRNAs in IBS is unknown. Further studies are required to determine functional targets of microRNAs and their potential link to intestinal permeability in IBS.

1.2.7. Mast cells as a potential target in IBS therapy

Increased mast cell numbers and evidence of mast cell activation has been reported in IBS patients (72). Mast cells appear to be a key component of enhanced immune activation in IBS patients and their alterations are linked to core IBS symptoms, namely abdominal pain and altered bowel habit. Thus, the regulation of mast cell activity and the activity of mast cell mediators may be considered as a therapeutic approach in IBS.

Animal studies demonstrated that a mast cell stabiliser, doxantrazole, prevented both stress- and chemically-induced visceral hypersensitivity (375). Recently, treatment of IBS patients with a mast cell stabiliser, ketotifen, increased the threshold of discomfort and improved abdominal pain together with other digestive symptoms such as bloating, diarrhoea and incomplete evacuation (268). However, ketotifen is a mast stabilising agent with histamine H1 receptor antagonistic properties, and further studies are required to investigate if mechanisms other than mast cell stabilisation are involved. In addition, in a pilot study, the anti-inflammatory compound mesalazine reduced mucosal mast cell numbers in the colon of IBS patients and the levels of related mediators such as tryptase and histamine. Furthermore, mesalazine treatment improved general well-being and reduced abdominal pain in IBS patients compared to placebo (376). However, further studies in larger patient cohorts are required to confirm the effects of mesalazine on IBS symptoms.

Mast cell proteases are attractive targets for inhibition due to their defined active sites and the relative ease to design inhibitors with high selectivity (377). Furthermore, as mast cell proteases are stored in mast cell granules under normal conditions, mast cell inhibitors might be designed to selectively target proteases released from activated mast cell at the site of pathologic condition (105). To date,

the efficacy of mast cell protease inhibition in IBS patients has not been investigated; however a growing interest particularly in terms of tryptase inhibition is noted. Recent *in vitro* study reported that tryptase inhibition with nafamostat mesilate (NM) significantly reduced permeability of rectal biopsy tissue of IBS-D patients compared to controls (176). Moreover, in experimental colitis anti-tryptase treatment, using NM significantly reduced intestinal inflammation (378) and NM also reduced mast cell infiltration and chymase activity (379). However, specific anti-protease treatment alone has limited therapeutic application since degranulating mast cells release an array of mediators besides proteases, including pro-inflammatory cytokines, which are involved in IBS pathophysiology. Therefore, a broader approach such as mast cell stabilisation may have stronger therapeutic potential for IBS.

1.3. Overall aims of the thesis

This thesis hypothesised that mast cells, and specifically the mast cell protease tryptase, may regulate intestinal permeability in IBS. Santos *et al.* (240) have shown that chronic stress resulted in increased mast cell numbers and activation in parallel with epithelial barrier dysfunction in animal models. More recent data suggest that tryptase may increase intestinal permeability (176) and down-regulate ZO-1 expression *in vitro* (57).

The aims of the thesis are:

- To investigate if tryptase disrupts the intestinal epithelial barrier *in vitro* using the intestinal epithelial cell line, Caco-2 (Chapter 3).

- To investigate if mast cell mediators, particularly tryptase, disrupt the intestinal epithelial barrier *in vitro* using a co-culture model of intestinal epithelial cells, Caco-2, and human mast cells, HMC-1 (Chapter 4).

- To investigate if the expression of TJ proteins (JAM-A, CLD-1 and ZO-1) is reduced in the caecal mucosa of IBS patients when compared to controls and if it associates with IBS symptoms (Chapter 5).

- To investigate if the expression of AJ protein E-cadherin is reduced in the caecal mucosa of IBS patients when compared to controls and if it associates with IBS symptoms (Chapter 6).

CHAPTER 2

MATERIALS AND METHODS

2.1. Materials

Reagents were purchased from Sigma-Aldrich (Ireland) unless stated otherwise. The mouse anti-ZO-1, rabbit anti-CLD-1, rabbit anti-CLD-3, mouse anti-CLD-2, rabbit anti-occludin, rabbit anti-JAM-A, and goat anti-rabbit Alexa Fluor® 633-conjugated antibodies were from Zymed Laboratories Inc. (UK). The mouse anti-E-cadherin was sourced from BD Biosciences (UK) and mouse anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) from Millipore (Ireland). The HRP-conjugated goat anti-mouse and anti-rabbit antibodies were purchased from Pierce (Ireland) and BD Pharmingen™ (UK), respectively. The FITC-conjugated goat anti-mouse and anti-rabbit antibodies were purchased from Jackson ImmunoResearch (USA).

2.2. Cell culture studies

Summary of methods used to investigate intestinal epithelial integrity in cell culture models is shown in Figure 2.1.

2.2.1. Cell lines

Caco-2 cells: The human colon adenocarcinoma cell line, Caco-2, was purchased from European Collection of Animal Cell Culture (ECACC, UK).

Caco-2 ("Carcinoma Colon") cells originate from human colon adenocarcinoma (380); however, they show phenotypic similarities to small intestinal enterocytes. They grow as a monolayer of cells that, at confluency, initiates a process of differentiation. They develop typical morphology of enterocytes with a brush

border with well-developed microvilli, AJC and a distinct apical and basolateral distribution of membrane components, including enzymes, receptors and transport systems (381). They can be grown on either polycarbonate or polyester filters to develop differentiated monolayers for permeability experiments (382, 383).

HMC-1 cells: The human mast cell line-1, HMC-1, was a generous gift from Professor Joseph H. Butterfield, Mayo Clinic (Rochester, MN, USA).

The HMC-1 cell line was derived from a patient with mast cell leukaemia (384). HMC-1 cells express a number of the known mast cell mediators including tryptase, histamine and heparin. Of note, HMC-1 cells express β-tryptase but not α-tryptase, and the former appears to be enzymatically active. The cells do not express a surface high-affinity IgE-receptor, FcεRI. Also, HMC-1 cells lack chymase expression and show a very low expression of carboxypeptidase A mRNA, indicating that the HMC-1 cell line represents, based on the protease content, a MC_T type of mast cells (385).

2.2.2. Cell culture

Cell maintenance

Caco-2 cells: Cells (P13 to P35) were cultured in Dulbecco's Modified Eagle Medium (DMEM) with 10% v/v fetal bovine serum (FBS), 2 mM L-Glutamine, 1% v/v non-essential amino acids, 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were cultured in 75 cm^2 tissue culture flasks until confluent, with feeding on alternate days (37 °C, 5% CO_2).

HMC-1 cells: Cells (P68 to P94) were routinely cultured in Iscove's medium [(+) 25 mM 4-(2-Hydroxyethyl)piperazine-1-ethanosulfonic acid (HEPES), (+) sodium

bicarbonate, (+) L-Glutamine] with 10% v/v iron supplemented calf serum (CS) and 1.2 mM α-thioglycerol. For co-culture studies, HMC-1 cells were cultured in DMEM medium with 10% v/v heat deactivated iron supplemented CS, 2 mM L-Glutamine, 1% v/v non-essential amino acids, 1.2 mM α-thioglycerol, 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were cultivated in 75 cm^2 tissue culture flasks with gentle shaking/resuspending every couple of days to break up small clumps in culture. Cells were passaged approximately once per week (37 °C, 5% CO^2).

Cell sub-culturing

Caco-2 cells: Sub-culturing was carried out by removing spent medium and twice washing with 12 ml of pre-warmed phosphate buffered saline (PBS) (37 °C). Pre-warmed trypsin-EDTA solution 1 X (37 °C) was added to the tissue culture flask (4 ml into 75 cm^2; 2 ml into 25 cm^2) and cells were incubated at 37 °C under 5% CO^2 for 8 min. Trypsin was deactivated by adding 8-12 ml of pre-warmed medium (37 °C) and cells were transferred to a sterile tube and pelleted by centrifugation at 200-400 g for 5 min. Cells were resuspended in pre-warmed medium (37 °C) and transferred to 75 cm^2 tissue culture flasks at reduced concentration. The viability of Caco-2 cells was typically > 99%.

HMC-1 cells: Cells were splitted when cell density reached at least 10^6 cells/ml. The cell suspension was transferred into a sterile tube and pelleted by centrifugation at 200g for 10 min. Cells were washed with pre-warmed PBS and transferred to 75 cm^2 tissue culture flasks at reduced concentration. The viability of HMC-1 cells was typically > 99%.

Cell culture in Transwells

Caco-2 cells: Cells (P16 to P30) were cultured in 75 cm^2 tissue culture flasks until confluent, then seeded on Transwells® polyester filters (0.4 μm pore, diam. 12

mm, surface area 1.12 cm^2) at a density of 5 x 10^5 cells/filter. Cells were cultured in DMEM medium with 2 mM L-Glutamine, 1% v/v non-essential amino acids, 10% v/v FBS until established a differentiated and polarised monolayer, from 21 to 26 days with feeding on alternate days (37 °C, 5% CO_2). The integrity of Caco-2 cells was determined by the measurement of TER on alternate days. TER was measured with a battery-powered EVOM voltohmmeter with chopstick-type electrode (World Precision Instruments, USA). EVOM produced current and the resistance across two electrodes was automatically determined using Ohm's law, which states that resistance is equal to the change in the transepithelial voltage divided by the change in the transepithelial current. TER (Ω x cm^2) was calculated by subtracting blank filter resistance from cell monolayer resistance and multiplied by the filter area.

Electric current travels through pathways of less resistance in general. The resistance of cell membranes (apical and basolateral) is typically much higher than that of paracellular pathway, defined by the composition and complexity of TJ. Therefore, TJs determine whether the overall resistance of the epithelium is high or low and measurement of electrical current across an epithelial monolayer serves as an indicator of tightness of TJ (386). With this method, confluent cells with tight TJs formed between cells are expected to block the current (cause resistance to the flow) and yield high resistance values. On the contrary, when poor TJs are formed between epithelial cells, most of the electric current passes through paracellular space and the epithelial cells yield low resistance values.

***Co-culture of Caco-2 and HMC-1 cells*:** Caco-2 cells (P22 to P35) were seeded at a density of 5 x 10^5 cells/filter in Transwells®. HMC-1 cells (P77-P95, 5 x 10^5 cells/filter) were added to the basolateral compartment of the Transwells®. Cells were co-cultured until Caco-2 established a differentiated and polarised monolayer, from 21 to 23 days, in DMEM medium with 5% v/v heat-deactivated

FBS, 5% v/v heat deactivated iron supplemented CS, 2 mM L-Glutamine, 1% v/v non-essential amino acids, 100 U/ml penicillin and 100 μg/ml streptomycin with feeding on alternate days. (37 °C, 5% CO_2). In parallel, Caco-2 cells were also cultured without HMC-1 cells as controls for the co-culture. The integrity of the Caco-2 monolayers was determined with the measurement of TER on alternate days.

Cell counting

Cell counting was carried out using a Neubauer's haemocytometer. For accurate cell counting, cells were resuspended to break up clumps in culture. Cells were prepared for live cell counting by mixing 1:1 with trypan blue. Cell suspensions (10 μl) were inserted under the coverslip into each chamber of the haemocytometer. Cells in the central large square were counted and the average cell number from both chambers of the haemocytometer was calculated. Cells showing blue dye within the cell membrane were excluded from the live cell count. The concentration (cells/ml) was determined using the equation:

Average cell number per square x 2 (trypan blue dilution factor) x 10^4 = cells/ml

Preparation of frozen cell stocks

Caco-2 cells: Cells were grown until 90-100% confluency, trypsinised, transferred to a sterile tube and pelleted by centrifugation at 200-400g for 5 min. Cells were resuspended in ice cold DMEM medium containing 20% FBS and 20% dimethyl sulfoxide, DMSO. The cell suspension (10^6 cells/ml) was immediately transferred into cryovials (kept on ice) at 1 ml/vial. Cryovials were cooled down to -80 °C overnight and then transferred to liquid nitrogen for long-term storage.

***HMC-1 cells*:** Cells were transferred to a sterile tube and pelleted by centrifugation at 200g for 10 min. Cells were resuspended at twice the desired

final concentration in ice cold DMEM/20% CS. Suspended cells were mixed with equal volume of ice cold DMEM medium containing 20% CS and 20% DMSO. Medium was sterile filtered by 0.2 μm filter. The suspended cells (5-10x10^6 cells/ml) were aliquoted to cryovials (kept on ice) at 1 ml/vial and snap-frozen in liquid nitrogen for long-term storage.

Revival of cells from frozen stocks

Caco-2 cells: Vials with frozen Caco-2 cells were removed from liquid nitrogen storage and transferred quickly (on ice) to a water bath set at 37 °C and left to thaw (~ 2 min). Thawed cells were transferred to a sterile tube containing 10 ml of warm DMEM medium, resuspended and pelleted by centrifugation at 200-400g for 5 min. The pellet was re-suspended in pre-warmed medium and placed into a 25 cm^2 tissue culture flask and incubated at 37 °C in a humidified atmosphere with 5% CO_2.

HMC-1 cells: Vials with frozen HMC-1 cells were removed from liquid nitrogen storage and transferred quickly (on ice) to a water bath set at 37 °C and left ~ 2 min until thawed. Cells were hold on ice for 2 min and transferred to a sterile tube. Thawing DMEM medium containing 20% CS and 50 μl 1:10 α-thioglycerol (sterile filtered by 0.2 μm filter) (RT) was added as follows:

- T0-T1 min: 1 drop;
- T1-T2 min: 2 drops;
- T2-T3 min: 4 drops;
- T3-T4 min: 8 drops;
- T4-T5 min: 16 drops.

Cells were kept in the medium for 5 min. The volume was brought to 10 ml and cells were pelleted by centrifugation at 200g for 10 min. Cells were washed twice in DMEM/20% CS to remove traces of DMSO, resuspended in pre-warmed medium (37 °C) and placed into a 25 cm^2 tissue culture flask.

2.2.3. Caco-2 monolayers incubated with tryptase

2.2.3.1. Integrity of Caco-2 monolayers determined by the measurement of TER

Polarised monolayers of Caco-2 cells were washed with pre-warmed (37 °C) Hank's Balanced Salt Solution (HBSS) buffer containing 11 mM glucose and 25 mM HEPES (HBSS/HEPES), and equilibrated with HBSS/HEPES buffer until resistance stabilised. Then tryptase (3 mU or 15 mU) was added to the apical compartment of the Transwell and incubated for up to 24 hours (37 °C, 5% CO_2). The integrity of Caco-2 monolayers was determined periodically by the measurement of TER over 24 hours and the results were displayed as the percentage change relative to untreated controls.

2.2.3.2. Paracellular permeability of Caco-2 monolayers determined by apical-to-basolateral flux of FITC-dextran

Polarised monolayers of Caco-2 cells were washed with pre-warmed (37 °C) HBSS/HEPES buffer and equilibrated with HBSS/HEPES buffer until resistance stabilised. Then, to assess epithelial permeability, FITC-dextran (4 kDa, 100 μg/ml) was added to the apical compartment of the Transwell followed by application of tryptase (3 mU or 15 mU) to the apical compartment of the Transwell for up to 4 hours of incubation (37 °C, 5% CO_2). Basolateral samples were taken at intervals and were replenished with fresh pre-warmed HBSS/HEPES buffer at each sample timepoint. The apical-to-basolateral flux of FITC-dextran was measured with a Thermo Fisher Varioskan Flash spectrophotometer (UK) using an external standard curve. Excitation and emission wavelengths were set at 495 and 520 nm, respectively, with bandwidth of 5 nm. The apparent permeability coefficient (Papp) of each treatment was

calculated according to Papp=dQ/dt (1/AC$_0$), where dQ/dt is the permeability rate derived from the slope of the line, A is the diffusion area and C$_0$ is the initial donor solution concentration (387).

2.2.4. Tryptase activity: effects of heparin

Previous studies have shown that tryptase is active as a tetramer when stabilised by heparin proteoglycan (158). To assess the effect of heparin on tryptase activity Caco-2 monolayers were treated with tryptase alone, heparin alone or tryptase in combination with heparin.

Polarised monolayers of Caco-2 cells were washed with pre-warmed (37 °C) HBSS/HEPES buffer. To assess the effect of heparin on tryptase activity, Caco-2 monolayers were equilibrated with HBSS/HEPES buffer containing heparin (50 μg/ml) until resistance stabilised. Then tryptase (3 mU or 15 mU) was added to the apical compartment of the Transwell and incubated for up to 4 hours (37 °C, 5% CO_2). TER was measured periodically over 4 hours and the results were displayed as the percentage change relative to untreated controls. TER was calculated as described previously (Section 2.2.2). To assess epithelial permeability, FITC-dextran (4 kDa, 100 μg/ml) was added to the apical compartment of the Transwell up to 4 hours. Basolateral samples were taken at intervals and were replenished with fresh pre-warmed HBSS/HEPES buffer (+/- heparin) at each sample timepoint. The apical-to-basolateral flux of FITC-dextran was measured as described previously (Section 2.2.3).

2.2.5. Caco-2 monolayers following mast cell stimulation with compound 48/80 in a Caco-2/HMC-1 co-culture model

TER value was determined prior the experiment. To assess the effects of mast cell degranulation of epithelial integrity, mast cells were challenged with commercially available synthetic compound 48/80 to initiate degranulation. Compound 48/80 activates mast cells through Ig-E independent receptors, i.e. G protein-coupled receptors (388). These receptors are 7-trans-membrane receptors, which are coupled to Guanosine-5'-triphosphate(GTP)-binding proteins, i.e. G proteins. After ligand binding, activation of phospholipase C (PLC) and protein kinase C (PKC) leads to massive influx of extracellular calcium whose high concentrations cause mast cell granule translocation and granule docking (389). Interaction between the integral membrane proteins called soluble N-ethylmaleimide-sensitive factor (NSF) attachment protein receptors (SNAREs), present on both granules and the plasma membrane, allows fusion between granules and cell membrane leading to the release of mast cell mediators (390).

2.2.5.1. Integrity of Caco-2 monolayers determined by the measurement of TER

HMC-1 cells were transferred from the basolateral compartment of the Transwell to a sterile tube and pelleted by centrifugation at 200g for 10 min. Meanwhile, fresh pre-warmed DMEM medium (37 °C) was added to the apical side of polarised monolayers of Caco-2 cells and Caco-2/HMC-1 monolayers. Then HMC-1 cells (5-6 x 10^6 cells/ml) were resuspended in 1 ml of medium containing compound 48/80 at a final concentration of 5 μg/ml, replaced in the Transwell and incubated for up to 24 hours (37 °C, 5% CO_2). TER was measured periodically over 24 hours and the results were displayed as the percentage change relative to the unstimulated control as described previously (Section 2.2.2).

2.2.5.2. Paracellular permeability of Caco-2 monolayers determined by apical-to-basolateral flux of FITC-dextran

HMC-1 cells were transferred from the basolateral compartment of the Transwell to a sterile tube and pelleted by centrifugation at 200g for 10 min. HMC-1 cells were washed twice with pre-warmed (37 °C) HBSS/HEPES containing heparin 25 μg/ml. Meanwhile, polarised monolayers of Caco-2 cells and Caco-2/HMC-1 monolayers were equilibrated with HBSS/HEPES containing heparin 25 μg/ml. FITC-dextran (4 kDa, 100 μg/ml) was added to the apical compartment of the Transwell. Then HMC-1 cells (5-6 x 10^6 cells/ml) were resuspended in 1 ml of warm HBSS/HEPES buffer containing compound 48/80 at a final concentration of 5 μg/ml, and put back to the Transwell and incubated for up to 24 hours (37 °C, 5% CO_2). Basolateral samples were taken at intervals and were replenished with fresh pre-warmed HBSS/HEPES buffer at each sample timepoint. FITC-dextran concentration was measured as described previously (Section 2.2.3). TER was measured to confirm that mast cell stimulation decreased epithelial integrity.

2.2.5.3. Transmission electron microscopy

To examine the ultrastructure of the co-culture model following mast cell stimulation, polarised monolayers of Caco-2 cells and Caco-2/HMC-1 monolayers were washed 3 times with pre-warmed (37 °C) HBSS/HEPES. Then the monolayers were fixed in HBSS/HEPES with 4% glutaraldehyde and stored at fixative at 4 °C. Cells were processed into resin, ultramicrotomy and contrasted in preparation for examination by TEM with a Tecnai 12 microscope (FEI UK Ltd., UK).

Cells were processed for examination by TEM in the Conway Institute, University College Dublin (Ireland) in collaboration with Professor Dimitri Scholz and Dr Cormac O 'Connell.

2.2.6. Inhibition of mast cell tryptase activity in a Caco-2/HMC-1 co-culture model

Mast cell tryptase activity was inhibited with NM. NM is a broad spectrum serine protease inhibitor; however, it is tryptase specific when used at low concentrations (10^{-9}-10^{-11} M) (378, 391). Other serine proteases that are inhibited by NM, though at higher concentrations (10^{-7}-10^{-8} M), include trypsin, kallikrein, thrombin and plasmin (378, 392). NM was also reported to inhibit experimentally-induced colonic inflammation in rats (378) and to attenuate allergen-induced airway inflammation in mice (393).

In this study, monolayers of Caco-2 cells and Caco-2/HMC-1 monolayers were treated with compound 48/80 alone, NM alone or compound 48/80 in combination with NM.

TER values were determined prior the experiment. HMC-1 cells were transferred from the basolateral compartment of the Transwell to a sterile tube and pelleted by centrifugation at 200g for 10 min. Meanwhile, fresh pre-warmed medium (37 °C) was added to the apical side of polarised monolayers of Caco-2 cells or Caco-2/HMC-1 monolayers. Then HMC-1 cells (5-6 x 10^6 cells/ml) were resuspended in 1 ml of warm DMEM medium containing compound 48/80 (5 μg/ml) and NM (10^{-10}-10^{-11} M), and put back to the Transwell and incubated for up to 24 hours (37 °C, 5% CO_2). TER was measured periodically and the results were displayed as the percentage change relative to corresponding unstimulated controls (+/- NM). TER was calculated as described previously (Section 2.2.2). To assess epithelial permeability, FITC-dextran (4 kDa, 100 μg/ml) was added to the apical compartment of the Transwell. Then HMC-1 cells (5-6 x 10^6 cells/ml) were resuspended in 1ml of warm HBSS/HEPES buffer containing heparin (25 μg/ml), compound 48/80 (5 μg/ml) and NM (10^{-10}-10^{-11} M), and put back to the Transwell

and incubated for up to 24 hours (37 °C, 5% CO_2). Basolateral samples were taken at intervals and were replenished with fresh pre-warmed buffer at each sample timepoint. FITC-dextran concentration was measured as described previously (Section 2.2.3). TER was measured to confirm that mast cell stimulation decreased epithelial integrity.

2.2.7. Expression of junctional proteins by western blotting

The effect of tryptase and mast cell stimulation with compound 48/80 (+/- NM) on the expression of junctional proteins was evaluated by western blot analysis. Transwell plates were put on ice, medium was removed and polarised monolayers of Caco-2 cells or Caco-2/HMC-1 monolayers were three times washed in ice cold PBS. To extract total protein content from the monolayers, cells were lysed in 150-200 µl radioimmunoprecipitation (RIPA) buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1 % sodium dodecyl sulfate, SDS) mixed at 19:1 ratio with 1X complete protease inhibitor cocktail (Roche Applied Science, UK). The cells were scraped from filters, transferred to eppendorfs and sonicated for 1 min. The cell lysates were stored at – 20 °C until required for use. Concentrations of total protein content were determined with Thermo Fisher Nanodrop 1000 Spectrophotometer (UK) in 2 µl of sample. Equal amounts (10-20 µg) were mixed with 5X loading buffer dye (0.06 M Tris pH 6.8, 25% glycerol, 2% SDS, 0.1% bromophenol blue, 0.7 M β-merkaptoethanol) followed by denaturation at 95 °C for 5 min. The samples were loaded to wells of 4% stacking gel [0.25 M Tris pH 6.8, 4% acrylamide (acrylamide:bisacrylamide 29:1), 0.1% SDS, 0.05% ammonium persulfate (APS), N,N,N′,N′-Tetramethylethylenediamine (TEMED)] and separated in 6-10% (depending on the protein) resolving gel [0.5 Tris pH 8.8, 6-10% acrylamide (acrylamide:bisacrylamide 29:1), 0.1% SDS, 0.05% APS, TEMED] in the presence of ColorBurst™ Electrophoresis Marker as a molecular weight control. Proteins

were separated in 1X Tris-glycine-SDS buffer (TGS) (25 mM Tris, 192 mM glycine, 0.1% SDS, pH 8.3) at 140 V for approximately 2 hours. Proteins were transferred to a nitrocellulose membrane (Whatman, VWR International Ltd., Ireland) using semi-dry system. Prior to transfer, blotting paper (Bio-rad, Ireland), nitrocellulose membrane and polyacrylamide gels were equilibrated in the transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol, 0.1% SDS). Proteins were transferred at 25 V for 1 to 1.5 hour. Membranes were blocked 1X Tris-buffered saline (TBS) buffer (25 mM Tris, 140 mM NaCl, 3 mM KCl, pH 7.4) with either 5% nonfat dry milk, 0.1% bovine serum albumin (BSA), 0.1% Tween 20 (CLD-1, CLD-3, E-cadherin) or 5% BSA and 0.1% Tween 20 (JAM-A, ZO-1, CLD-2) or 10% non-fat dry milk, 0.1% BSA, 0.1% Tween 20 (occludin) or 5% nonfat dry milk and 0.1% Tween 20 (GAPDH). Blots were incubated with a primary antibody (0.05-1 μg/ml) (Table 2.1) overnight at 4 °C , were washed 3 times with TBS containing 1% Tween-20 and were incubated with goat anti-mouse or anti–rabbit conjugated to HRP for 1 hour at RT (0.01-0.016 μg/ml) (Table 2.1). Proteins were detected by chemiluminescence (Millipore, Ireland). The density of each individual band was compared to the corresponding control band and normalised against GAPDH (loading control protein) by densitometry. ImageJ software was used to analyse western blot signals and to adjust contrast and brightness of the images (http://rsbweb.nih.gov/ij/). The results were expressed as a change relative to untreated controls. The results were based on data from a minimum of 2 independent experiments. More than 2 independent experiments were performed when the findings were unclear and further study was required to confirm either a positive or negative result.

Junctional protein	Primary antibody	Secondary antibody
ZO-1	1 μg/ml	0.01 μg/ml
E-cadherin	0.05 μg/ml	0.01 μg/ml
Occludin	0.25 μg/ml	0.16 μg/ml
JAM-A	0.1 μg/ml	0.16 μg/ml
CLD-1	0.12 μg/ml	0.16 μg/ml
CLD-2	1 μg/ml	0.01 μg/ml
CLD-3	0.12 μg/ml	0.16 μg/ml

Table 2.1 Concentrations of antibodies that were used to detect junctional proteins by western blotting.

2.2.8. Expression of junctional proteins by immunofluorescence confocal microscopy

The effect of tryptase and mast cell stimulation with compound 48/80 (+/- NM) on the expression and localisation of junctional proteins was evaluated by immunofluorescence confocal microscopy analysis. Polarised monolayers of Caco-2 cells were rinsed with pre-warmed PBS and permeabilised with cold methanol (-20 °C) for 30 min. Non-specific binding sites were blocked with 1% BSA in PBS for 10 min. Cells were incubated in 1% BSA in PBS with primary antibodies as follows: anti-JAM-A, anti-ZO-1, anti-CLD-1, anti-CLD-3 and anti-occludin (5-20 μg/ml) (Table 2.2) for 1 hour at RT. Cells were washed 3 times with 1% in BSA in PBS and incubated with FITC-conjugated goat anti-mouse or anti–rabbit (1-2 μg/ml) (Table 2.2) as appropriate for 1 hour at RT. Cells were washed 3 times with 1% BSA–PBS and postfixed with 4% paraformaldehyde for

10 min. Monolayers were mounted on slides with vectashield containing 4',6-diamidino-2-phenylindole (DAPI, Vector Laboratories, USA), and covered with a coverslip. Specimens were stored at 4 °C prior to analysis on an Olympus Fluoview confocal FV100 microscope with *UPlan FLN* 40×/1.30 lenses and with FV10-ASW 2.0 Viewer software (Germany). Specimens for which the primary antibody was omitted were used as negative controls for junctional proteins (Figure 2.2).

Junctional protein	Primary antibody	Secondary antibody
ZO-1	10 μg/ml	2 μg/ml
Occludin	20 μg/ml	1 μg/ml
JAM-A	10 μg/ml	1 μg/ml
CLD-1	7.5 μg/ml	2 μg/ml
CLD-3	5 μg/ml	1 μg/ml

Table 2.2 Concentrations of antibodies that were used to detect junctional proteins by immunofluorescence confocal microscopy.

2.2.9. Statistical analysis – cell culture

Statistical analysis was performed on cell culture data using SPSS software (IBM Corporation, USA). Differences between 2 groups were compared with parametric 2-tailed Student's t-test. Differences between more than 2 groups were compared with 1-way analysis of variance (1-way ANOVA) followed with the post hoc Tukey's test. Results are expressed as mean and standard error of the mean (SEM). $P \leq 0.05$ was considered statistically significant.

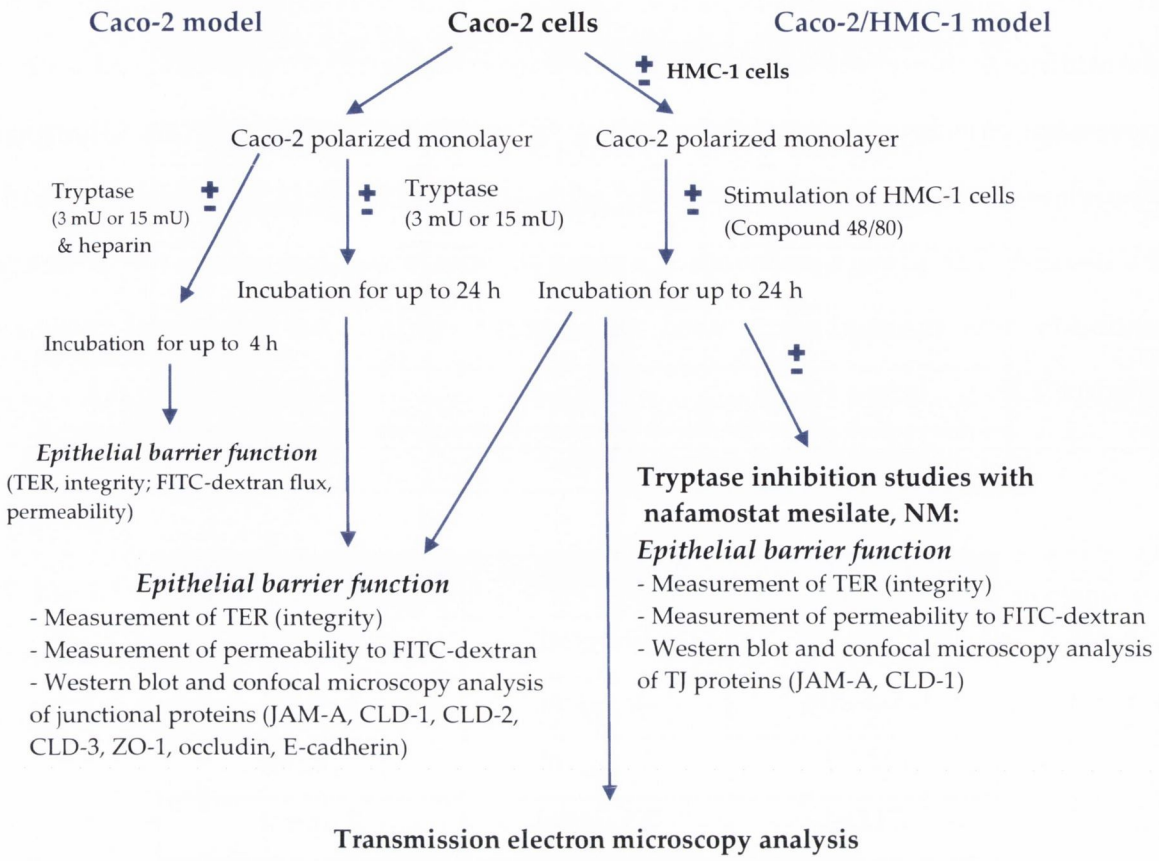


Figure 2.1 Summary of methods used to investigate epithelial barrier function in the Caco-2 cell model and in the Caco-2/HMC-1 co-culture model.

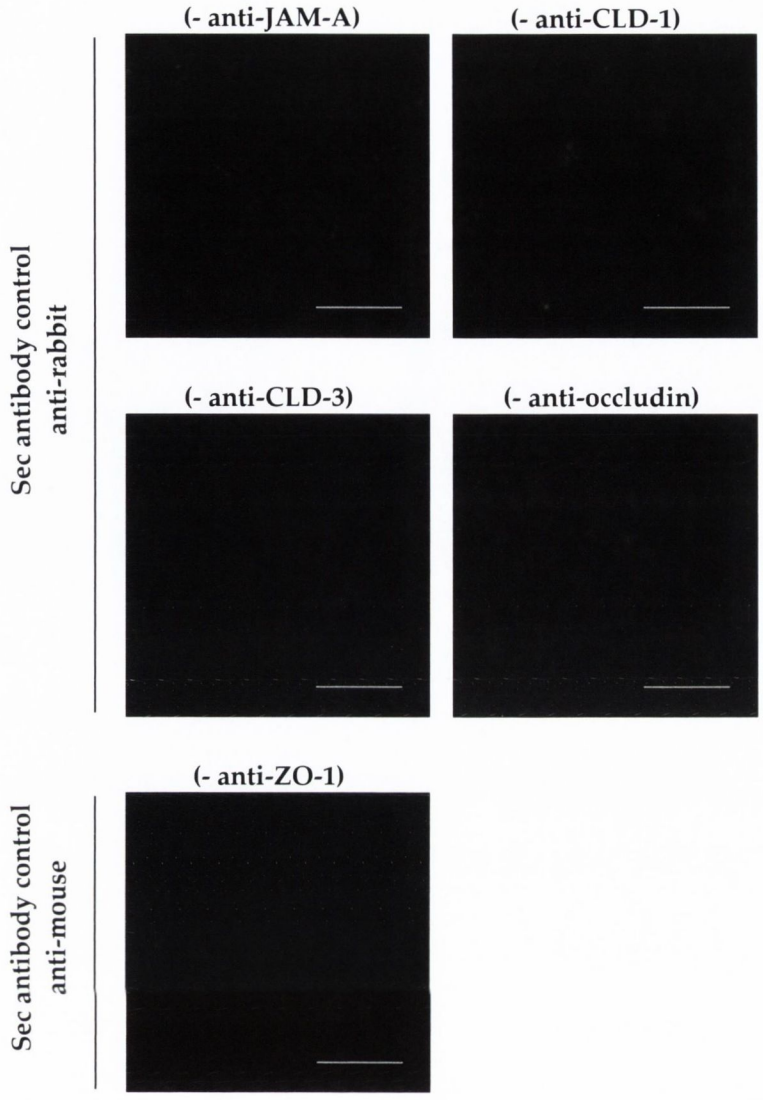


Figure 2.2 Representative photomicrographs showing negative controls (secondary antibody controls) for junctional proteins in Caco-2 monolayers. Bars=20 μm.

2.3. Clinical studies

A summary of methods used to investigate the mechanism underlying increased paracellular permeability in IBS is shown in Figure 2.3.

2.3.1. Patient cohort

IBS patients and control participants were recruited through the Endoscopy Unit in the Department of Gastroenterology of the Adelaide and Meath Hospital, Dublin (Ireland). All patients had a colonoscopy and a biopsy for clinical reasons. Ethical approval was received from the Adelaide and Meath Hospital Ethics Committee and informed consent was obtained from participants.

Junctional proteins were immunostained in biopsy tissue from the caecum of patients with IBS (n=34), asymptomatic patient controls (n=12) and inflammatory controls with confirmed IBD (n=8). Clinical and demographic characteristics are described in the results chapters. All patients had Rome II criteria compatible IBS (394). On a colonoscopy, patients and controls had macroscopically and histologically normal colonic mucosa and no evidence of organic bowel disease as evaluated by a pathologist.

Control patients were also undergoing a colonoscopy for clinical reasons (colon cancer screening, haemorrhoids, anaemia, vomiting) and were free from organic bowel disease and IBS. Other exclusion criteria applied to IBS and normal controls were current use of the following medications: non-steroidal anti-inflammatory drugs, corticosteroids, aspirin, mast-cell stabilisers or antibiotics. In the IBD group, 5 patients had clinically confirmed Crohn disease (CD) while 3 had ulcerative colitis (UC) (395).

Symptom assessment: severity of abdominal pain was assessed in IBS using a pain rating of 0-4, where patients rated pain as: 0, absent; 1, mild (not influencing usual activities); 2, relevant (diverting from, but not urging modification of, usual activities); 3, severe (influencing usual activities markedly enough to urge modifications); 4, extremely severe (precluding daily activities) (396). Severity of diarrhoea was quantified in IBS by the reported number of soft/liquid stools per week, based on a similar variable that is in use in IBD, namely the Crohn's Disease Activity Index (CDAI) (397). The duration of symptoms was recorded for IBS as time since symptom onset as reported by the patient. Clinical and demographic data were recorded on all participants. The analysis was performed on a previously recruited cohort with clinical and demographic data available in the research database.

2.3.2. Expression of junctional proteins by immunofluorescence confocal microscopy in human caecal tissue

2.3.2.1. Sample processing

Biopsy specimens of the caecum were obtained during routine colonoscopies and fixed in formalin. For the current study, 3-4 um sections were cut from the paraffin-embedded blocks and fixed on slides. Sections were dewaxed in xylene (first for 8 and then 5 min) and passed through decreasing concentration of graded alcohol (twice for 5 min in 100%, 5 min in 90%, 5 min in 70%) to water (twice for 5 min) with 10-20 s agitation every 30 s. Antigen retrieval was performed using target retrieval solution (Dako, UK) at 95 °C-96 °C for 30 min (water bath) and allowed to cool at RT for 20 min.

2.3.2.2. Immunofluorescence labelling

Non-specific binding was blocked with 20% goat serum in PBS (JAM-A, ZO-1, E-cadherin) or 2% BSA in PBS (CLD-1) for 1 hour at RT. Sections were incubated in 2% BSA in PBS with primary antibodies as follows: anti-JAM-A, anti-CLD-1, anti-ZO-1 and anti-E-cadherin (2-20 μg/ml) (Table 2.3) for 1 hour at RT. Sections were washed 3 times with 0.2% in BSA in PBS and incubated with Alexa Fluor® 633-conjugated goat anti-mouse or anti–rabbit (2 μg/ml) (Table 2.3) for 1 hour at RT. Sections were washed in PBS, 3 times in PBS containing DAPI (1.43 μM) and mounted in vectashield (Vector Laboratories, UK). Slides were visualised using Olympus Fluoview FV100 microscope with *UPlan FLN* 40×/1.30 lenses and FV10-ASW 2.0 Viewer software. Slides were assessed semiquantatively while blind to the sample identity. Specimens for which the primary antibody was omitted were used as negative controls for junctional proteins (Figure 2.4).

Junctional protein	Primary antibody	Secondary antibody
JAM-A	20 μg/ml	2 μg/ml
CLD-1	10 μg/ml	2 μg/ml
ZO-1	20 μg/ml	2 μg/ml
E-cadherin	2 μg/ml	2 μg/ml

Table 2.3 Concentrations of antibodies that were used to detect junctional proteins by immunofluorescence confocal microscopy.

2.3.3. Expression of E-cadherin by immunohistochemistry in human caecal tissue

In parallel, E-cadherin was immunostained commercially at Source BioScience company (UK) using Super Sensitive™ Polymer HRP IHC Detection System (Biogenex, UK). Breast cancer tissue was used as a positive control for E-cadherin staining (data not shown).

In the present study, the slides were visualised using Nikon eclipse 80i fluorescence microscope with Plan Apo 20×/0.75 lenses and with ACT-1 software (Micron Optical Ltd., Ireland). Slides were assessed semiquantatively while blind to the sample identity.

2.3.4. Semiquantitative analysis of junctional protein expression in human caecal tissue

Immunofluorescence staining was graded semiquantatively on a 4-point scale: 1-no specific staining, 2-mild specific staining, 3-moderate specific staining, 4-strong specific staining. Protein staining in all fields of view of epithelium (surface and crypt) was assessed and the median staining score was calculated separately for the surface epithelium (primary variable) and crypt epithelium (secondary variable). Immunohistochemical staining for E-cadherin was similarly assessed. Representative photomicrographs showing each grade of staining for JAM-A are displayed in Figure 2.5, for CLD-1 in Figure 2.6, for ZO-1 in Figure 2.7 and for E-cadherin in Figures 2.8 (immunofluorescence) and 2.9 (immunohistochemistry). Slides were assessed semiquantatively by one researcher in a blinded manner. A second skilled observer performed parallel grading of immunofluorescence staining for training.

2.3.5. Statistical analysis – clinical data

Statistical analysis was performed on the clinical data using SPSS software (IBM Corporation, USA). Differences between 2 groups were compared using 2-tailed Student's t-test or Mann-Whitney U test for parametric and non-parametric data respectively. Differences between more than 2 groups were compared with non-parametric tests: Kruskal–Wallis or Jonckheere-Terpstra (J-T, order of groups is meaningful) and followed by Mann-Whitney U test for single comparisons. Associations between protein expression and symptoms were assessed using Spearman's correlation. Pearson Chi-square test was used to compare percentage differences in gender between controls, IBS and IBD patients. A general linear model was employed to determine the potential influence of age and gender on junctional protein expression in controls, IBS and IBD patients. Results are expressed as mean and standard error of the mean (SEM) or as median and range. $P \leq 0.05$ was considered statistically significant.

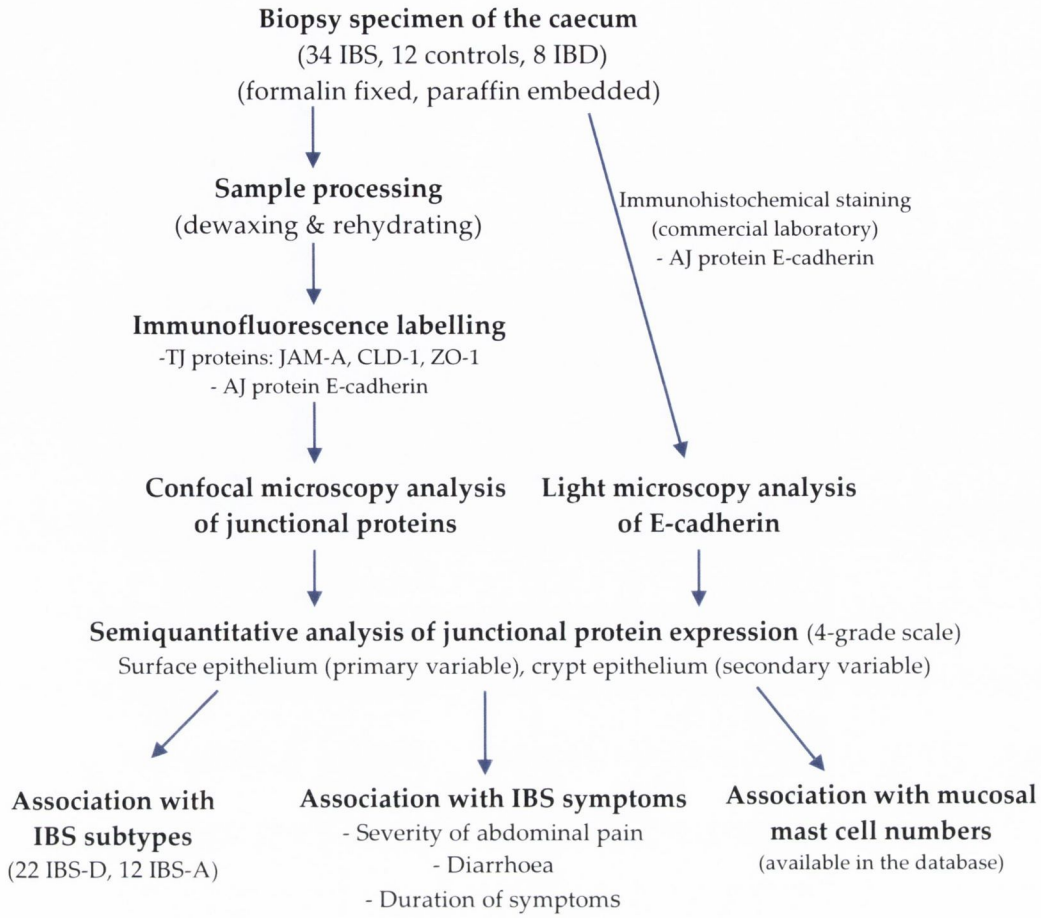


Figure 2.3 Summary of methods used to investigate the expression of junctional proteins in the caecal mucosa of IBS, IBD and control patients. The expression of junctional proteins was associated with 1) IBS subtypes according to bowel predominance: IBS-D and IBS-A 2) IBS symptoms 3) mucosal mast cell numbers.

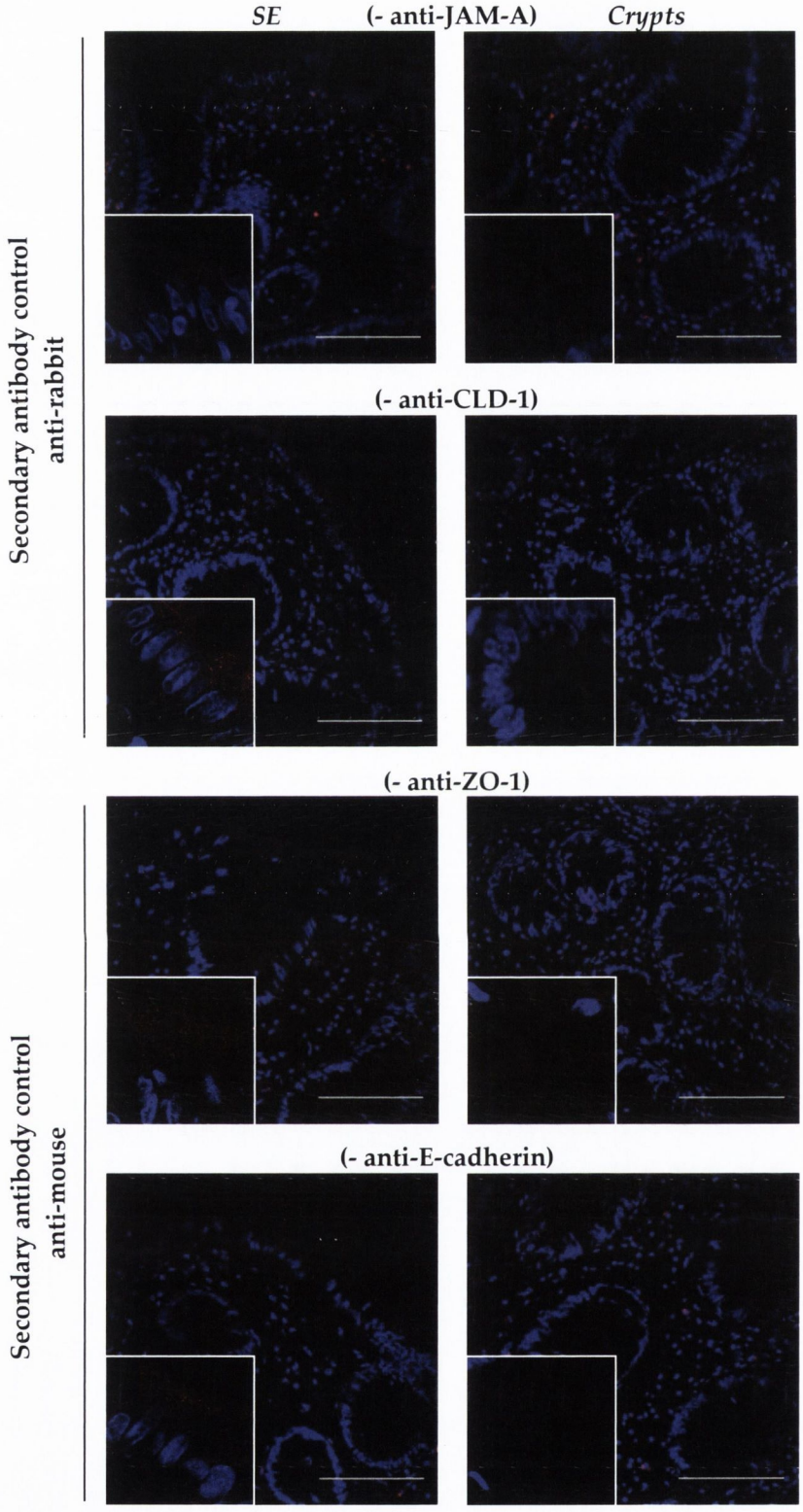


Figure 2.4 Representative photomicrographs showing negative controls (secondary antibody controls) for junctional proteins in human caecal tissue. SE, surface epithelium. Bars=50 μm.

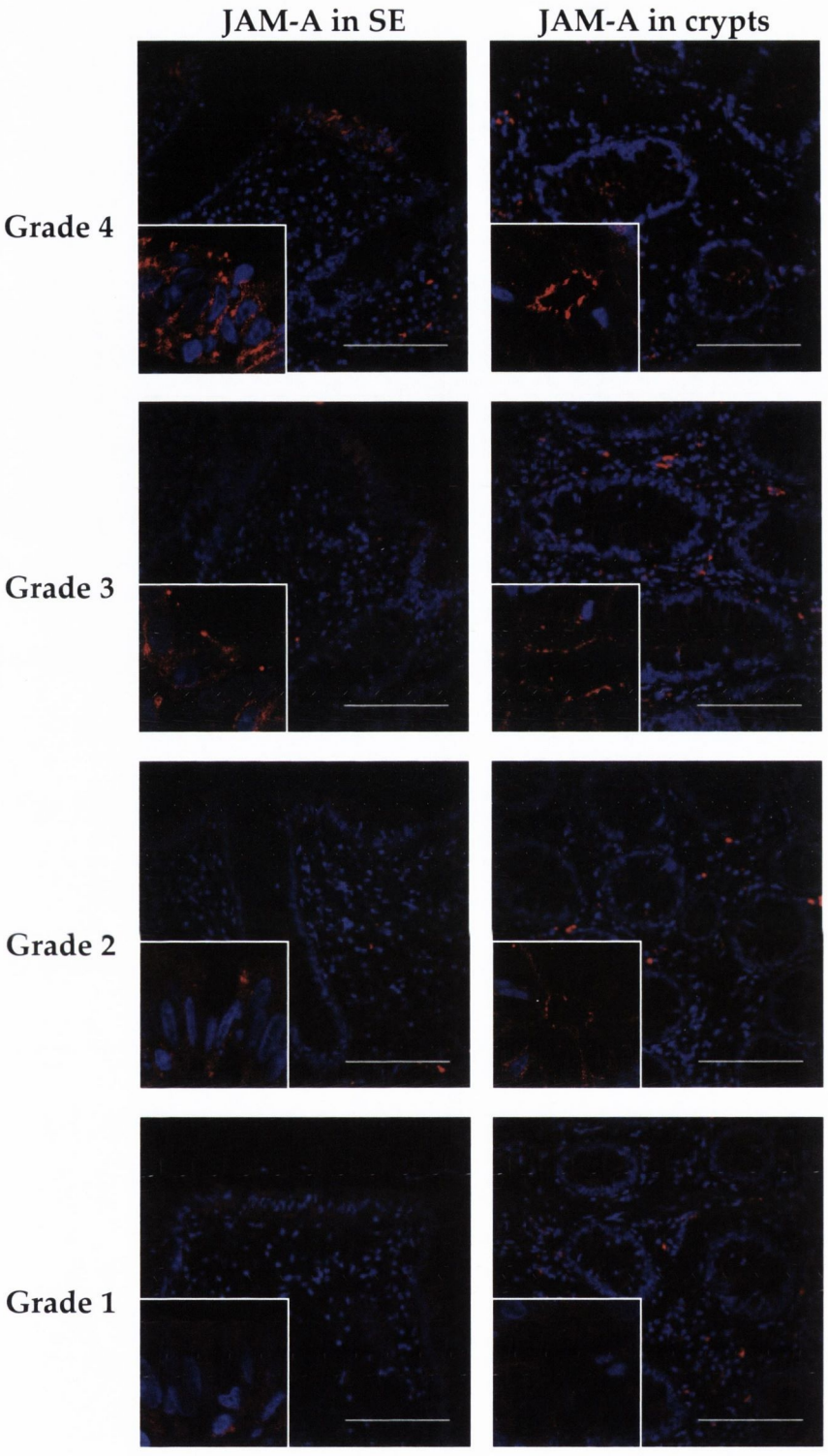


Figure 2.5 Representative photomicrographs showing each grade of staining for JAM-A protein. Immunofluorescence staining for JAM-A is shown in red, while nuclear staining is blue. SE, surface epithelium. Bars=50 μm.

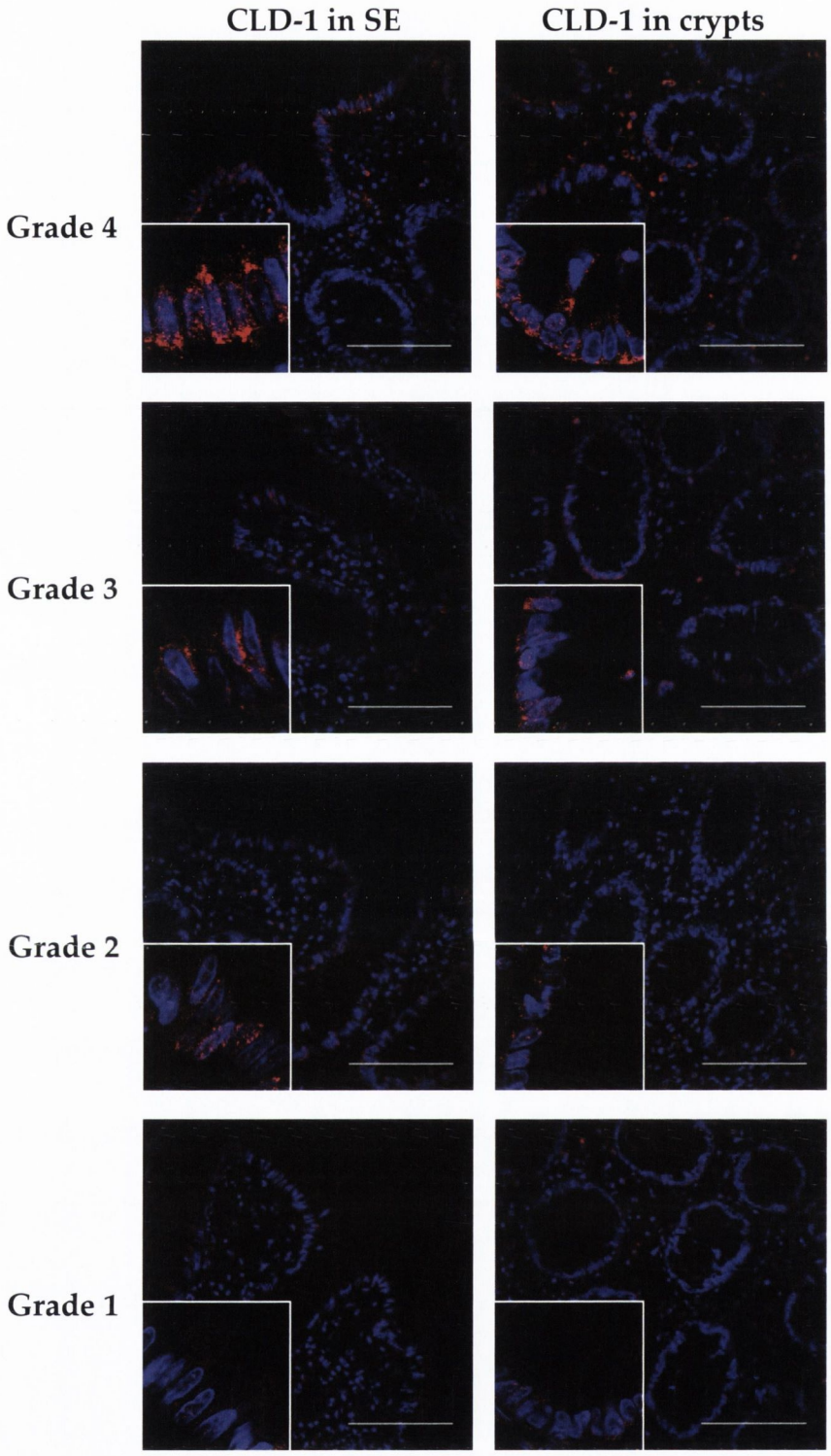


Figure 2.6 Representative photomicrographs showing each grade of staining for CLD-1 protein. Immunofluorescence staining for CLD-1 is shown in red, while nuclear staining is blue. SE, surface epithelium. Bars=50 μm.

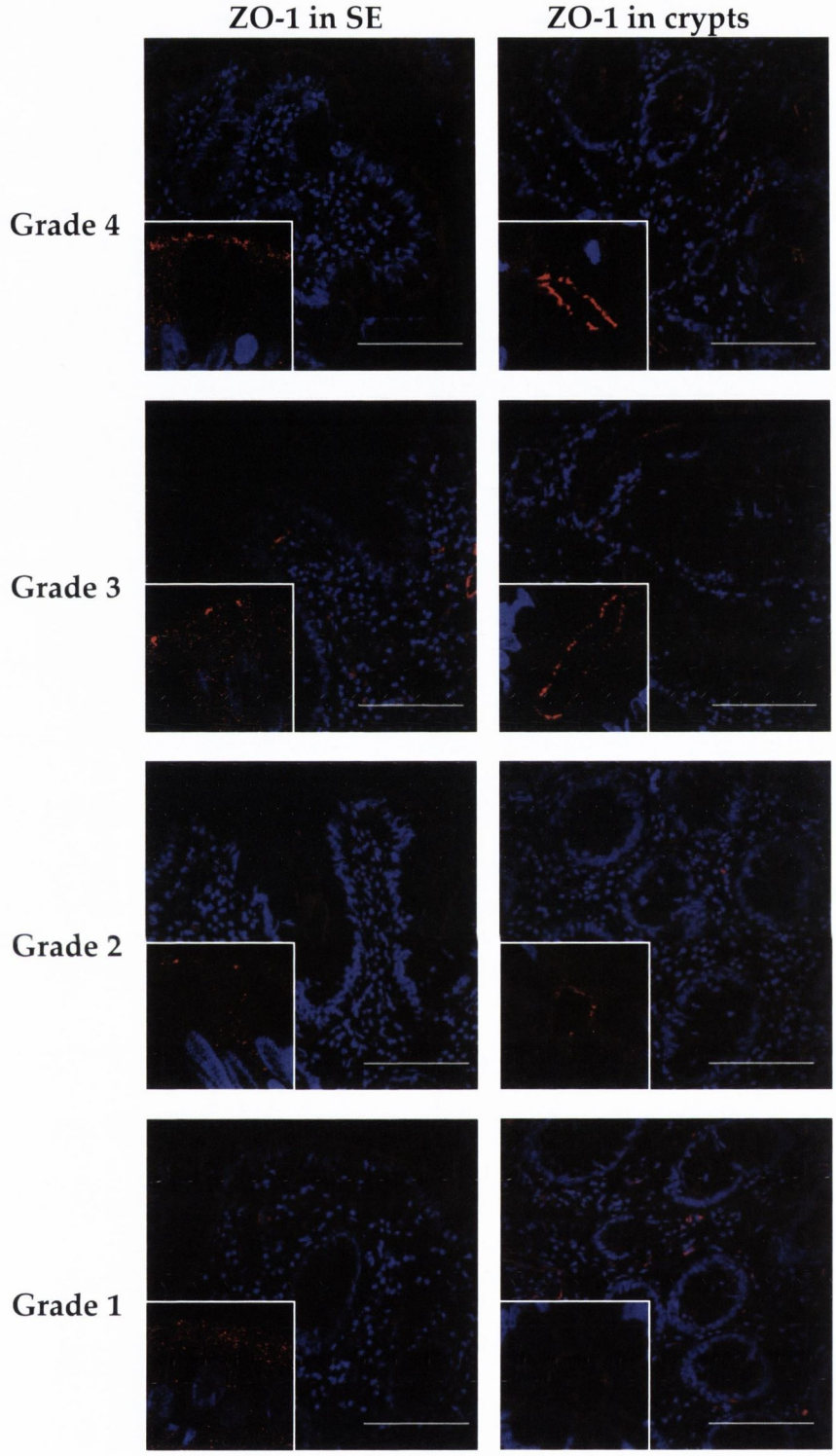


Figure 2.7 Representative photomicrographs showing each grade of staining for ZO-1 protein. Immunofluorescence staining for ZO-1 is shown in red, while nuclear staining is blue. SE, surface epithelium. Bars=50 μm.

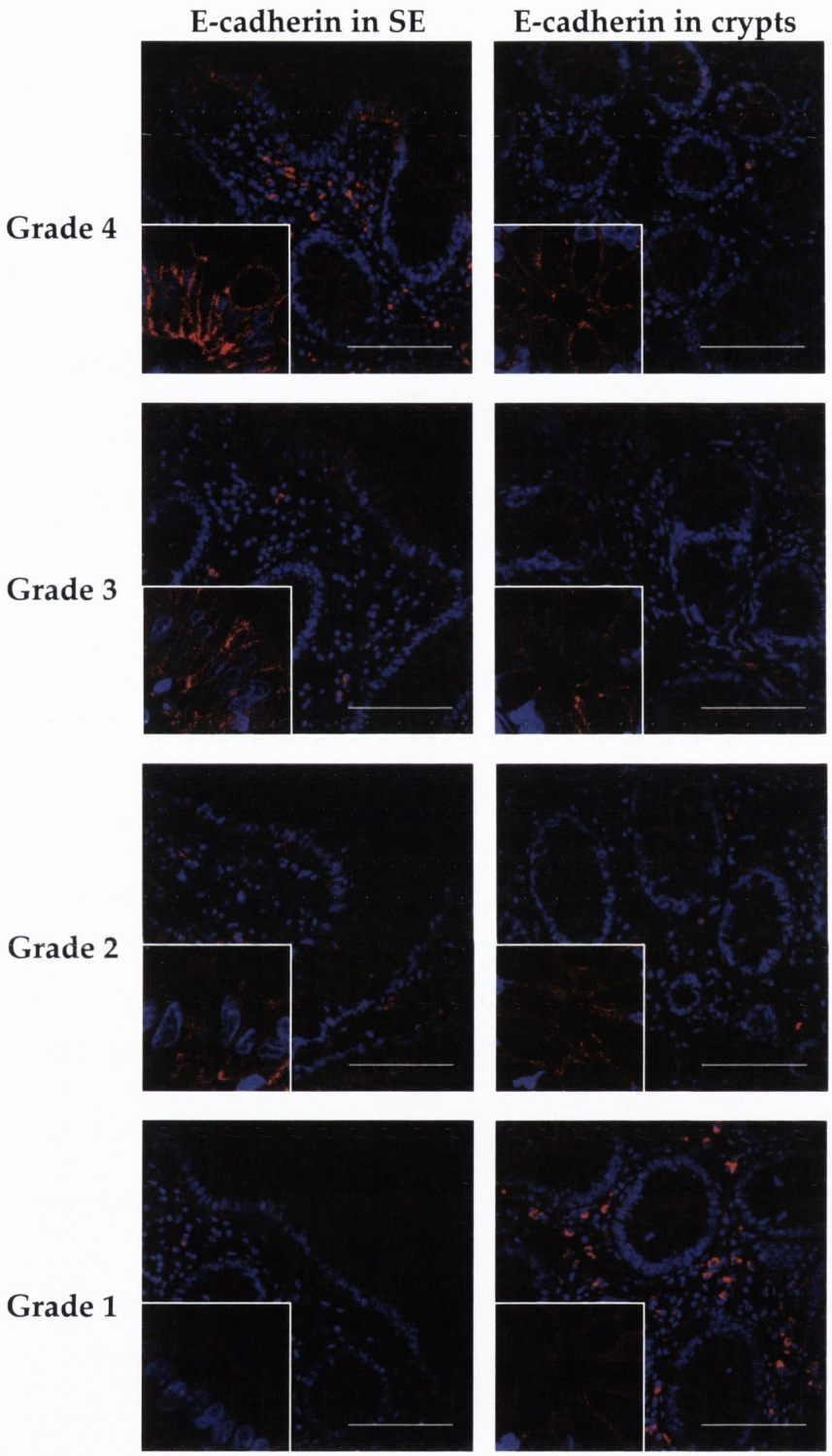


Figure 2.8 Representative photomicrographs showing each grade of staining for E-cadherin protein. Immunofluorescence staining for E-cadherin is shown in red, while nuclear staining is blue. SE, surface epithelium. Bars=50 μm.

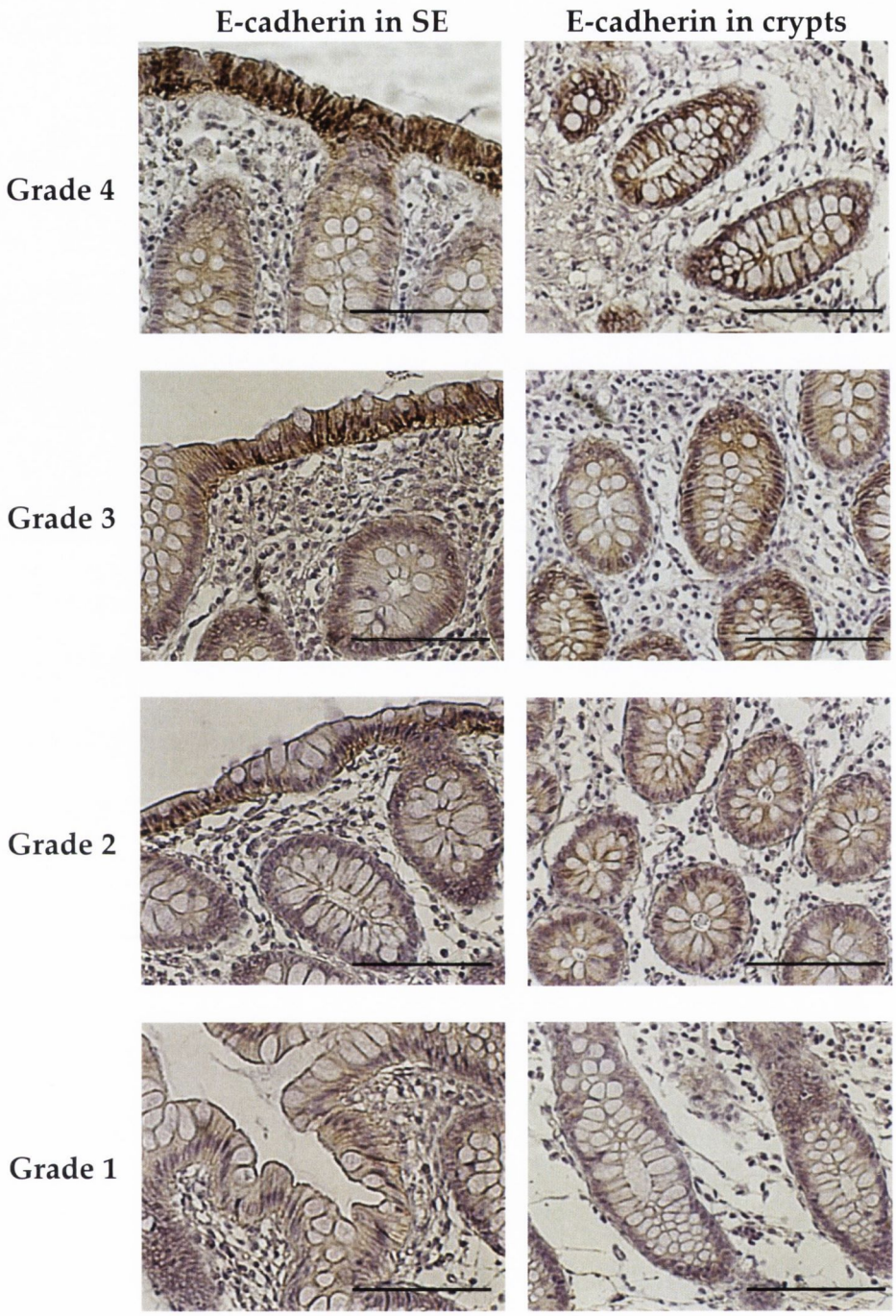


Figure 2.9 Representative photomicrographs showing each grade of staining for E-cadherin protein. Immunohistochemical staining for E-cadherin is shown in brown, nuclei are stained blue. Bars=50 μm.

CHAPTER 3

TRYPTASE DISRUPTS THE INTESTINAL EPITHELIAL BARRIER IN THE CACO-2 CELL MODEL

3.1. Introduction

3.1.1. Rationale

Permeability of the intestinal epithelial barrier is regulated by the intercellular AJC composing TJ and AJ proteins (8). Claudins and occludin make up the structural core of TJ and associate with the cytoskeleton through ZO proteins. CLD-2 creates cation-selective channels and is associated with increased permeability (398), whereas CLD-1 and CLD-3 tighten the barrier and reduce epithelial permeability (47). JAM-A in epithelial junctions is associated with the regulation of intestinal permeability and its loss leads to enhanced permeability and inflammation (53). E-cadherin in the AJs mediates adhesion between intestinal epithelial cells (60).

Barrier dysfunction might lead to excessive penetration of luminal macromolecules and bacteria, stimulating disruptive immunological responses resulting in inflammation (19). A number of studies have suggested an increase in intestinal permeability in IBS patients (148, 212, 335, 337, 339) (Section 1.2.5.1). Recently, a decrease in ZO-1, occludin and CLD-1 was identified in colonic tissue from IBS patients (213) whereas CLD-2 appeared to be increased (344). Reduced ZO-1 expression and increased paracellular permeability was also reported by Piche *et al.* (212) in intestinal epithelial cells incubated with tissue supernatants from IBS patients. However, the soluble mediators involved in these changes were not identified. The role of E-cadherin and JAM-A in barrier function in IBS remains largely unexplored. Interestingly, variants in the CDH1 gene, which encodes the junctional protein E-cadherin, may be associated with the risk of PI-IBS (399). While JAM-A is reduced at sites of active inflammation in IBD colonic tissue (53), its role in IBS is unknown.

The present study suggests that tryptase might contribute to barrier dysfunction in IBS. Tryptase content has been shown to be increased in IBS both in jejunal fluids (228) and in colonic (144, 175) and jejunal mucosa (285, 344), and was associated with enhanced permeability in mucosal tissue of IBS patients (176) and in intestinal epithelial cells (57). This study aims to investigate if tryptase disturbs intestinal epithelial integrity and if the potential effects involve alterations in the expression of junctional proteins.

3.1.2. Aims of the study

General aim:

To investigate if tryptase disrupts the intestinal epithelial barrier *in vitro* using the human colonic epithelial cell line, Caco-2.

Specific aims:

1. To investigate the effects of tryptase on:
    - epithelial integrity by measurement of TER;
    - paracellular permeability to FITC-dextran;
    - expression of junctional proteins by western blotting;
    - expression and localisation of junctional proteins by immunofluorescence confocal microscopy.
2. To investigate the effects of heparin on tryptase activity in terms of:
    - epithelial integrity by measurement of TER;
    - paracellular permeability to FITC-dextran.

3.1.3. Methods to achieve the aims

Caco-2 cells were grown on semipermeable filters until they established differentiated and polarised monolayers, for 21 to 26 days; the integrity of monolayers was monitored by the measurement of TER. Tryptase (3 mU to 15 mU) was added to the apical side of Caco-2 polarised monolayers for between 1 to 24 hours (+/- 50 μg heparin). The integrity of Caco-2 monolayers was analysed by the measurement of TER and paracellular permeability to molecular marker FITC-dextran. The expression of junctional proteins was assessed by western blot analysis and confocal immunofluorescence microscopy.

Caco-2 cells were the model of choice to study intestinal epithelial permeability. Caco-2 cells, when grown on semipermeable filters, form polarised, high resistance monolayers expressing many small intestinal functions. Caco-2 cells undergo spontaneous differentiation at confluency: AJC are formed between epithelial cells, microvilli are formed on the apical cell surface, and they are characterised by a polarised distribution of brush border enzymes, receptors and transporters (381). Caco-2 cells appear to be most widely studied and applied cell model for intestinal permeability studies (400). This cell line was also successfully used in my laboratory (401) and thus was readily available for my studies. Other human intestinal epithelial cell lines such as HT-29 and T84 have also been used to study intestinal barrier functions (402) and could be considered as alternatives to Caco-2 cells.

3.2. Results

3.2.1. Tryptase decreases TER of Caco-2 monolayers

Application of tryptase (15 mU) to Caco-2 cell monolayers had a dramatic effect on epithelial integrity as evidenced by a significant drop in TER within 1 hour of incubation that was maximal at 4 hours (36±11%, P=0.008) compared to controls (Figure 3.1A). The effect was partially reversible within 24 hours following tryptase administration. For the lower concentration of tryptase (3 mU) there was a tendency towards the reduction in TER levels within 24 hours of incubation compared to controls, however, the difference was not statistically significant.

3.2.2. Tryptase increases permeability of Caco-2 monolayers to FITC-dextran

Consistent with TER measurements, the higher concentration of tryptase, 15 mU, induced a 2-fold (P=0.048) increase in permeability of Caco-2 monolayers to FITC-dextran (Figure 3.2) at 4 hours compared to controls. However, the lower concentration of tryptase, 3 mU, did not significantly alter permeability to FITC-dextran within 4 hours of incubation.

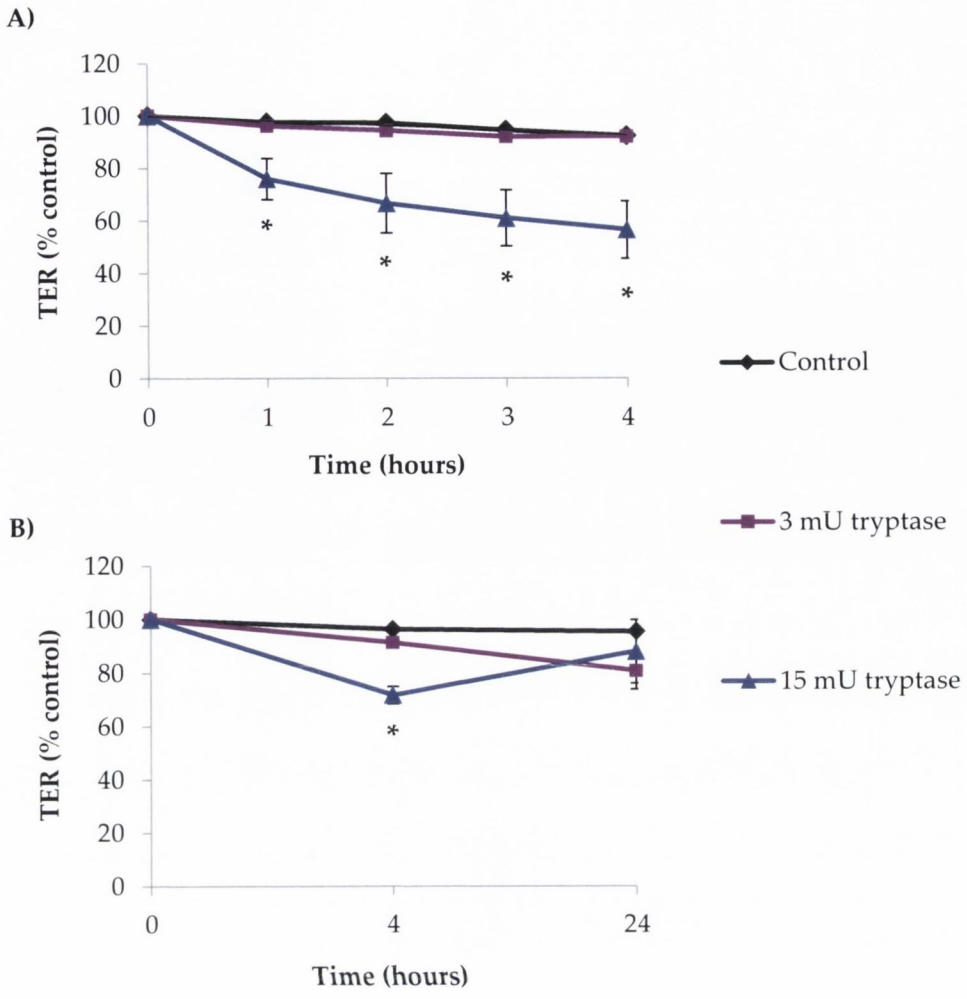


Figure 3.1 Effects of tryptase (3 mU or 15 mU) on the epithelial barrier. TER of Caco-2 monolayers incubated with tryptase **(A)** up to 4 hours or **(B)** up to 24 hours compared to untreated controls. Results represent the mean ± SEM of 4 independent experiments. *P<0.05 (1-way ANOVA followed by Tukey's post hoc test).

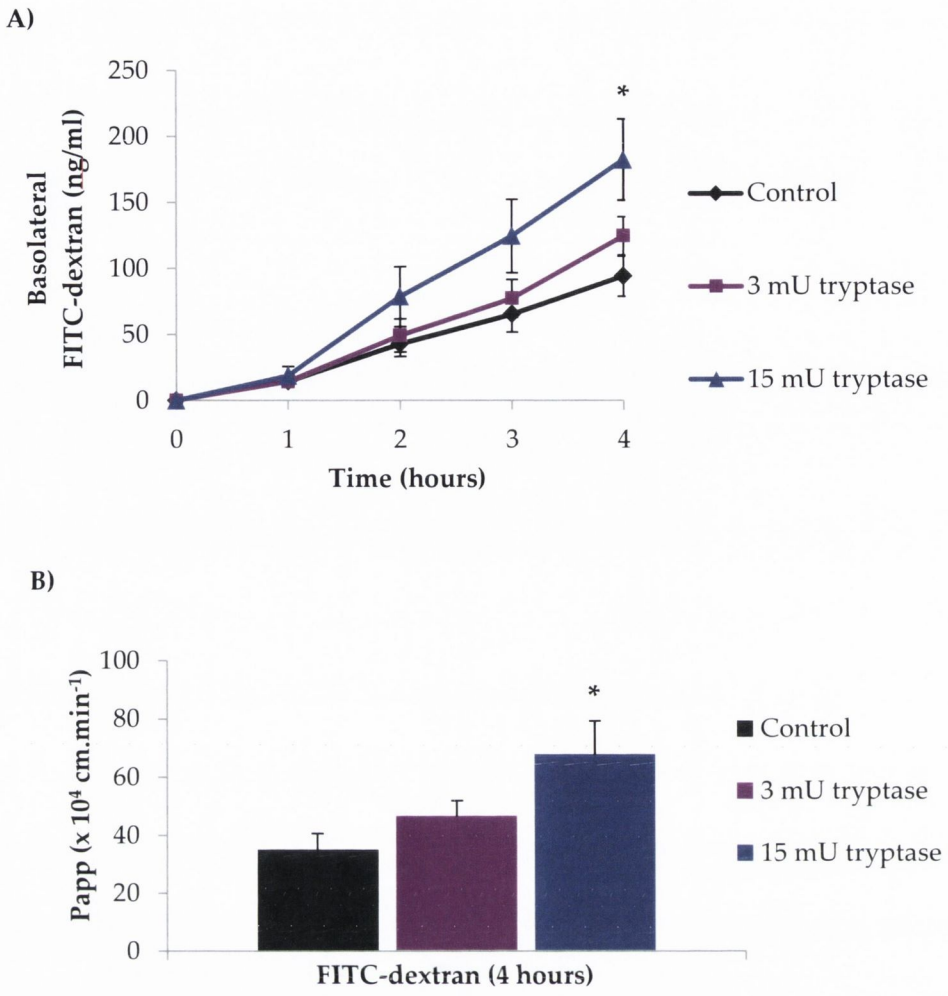


Figure 3.2 Effects of tryptase (3 mU or 15 mU) on the epithelial barrier. **A)** Apical-to-basolateral flux of FITC-dextran through Caco-2 monolayers incubated with tryptase up to 4 hours compared to untreated controls. **B)** Permeability coefficients of FITC-dextran. Results represent the mean ± SEM of 4 independent experiments. *P<0.05 (1-way ANOVA followed by Tukey's post hoc test).

3.2.3. Tryptase alters junctional protein expression as determined by western blotting

JAM-A expression was significantly decreased when Caco-2 monolayers were exposed to tryptase (15 mU) for 4 hours (0.68±0.12 fold, P=0.04) (Figures 3.3, 3.4) which was concomitant with the drop in TER and increased paracellular permeability. The effect of tryptase on JAM-A expression was significantly reversible after prolonged incubation (24 hours) and was in line with the observed partially reversible effect on TER. CLD-1 was also significantly reduced (0.68±0.01 fold, P=0.03) relative to controls after 24 hours of incubation with 15 mU tryptase, although initially an elevated expression (1.97±0.35 fold, P=0.05) was observed at 4 hours (Figures 3.3, 3.4). The lower concentration of tryptase (3 mU) did not affect JAM-A or CLD-1 expression. Prolonged exposure (24 hours) of Caco-2 monolayers to tryptase (3 mU or 15 mU) significantly decreased ZO-1 levels (0.44±0.05 fold, P=0.0004 and 0.20±0.02 fold, P<0.00001, respectively). ZO-1 expression appeared to be elevated, but not significantly, following 4 hours exposure to either 3 mU or 15 mU tryptase (2.40±1.06 fold, P=0.22 and 3.80±1.91 fold, P=0.17 respectively) (Figures 3.3, 3.4). CLD-3 levels increased after 4 hours of exposure to 15 mU tryptase (2.62±0.40 fold, P=0.06), returning to basal levels within 24 hours (Figures 3.3, 3.4) and remained unchanged in response to the lower concentration of tryptase (3 mU). Occludin expression increased after 4 hours of incubation with tryptase [either 3 mU (1.36±0.05 fold, P=0.001) or 15 mU (2.15±0.37 fold, P=0.02)] but there was no detectable change in expression after 24 hours of incubation. Tryptase did not alter CLD-2 or E-cadherin expression in Caco-2 monolayers (Figures 3.3, 3.4).

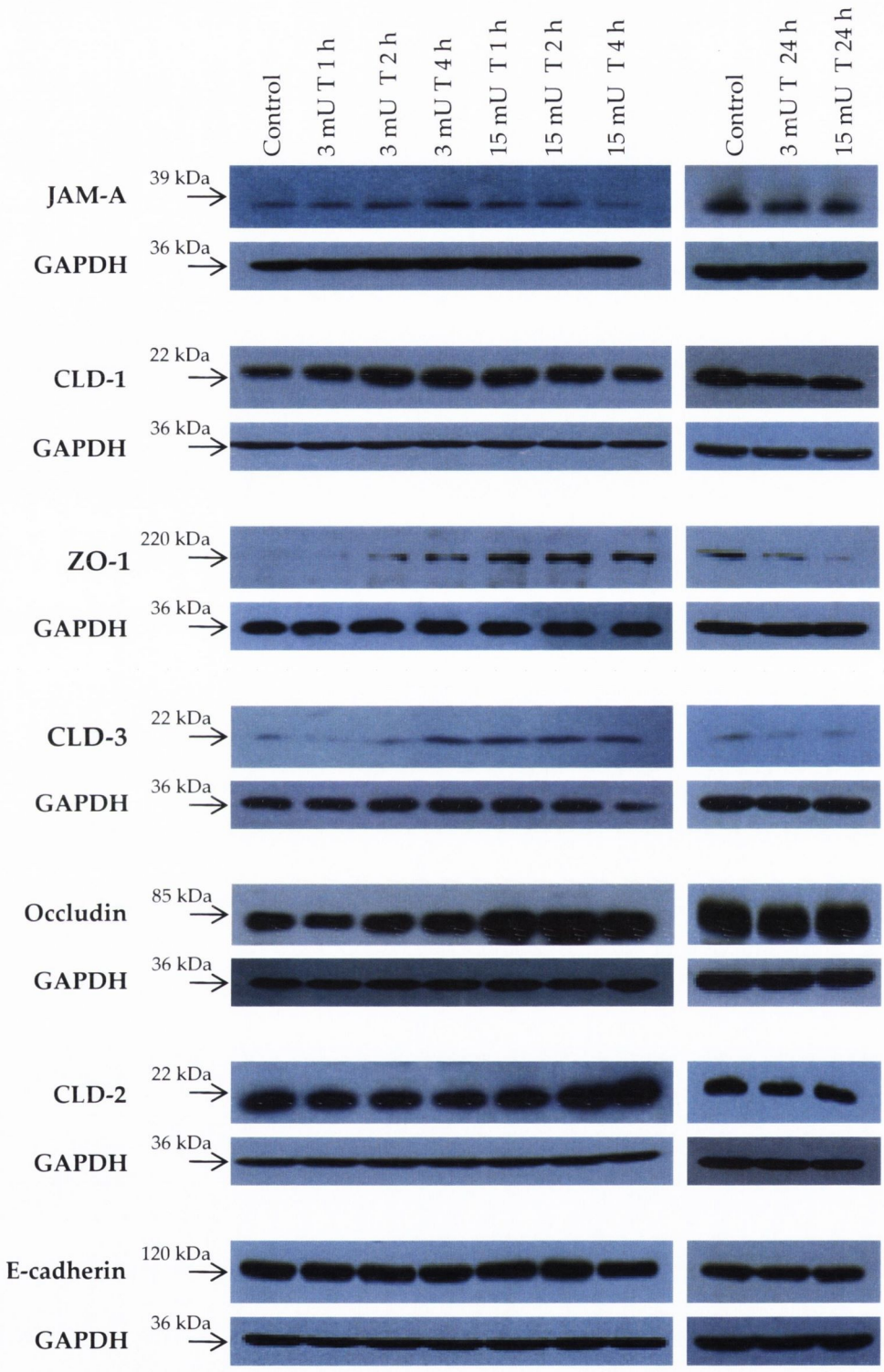


Figure 3.3 Western blot analysis of the expression of junctional proteins in Caco-2 monolayers incubated with tryptase, T, (3 mU or 15 mU) for up to 24 hours. Equal protein loading was verified with corresponding GAPDH levels. The blots shown are a representative of a minimum of 2 independent experiments.

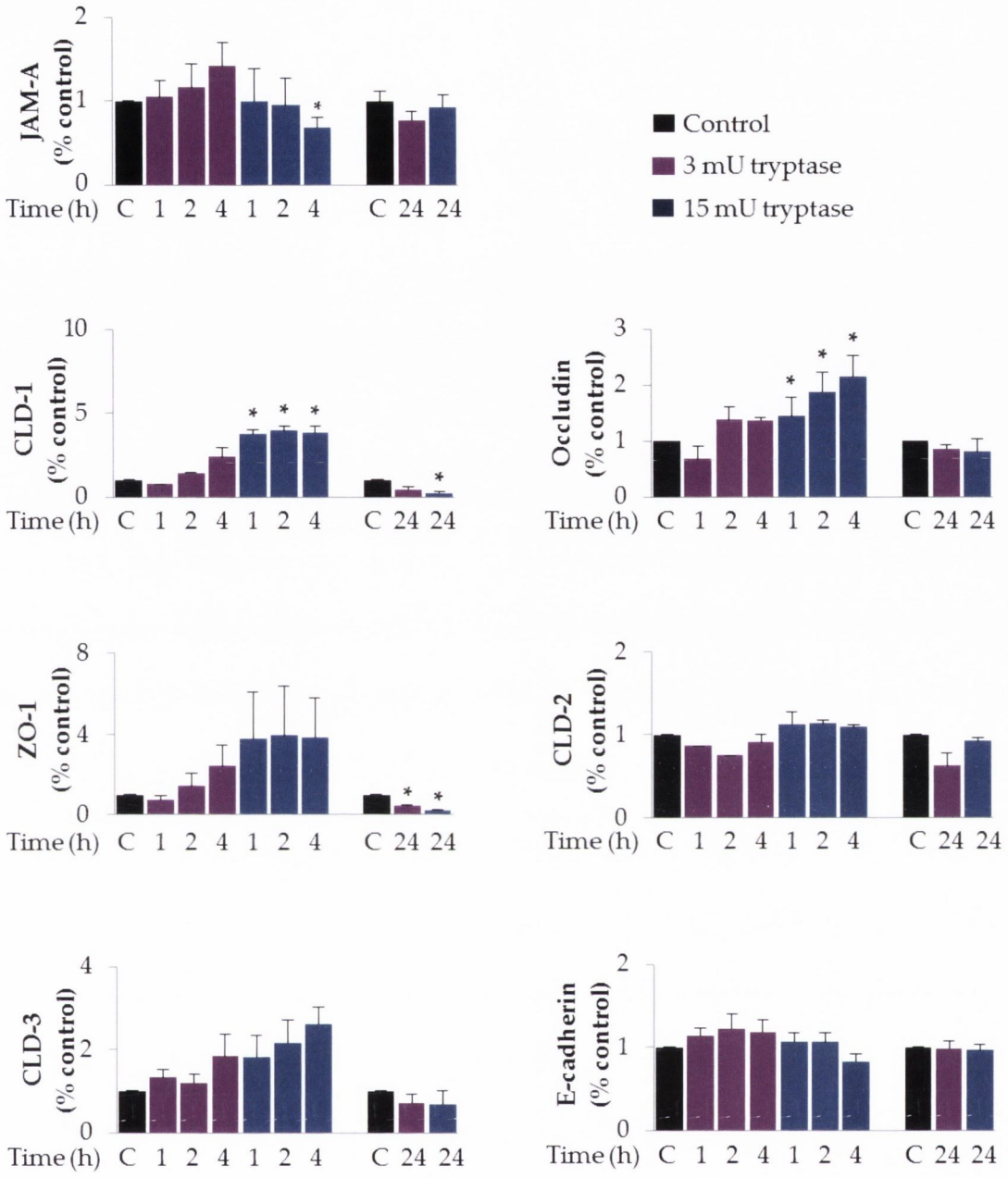


Figure 3.4 Expression of junctional proteins in Caco-2 monolayers incubated with tryptase (3 mU or 15 mU) for up to 24 hours as determined by densitometry. The results are expressed as a change relative to untreated controls. The density of each individual band was compared with the corresponding control band and normalised against GAPDH by densitometry. Results represent the mean ± SEM of a minimum of 2 independent experiments. *P≤0.05 (Student's t-test).

3.2.4. Tryptase alters junctional protein expression as determined by confocal microscopy

The distribution of junctional proteins was analysed further by confocal immunofluorescence microscopy. Z-stack projections showed that all proteins were localised in the apical TJ region of Caco-2 monolayers prior to treatment, as expected (Figures 3.5, 3.6).

In line with the western blot results, in Caco-2 monolayers treated with 15 mU tryptase for 4 hours the intensity of staining for JAM-A in TJs was clearly diminished, and JAM-A was redistributed towards tricellular junctions, (Figure 3.5). CLD-1 intensity within TJs did not change significantly; however, additional CLD-1 staining appeared in punctate cytoplasmic areas in cells exposed to 15 mU tryptase for 4 hours which were not observed in controls. This cytoplasmic CLD-1 localisation was not apparent following prolonged incubation (24 h, 15 mU tryptase) and, consistent with the western blot analysis, overall CLD-1 staining in TJs was lower at 24 hours (Figure 3.5). Also consistent with the western blot findings, the intensity of staining for ZO-1 in TJs increased after 4 hours, but decreased after 24 hours of incubation with 15 mU tryptase compared to controls (Figure 3.6). The levels of CLD-3 and occludin in TJs increased after 4 hours of incubation with tryptase (15 mU) (Figure 3.6) in line with the western blot results.

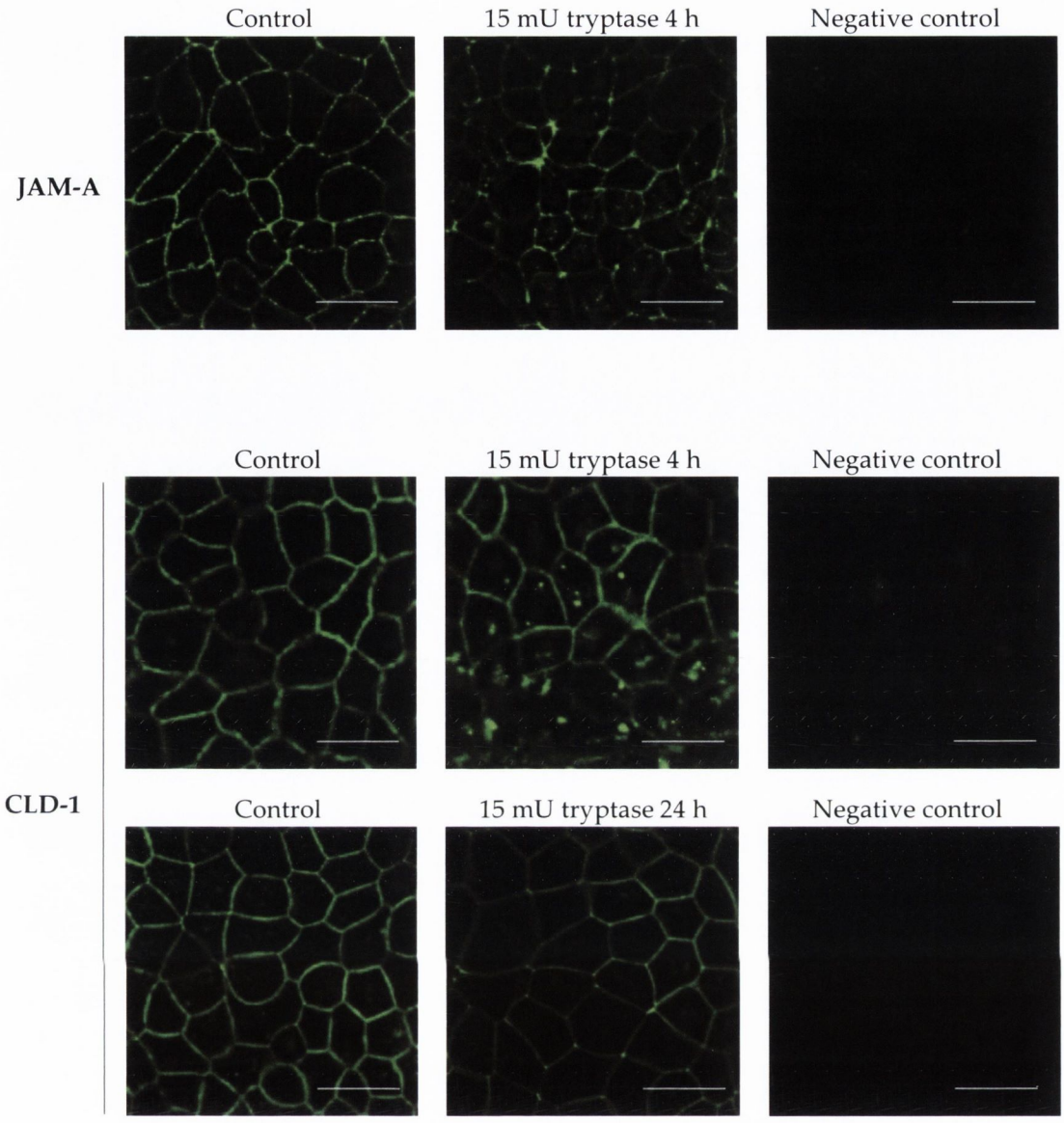


Figure 3.5 Expression of TJ proteins in Caco-2 monolayers incubated with tryptase (15 mU) either 4 or 24 hours compared to untreated controls. Immunofluorescence staining was heterogeneous with small differences in the intensity of staining observed. Staining in Caco-2 monolayers exposed to tryptase shown here was not identified in Caco-2 controls. No staining was seen in negative controls for which the primary antibody was omitted. Photomicrographs shown are representative of 2 independent experiments. Bars=20 μm.

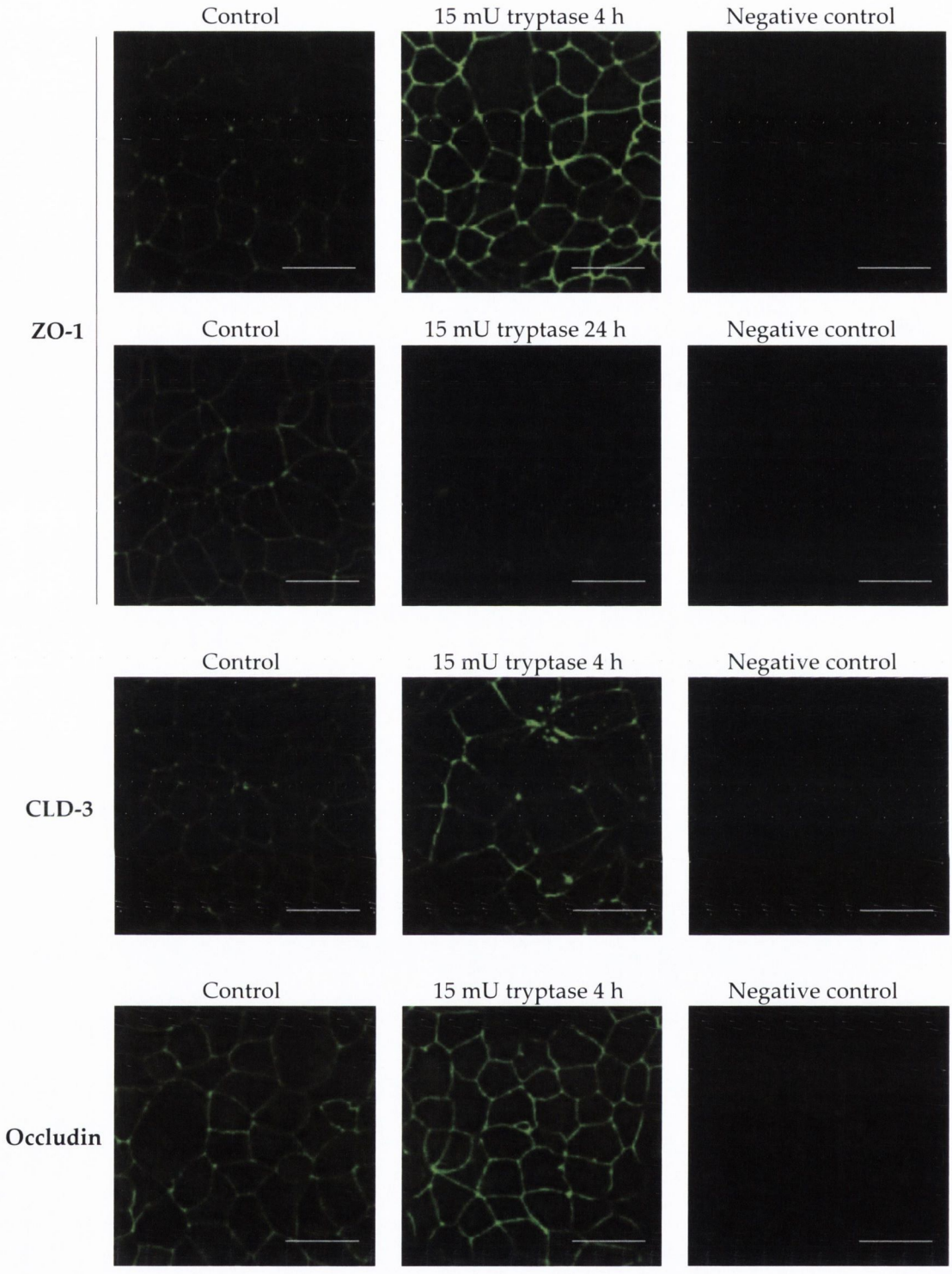


Figure 3.6 Expression of TJ proteins in Caco-2 monolayers incubated with tryptase (15 mU) either 4 or 24 hours compared to untreated controls. Immunofluorescence staining was heterogeneous with small differences in the intensity of staining observed. Staining in Caco-2 monolayers exposed to tryptase shown here was not identified in Caco-2 controls. No staining was seen in negative controls for which the primary antibody was omitted. Photomicrographs shown are representative of 2 independent experiments. Bars=20 μm.

3.2.5. Heparin does not affect tryptase activity

Previous studies have shown that tryptase is active as a tetramer and that this tetrameric form is stabilised by heparin proteoglycan or other highly negatively charged polymers (157-159). Therefore, in the present study it was of interest to investigate if heparin might affect tryptase activity. However, the apical co-administration of heparin (50 μg/ml) with tryptase did not have any significant effect on tryptase activity as determined by the measurement of TER (Figure 3.7) or permeability to FITC-dextran (Figure 3.8).

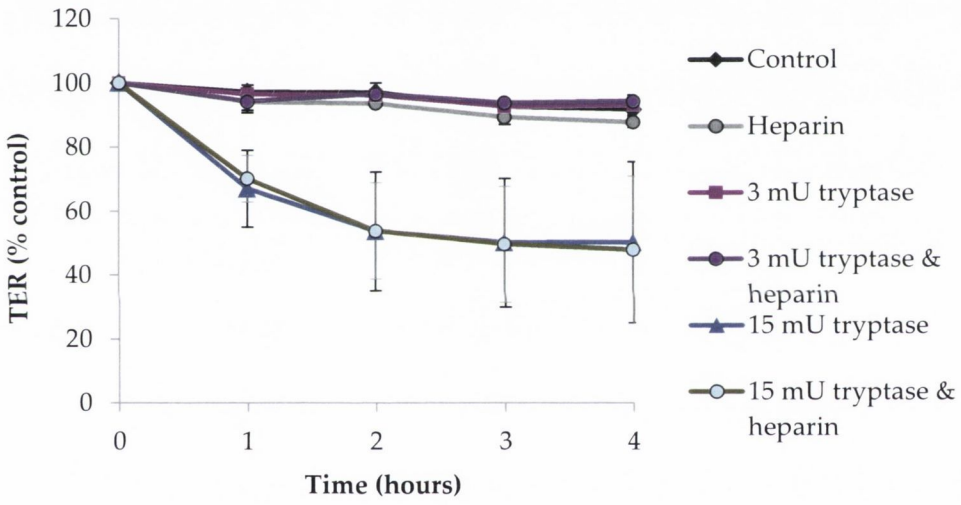


Figure 3.7 Effects of heparin on tryptase activity (3 mU or 15 mU). TER of Caco-2 monolayers incubated with tryptase +/- heparin for up to 4 hours compared to untreated controls. Results represent the mean ± SEM of 2 independent experiments.

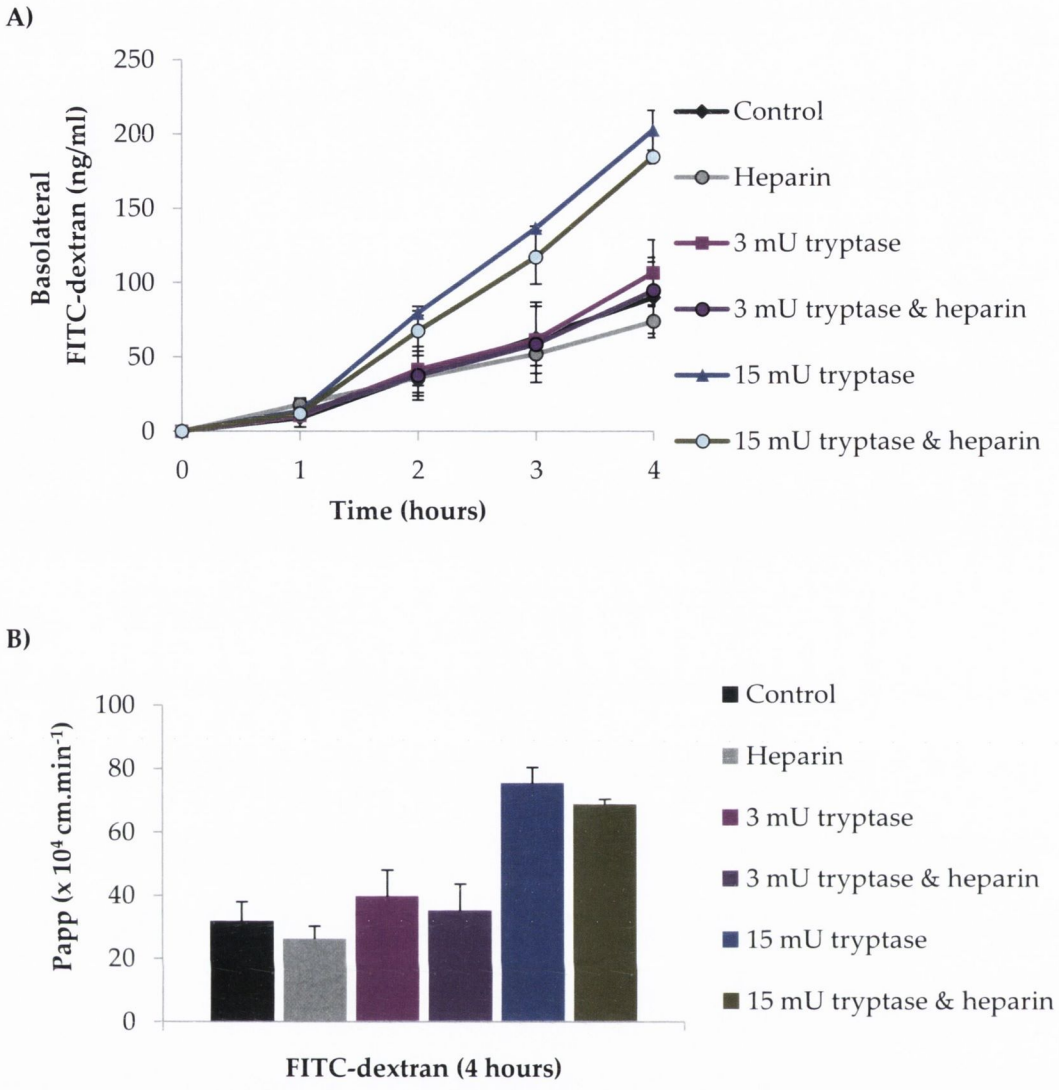


Figure 3.8 Effects of heparin on tryptase activity (3 mU or 15 mU). **A)** Apical-to-basolateral flux of FITC-dextran through Caco-2 monolayers incubated with tryptase +/- heparin for up to 4 hours compared to untreated controls. **B)** Permeability coefficients of FITC-dextran. Results represent the mean ± SEM of 2 independent experiments.

3.3. Discussion

Previous evidence demonstrates increased content of tryptase in intestinal mucosa (175, 285) of IBS patients and in jejunal luminal fluids (228), and its association with intestinal permeability (176). However, mechanisms behind these alterations have not been fully elucidated. Mast cell tryptase was shown to activate PAR-2 receptors (168) (reviewed in Section 1.1.5) that are expressed both on the apical and basolateral side of enterocytes (170). Furthermore, PAR-2 agonists were shown to increase paracellular permeability of intestinal epithelial cells *in vitro* (57) and of the murine colon *in vivo* (173, 403). The present study demonstrates that tryptase may impair integrity of intestinal epithelial cells and alter the expression of junctional proteins. Tryptase (15 mU) appeared to disrupt epithelial integrity as evidenced by a decrease of TER, an increase in paracellular permeability to FITC-dextran and a decrease in the expression of junctional proteins JAM-A, CLD-1 and ZO-1 within 24 hours of incubation.

In this study, to assess the effects of tryptase on the intestinal epithelial barrier, tryptase (3 mU & 15 mU) was administered apically to polarised and differentiated Caco-2 monolayers and incubated for up to 24 hours. It appeared that tryptase (15 mU) induced a dramatic loss of epithelial integrity within 1 hour of incubation as evidenced by a significant drop in TER. The effect was partially reversible within 24 hours, showing that this treatment did not have permanent deleterious effects on Caco-2 integrity. Also, enhanced permeability to FITC-dextran was observed at 4 hours of incubation. The effect of 3 mU tryptase was less dramatic and appeared to only induce a drop in TER ($P>0.05$) after prolonged incubation (24 hours). Initially (for up to 4 hours) TER levels were comparable to untreated controls. These findings are in line with the recent data where tryptase administered to rectal biopsy tissue from healthy controls increased macromolecular permeability and the degree of the increase was proportional to

tryptase concentration (176). This study also extends the previous data by Jacob *et al.* (57) showing that activation of PAR-2 on both the apical and basolateral side of intestinal epithelial cells resulted in decreased TER and increased permeability to macromolecules.

Evaluation of junctional protein expression revealed that tryptase (15 mU) significantly reduced JAM-A expression in Caco-2 monolayers following 4-hour exposure as shown by western blotting. Furthermore, confocal microscopy analysis demonstrated that JAM-A appeared to be redistributed from TJ regions. The drop in JAM-A expression was concomitant with the drop in TER and an increase in paracellular permeability to FITC-dextran. After prolonged incubation (24 hours) the effect was significantly reversible and was in line with the partially reversible effect on TER. This fits well with the previous studies on mice where JAM-A$^{-/-}$ mice exhibited an increase in intestinal permeability and a decrease in TER (53, 54). Furthermore, these mice appeared to have an elevated level of inflammatory mediators in intestinal mucosa (53) and to be more susceptible to developing dextran sulfate sodium (DSS)-induced colitis (53, 54) compared to wild type mice. In addition, JAM-A$^{-/-}$ mice exhibited higher expression of CLD-10 and CLD-15 (53, 54) but not CLD-1, CLD-2 and CLD-3 (53) compared to healthy wild type mice. Therefore, it is possible that particular claudins enhance intestinal permeability in JAM-A$^{-/-}$ mice. Interestingly, the experiments on mice with selective knock-out of endothelial/hematopoietic JAM-A (but not epithelial) demonstrated that these mice exhibited comparable susceptibility to develop DSS colitis as wild type mice and indicated that epithelial JAM-A regulates both intestinal permeability and gut homeostasis (53).

This study reports lower CLD-1 expression at 24 hours following tryptase (15 mU) treatment, without any significant changes for CLD-2 and CLD-3 protein expression. CLD-1 initially (4 hours) appeared in punctate cytoplasmic areas in

cells; however, its expression in TJs was comparable to untreated controls as shown by confocal microscopy analysis. After prolonged incubation (24 hours) CLD-1 was clearly reduced in TJs compared to controls, further confirming the role of tryptase in the disruption of junctional integrity. This is in agreement with studies showing that redistribution of CLD-1 from TJ regions associated with increased permeability of epithelial cells *in vitro* (352, 353). In contrast, CLD-1 expression was not altered following PAR-2 activation in intestinal epithelial cells (57). In patients with IBS, a trend towards reduced expression of colonic CLD-1 was previously shown (213), however, in a more recent study by Martínez *et al.* (344) CLD-1 jejunal expression was comparable to controls. Thus, further studies are needed to determine the role of CLD-1 in the intestinal barrier function.

ZO-1 expression decreased after prolonged incubation (24 hours) with tryptase (3 mU & 15 mU). Previously both ZO-1 protein and mRNA expression was shown to be decreased in jejunal tissue from IBS-D patients, and ZO-1 mRNA levels negatively associated with increased tryptase mRNA levels in the tissue (285). Also, PAR-2 agonists induced a redistribution of ZO-1 from TJs which associated with an increase in paracellular permeability and a decrease in TER of intestinal epithelial cells (57). Considering these data, it might be speculated that tryptase contributes to increased permeability in IBS patients through down-regulation of epithelial ZO-1.

This study shows that occludin expression appeared to be significantly and consistently increased following short-time exposure (4 hours) to 15 mU tryptase treatment, although the effect was reversible within 24 hours of incubation. Therefore, the role of occludin in maintaining barrier function appears to be secondary in these experimental settings. This is in agreement with previous studies showing an intact intestinal barrier in occludin-deficient mice (40). Jacob *et al.* (57), however, demonstrated that occludin was redistributed from TJs upon

PAR-2 activation *in vitro*. In IBS, occludin expression was shown to be lower and redistributed from TJs in intestinal mucosa (213, 265, 344), and its lower expression was suggested be due to enhanced proteasome trypsin-like activity (265). Therefore, future work is necessary to define the role of occludin in TJ regulation.

Besides TJ proteins this study also focused on the expression of AJ protein E-cadherin. E-cadherin was of interest because of its association with the risk of PI-IBS in large scale genetic studies (399), but in keeping with other *in vitro* data (57) this study showed no alterations in E-cadherin. Indeed, this junctional protein may be important in IBS, but tryptase may not be central to its regulatory mechanisms.

In the present study, tryptase was applied apically to Caco-2 monolayers to mimic the reported increased tryptase levels in luminal fluids of IBS patients (228). Additionally, a statistically significant increase in tryptase content was found in the mucosal tissue of IBS patients (175, 285). This study initially sought to focus on the role of luminal tryptase, and the interesting findings described here prove that tryptase disrupts intestinal epithelial barrier function *in vitro*. Noteworthy the concentration of tryptase used in this study was higher (15 mU=5 ng/ml; 3 mU=2 ng/ml) than the reported levels of luminal tryptase in IBS patients (0.45 ng/ml) and only 15 mU tryptase significantly reduced integrity of Caco-2 monolayers, and the effects of tryptase were rapid and dramatic. In IBS impaired intestinal barrier dysfunction is likely to be the result of a chronic process and occurs due to longstanding exposure to enhanced tryptase levels and other immune mediators. Nevertheless, this study focussed on the potential role of tryptase in the regulation of intestinal epithelial barrier and on the mechanisms behind this regulation *in vitro*.

Based on previous evidence (57, 168) it is likely that tryptase affected intestinal permeability via PAR-2 activation. Mast cell tryptase was shown to cleave a synthetic peptide corresponding to the N terminus of PAR-2 that in the intact receptors lead to exposure of the receptor tethered ligand domain (SLIGKV) and receptor activation (168) (Figure 1.7). How PAR-2 activation mediates increased epithelial permeability still needs to be elucidated. Jacob *et al.* (57) suggested that activated PAR-2 coupled to extracellular signal-regulated kinases 1/2 to regulate the cytoskeleton and thus the permeability of TJs in intestinal epithelial cells. Indeed, further studies reported that PAR-2 activation by its agonists increased intestinal paracellular permeability through MLC phosphorylation by MLCK in mice (6). In line with this, phosphorylation/contraction of peri-junctional cytoskeleton has been associated with TJ disassembly and barrier dysfunction (344). In this study, both immunofluorescence and western blot analysis showed that tryptase altered the expression of transmembrane proteins CLD-1 and JAM-A, and scaffolding protein ZO-1, which directly associates with cytoskeleton. Though it was not studied here, contraction of the peri-junctional cytoskeleton could cause a tension on the lateral membrane of adjacent epithelial cells impairing TJ barrier function (4) through the opening of TJs and redistribution of CLD-1, JAM-A and ZO-1 from TJ regions. The role of either PAR-2 or cytoskeleton, however, was not investigated in this study and a future analysis aimed e.g. at blocking PAR-2 or MLCK expression may further confirm tryptase role in the tryptase-mediated epithelial barrier dysfunction.

In view of the recent evidence showing increased tryptase content in the gut of IBS patients and its association with increased permeability, this study supports the hypothesis that tryptase might be involved in barrier disruption in IBS. Tryptase appears to disrupt an intestinal epithelial barrier *in vitro* as evidenced by a decrease in TER, increased paracellular permeability to FITC-dextran and the alterations of the expression of junctional proteins JAM-A, CLD-1 and ZO-1.

Key findings

Tryptase (15 mU) disrupted the barrier function of Caco-2 monolayers as determined by:

1. a significant 40% drop in TER (4 h);

2. a significant 2-fold increase in paracellular permeability to FITC-dextran (4 h);

3. a significant decrease in JAM-A, CLD-1 and ZO-1 expression within 24 hours of incubation;

4. a redistribution of JAM-A and CLD-1 from TJs into cellular compartments within 4 hours of incubation.

CHAPTER 4

MAST CELL STIMULATION WITH COMPOUND 48/80 DISRUPTS THE INTESTINAL EPITHELIAL BARRIER IN A CACO-2/HMC-1 CO-CULTURE MODEL

4.1. Introduction

4.1.1. Rationale

An increase in mast cell numbers in the colonic mucosa of IBS patients is generally well documented (72), as is their interaction with enteric nerves and association with abdominal pain, although not all studies support these findings (404). Beyond brain-gut interactions, the involvement of mast cells in other pathogenic mechanisms, such as enhanced intestinal permeability, are incompletely understood. The present study suggests that mast cells, and specifically the mast cell protease tryptase, may regulate intestinal permeability in IBS. Santos *et al.* (240) have shown that chronic stress resulted in increased mast cell numbers and activation in parallel with intestinal epithelial barrier dysfunction in animal models. Moreover, an increase in intestinal permeability was prevented by mast cell stabilisers (240, 349) demonstrating that mast cell activation is crucial to barrier dysfunction.

The mechanisms by which the increased mast cell activity documented in IBS may alter intestinal permeability and TJ proteins are not clear. Activated mast cells release a plethora of potent biological mediators to the surrounding environment, some of which might be implicated in the regulation of the intestinal barrier function and the generation of IBS symptoms. Based on emerging data, tryptase released from mast cells might reduce colonic epithelial integrity and alter the expression of junctional proteins. To study this hypothesis a human *in vitro* intestinal epithelial-mast cell model was developed, and the effects of compound 48/80-stimulated mast cells and mast cell tryptase on epithelial integrity and on the expression of the junctional proteins JAM-A, ZO-1, claudins, occludin and E-cadherin were investigated.

4.1.2. Aims of the study

General aim

To investigate if mast cell mediators, particularly tryptase, disrupt the intestinal epithelial barrier *in vitro* using a co-culture model of intestinal epithelial cells, Caco-2, and human mast cells, HMC-1.

Specific aims

1. To develop a co-culture model of intestinal epithelial and mast cells, Caco-2/HMC-1.
2. To investigate the effects of mast cell stimulation with compound 48/80 on the intestinal epithelial barrier in the Caco-2/HMC-1 model in terms of:
   - epithelial integrity by measurement of TER;
   - paracellular permeability to FITC-dextran;
   - ultrastructure of Caco-2 monolayers;
   - expression of junctional proteins by western blotting;
   - expression and localisation of junctional proteins by immunofluorescence confocal microscopy.
3. To investigate the role of mast cell tryptase in the regulation of the intestinal epithelial barrier using tryptase inhibitor, NM, in terms of:
   - epithelial integrity by measurement of TER;
   - paracellular permeability to FITC-dextran;
   - expression of junctional proteins by western blotting;
   - expression and localisation of junctional proteins by immunofluorescence confocal microscopy.

4.1.3. Methods to achieve the aims

Caco-2 cells were co-cultured with HMC-1 cells until Caco-2 cells established differentiated and polarised monolayers, from 21 to 23 days, and the integrity of monolayers was monitored by the measurement of TER. HMC-1 cells were stimulated with compound 48/80 (+/- tryptase inhibitor, NM) and incubated for between 1 to 24 hours. Epithelial integrity was assessed by TER, permeability to molecular marker FITC-dextran and TEM. The expression of junctional proteins ZO-1, JAM-A, CLD-1, CLD-2, CLD-3, occludin and E-cadherin was determined by western blotting and immunofluorescence confocal microscopy.

HMC-1 cells were selected to study the effects of mast cell stimulation on intestinal epithelial integrity. HMC-1 cell line is well characterised and exhibits a phenotype similar to that of human mast cells (385). For this study it was particularly important that HMC-1 cells express enzymatically active β-tryptase (~50 ng/10^6 cells) (405, 406). The other established human mast cell line, LAD2, also expresses an active form of tryptase and could be used alternatively to HMC-1 cells (407).

4.2. Results

4.2.1. Development of a Caco-2/HMC-1 co-culture model

Optimisation of co-culture medium

For the co-culture of Caco-2 cells with HMC-1 cells the effects of several media combinations on cell culture were investigated (Table 4.1). The morphology of both cell lines was analysed with a light microscope, and the viability of HMC-1 was assessed periodically with trypan blue staining. Based on these parameters, DMEM medium with 2 mM L-Glutamine, 1% v/v non-essential amino acids, 5% heat-deactivated FBS, 5% heat-deactivated iron supplemented CS, 100 U/ml penicillin and 100 μg/ml streptomycin was selected for further studies. For HMC-1 cells culture serum was heat deactivated to inactivate complement components which trigger mast cell degranulation (102, 408). In media with non-heat deactivated serum, mast cells did not proliferate and subsequently died as evidenced by cell counting and assessment of their viability with trypan blue staining. Caco-2 cells did not attach to the surface of tissue culture flasks when media (either DMEM or Iscove's) were supplemented with α-thioglycerol (used in most cell-cultures to stimulate cell proliferation) and a significant number of floating cells were observed under the microscope.

Co-culture in Transwells

The co-culture model of Caco-2/HMC-1 cells was developed to investigate the role of mast cells in the disruption of epithelial barrier integrity. Caco-2 cells (5 x 10^5 cells/ml) (401) were seeded on filters and HMC-1 cells (5 x 10^5 cells/ml) (57) were added to the basolateral compartment of the Transwells® either on the 1st, 15th or 18th day of culture. The integrity of Caco-2 monolayers was monitored by the measurement of TER on alternate days and appeared to be maintained

throughout the co-culture (Figure 4.1). The morphology of both cell lines was analysed with a light microscope, and the viability of HMC-1 cells was assessed periodically with trypan blue staining. The viability of HMC-1 cells was greater than 99%. After optimisation, the novel co-culture model based on the addition of Caco-2 and HMC-1 cells on the 1st day was selected for the following reasons: 1) TER of Caco-2 was maintained throughout the co-culture 2) the viability of HMC-1 cells was greater than 99% 3) this model enabled to investigate the effect of undegranulated mast cells on the development of Caco-2 monolayers including TJ assembly.

Medium	Caco-2 cells	HMC-1 cells
Iscove's with 5% iron supplemented CS, 5% FBS (serum non heat-deactivated), (-) α-thioglycerol	+	-
Iscove's with 5% iron supplemented CS, 5% FBS (serum heat-deactivated), (-) α-thioglycerol	+	+
Iscove's with 5% iron supplemented CS, 5% FBS (serum non heat-deactivated) (+) α-thioglycerol	-	-
Iscove's with 5% iron supplemented CS, 5% FBS (serum heat-deactivated) (+) α-thioglycerol	-	+
DMEM with 5% iron supplemented CS, 5% FBS (serum heat-deactivated) (-) α-thioglycerol	+	+
DMEM with 5% iron supplemented CS, 5% FBS (serum heat-deactivated), (+) α-thioglycerol	-	+

Table 4.1 Combinations of different media that were tested for Caco-2/HMC-1 cells co-culture. All the media were supplemented with 2 mM L-Glutamine, 1% v/v non-essential amino acids, 100 U/ml penicillin and 100 μg/ml streptomycin. + means: *Caco-2 cells* proliferated and attached to the surface of tissue culture flasks; *HMC-1 cells* proliferated and their viability was greater than 99%. – means: *Caco-2 cells* did not attach to the surface of tissue culture flasks; *HMC-1 cells* did not proliferate and their viability was lower than 99% and gradually decreased throughout the culture. The medium that was chosen for co-culture studies is marked with the blue background.

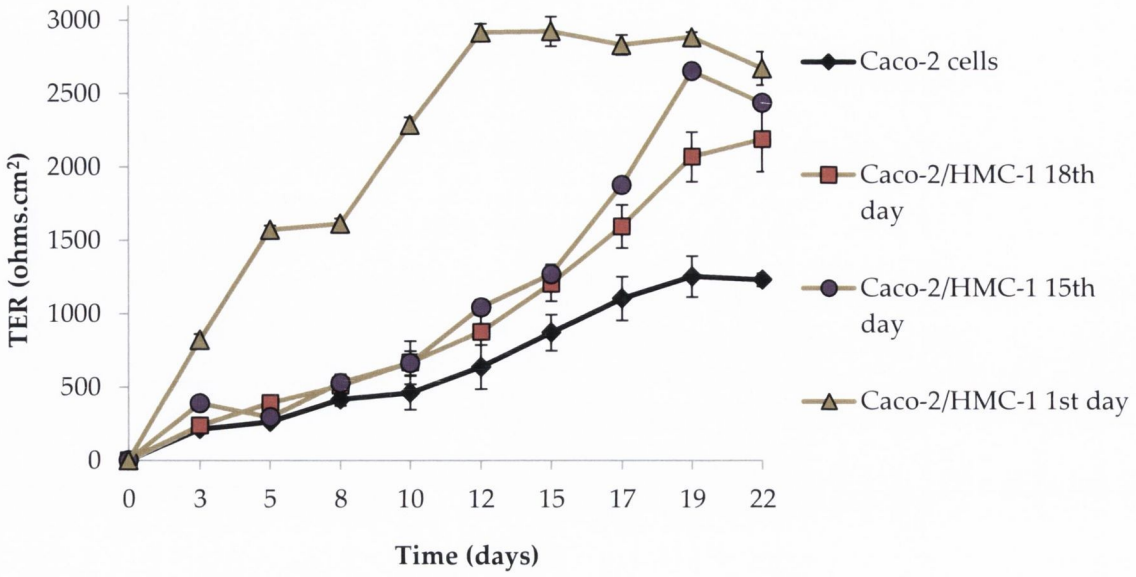


Figure 4.1 TER of Caco-2 cells co-cultured with HMC-1 cells for 22 days compared to Caco-2 cells cultured alone. The Caco-2/HMC-1 18th day line represents the mean ± SEM of 2 samples while the other lines represent the mean ± SEM of 3 samples.

Characterisation of the co-culture model

In this study Caco-2 cells were continuously co-cultured with unstimulated HMC-1 cells for approximately 3 weeks until Caco-2 cells established differentiated and polarised monolayers. The morphology of the cells was regularly evaluated with light microscopy. HMC-1 cells were typically homogenous in size and were forming small clumps. The cells looked healthy, they had regular cell membranes and their viability was typically greater than 99% as determined by trypan blue staining. HMC-1 cells were proliferating and their numbers significantly increased (10-fold) after a 3 week co-culture. The proliferation rates of Caco-2 cells were not determined, however cells continuously co-cultured with unstimulated mast cells formed a tight epithelial barrier as demonstrated by a gradual increase in TER over time, which was

higher when compared to Caco-2 cells alone (day 22nd, 150%, P=0.0002) (Figure 4.2). In line with this, the permeability to FITC-dextran of Caco-2 monolayers which were co-cultured with mast cells for up to 23 days was significantly lower compared to Caco-2 cells alone (1.34±0.12 fold, P=0.02, 24 h) (Figure 4.3). No significant changes for either HMC-1 or Caco-2 cells were observed between cell passages.

Cell culture medium was changed on alternate days for up to 2 weeks of a co-culture, and since then every day to reduce the potential detrimental effects arising due to competition for oxygen and nutrients between cell lines.

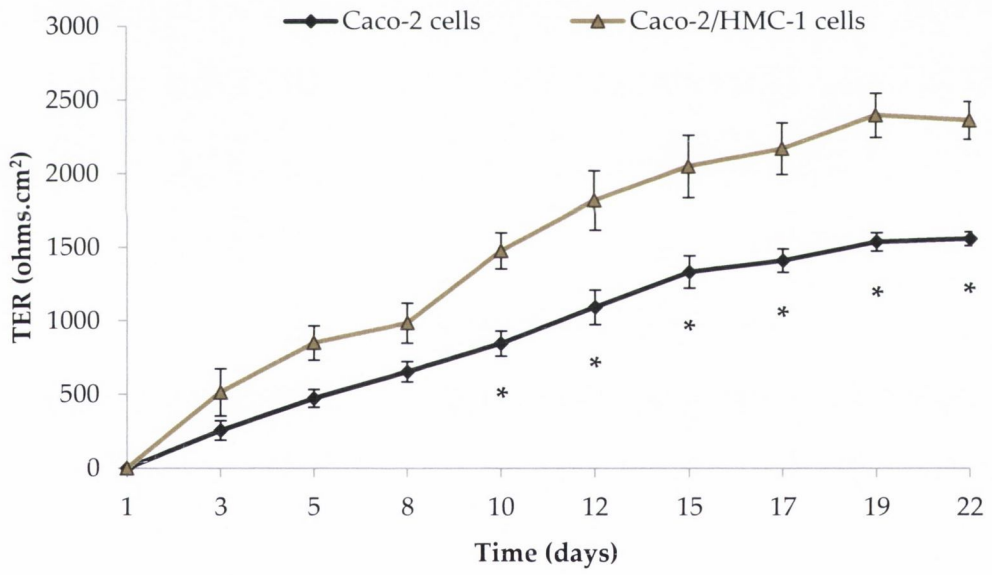


Figure 4.2 TER of Caco-2 cells co-cultured with HMC-1 cells for 22 days compared to Caco-2 cells cultured alone. Results represent the mean ± SEM of 6 independent experiments. *P<0.05 (Student's t-test).

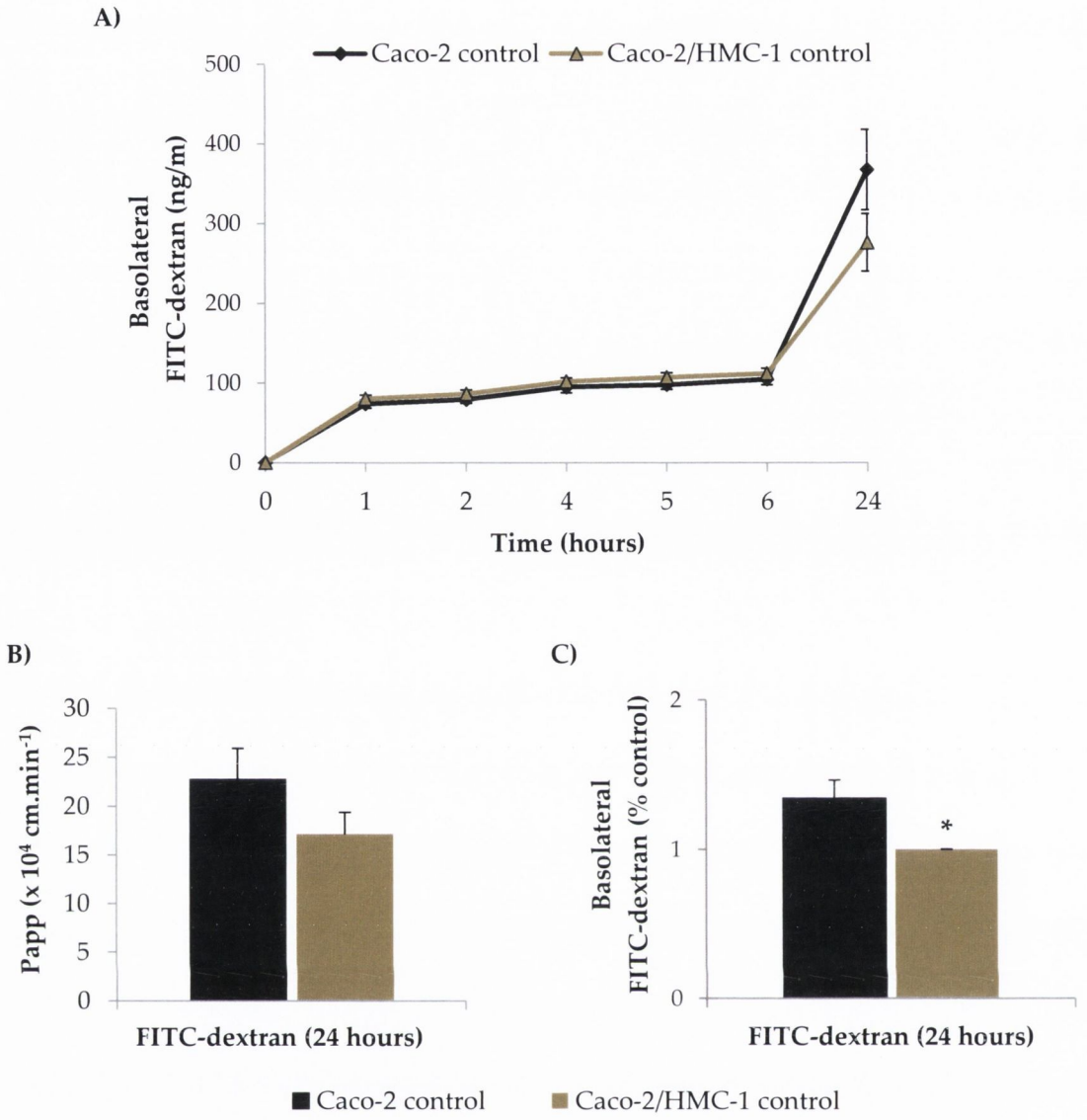


Figure 4.3 Epithelial barrier function of Caco-2 cells co-cultured with HMC-1 cells. **A)** Paracellular permeability to FITC-dextran of Caco-2 cells co-cultured with HMC-1 cells for up to 23 days compared to Caco-2 cells cultured alone **B)** Permeability coefficients of FITC-dextran. **C)** Basolateral FITC-dextran content at 24 hours expressed as a change relative to Caco-2/HMC-1 control. **(A-C)** Results represent the mean ± SEM of 6 independent experiments. *P<0.05 (Student's t-test).

4.2.2. Mast cell stimulation with compound 48/80 decreases TER of Caco-2 monolayers

To induce degranulation, mast cells were stimulated with synthetic compound 48/80 which activates G protein-coupled receptors (388) and was previously shown to induce degranulation of HMC-1 cells (57). When HMC-1 cells were challenged with compound 48/80 to initiate degranulation, Caco-2 monolayers showed a significant decrease in TER within 2 hours of incubation (19±2%, $P<0.0001$) compared to untreated controls - this effect was maintained at a comparable level for up to 24 hours (Figure 4.4). Compound 48/80 had no effect on TER of Caco-2 cells cultured alone.

4.2.3. Mast cell stimulation with compound 48/80 increases permeability of Caco-2 monolayers to FITC-dextran

In addition to a significant drop in TER, mast cell stimulation with compound48/80 induced a significant increase in paracellular permeability to FITC-dextran within 6 hours of incubation (1.22±0.08 fold, $P=0.03$) with a maximal difference at 24 hours (1.68±0.07 fold, $P=0.002$) (Figure 4.5) demonstrating that mast cell stimulation disrupted the epithelial barrier. Compound 48/80 had no effect on FITC-dextran flux of Caco-2 cells cultured alone.

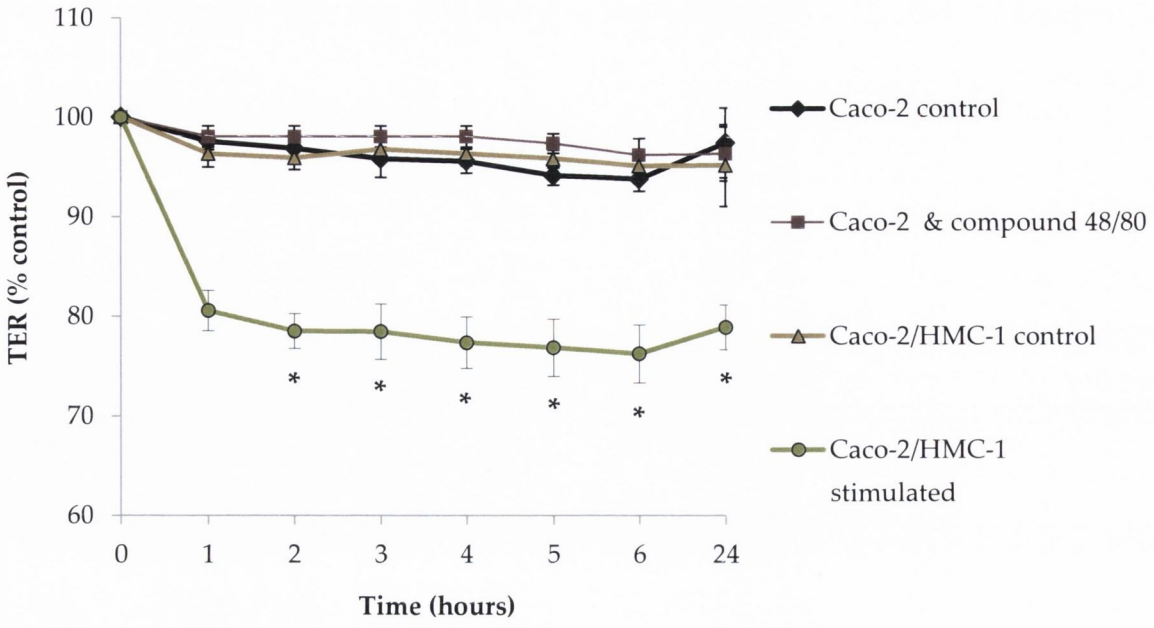


Figure 4.4 Effects of HMC-1 stimulation with compound 48/80 on the epithelial barrier. TER of Caco-2/HMC-1 co-cultures monitored for up to 24 hours following HMC-1 stimulation and compared to untreated controls (Caco-2/HMC-1 controls). Results represent the mean ± SEM of 6 independent experiments. *P<0.05 (1-way ANOVA followed by Tukey's post hoc test).

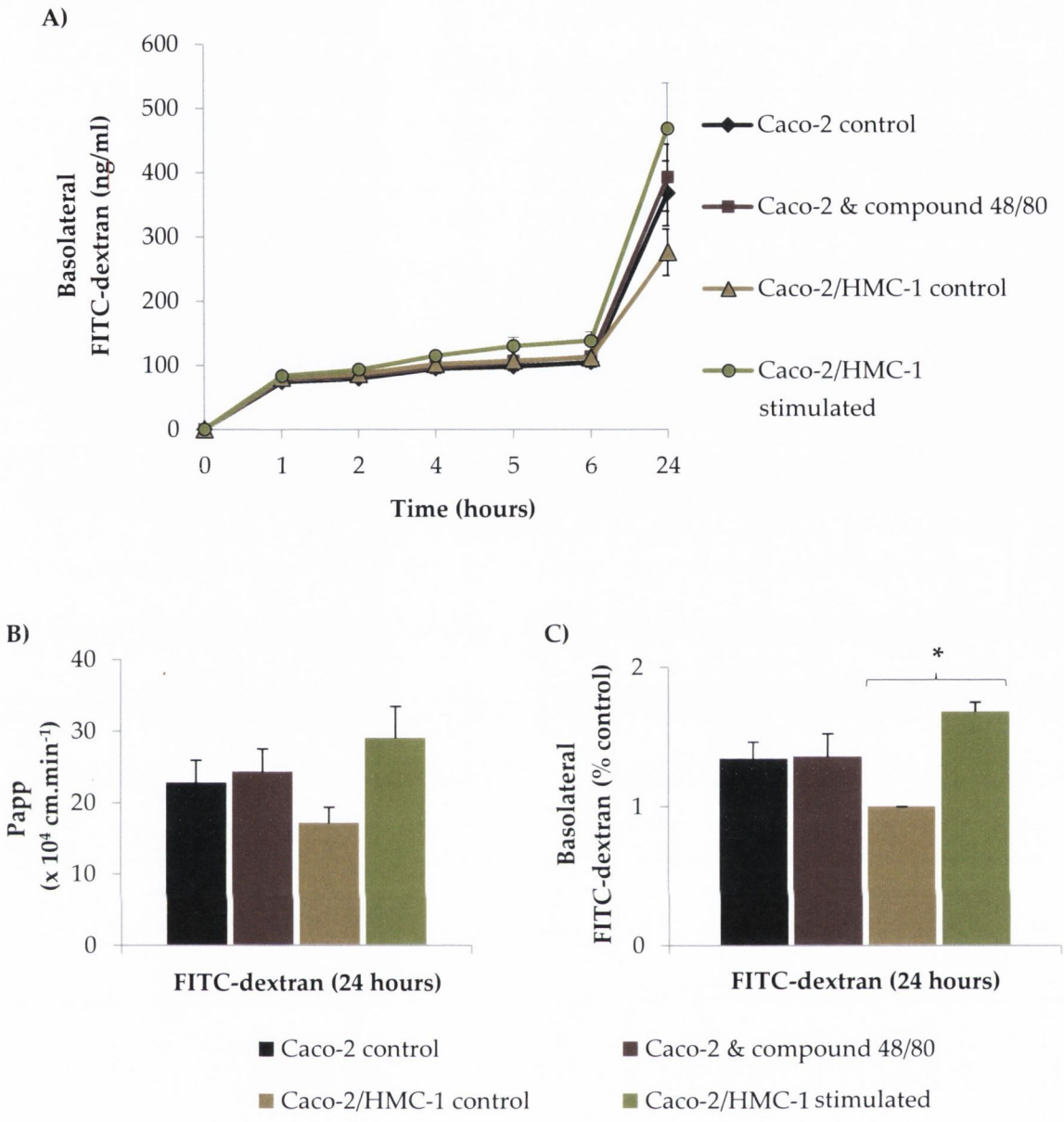


Figure 4.5 Effects of HMC-1 stimulation with compound 48/80 on the epithelial barrier. **A)** Apical-to-basolateral flux of FITC-dextran through Caco-2 monolayers monitored up to 24 hours following HMC-1 stimulation compared to untreated controls (Caco-2/HMC-1 controls). **B)** Permeability coefficients of FITC-dextran. **C)** Basolateral FITC-dextran content 24 hours after HMC-1 stimulation expressed as a change relative to untreated controls. Results represent the mean ± SEM of 6 independent experiments. *P<0.05 (1-way ANOVA followed by Tukey's post hoc test).

4.2.4. Mast cell stimulation with compound 48/80 alters the ultrastructure of Caco-2 monolayers as shown by TEM

TEM analysis of Caco-2/HMC-1 co-cultures confirmed that there was no difference in TJ ultrastructure between Caco-2 cells alone and those co-cultured with unstimulated mast cells for 21 days (Figure 4.6). Stimulation of the mast cells with compound 48/80 in the co-culture, however, clearly disrupted epithelial integrity as determined by the presence of open TJs at 6 and 24 hours in TEMs (Figure 4.6), which showed a distinct lack of electronegativity in the TJ complex.

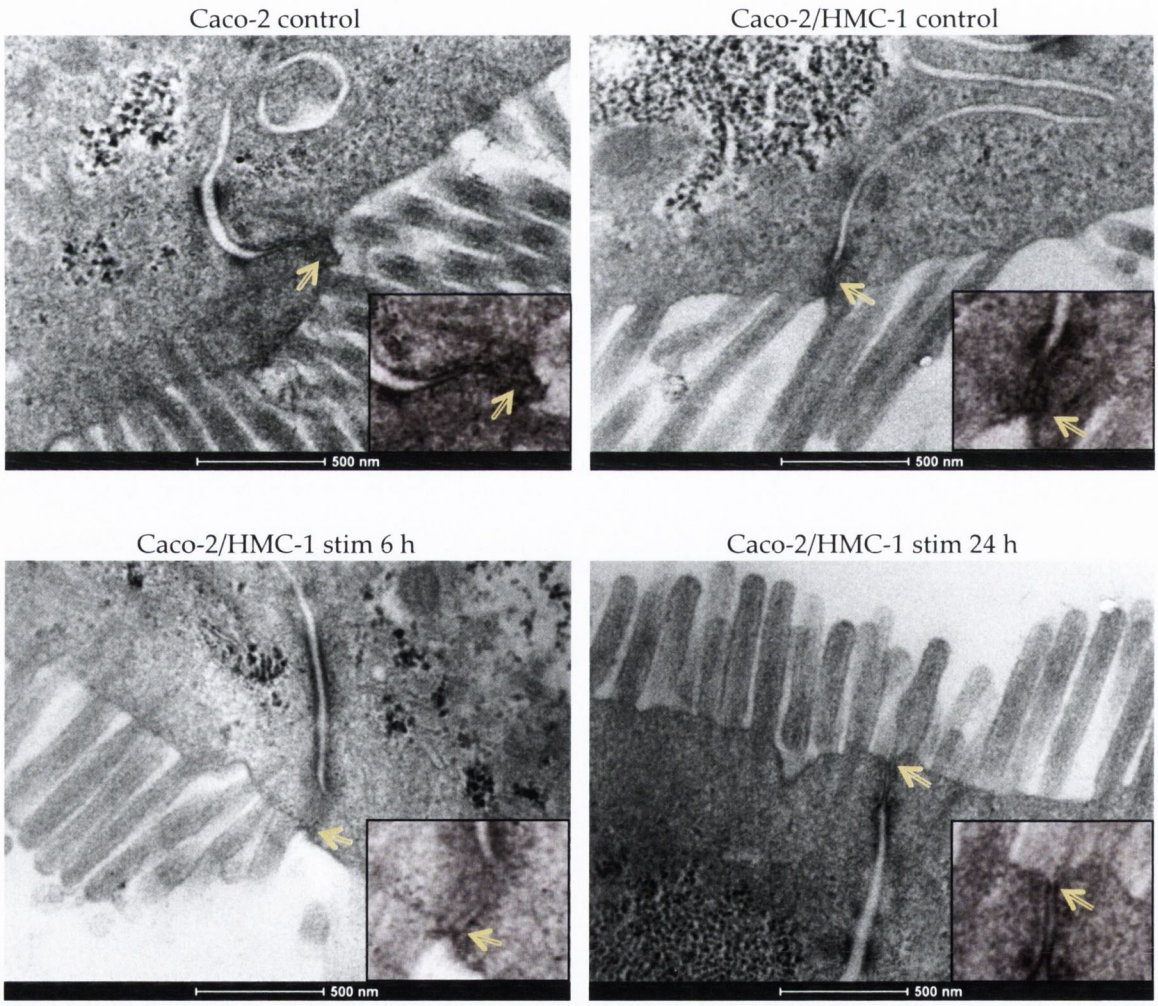


Figure 4.6 Representative photomicrographs showing the ultrastructure of AJC of Caco-2 cells co-cultured with HMC-1 cell for 21 days either 6 or 24 hours after mast cell stimulation (stim). TJs are marked with an arrow. The experiment was performed once.

In addition, mast cell stimulation induced a release of microvesicles from Caco-2 monolayers that were observed both at 6 and 24 hours following stimulation (Figure 4.7).

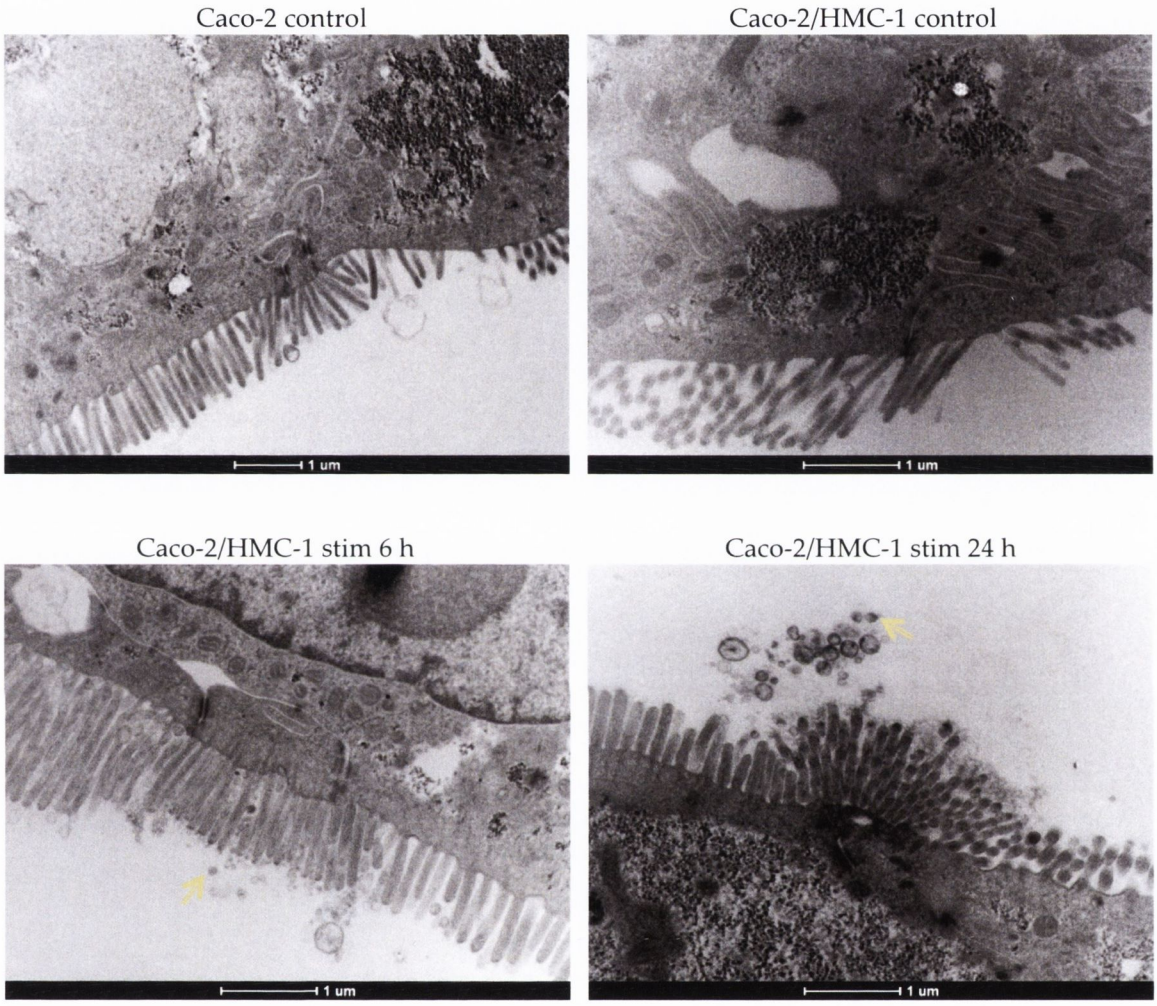


Figure 4.7 Representative photomicrographs showing the ultrastructure of the apical side of Caco-2 cells co-cultured with HMC-1 cell for 21 days either 6 or 24 hours after mast cell stimulation (stim). The microvesicles (marked with an arrow) were observed in close proximity to the apical surface. The experiment was performed once.

Furthermore, on the apical side of Caco-2 cells co-cultured with unstimulated HMC-1 cells, organised areas, appearing as cytoskeletal condensation, were observed which were distinctly darker than the surrounding cytoplasm. The areas were not detected in Caco-2 cells cultured alone or in co-cultures of Caco-2-HMC-1 cells following mast cell stimulation (Figure 4.8).

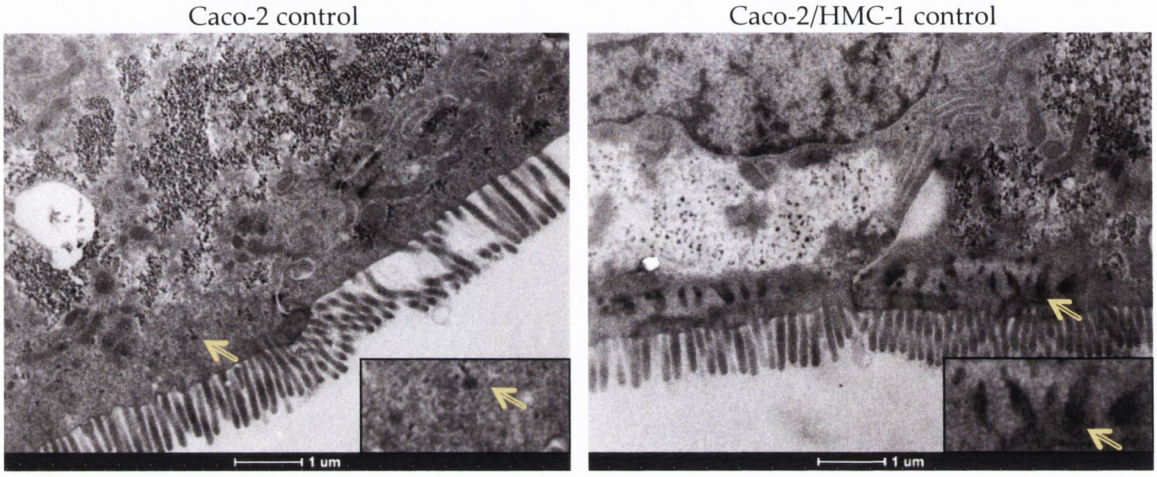


Figure 4.8 Representative photomicrographs showing the ultrastructure of the apical side of Caco-2 cells co-cultured with unstimulated HMC-1 cells for 21 days. Organised electron-dense areas (marked with an arrow) were observed on the apical side. The experiment was performed once.

4.2.5. Mast cell stimulation with compound 48/80 alters junctional protein expression as determined by western blotting

In the Caco-2/HMC-1 model, JAM-A expression was significantly reduced within 6 hours of mast cell stimulation with compound 48/80 (0.67±0.07 fold, P=0.01) compared to controls, and the effect was maintained for up to 24 hours (0.63±0.06 fold, P=0.0008) (Figures 4.9, 4.10). Interestingly, the level of CLD-1 expression in Caco-2/HMC-1 co-cultures for up to 6 hours after stimulation was comparable to controls. However, 24 hours after stimulation, CLD-1 expression appeared to be significantly reduced (0.57±0.08 fold, P<0.0001) (Figures 4.9, 4.10). ZO-1 expression was dramatically reduced within 1 hour (0.34±0.09 fold, P=0.016) following mast cell stimulation and was maintained at a comparable level for up to 4 hours. After prolonged incubation there was no significant difference relative to the unstimulated controls (Figures 4.9, 4.10). CLD-2 expression increased significantly within 24 hours (2.30±0.17 fold, P=0.037) following mast cell stimulation, though initially (for up to 6 hours) there was no detectable change in its expression (Figures 4.9, 4.10). Mast cell stimulation did not alter CLD-3, occludin or E-cadherin expression (Figures 4.9, 4.10).

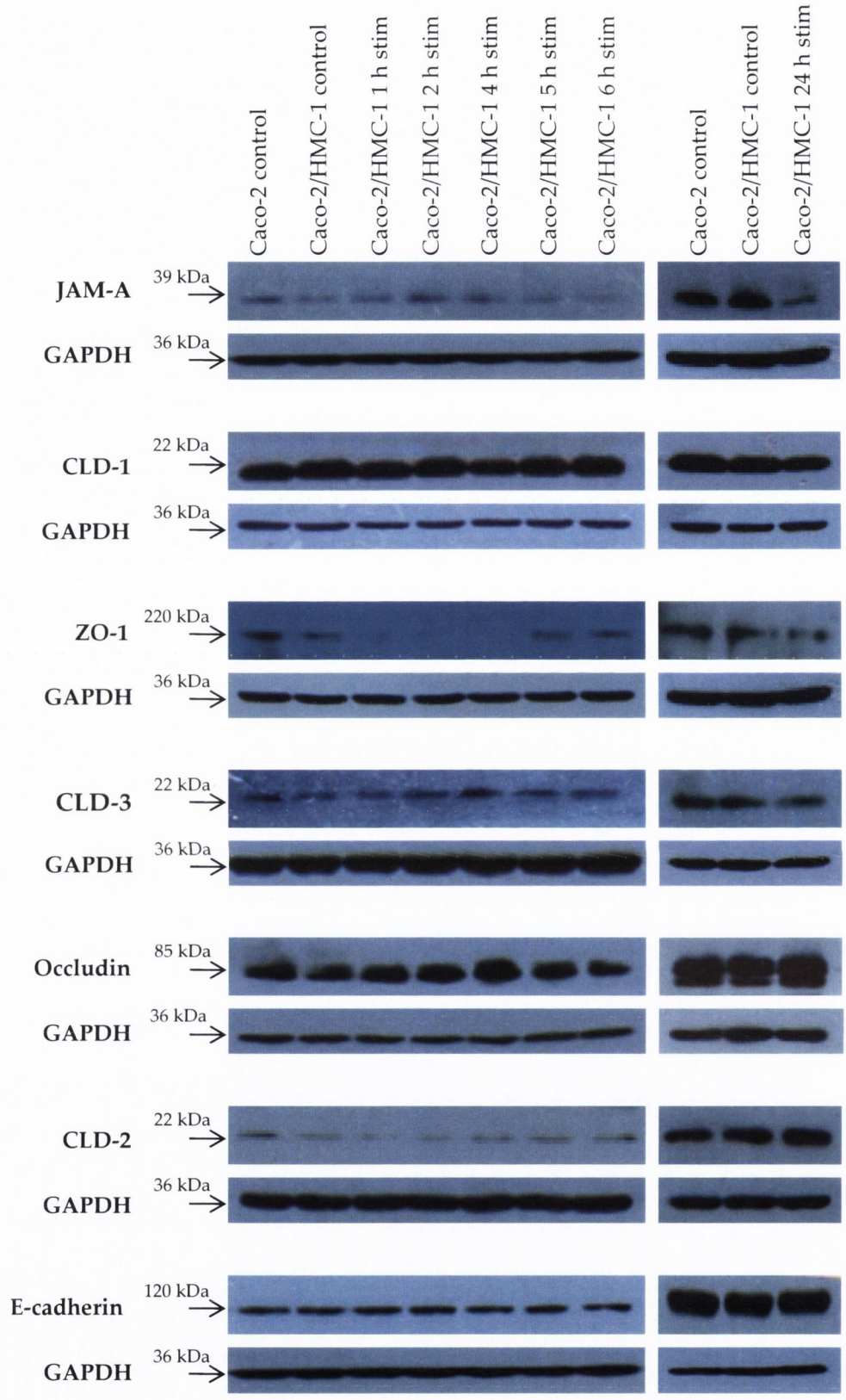


Figure 4.9 Western blot analysis of the expression of junctional proteins in Caco-2/HMC-1 co-cultures for up to 24 hours after mast cell stimulation (stim) with compound 48/80. Equal protein loading was verified with corresponding GAPDH levels. The blots shown are a representative of a minimum of 2 independent experiments.

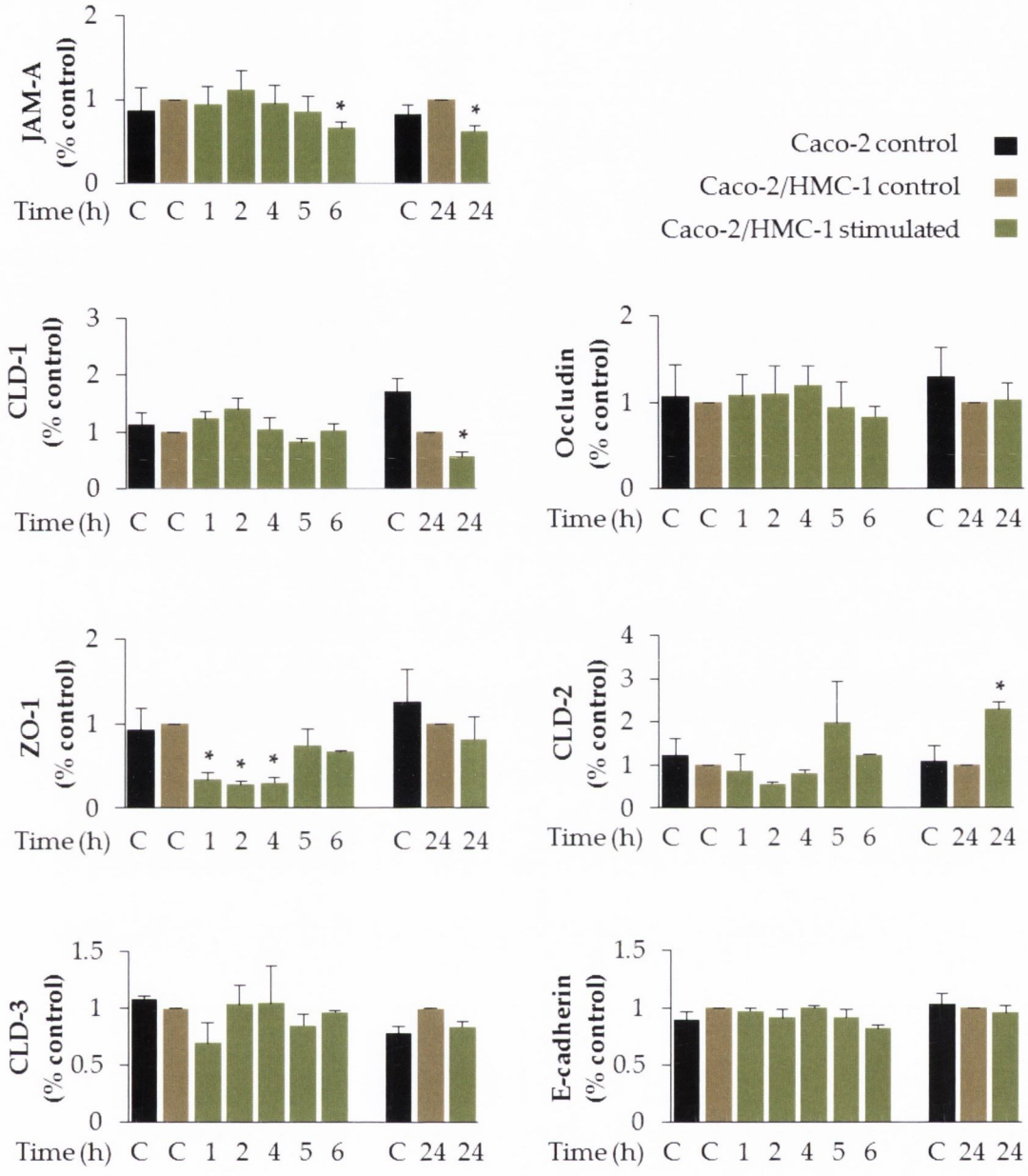


Figure 4.10 Expression of junctional proteins in the Caco-2/HMC-1 co-culture model for up to 24 hours after mast cell stimulation with compound 48/80 as determined by densitometry. The results are expressed as a change relative to the untreated control. The density of each individual band was compared with the corresponding control band and normalised against GAPDH by densitometry. Results represent the mean ± SEM of a minimum of 2 independent experiments. *P<0.05 (Student's t-test).

4.2.6. Mast cell stimulation with compound 48/80 alters junctional protein expression as determined by confocal microscopy

In line with western blot analysis, JAM-A expression following mast cell stimulation with compound 48/80 was clearly diminished in TJs of Caco-2 monolayers as shown in Figure 4.11. Immunofluorescence confocal microscopy also confirmed that CLD-1 expression was reduced in TJs following stimulation (Figure 4.12). Since both JAM-A and CLD-1 showed a consistent and significant decrease at 24 hours following mast cell stimulation, they were selected as a focus for follow-up experiments.

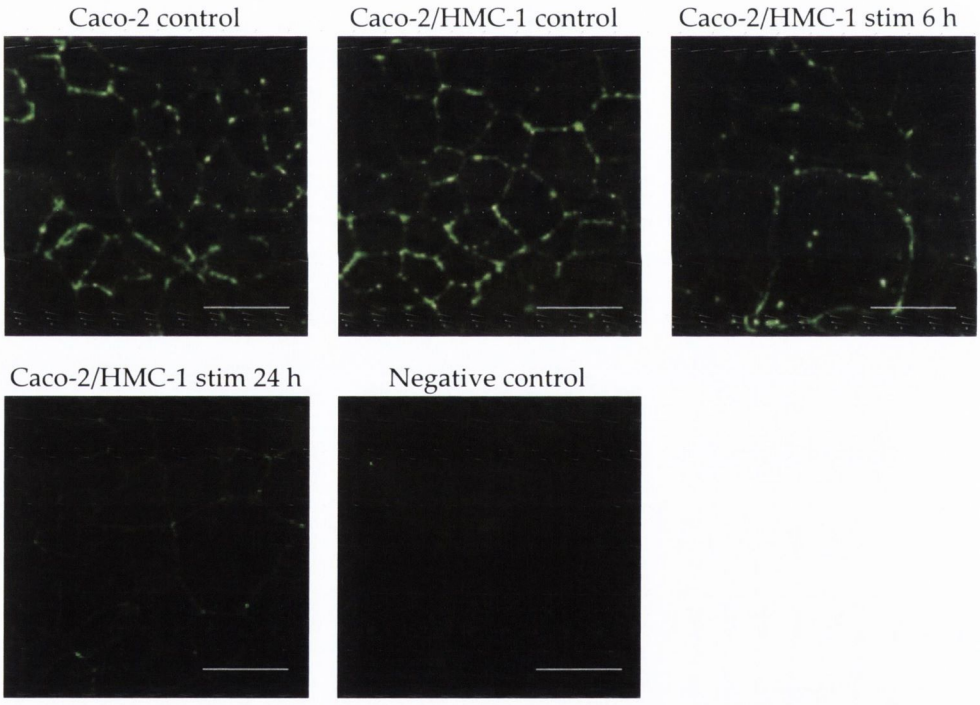


Figure 4.11 JAM-A expression in Caco-2/HMC-1 co-cultures either 6 or 24 hours after mast cell stimulation (stim) with compound 48/80. Immunofluorescence staining was heterogeneous with small differences in the intensity of staining observed. Staining in Caco-2/HMC-1 co-cultures following mast cell stimulation with compound 48/80 shown here was not identified in corresponding controls. No staining was seen in negative controls for which the primary antibody was omitted. Photomicrographs shown are representative of 2 independent experiments. Bars=20 μm.

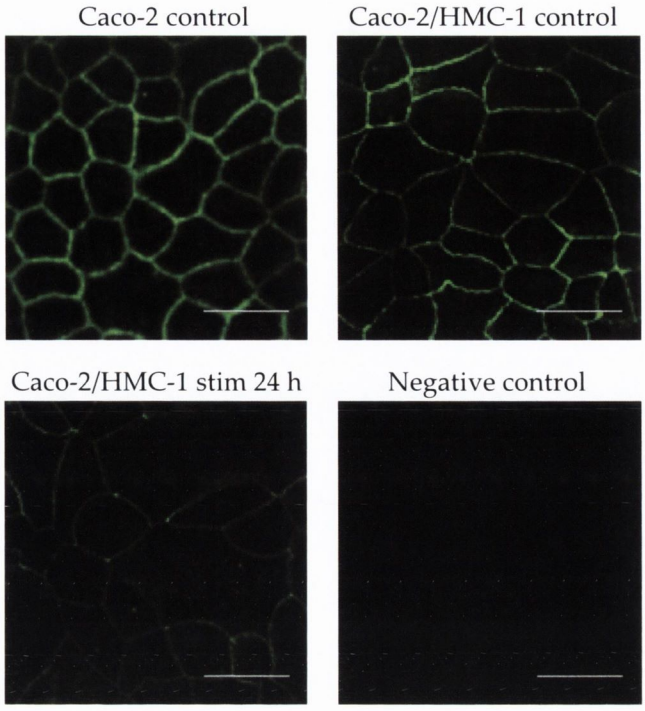


Figure 4.12 CLD-1 expression in Caco-2/HMC-1 co-cultures 24 hours after mast cell stimulation (stim) with compound 48/80. Immunofluorescence staining was heterogeneous with small differences in the intensity of staining observed. Staining in Caco-2/HMC-1 co-cultures following mast cell stimulation with compound 48/80 shown here was not identified in corresponding controls. No staining was seen in negative controls for which the primary antibody was omitted. Photomicrographs shown are representative of 2 independent experiments. Bars=20 μm.

4.2.7. Mast cell tryptase impairs the intestinal epithelial barrier - a tryptase inhibition study

In order to better understand the role of tryptase on epithelial permeability, the effect of mast cells tryptase from the array of other mediators on epithelial integrity was elucidated by specifically blocking tryptase with a competitive inhibitor, NM (391).

4.2.7.1. Mast cell tryptase decreases TER

NM did not have a significant effect on TER of Caco-2/HMC-1 co-cultures for up to 6 hours following mast cell stimulation with compound 48/80 compared to untreated controls but the general reduction in TER was clearly attenuated in the presence of NM (Figure 4.13). After 24 hours, however, the reduction in TER following mast cell stimulation was significantly inhibited returning to almost control levels (P=0.046). NM had no effect on TER of Caco-2 cells co-cultured with unstimulated HMC-1 cells (Caco-2/HMC-1 control).

4.2.7.2. Mast cell tryptase increases permeability to FITC-dextran

Consistent with the TER results, NM significantly inhibited the effect of mast cell stimulation on paracellular permeability to FITC-dextran over 24 hours of incubation back to the levels of the unstimulated co-cultures (1.15±0.11 fold, P=0.016) (Figure 4.14). NM had no effect on FITC-dextran flux of Caco-2 cells co-cultured with unstimulated HMC-1 cells.

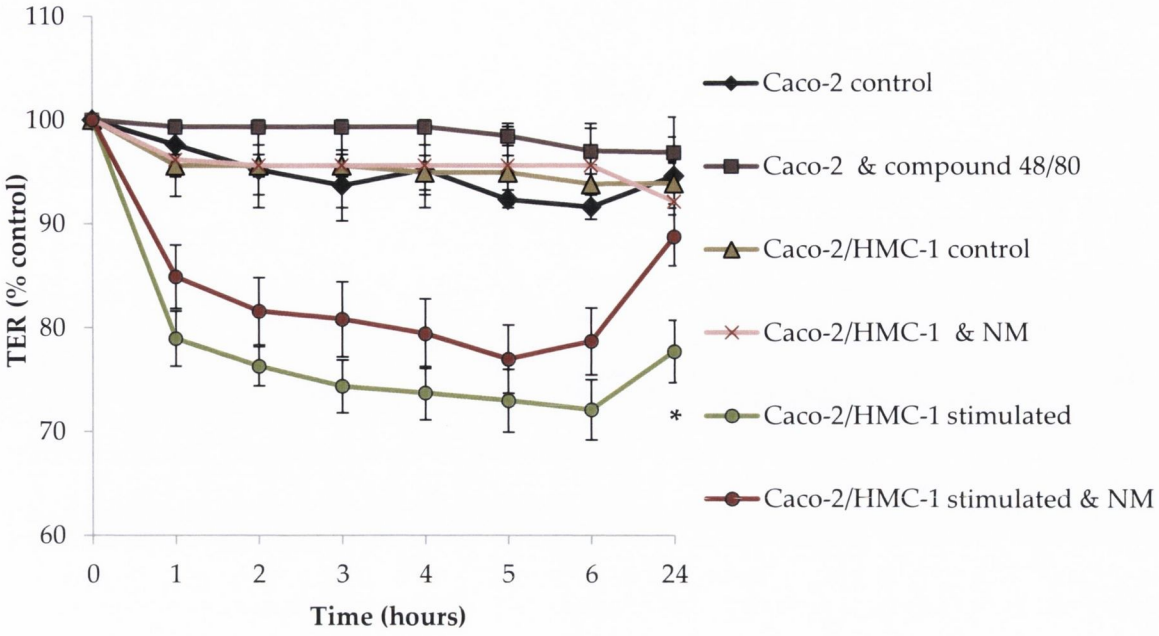


Figure 4.13 Effects of tryptase inhibition on the TER of Caco-2/HMC-1 co-cultures for up to 24 hours after mast cell stimulation with compound 48/80 compared to untreated controls. Mast cell tryptase activity was inhibited with NM. Results represent the mean ± SEM of 3 independent experiments. *P<0.05 (1-way ANOVA followed by Tukey's post hoc test).

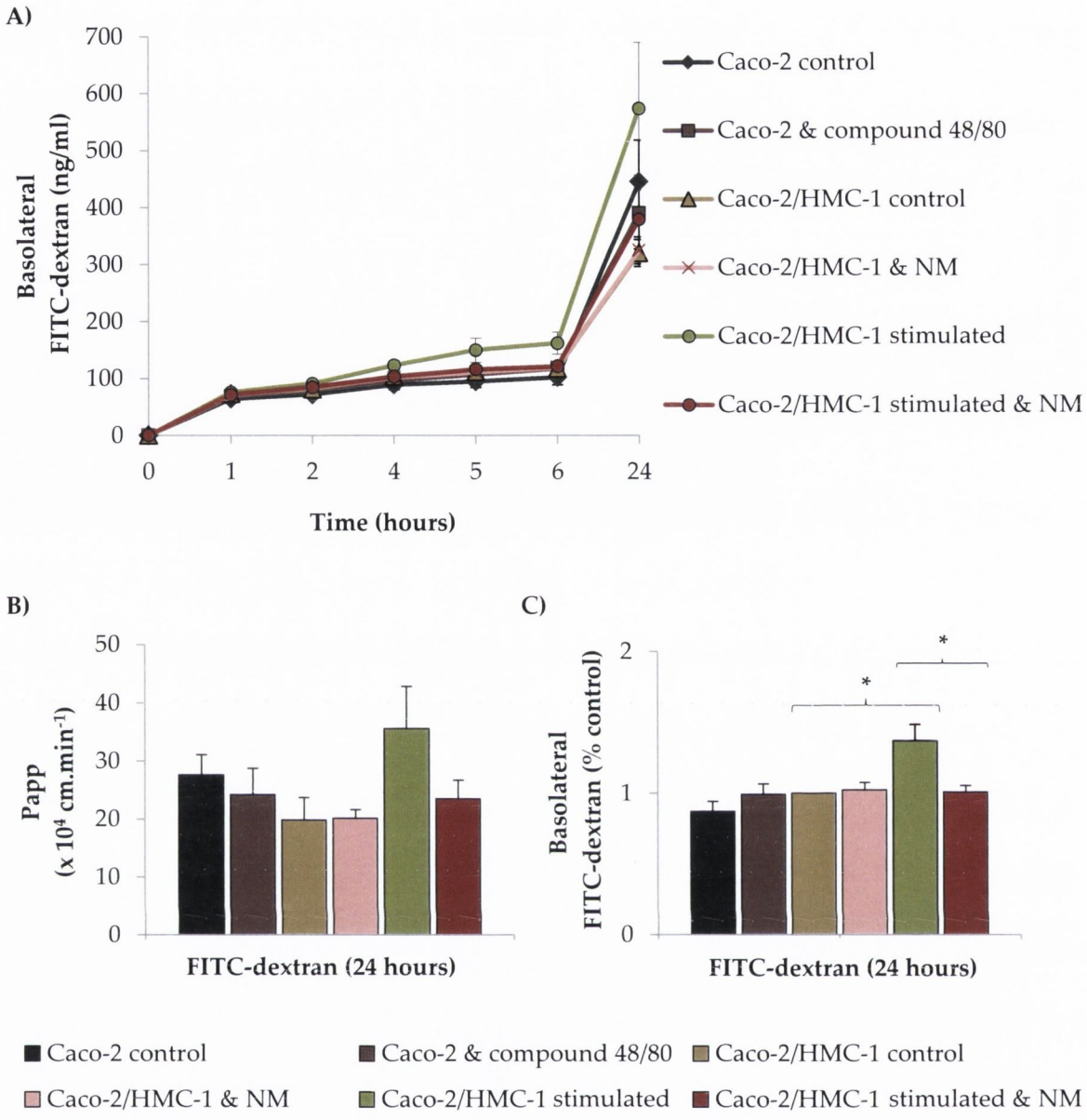


Figure 4.14 Effects of tryptase inhibition on permeability to FITC-dextran of Caco-2/HMC-1 co-cultures for up to 24 hours after mast cell stimulation with compound 48/80. Mast cell tryptase activity was inhibited with NM. **A)** Apical-to-basolateral flux of FITC-dextran through Caco-2 monolayers. **B)** Permeability coefficients of FITC-dextran. **C)** Basolateral FITC-dextran content 24 hours after mast cell stimulation expressed as a change relative to untreated controls. Results represent the mean ± SEM of 3 independent experiments. *P<0.05 (1-way ANOVA followed by Tukey's post hoc test).

4.2.7.3. Mast cell tryptase reduces the expression of JAM-A as determined by western blotting

The effect of tryptase inhibition on JAM-A and CLD-1 was investigated since, in experiments to date, both were consistently reduced at 24 hours in response to mast cell stimulation with compound 48/80 which was accompanied by a decrease in TER and an increase in FITC-dextran flux. As shown previously, stimulation of mast cells significantly reduced the expression of JAM-A in Caco-2/HMC-1 co-cultures within 24 hours (0.69±0.02 fold, $P<0.0001$). Incubation of tryptase with NM significantly reversed the effect of mast cell stimulation on JAM-A expression (0.87±0.05 fold, $P=0.02$) to that of unstimulated controls as shown by western blotting (Figures 4.15, 4.16). This finding supports the conclusion that the reduction in JAM-A was due, at least in part, to the effects of mast cell tryptase. While CLD-1 expression was also significantly reduced within 24 hours of mast cell stimulation (0.50±0.07 fold, $P<0.0001$) (Figures 4.15, 4.16), it was unaltered by tryptase inhibition in this model.

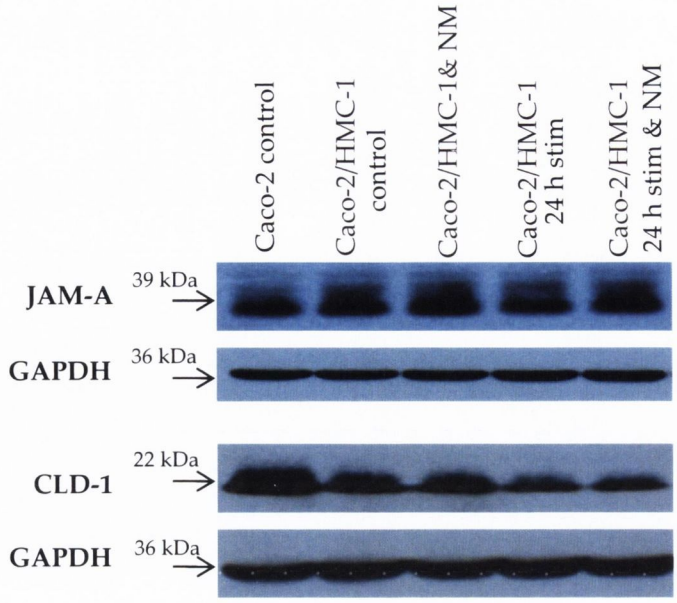


Figure 4.15 Western blot analysis of the expression of JAM-A and CLD-1 in the Caco-2/HMC-1 model 24 hours after mast cell stimulation (stim) with compound 48/80. Tryptase activity was inhibited with NM. Equal protein loading was verified with corresponding GAPDH levels. The blots shown are a representative of 3 independent experiments.

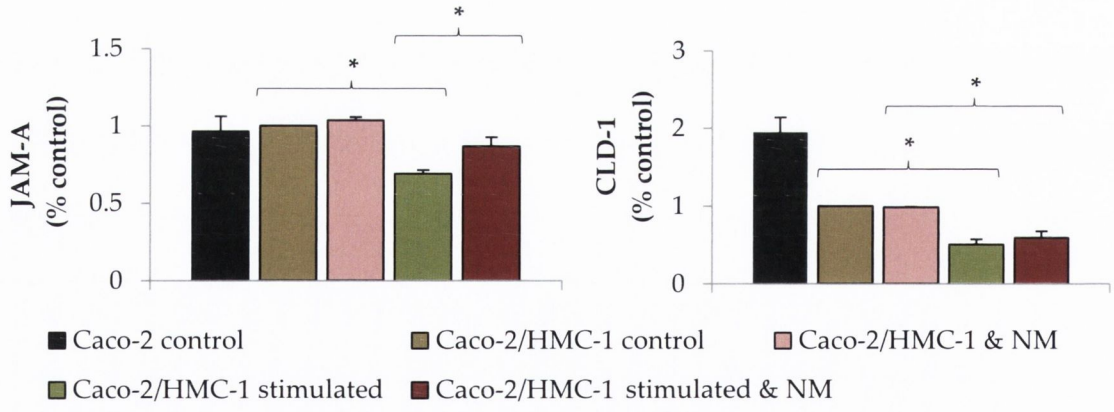


Figure 4.16 Effects of tryptase inhibition on the expression of JAM-A and CLD-1 in the Caco-2/HMC-1 model 24 hours after mast cell stimulation with compound 48/80 as determined by densitometry. Tryptase activity was inhibited with NM. The results are expressed as a change relative to untreated controls. The density of each individual band was compared with the corresponding control band and normalised against GAPDH by densitometry. Results represent the mean ± SEM of 3 independent experiments. *P<0.05 (Student's t-test).

4.2.7.4. Mast cell tryptase reduces junctional JAM-A expression as determined by confocal microscopy

As shown by western blotting, tryptase inhibition significantly reduced the effect of mast cell stimulation on JAM-A protein but not on CLD-1. Therefore, further studies investigated if the effect of tryptase inhibition on the expression of JAM-A would be confirmed by immunofluorescence confocal microscopy. In agreement with the western blot findings, JAM-A expression appeared to be reduced in TJs in Caco-2/HMC-1 co-cultures within 24 hours of mast cell stimulation and this effect was mitigated by NM treatment (Figure 4.17). These data further confirm that mast cell tryptase decreases junctional JAM-A expression.

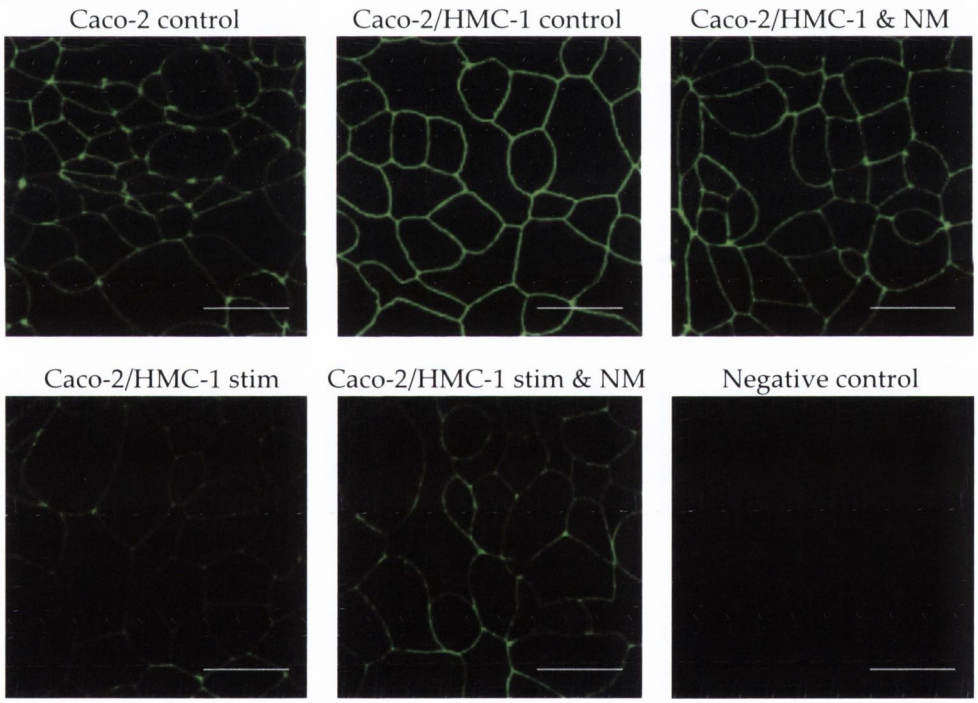


Figure 4.17 Effects of tryptase inhibition on JAM-A expression in the Caco-2/HMC-1 model 24 hours after mast cell stimulation (stim) with compound 48/80. Tryptase activity was inhibited with NM. Immunofluorescence staining was heterogeneous with small differences in the intensity of staining observed. Staining in Caco-2/HMC-1 co-cultures after mast cell stimulation +/- NM shown here was not identified in corresponding controls. No staining was seen in negative controls for which the primary antibody was omitted. Photomicrographs shown are representative of 2 independent experiments. Bars=20 μm.

4.3. Discussion

Low-grade inflammation is now widely recognised in the pathogenesis of IBS, with increased mast cell numbers as the most consistent finding (72). Furthermore, enhanced activation of mast cells has been repeatedly reported in IBS (175, 344, 409). Mast cell activation, given that mast cells are the only significant source of tryptase in the intestinal mucosa, may be determined by the measurement of the tryptase activity that is spontaneously released from intestinal mucosal tissue (144, 175). Also, tryptase content measured in various biological fluids may serve as a marker of mast cell activation (410). In the previous chapter tryptase was shown to significantly impair intestinal epithelial integrity in the Caco-2 cell model. In this study a novel co-culture model of intestinal epithelial and mast cells was developed to further investigate the mechanisms that might be involved in the regulation of epithelial permeability, with a view to translate these findings to IBS. To date, there are several studies indicating the role of mast cells in TJ protein regulation. This study, however, is the first to show the effects of mast cell stimulation on the expression of TJ proteins. In this model, compound 48/80-stimulated mast cells significantly increased intestinal epithelial permeability, disrupted epithelial integrity and decreased the expression of junctional proteins JAM-A, ZO-1 and CLD-1 *in vitro*. Tryptase inhibition (391) significantly reduced the effects of mast cell stimulation on epithelial integrity and on the junctional protein JAM-A.

To assess the effects of mast cell stimulation with compound 48/80 on epithelial permeability, Caco-2 cells were continuously co-cultured with HMC-1 for approximately 3 weeks. The integrity of the epithelial barrier was determined by the measurement of TER, which appeared to increase with the duration of the co-culture. However, when the HMC-1 cells were challenged with compound 48/80 to initiate degranulation, the TER significantly decreased within 2 hours and the

effect was maintained for up to 24 hours. Also, epithelial permeability to FITC-dextran appeared to be significantly higher at 24 hours following mast cell stimulation compared to unstimulated controls. These results are in line with the previous findings by Jacob *et al.* (57), who reported increased permeability of colonic epithelial cells T84 following 1) the incubation with supernatants from degranulated HMC-1 cells 2) degranulation of co-cultured (for 24 hours) HMC-1 cells. The Caco-2/HMC-1 co-culture model extends the Jacob *et al.* study (57) by allowing investigation of the interactions between both cell lines throughout the continuous 3-week co-culture. Based on this model, TEM analysis showed that the presence of intact (unstimulated) mast cells co-cultured with the epithelial cells for 3 weeks did not disturb epithelial integrity. Ultrastructural alterations, however, were found in Caco-2 monolayers when the underlying HMC-1 cells were stimulated, namely dilated junctions at 6 and 24 hours of incubation. Interestingly, in a recently published study, Martínez *et al.* (344) demonstrated by TEM that the paracellular space between adjacent enterocytes was significantly increased in the jejunal mucosa of IBS-D patients compared to controls. Also, IBS patients exhibited increased mast cell numbers and activation. These findings are consistent with the results found in this study by TEM suggesting that similar mechanisms (mast cell activation) might be involved in TJ disassembly.

The evaluation of the effects of mast cell stimulation with compound 48/80 on junctional proteins revealed significant changes in their expression. JAM-A expression was reduced at 6 hours following mast cell stimulation and these lower levels were maintained for up to 24 hours, as shown by both western blot and confocal microscopy analysis. This finding was consistent with the drop in TER and enhanced permeability to FITC-dextran. Initially, compound 48/80-stimulated mast cells (for up to 6 hours) did not affect CLD-1 expression, however, after prolonged incubation (24 hours) CLD-1 was significantly reduced and its levels were clearly lower in TJ regions. Also, ZO-1 expression appeared to

be reduced following mast cell stimulation, though the pattern of its expression differed from JAM-A and CLD-1. ZO-1 reduction was found at 1 to 4 hours but its expression was comparable to controls after prolonged incubation. In IBS, ZO-1 was shown to be significantly reduced in intestinal mucosal tissue (212, 285) and ZO-1 mRNA negatively correlated with increased tryptase mRNA levels in IBS tissue (285). The present study, however, suggests that reduced ZO-1 expression in IBS might be maintained by other non-mast related factors.

In the previous chapter, tryptase was shown to significantly decrease TER and increase permeability to FITC-dextran of Caco-2 monolayers via the alterations of TJ protein expression. In order to further confirm the role of tryptase in the regulation of barrier integrity, co-cultured mast cell were stimulated with compound 48/80 and incubated with the tryptase inhibitor, NM, for up to 24 hours. In line with previous studies (57) tryptase inhibition significantly diminished the effect of mast cell stimulation on TER and FITC-dextran flux within 24 hours after mast cell stimulation, confirming the role of mast cell tryptase in the disruption of barrier integrity. Noteworthy, irrespectively of the presence of NM, TER of Caco-2 monolayers initially dropped following mast cell stimulation and the inhibitory effect of NM was detected at 24 hours but not earlier. This suggests that, in the presence of NM, other short acting mast cell mediators induced and maintained a significant drop in TER for at least up to 6 hours. These might include pro-inflammatory cytokines such as TNF-α and IFN-γ which appeared to be implicated in the disruption of intestinal epithelial barrier (352, 411-413) through MLCK-dependent mechanisms (352). Another mediator, i.e. histamine, was shown to induce a secretory response in intestinal epithelial cells and mouse colon (111), however it did not affect paracellular permeability of intestinal epithelial cells (212).

The role of mast cell tryptase in the regulation of junctional proteins JAM-A and CLD-1 was also investigated in tryptase inhibition experiments. It appeared that NM significantly diminished the effect of mast cell stimulation on the expression of JAM-A protein within 24 hours of incubation. However, based on the Caco-2 cell model findings, it is also possible that mast cell tryptase modulate the expression of CLD-1 and ZO-1 proteins. In the co-culture model both of these proteins appeared to be lower upon mast cell stimulation, though this study did not associate these alterations with mast cell activity. ZO-1 expression was reduced initially (from 1 to 4 hours) following mast cell stimulation, and during this period no significant inhibitory effect of NM on TER and FITC-dextran flux was detected. CLD-1 expression was reduced following mast cell stimulation at 24 hours independently of tryptase inhibition suggesting that mast cell mediators other than tryptase contributed to the maintenance of its low levels. Previously, inflammatory cytokines TNF-α and IFN-γ were shown to redistribute TJ proteins including CLD-1, ZO-1 and occludin in epithelial cell lines (352). Of note, also JAM-A was shown to be down-regulated by TNF-α and IFN-γ *in vitro* (353, 414); however in this study mast cell tryptase appears to play a central role in the regulation of JAM-A expression. Of note, mast cell mediators may act synergistically to modulate epithelial barrier function. For example, TNF-α and IFN-γ were reported to synergise to induce barrier dysfunction even when used at relatively low concentrations (352), while TNF-α and histamine synergise to stimulate ion secretion in intestinal epithelium (111). A synergy between tryptase and other mast cell mediators is also possible; this however needs yet to be investigated.

Interestingly, mast cell stimulation with compound 48/80 induced a significant increase in CLD-2 expression at 24 hours following incubation. This finding might be clinically relevant since Martínez *et al.* (344) have recently showed CLD-2 up-regulation in the jejunal mucosa of IBS patients. Furthermore, Martínez *et al.*

(344) found a positive correlation between CLD-2 protein expression and tryptase protein expression, levels of which appeared to be higher compared to controls. However, since no significant change in CLD-2 expression was detected upon tryptase treatment in the Caco-2 cell model, other mast cell mediators seem to be involved in CLD-2 up-regulation. Previously, IL-6 was shown to up-regulate CLD-2 expression in intestinal epithelial cells that was paralleled by increased TJ permeability to ionic solutes (82). Therefore, the correlation between CLD-2 expression and tryptase content in IBS tissue (344) might indicate the association with mast cell activation rather than with tryptase *per se*.

The previous chapter reports an increase in occludin expression and a trend towards a decrease in expression of CLD-3 following "short-term" incubation with tryptase in the Caco-2 cell model. To extend these previous results, the expression of occludin and CLD-3 was further assessed in the co-culture model. However, it appeared that mast cell stimulation with compound 48/80 did not induce any significant change in CLD-3 or occludin as determined by western blotting. The lack of evidence of mast cell mediators significantly altering CLD-3 expression is in line with a recent study where CLD-3 expression in the jejunal mucosa of IBS was comparable to controls, suggesting that this protein might not be involved in barrier dysfunction in IBS (344). Occludin, on the other hand, appeared to be down-regulated in IBS tissue and redistributed from the membrane to the cytoplasm of enterocytes (213, 265, 344). However, the findings of the present study suggest that mast cells do not contribute to these occludin changes. They might be related to the previously observed lower occludin expression due to enhanced proteasome activity (265). Also, the regulation by other inflammatory mediators is possible (352).

Consistent with the data from the Caco-2 cell model, stimulation of mast cells with compound 48/80 did not have any effect on the expression of E-cadherin.

The importance of E-cadherin in the pathogenesis of IBS (211) suggested that there might be possible alterations in its expression in IBS. However, in this study no evidence was found to suggest that mast cell mediators modulate E-cadherin protein expression, highlighting the complexity of the junctional protein regulation in this disease.

In addition to the disruption of epithelial integrity, mast cell stimulation with compound 48/80 also induced the release of microvesicles (circular membrane fragments containing cytosol) (415) from Caco-2 monolayers, observed at 6 and 24 hours in TEMs. Though this observation is interesting, this study was not designed to investigate the nature of these structures. However, based on the previous evidence, the microvesicles might be distinguished in shedding vesicles or exosomes depending on e.g. their intracellular origin and mechanism of formation (416). Shedding vesicles are formed by direct budding from the plasma membrane, whereas exosomes are stored within the lumen of multivesicular bodies and are released from cells by exocytosis (416). The function of these vesicles is largely unexplored but they appear to play a role in a broad range of biological processes, including stimulation of the immune system or intercellular signalling mediated by their specific interaction with target cells (416, 417). Therefore, future studies investigating the nature of these microvesicles might add insights into the mechanisms underlying mast cell activation.

To induce degranulation mast cells were stimulated with synthetic compound 48/80. Compound 48/80 was first characterised as a histamine releaser from mast cells (418) while more recently it was also shown to activate directly enteric neurons and visceral afferents *in vitro* (419). The concentration of compound 48/80 may determine the degree of mast cell activation. For example, human skin mast cells responded significantly to 3-10 ug/ml of compound 48/80 as evidenced by histamine release, but not when exposed to lower concentrations (0.2-1 ug/ml)

(420). Other study reported that 1 ug/ml of compound 48/80 induced only a partial degranulation of rat peritoneal mast cells (421). Importantly, mast cells exhibit heterogeneity at distinct anatomical locations and between different species (100). In this manner, compound 48/80 appears not to activate mucosal mast cells (146). It was however reported by Jacob *et al.* (57) that compound 48/80 degranulated HMC-1 cells at the concentration of 5 μg/ml, which matched the concentration used in this study. Compound 48/80 appears to induce anaphylactic type of degranulation, which means a rapid release of mediators from the mast cell granules (114). Interestingly, mast cells undergoing massive degranulation can rebuild their granular stores within 24 hours, i.e. regranulate (110). Human skin mast cells, however, were reported to reacquire their susceptibility to reactivation with compound 48/80 in 2-3 days (420). However, in this study the status of mast cell activation or whether compound 48/80 actually degranulated HMC-1 was not validated. Therefore, in order to better understand mechanisms following mast cell stimulation with compound 48/80 in this model, in future studies mast cell degranulation should be validated with enzyme assay for tryptase (57, 112) or β-hexosaminidase (422, 423). Staining of mast cells using e.g. May–Grünwald/Giemsa could be also performed to visualise degranulation (100).

Interestingly, while stimulation of mast cells with compound 48/80 induced the disruption of epithelial integrity, Caco-2 cells co-cultured with unstimulated mast cell for 3 weeks had higher TER and lower permeability to FITC-dextran compared to Caco-2 cells alone. This suggests a protective role of intact mast cells in the regulation of intestinal barrier function. Unstimulated mast cells appeared to activate distinct mechanisms promoting tightening of TJs. Mediators released from intact mast cells responsible for the enhanced tightness of epithelial cells remain to be identified. Previous studies indicated a role for phosphorylation in the assembly of TJs that is mediated by mechanisms involving selective activity of

phospholipase C (PLC), PKC, and mitogen-activated protein kinase (MAPK) (424). Interestingly, PKC activation is associated with the apical tightening of ZO-1 protein, leading to enhancement of intestinal epithelial integrity (363). Also, PKC influences actin cytoskeleton and therefore regulates processes that involve remodelling of the microfilaments (425). Peri-junctional cytoskeleton is structurally associated with TJs and regulates epithelial permeability (4). In the present study, organised electron-dense areas in of Caco-2 co-cultured with unstimulated mast cells were observed with TEM which might indicate cytoskeletal condensation. These, however, were not focused around TJs but were seen along the apical side of Caco-2 monolayers. Therefore, future analysis might attempt to explore the mechanism underlying these observations. Overall, these data demonstrate that mast cell stimulation with compound 48/80 impairs the intestinal epithelial barrier as evidenced by a decrease in TER, increased paracellular permeability to FITC-dextran, ultrastructural features of TJ disassembly and alteration in the expression of junctional proteins JAM-A, CLD-1 and ZO-1. Furthermore, these data confirm that mast cell tryptase contributes to barrier dysfunction, since tryptase inhibition significantly reduced the disrupting effects of mast cell stimulation on TER, permeability to FITC-dextran and on the expression of JAM-A protein. These findings may offer further insights into underlying mechanisms of intestinal barrier dysfunction in IBS.

Key findings

1. Caco-2 cells continuously co-cultured with unstimulated HMC-1 cells formed tight monolayers as evidenced by the measurement of TER, FITC-dextran flux and TEM analysis.

2. Stimulation of co-cultured HMC-1 cells with compound 48/80 disrupted the barrier function of Caco-2 monolayers as determined by:
   - a significant 20% drop in TER within 2 hours of incubation that was sustained up to 24 hours;
   - a significant increase in paracellular permeability to FITC-dextran within 6 hours of incubation that was sustained up to 24 hours (1.68-fold, 24 h);
   - a significant decrease in the expression of JAM-A (6 h & 24 h), CLD-1 (24 h), ZO-1 (1 h to 4 h);
   - alterations in ultrastructure of Caco-2 monolayers as evidenced by open TJs in TEMs (6 h, 24 h).

3. Inhibition of mast cell tryptase significantly diminished the effect of mast cell stimulation on the barrier function of Caco-2 monolayers within 24 hours in terms of:
   - epithelial integrity measured by TER;
   - epithelial permeability to FITC-dextran;
   - JAM-A expression.

CHAPTER 5

THE EXPRESSION OF TJ PROTEINS JAM-A, CLD-1 AND ZO-1 IS REDUCED IN THE CAECAL MUCOSA OF IBS PATIENTS AND IS ASSOCIATED WITH IBS SYMPTOMS

5.1. Introduction

5.1.1. Rationale

An increase in intestinal permeability has been reported in IBS (148) (Section 1.2.5.1). Junctional complexes sealing epithelial cells are key determinant of intestinal permeability. Thus, increased intestinal permeability in IBS is likely to be due to altered expression of junctional proteins. Data reported in chapters 3 and 4 suggested the role of altered expression of TJ proteins JAM-A, CLD-1 and ZO-1 in IBS. Further investigation of these TJ proteins in clinical tissue samples of IBS patients would confirm their importance in IBS pathogenesis and might suggest mechanisms of intestinal permeability and barrier dysfunction in this condition.

The expression of junctional proteins in IBS has not been widely studied (Section 1.2.5.2). Recent data have demonstrated that ZO-1 and CLD-1 levels were lower in the colonic mucosa of IBS-D patients compared to controls (213, 346) whereas other studies reported lower ZO-1 expression in the jejunum (285) but CLD-1 expression was comparable to controls (344). JAM-A expression has not been reported in IBS to date, to my knowledge, whereas its expression in IBD, a disease also associated with increased barrier permeability, appeared to be lower in the colonic mucosa of active IBD (53). This chapter investigated if the expression of TJ proteins is lower in IBS tissue relative to controls, and the study for JAM-A in this disease was novel. While immune activation has been shown in the caecum of IBS (Section 1.2.4.4), the expression of TJ proteins has not yet been investigated at the caecal site and the study of TJ proteins in caecal IBS tissue was novel. The clinical relevance of TJ protein expression in IBS in terms of their relationship with IBS symptoms and severity was also determined in the present study. Altered

expression of junctional proteins has been reported to negatively associate with the severity of abdominal pain (213) and bowel movements (344) in IBS patients. Thus investigation of junctional protein expression in IBS and implications for symptoms might shed light on the aetiology of increased intestinal permeability and contribute to a better understanding of related IBS symptoms. In addition, given that the expression of JAM-A, CLD-1 and ZO-1 was reduced in response to mast cell mediators in the cell models, this chapter explored if there was an association between the expression of TJ proteins in IBS tissue and mast cell numbers in the database.

5.1.2. Aims of the study

1. To investigate if the expression of JAM-A, CLD-1 and ZO-1 in caecal biopsy tissue of IBS patients is reduced when compared with tissue from controls.

2. To investigate if there is an association between TJ protein expression and IBS symptoms such as abdominal pain and diarrhoea, and duration of symptoms.

5.1.3. Methods to achieve the aims

Methods are described in detail in chapter 2. In brief, for this study TJ proteins JAM-A, CLD-1 and ZO-1 were immunostained in formalin fixed, paraffin embedded biopsy tissue from the caecum of Rome II IBS patients (n=34) and normal controls (n=12). Severity of abdominal pain and diarrhoea were assessed in IBS using a pain rating of 0-4 (0-no pain, 1-mild, 2-relevant, 3-severe, 4-extremely severe) (396) and number of liquid stools per week (397), respectively.

In addition, JAM-A expression was analysed in caecal biopsy tissue from inflammatory controls with confirmed IBD (n=8). The expression of CLD-1 and

ZO-1 was not analysed in IBD due to limited quantity of tissue. The analysis of JAM-A expression in IBD was of priority as it enabled comparison of IBS cohort with IBD in the absence of published data on IBS. Furthermore, JAM-A cell culture findings were novel and interesting, and pursue of JAM-A expression in translational studies was particularly relevant.

Expression of junctional proteins was analysed with immunofluorescence confocal microscopy. This method was chosen since immunofluorescence is highly sensitive and provides high resolution of the images. Good quality images can be taken due to higher-resolution microscopy performed with confocal immunofluorescence microscope. Secondary, confocal microscopy allows performing Z-stack scanning of specimens to determine cellular localisation of stained proteins. Tertiary, this method was used in cell culture studies and with this experience the analysis of junctional proteins in mucosal biopsy tissues was expected to be facilitated.

Immunofluorescence staining was assessed semiquantatively using a 4-grade scale (1-no specific staining, 2-mild specific staining, 3-moderate specific staining, 4-strong specific staining). Differences in the grade of TJ proteins in the surface epithelium were the primary outcome measure, while these in the crypt epithelium were the secondary one. Specimens were processed in several batches of experiment and biopsy tissues from control patients were used as internal sample controls to detect possible heterogeneity in protein expression related to differences in staining procedure.

5.2. Results

5.2.1. Study population

Demographic and clinical characteristics of the study group (n=54) are displayed in Table 5.1. The study comprised 34 Rome II Criteria IBS patients, the majority (n=22) were classified as IBS-D (65%) and 12 as IBS-A (35%). In the IBD group, 3 patients had clinically confirmed UC (37%) while 5 had CD (63%), whereas according to disease activity status 3 patients (37%; n=2, CD; n=1, UC) were identified with active IBD and had histological evidence of inflammation in the caecum, while 5 (63%; n=3, CD; n=2, UC) with inactive IBD and had no evidence of inflammation in the caecum. The IBS group had a female predominance and was younger in age compared to controls. IBD patients and controls were gender matched but IBD patients were younger in age. Therefore, potential age and gender effects on TJ protein expression were addressed in further analysis using a general linear model, which confirmed that neither gender nor age significantly influenced TJ protein expression in the disease group (controls, IBS, IBD) (Supplementary Table 5.1). In line with this, analysis of the control group, similarly to previously published (213), confirmed no gender or age influences on TJ protein expression in controls (Supplementary Tables 5.2, 5.3).

Disease group	IBS (n=34)	Controls (n=12)	IBD, inflammatory controls, (n=8)
Gender			
Male	18% (n=6)	58% (n=7)	62% (n=5)
Female	82% (n=28)	42% (n=5)	38% (n=3)
	P*=0.007, P**=0.009		P*=0.85
Age (years)			
Mean±SEM (range)	32.1±1.4 (21-54)	43.1±4.7 (18-64)	29.0±2.4 (22-42)
	P*=0.004, P**=0.32		P*=0.033
Abdominal pain severity (1-4 scale)			
Median score (range)	2 (1-4)	NA	NA
Mild (1)	27% (n=9)		
Relevant (2)	52% (n=17)		
Severe (3)	12% (n=4)		
Extremely severe (4)	9% (n=3)		
Diarrhoea severity:			
No. of liquid stools/week	14.9±3.7 (0.2-52.5) (20.7±4.7 IBS-D; 1.8±1.1, IBS-A; P=0.001)	NA	NA
Duration of IBS symptoms (years)			
Mean±SEM (range)	3.2±0.7 (0.3-20)		NA
	<1 year-32% (n=11)	NA	
	1-5 year-53% (n=18)	NA	
	>5 year-15% (n=5)	NA	
	(2.1±0.5, IBS-D; 5.3±1.7, IBS-A; P=0.10)		

Table 5.1 Characteristics of IBS patients and controls. Intergroup comparisons were performed by using Pearson Chi-square test (gender) or Student's t-test (age). P*, compared with controls. P**, compared with IBD. NA, not applicable.

5.2.2. JAM-A, CLD-1 and ZO-1 expression is reduced in the caecal mucosa of IBS patients

JAM-A expression: In controls JAM-A expression was typically abundant at the apex but also along the lateral membrane of the surface epithelium of the caecal mucosa. In IBS patients, however, overall JAM-A expression was significantly lower compared to controls (median: IBS grade 3 v control grade 4, P=0.012) (Figures 5.1, 5.4A; Table 5.2). Noteworthy, strong JAM-A expression (grade 4) was found in 27% (n=9) of IBS patients compared to 67% (n=8) of controls; however, in 18% (n=6) of IBS patients JAM-A staining was clearly abolished (grade 1) compared to 8% (n=1) of controls. The reduction in JAM-A was consistent in both IBS-D (median: grade 2.5, P=0.016) and IBS-A (median: grade 3, P=0.041) compared to controls (Figures 5.1, 5.4B; Table 5.2).

Since there were no published data on JAM-A expression in IBS, to place these changes in perspective JAM-A was also investigated in the caecal mucosa of inflammatory controls (IBD) and the findings were compared with recently published data (53). In agreement with previous studies, JAM-A expression was significantly lower compared to controls (median: IBD grade 2 v controls grade 4, P=0.01) (Figures 5.1, 5.9A-C; Table 5.3). However, no difference was found when compared to IBS patients. Interestingly, looking at the disease activity status, the significant reduction in JAM-A expression was shown for active IBD (median: grade 1, P=0.021) whereas in inactive IBD there was a trend towards reduction (median grade of 3, P=0.063) compared to controls. Analysis according to inflammatory disease type demonstrated that lower JAM-A levels were found only in patients with CD (median grade of 1, P=0.008) but not in those with UC.

CLD-1 expression: In the caecal mucosa of controls CLD-1 was typically abundantly expressed in the surface epithelium. CLD-1 did not localise

specifically to the apical region but was expressed basolaterally (Figure 5.2). Altered CLD-1 expression was observed in IBS patients where overall CLD-1 staining appeared to be more diffused and less intense compared to controls. CLD-1 expression appeared to be reduced in IBS patients compared to controls, however, the difference did not reach statistical significance (median: IBS grade 3 v control grade 3.5, P=0.074) (Figures 5.2, 5.4C; Table 5.2). The lowest specific staining that was observed was mild (grade 2) and this was detected at comparable levels in both IBS patients (32%, n=11) and controls (25%, n=3). On the other hand, the strong CLD-1 staining (grade 4) was noted only in 12% (n=4) of IBS patients compared to 50% (n=6) of controls. Looking at the IBS bowel subtype, CLD-1 expression showed a tendency towards a reduction in IBS-D patients compared to controls (median grade of 3, P=0.08) but not in IBS-A patients (Figures 5.2, 5.4D; Table 5.2).

ZO-1 expression: In controls, ZO-1 was focally expressed at the apical region of caecal epithelial cells. However, it was significantly reduced in the caecal surface epithelium of IBS patients compared to controls (P=0.007), although the median grade of expression in both groups was the same (median grade of 2) (Figures 5.3, 4E; Table 5.2). The lack of ZO-1 expression (grade 1) was found in 47% (n=16) of IBS patients compared to 8% (n=1) of controls. Significantly reduced ZO-1 expression was a feature of both subtypes and appeared to be lower both in IBS-D (median grade of 2, P=0.015) and IBS-A (median grade of 2, P=0.022) patients compared to controls (Figures 5.3, 5.4F, Table 5.2).

TJ protein expression by secondary outcome (crypts): The expression of TJ proteins in IBS and controls was further analysed by the secondary endpoint, namely expression in the crypt epithelium. In caecal tissue of controls both JAM-A (Figure 5.5) and ZO-1 (Figure 5.7) proteins were concentrated at the apical tip of lateral membrane while CLD-1 was particularly expressed at the basolateral

side (Figure 5.6). In the crypt epithelium the key finding was a reduction in JAM-A expression in IBS patients compared to controls (median: IBS grade 2 v control grade 3, P=0.013) (Figures 5.5, 5.8A; Table 5.2) and this finding matched that of the surface epithelium, whereas levels of both CLD-1 and ZO-1 were comparable to controls (Figures 5.6-5.8; Table 5.2). Similarly to the surface epithelium JAM-A expression appeared to be reduced in both IBS subtypes IBS-D (median grade of 2, P=0.004) and IBS-A (median grade of 2.5, P=0.027) (Figure 5.2C) compared to controls (Figures 5.5, 5.8B). JAM-A appeared to be also reduced in IBD patients compared to controls (median: IBD grade 2 v control grade 4, P=0.016) (Figures 5.1, 5.9D-F; Table 5.3) whereas not different to IBS patients. Based on activity status, JAM-A levels were lower both in active and inactive IBD, however, the significant cut off was reached only in the latter subgroup (median grade of 2, P=0.018). Based on inflammatory disease type, comparable to the surface epithelium, a significant decrease in JAM-A was observed only in patients with CD (median grade of 2, P=0.018) but not in those with UC.

To sum up, this study showed for the first time JAM-A expression in IBS patients, and that it was significantly lower in the surface epithelium of the caecal mucosa compared to controls. The consistent reduction in JAM-A was found both in IBS-D and IBS-A subtypes. JAM-A expression was also clearly reduced in IBD patients compared to controls, but not different to IBS patients. Furthermore, this study demonstrates significantly reduced ZO-1 expression and a trend towards decreased expression of CLD-1 in the surface epithelium of the caecal mucosa of IBS patients. Findings using the secondary endpoint, i.e. crypts, corresponded for JAM-A expression in the surface epithelium, whereas CLD-1 and ZO-1 levels in crypts were comparable to controls. In line with the surface epithelium data, JAM-A was significantly reduced in crypts in IBD patients compared to controls but no difference was found when compared to IBS patients. The summary of TJ protein expression is displayed in Tables 5.7 and 5.8.

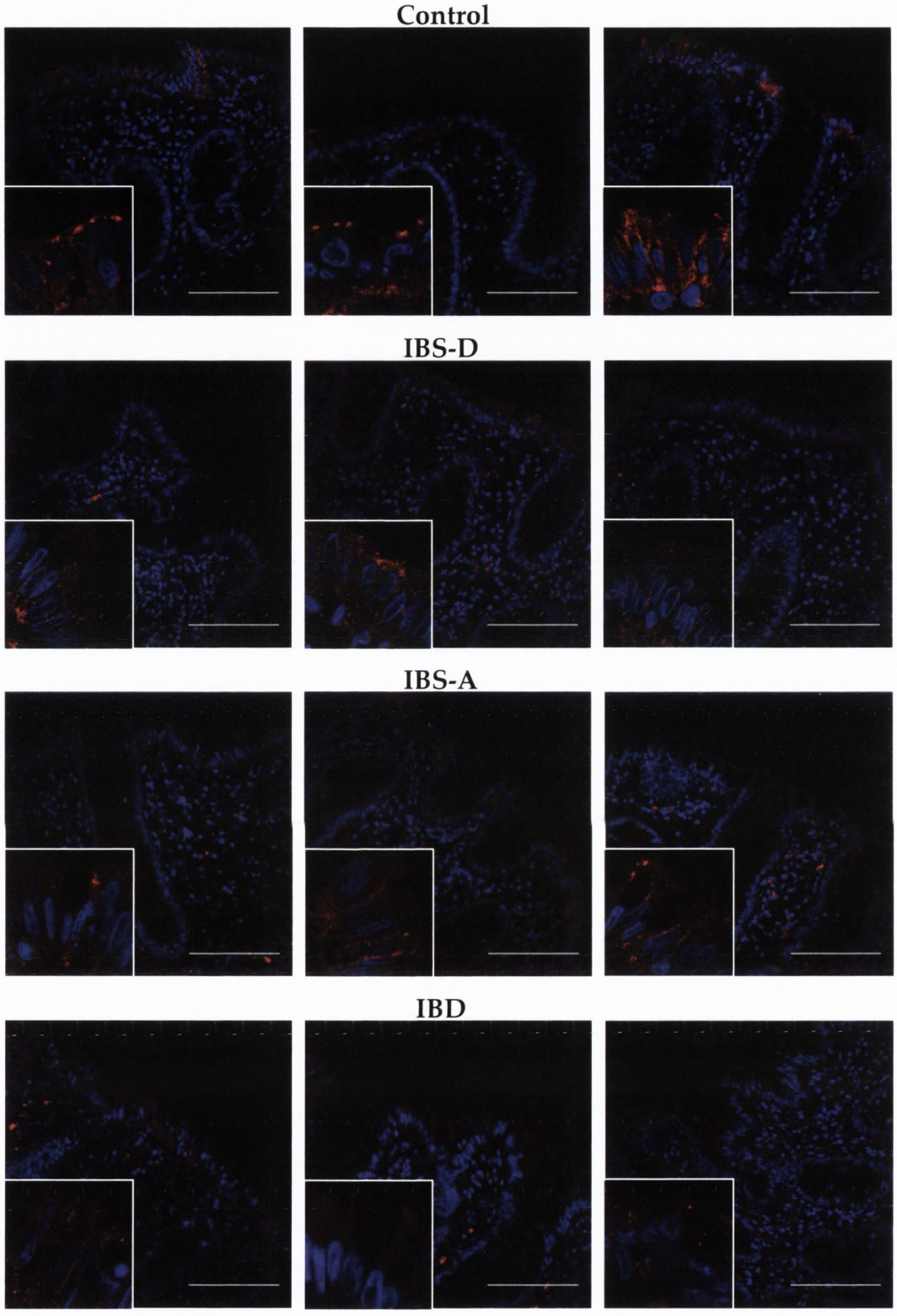


Figure 5.1 Representative photomicrographs showing JAM-A expression in the surface epithelium of the caecal mucosa of controls, IBS subtypes according to bowel predominance: IBS-D and IBS-A, and IBD patients. Immunofluorescence staining for JAM-A is shown in red, while nuclear staining is blue. Bars=50 μm.

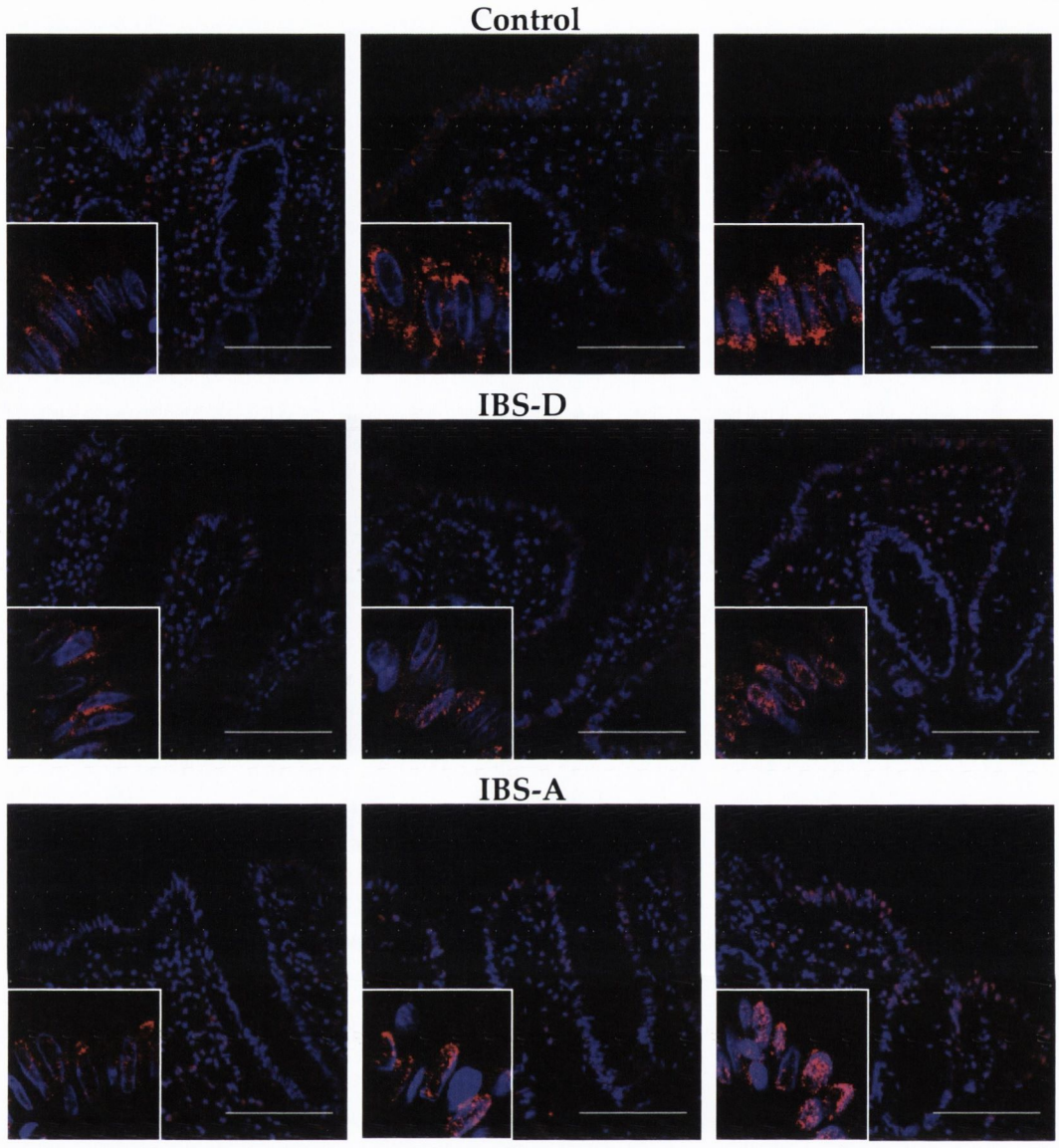


Figure 5.2 Representative photomicrographs showing CLD-1 expression in the surface epithelium of the caecal mucosa of controls and IBS subtypes according to bowel predominance: IBS-D and IBS-A. Immunofluorescence staining for CLD-1 is shown in red, while nuclear staining is blue. Bars=50 μm.

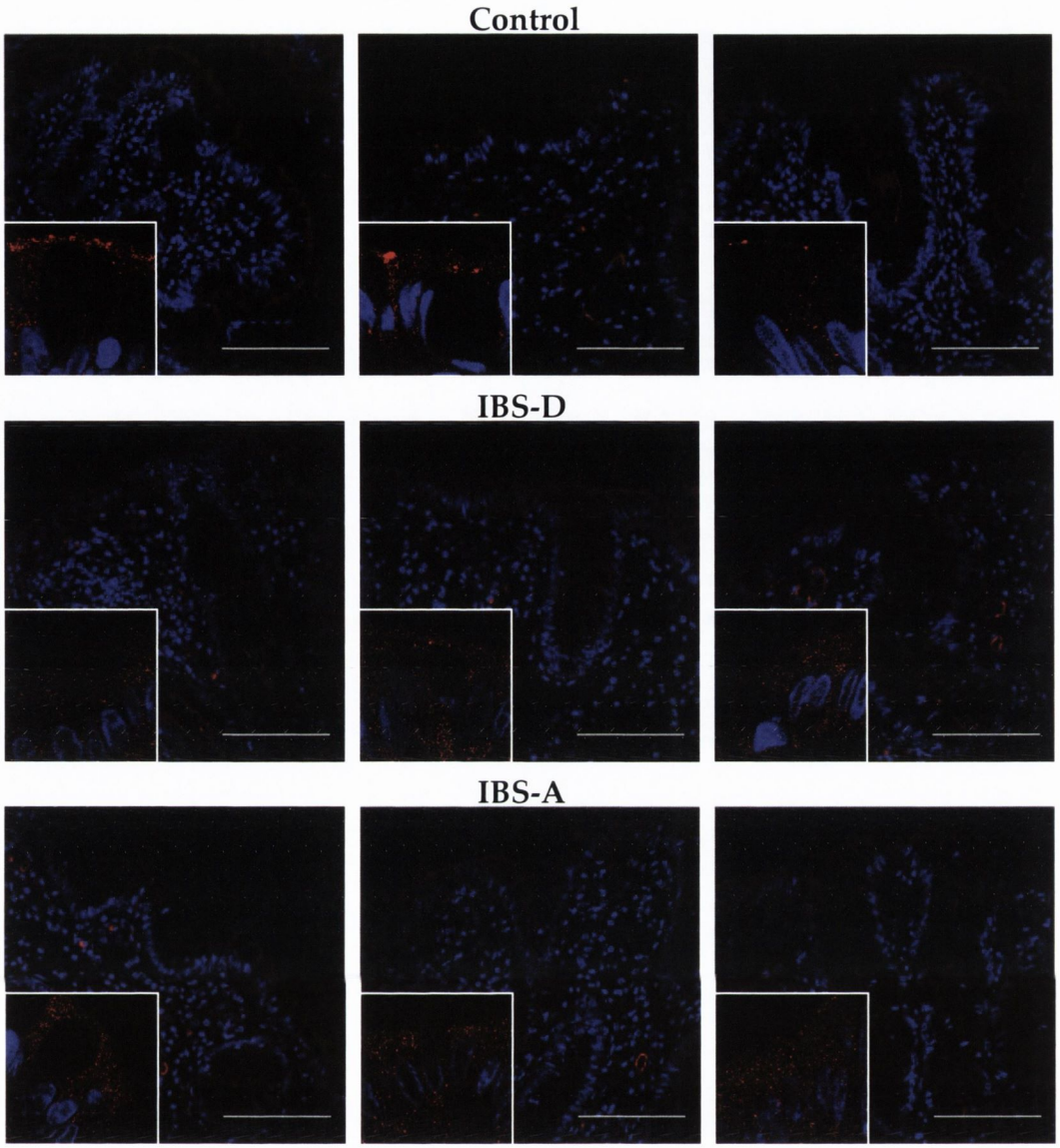


Figure 5.3 Representative photomicrographs showing ZO-1 expression in the surface epithelium of the caecal mucosa of controls and IBS subtypes according to bowel predominance: IBS-D and IBS-A. Immunofluorescence staining for ZO-1 is shown in red, while nuclear staining is blue. Bars=50 μm.

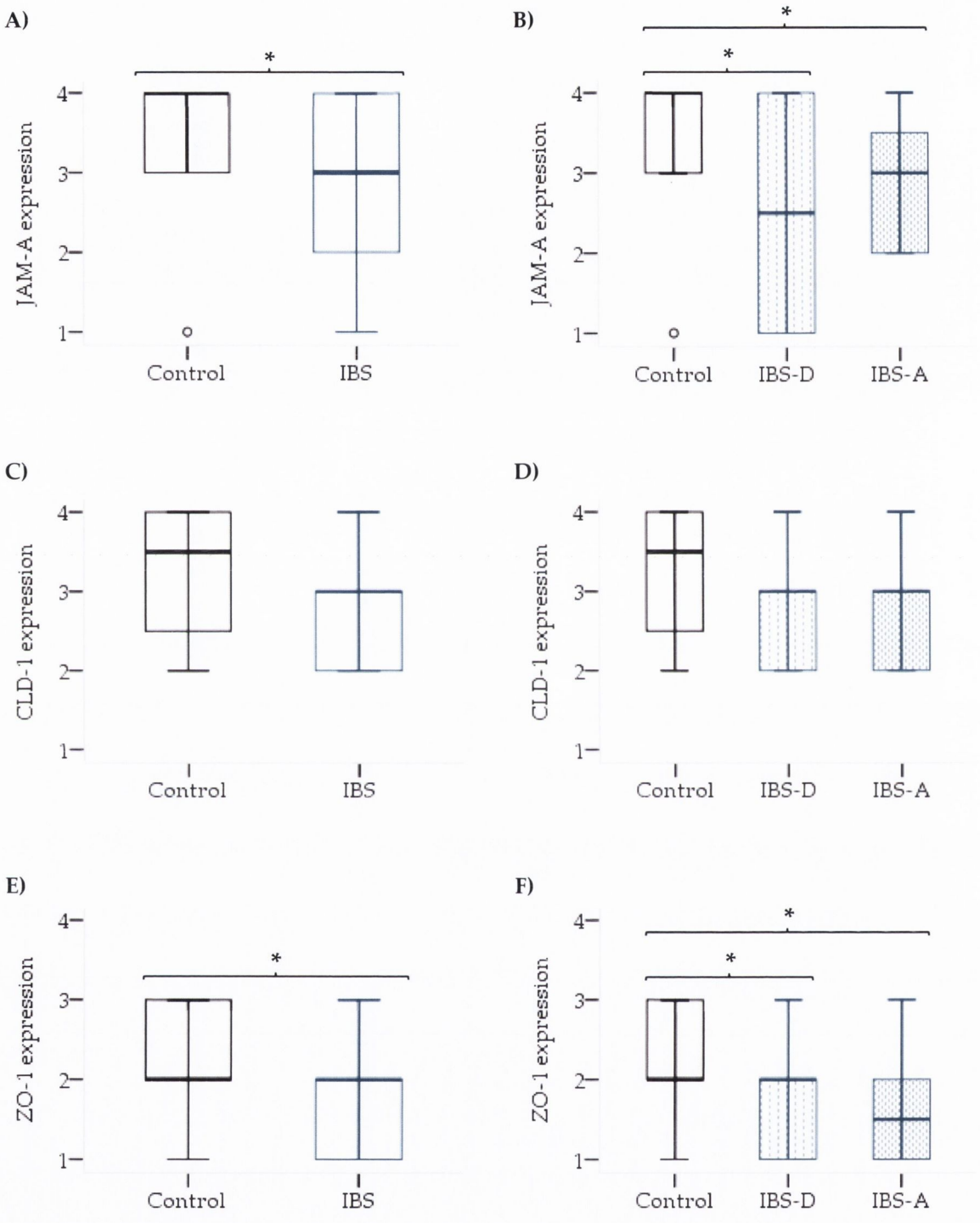


Figure 5.4 Expression of TJ proteins in the surface epithelium of the caecal mucosa of **A,C,E)** controls and IBS patients **B,D,F)** controls and IBS patients according to subtype: IBS-D and IBS-A. Values are represented as a median with interquartile ranges. *P<0.05 Mann-Whitney U test. Kruskal-Wallis P values (not marked in the graphs): **B**, P=0.034; **D**, P=0.20; **F**, P=0.026.

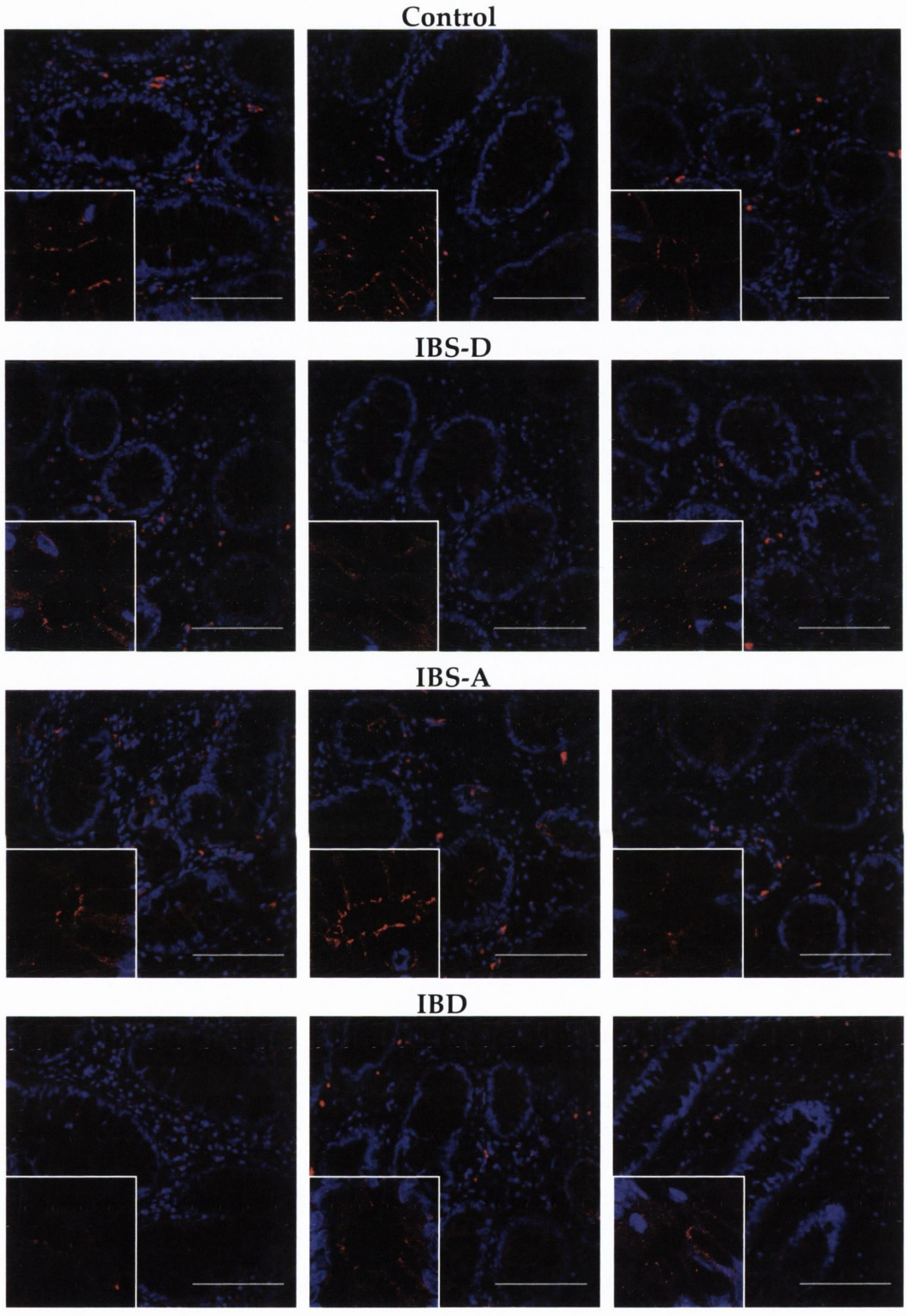


Figure 5.5 Representative photomicrographs showing JAM-A expression in the crypt epithelium of caecal mucosa of controls, IBS subtypes according to bowel predominance: IBS-D and IBS-A, and IBD patients (inflammatory controls). Immunofluorescence staining for JAM-A is shown in red, while nuclear staining is blue. Bars=50 μm.

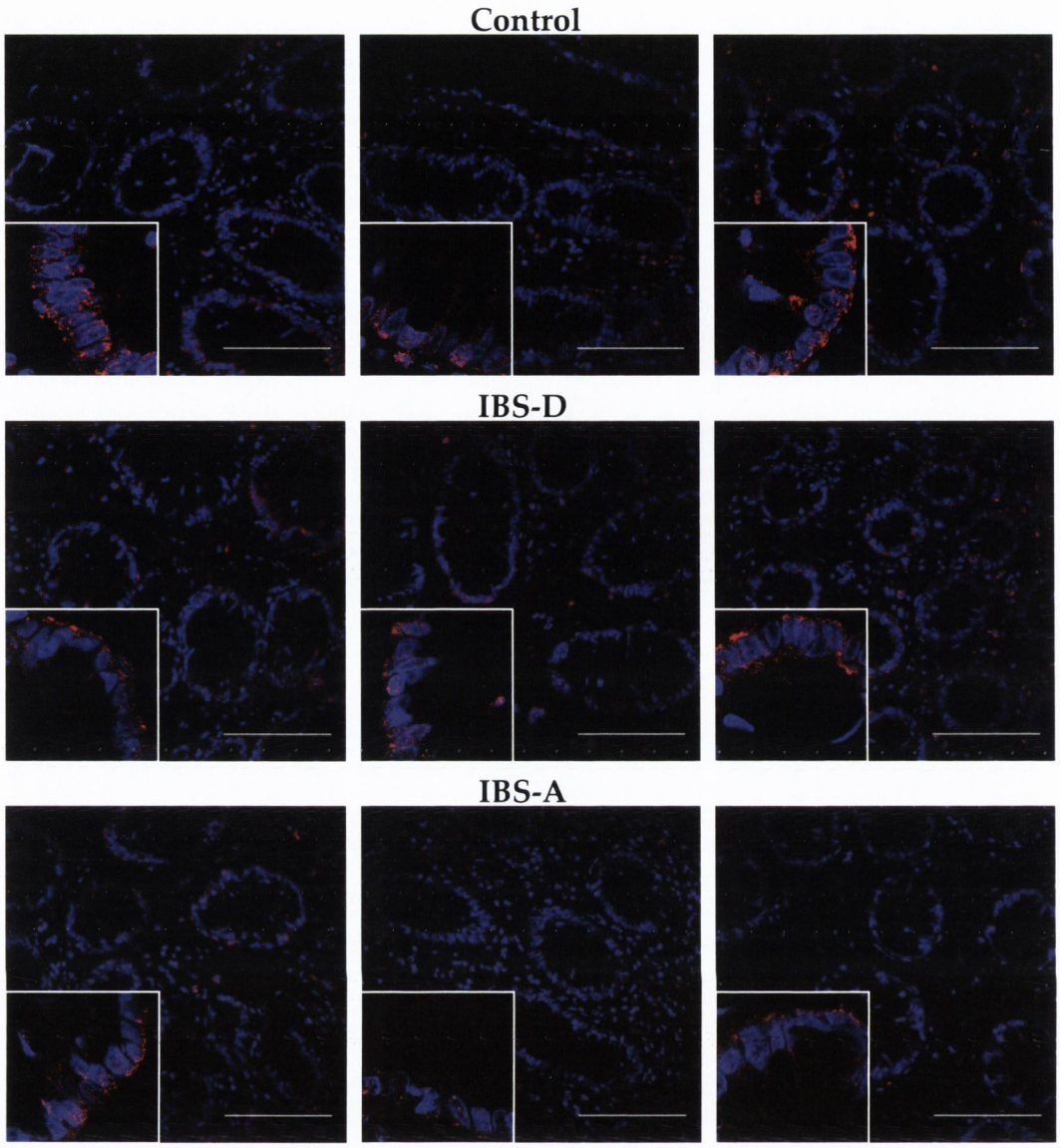


Figure 5.6 Representative photomicrograph showing CLD-1 expression in the crypt epithelium of caecal mucosa of controls and IBS subtypes according to bowel predominance: IBS-D and IBS-A. Immunofluorescence staining for CLD-1 is shown in red, while nuclear staining is blue. Bars=50 μm.

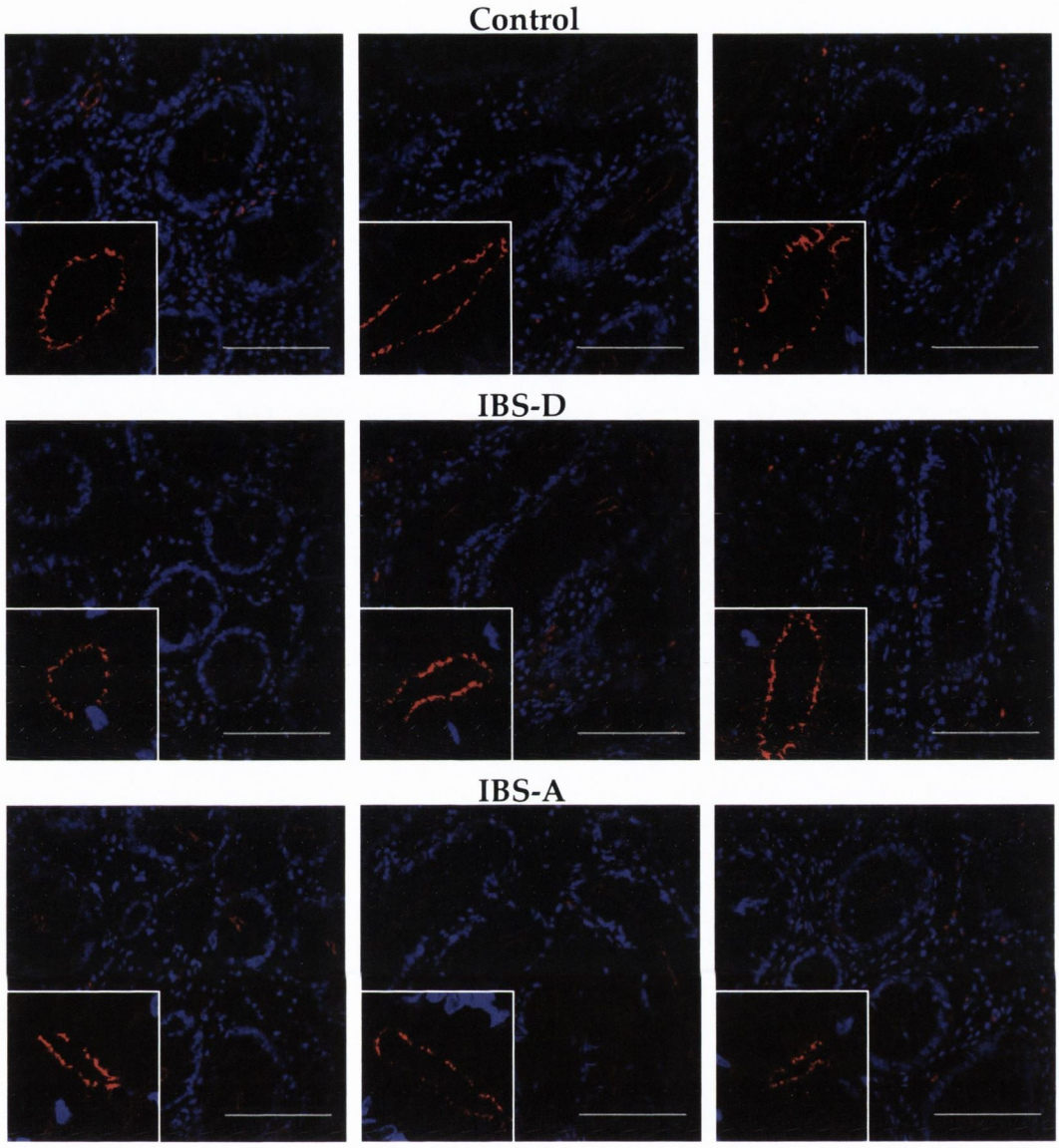


Figure 5.7 Representative photomicrographs showing ZO-1 expression in the crypt epithelium of caecal mucosa of controls and IBS subtypes according to bowel predominance: IBS-D and IBS-A. Immunofluorescence staining for ZO-1 is shown in red, while nuclear staining is blue. Bars=50 μm.

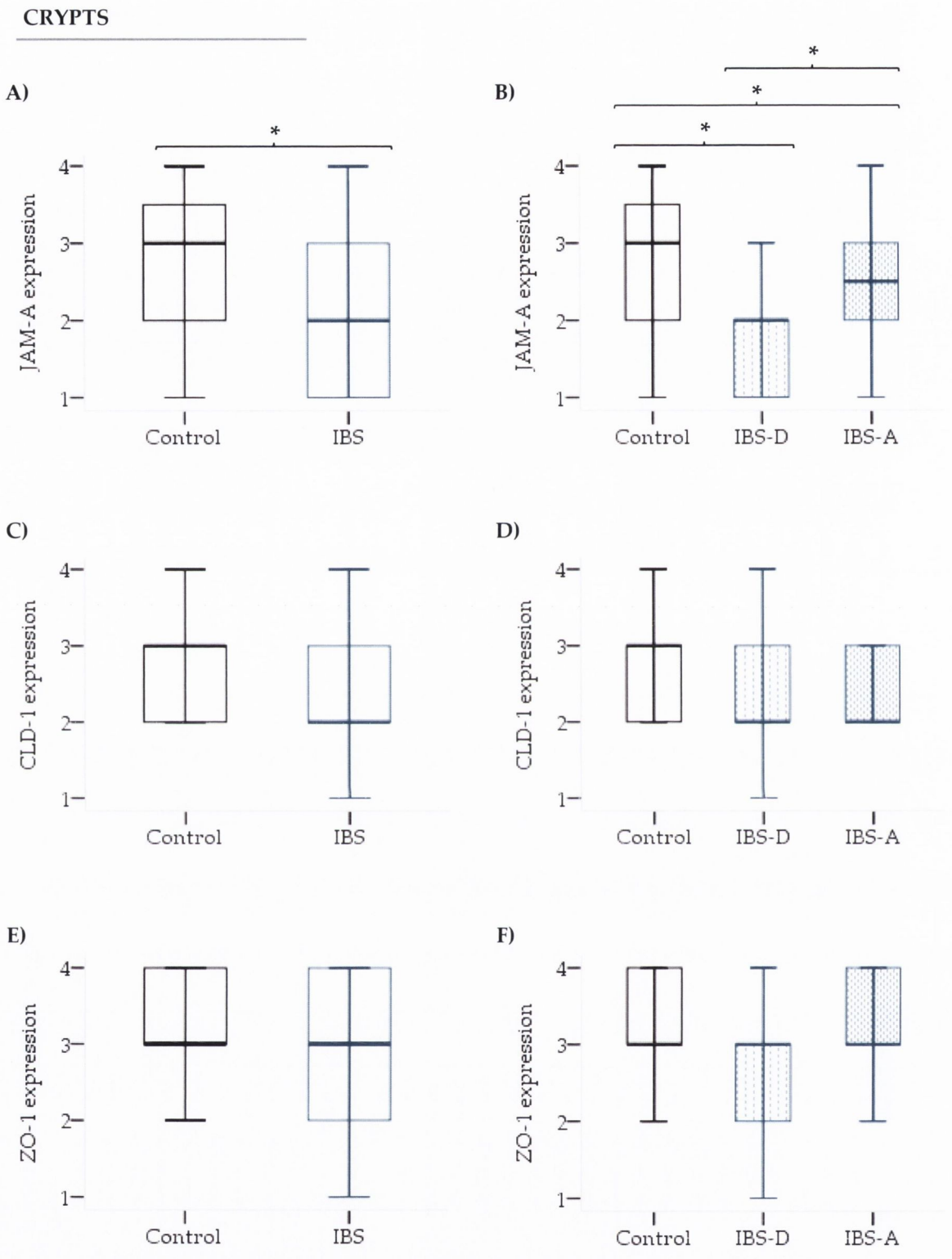
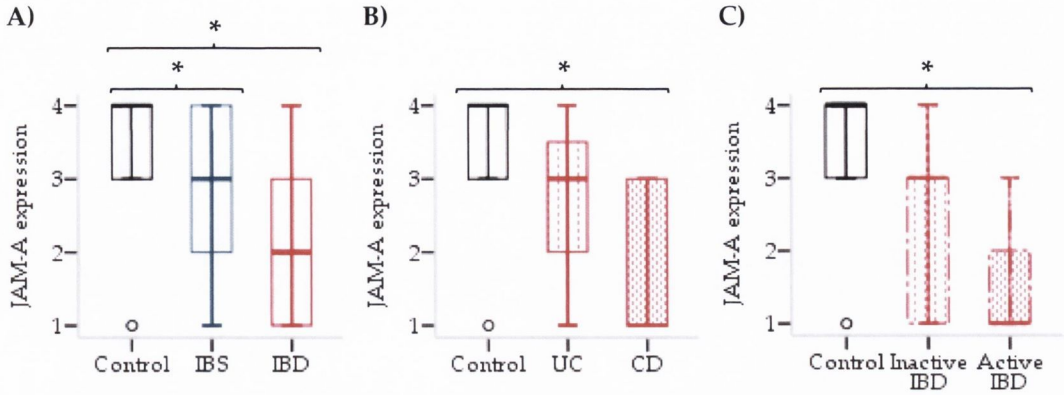


Figure 5.8 Expression of TJ proteins in crypts of the caecal mucosa of **A,C,E)** controls and IBS patients **B,D,F)** controls and IBS patients according to subtype: IBS-D and IBS-A. Values are represented as a median with interquartile ranges. *P≤0.05 Mann-Whitney U test. Kruskal-Wallis P values (not marked in the graphs): **B**, P=0.008; **D**, P=0.20; **E**, P=0.19.

SURFACE EPITHELIUM



CRYPTS

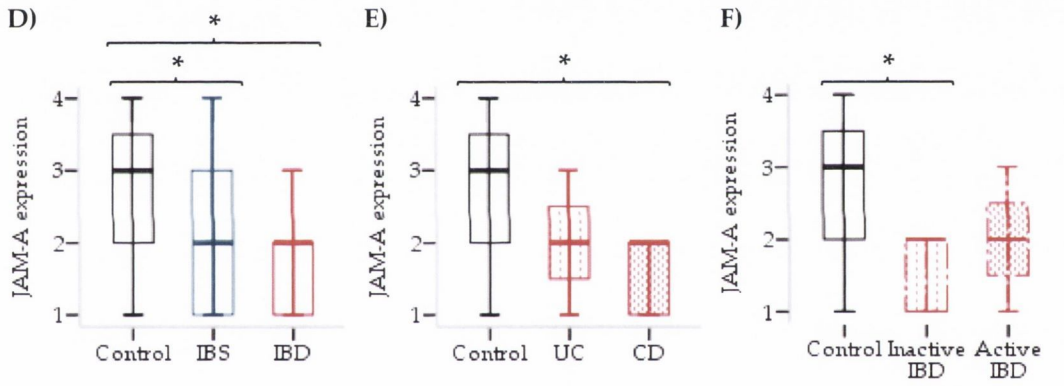


Figure 5.9 Expression of JAM-A in **A, B, C)** the surface epithelium and **D, E, F)** crypts of the caecal mucosa of controls, IBS and IBD patients. IBD patients are also shown according to disease activity status and inflammatory disease type (UC, CD). Values are represented as a median with interquartile ranges. *P<0.05 Mann-Whitney U test. Kruskal-Wallis P values (not marked in the graphs): **A**, P=0.014; **B**, P=0.025; **C**, P= 0.031; **D**, P=0.019; **E**, P=0.045; **F**, P= 0.045.

A)

Surface epithelium			
TJ protein	**JAM-A**	**CLD-1**	**ZO-1**
Group	Median (range), mean±SEM		
Control	4.0 (1-4), 3.5±0.3	3.5 (2-4), 3.2±0.2	2.0 (1-3), 2.2±0.2
IBS	3.0 (1-4), 2.6±0.2	3.0 (2-4), 2.8±0.1	2.0 (1-3), 1.6±0.1
v control	**P=0.012**	P=0.074	**P=0.007**
IBS-D	2.5 (1-4), 2.5±0.2	3.0 (2-4), 2.8±0.1	2.0 (1-3), 1.6±0.1
v control	**P=0.016**	P=0.08	**P=0.015**
IBS-A	3.0 (2-4), 2.8±0.2	3.0 (2-4), 2.8±0.2	1.5 (1-3), 1.6±0.2
v control	**P=0.041**	P=0.2	**P=0.022**
v IBS-D	P=0.42	P=0.84	P=0.81

B)

Crypts			
TJ protein	**JAM-A**	**CLD-1**	**ZO-1**
Group	Median (range), mean±SEM		
Control	3.0 (1-4), 2.8±0.3	3.0 (2-4), 2.7±0.2	3.0 (2-4), 3.2±0.2
IBS	2.0 (1-4), 2.0±0.1	3.0 (1-4), 2.3±0.1	3.0 (1-4), 2.8±0.1
v control	**P=0.013**	P=0.092	P=0.63
IBS-D	2.0 (1-3), 1.8±0.2	2.0 (1-4), 2.3±0.2	3.0 (1-4), 2.7±0.2
v control	**P=0.004**	P=0.11	P=0.14
IBS-A	2.5 (1-4), 2.3±0.3	2.0 (2-3), 2.3±0.1	3.0 (2-4), 3.2±0.2
v control	**P=0.027**	P=0.18	P=1.00
v IBS-D	**P=0.05**	P=0.72	P=0.14

Table 5.2 Expression of TJ proteins in **A)** the surface epithelium **B)** crypts of the caecal mucosa of controls (n=12) and IBS patients (n=34). IBS patients are also shown according to subtypes: IBS-D (n-22) and IBS-A (n=12). The expression of TJ proteins was assessed semiquantitatively using a 4-grade scale. Intergroup comparisons were performed by using Mann-Whitney U test.

Group	JAM-A in SE	JAM-A in crypts
	Median (range), mean±SEM	
Control	4.0 (1-4), 3.5±0.3	3.0 (1-4), 2.8±0.3
IBD	2.0 (1-4), 2.1±0.4	2.0 (1-3), 1.7±0.2
v control	**P=0.012**	**P=0.016**
v IBS	P=0.27	P=0.5
Active IBD	1.0 (1-3), 1.7±0.7	2.0 (1-3), 2±0.6
v control	**P=0.021**	P=0.2
v IBS	P=0.16	P=0.98
Inactive IBD	3.0 (1-4), 2.4±0.6	2.0 (1-2), 1.6±0.2
v control	**P=0.063**	**P=0.018**
v IBS	P=0.71	P=0.36
v active	P=0.41	P=0.51
UC	3.0 (1-4), 2.7±0.9	2.0 (1-3), 2±0.6
v control	P=0.25	P=0.2
v IBS	P=0.91	P=0.98
CD	1.0 (1-3), 1.8±0.5	2.0 (1-2), 1.6±0.2
v control	**P=0.008**	**P=0.018**
v IBS	P=0.13	P=0.36
v UC	P=0.33	P=0.51

Table 5.3 JAM-A expression in the mucosa of controls (n=12), IBS (n=34) and IBD (n=8) patients. IBD patients are also shown according to disease activity status (active, inactive) and inflammatory disease type (UC n=3; CD n=5). JAM-A expression was assessed semiquantitatively using a 4-grade scale. Intergroup comparisons were performed by using Mann-Whitney U test. SE, surface epithelium.

5.2.3. JAM-A expression is significantly associated with IBS symptoms

Abdominal pain: JAM-A expression in surface epithelial cells of the caecal mucosa significantly negatively correlated with abdominal pain severity in the IBS-A subgroup (r_s=-0.69, P=0.018) (Tables 5.4, 5.7). The lowest JAM-A expression was observed in patients reporting severe abdominal pain (pain severity score 3; JAM-A expression median of 2) and the highest in those reporting mild abdominal pain (pain severity score 1; JAM-A expression median of 4) (r_J=-0.64, P=0.025, J-T test) (Table 5.5, Figure 5.10). This finding was specific to IBS-A and no significant associations were found between abdominal pain and JAM-A expression in the IBS group overall or in the IBS-D subtype (Table 5.4, 5.5). While it was evident that JAM-A expression in the surface epithelium in IBS-A was linked to abdominal pain severity, pain severity was not associated with either CLD-1 or ZO-1 expression (Tables 5.4, 5.5)

Diarrhoea: Looking at diarrhoea as quantified by the reported number of liquid stools per week, no association was found for JAM-A, CLD-1 or ZO-1 expressed in the surface epithelium of the caecal mucosa of IBS patients overall or in subtypes (Tables 5.4, 5.5, 5.7).

Duration of symptoms: A strong relationship between duration of symptoms (years since symptom onset) and JAM-A protein was shown in IBS-A patients. There was a significant negative correlation between JAM-A expression in the surface epithelium of the caecal mucosa and duration of IBS symptoms (r_s=-0.7, P=0.012) (Tables 5.4, 5.7), with lower expression being associated with longer duration of symptoms. The highest expression of JAM-A was noted in IBS-A patients with relatively short duration of IBS symptoms (up to one year) (r_J=-0.60, P=0.017, J-T test) (Table 5.5, Figure 5.11). In line with the abdominal pain findings,

the association with duration of symptoms and JAM-A expression was specific to IBS-A, and an association with grade of JAM-A expression was not found in the IBS-D subtype (Tables 5.4, 5.5). Duration of IBS symptoms did not associate with the expression of CLD-1 or ZO-1 in the surface epithelium of the caecal mucosa of IBS patient (Tables 5.4, 5.5, 5.7).

IBS symptoms by TJ protein expression in crypts: There was no significant association between JAM-A, CLD-1 and ZO-1 in the crypt epithelium of caecal mucosa of IBS patients and abdominal pain severity, diarrhoea and duration of IBS symptoms (Table 5.8, Supplementary Tables 5.4, 5.5).

In summary, lower JAM-A expression in the surface epithelium was significantly associated both with more severe abdominal pain and longstanding symptoms in patients with the alternating subtype of IBS. No association with IBS symptoms was found either for CLD-1 or ZO-1.

TJ protein	Group	Abdominal pain severity (scale1-4)		Diarrhoea (No. of liquid stools/week)		Duration of symptoms (years)	
		r_s value	P value	r_s value	P value	r_s value	P value
JAM-A	IBS	0.09	0.63	-0.19	0.32	0.09	0.59
	IBS-D	0.35	0.11	-0.28	0.24	0.19	0.38
	IBS-A	**-0.69**	**0.018**	0.45	0.22	**-0.7**	**0.012**
CLD-1	IBS	0.23	0.21	0.18	0.34	-0.15	0.39
	IBS-D	0.35	0.11	0.18	0.44	-0.19	0.40
	IBS-A	-0.03	0.92	0.26	0.50	-0.19	0.30
ZO-1	IBS	0.25	0.15	-0.22	0.91	0.20	0.25
	IBS-D	0.30	0.16	-0.10	0.66	0.27	0.22
	IBS-A	0.13	0.71	-0.15	0.69	0.01	0.97

Table 5.4 Correlation between TJ protein expression in the surface epithelium of the caecal mucosa of IBS overall and IBS according to subtypes: IBS-D and IBS-A and IBS symptoms. Data were correlated with Spearman correlation.

TJ protein	Group	Abdominal pain severity (scale1-4)		Diarrhoea (No. of liquid stools/week)		Duration of symptoms (years)	
		r_J value	P value	r_J value	P value	r_J value	P value
JAM-A	IBS	0.08	0.61	-0.14	0.34	-0.07	0.61
	IBS-D	0.32	0.086	-0.21	0.24	0.16	0.36
	IBS-A	**-0.64**	**0.025**	0.42	0.19	**-0.60**	**0.017**
CLD-1	IBS	0.19	0.24	0.16	0.31	-0.12	0.40
	IBS-D	0.31	0.12	0.15	0.42	-0.16	0.39
	IBS-A	-0.03	0.92	0.24	0.44	-0.25	0.32
ZO-1	IBS	0.22	0.16	-0.02	0.90	0.18	0.21
	IBS-D	0.27	0.17	-0.09	0.64	0.25	0.17
	IBS-A	0.11	0.695	-0.13	0.68	0.04	0.88

Table 5.5 Association of TJ protein expression in the surface epithelium of the caecal mucosa of IBS overall and IBS according to subtypes: IBS-D and IBS-A and IBS symptoms. Data were analysed with J-T test.

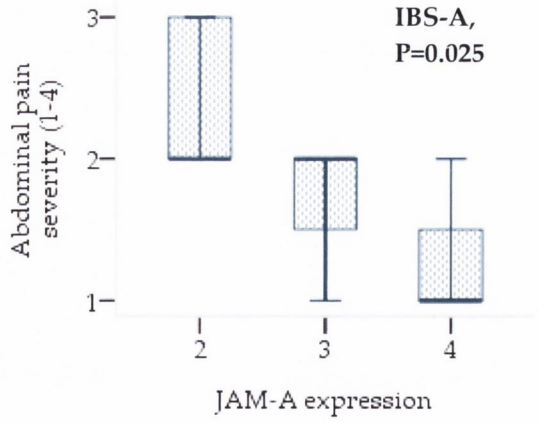


Figure 5.10 Abdominal pain severity as a function of JAM-A expression in surface epithelial cells of the caecal mucosa of IBS-A patients. Lines represent individual data and medians values. Data were analysed with J-T test.

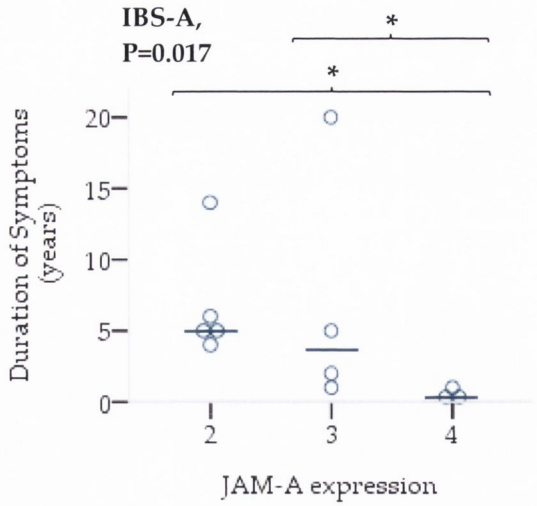


Figure 5.11 Duration of symptoms as a function of JAM-A expression in surface epithelial cells of the caecal mucosa of IBS-A patients. Lines represent individual data and medians values. Data were analysed with J-T test. *P<0.05 Mann-Whitney U test.

5.2.4. The expression of TJ proteins and mast cell numbers in IBS patients

As demonstrated in the results of the cell culture chapters (3 and 4) JAM-A, CLD-1 and ZO-1 expression was reduced in response either to tryptase or to compound 48/80-mediated mast cell stimulation. In the clinical studies to date TJ proteins JAM-A, CLD-1 and ZO-1 appeared to be reduced in IBS tissue. The study went on to explore if there was an association between the expression of these TJ proteins in the caecal mucosa of IBS patients and mast cell numbers that had been previously assessed in my laboratory and were available in the database. Mast cells in the lamina propria of the caecum were counted per field of view and normalised to mm^2 of the lamina propria.

In the present IBS study cohort, mast cell numbers in caecal biopsy tissue (426) were significantly increased, compared to controls (59.5±6 MC/mm^2 lamina propria v 40±7.5 MC/mm^2 lamina propria, P=0.039) with mast cell counts significantly associated with IBS-A (58±7.5 MC/mm^2 lamina propria, P=0.021) (Supplementary Table 5.6). On analysis, however, no association was found between mast cell numbers in the lamina propria and the expression of JAM-A, CLD-1 or ZO-1 proteins either in the surface epithelium (Tables 5.6, 5.7) or the crypt epithelium (Table 5.8, Supplementary Table 5.7) of the caecal mucosa of IBS patients. The analysis by subtypes IBS-D and IBS-A (Table 5.6) or by gender (Supplementary Table 5.8) did not alter this finding. Of note, in the present study the analysis was confined to mast cells only in formalin fixed tissue, and the tryptase-based staining technique and quantification did not distinguish between intact and degranulated mast cells.

Statistical test	Group	JAM-A		CLD-1		ZO-1	
Spearman correl.	IBS	r_s=0.08	P=0.65	r_s=0.17	P=0.33	r_s=0.16	P=0.36
	IBS-D	r_s=0.19	P=0.40	r_s=0.32	P=0.15	r_s=0.16	P=0.46
	IBS-A	r_s=-0.34	P=0.28	r_s=-0.16	P=0.62	r_s=0.21	P=0.52
J-T test	IBS	r_J=0.05	P=0.72	r_J=0.14	P=0.31	r_J= 0.13	P=0.35
	IBS-D	r_J=0.14	P=0.40	r_J=0.25	P=0.15	r_J=0.14	P=0.43
	IBS-A	r_J=-0.30	P=0.21	r_J= -0.11	P=0.65	r_J=0.17	P=0.49

Table 5.6 Association between the expression of TJ proteins: JAM-A, CLD-1 or ZO-1 in surface epithelial cells of the caecal mucosa of IBS overall and IBS according to subtypes (IBS-D, IBS-A) and mucosal mast cell numbers. Data were analysed by using Spearman correlation and J-T test.

Surface epithelium				
TJ protein		JAM-A	CLD-1	ZO-1
Group	IBS v controls	**decreased**	no change#	**decreased**
	IBS-D v controls	**decreased**	no change#	**decreased**
	IBS-A v controls	**decreased**	no change	**decreased**
	IBS-D v IBS-A	no change	no change	no change
	IBD v controls	**decreased**	NA	NA
	IBD v IBS	no change	NA	NA
IBS symptoms	Abdominal pain severity	**neg. correl. (IBS-A); P<0.05 (J-T test)**	no correl.	no correl.
	Diarrhoea	no correl.	no correl.	no correl.
	Duration of symptoms	**neg. correl. (IBS-A); P<0.05 (J-T test)**	no correl.	no correl.
Immune cells	Mast cells	no correl.	no correl.	no correl.

Table 5.7 Summary of the expression of TJ proteins: JAM-A, CLD-1 and ZO-1 in the surface epithelium of the caecal mucosa of controls, IBS and IBD patients. TJ protein expression was associated with IBS symptoms and mucosal mast cell numbers. # means a trend towards decreased expression. P value ≤0.05 was considered as a statistically significant difference. NA, not applicable.

Crypts				
TJ protein		JAM-A	CLD-1	ZO-1
Group	IBS v controls	**decreased**	no change	no change
	IBS-D v controls	**decreased**	no change	no change
	IBS-A v controls	**decreased**	no change	no change
	IBS-A v IBS-D	**increased**	no change	no change
	IBD v controls	**decreased**	NA	NA
	IBD v IBS	no change	NA	NA
IBS symptoms	Abdominal pain severity	no correl.	no correl.	no correl.
	Diarrhoea	no correl.	no correl.	no correl.
	Duration of symptoms	no correl.	no correl.	no correl.
Immune cells	Mast cells	no correl.	no correl.	no correl.

Table 5.8 Summary of the expression of TJ proteins: JAM-A, CLD-1 and ZO-1 in the crypt epithelium of the caecal mucosa of controls, IBS and IBD patients. TJ protein expression was associated with IBS symptoms and mucosal mast cell numbers. P value ≤0.05 was considered as a statistically significant difference. NA, not applicable.

Statistical power analysis

A)

JAM-A						
Group	**IBS & controls**		**IBD & controls**		**IBD & IBS**	
Region	**SE**	**crypts**	**SE**	**crypts**	**SE**	**crypts**
Effect size (d)	0.82*	0.83*	1.1*	1.0*	0.44	0.34
Statistical power	0.78*	0.78*	0.62*	0.54*	0.19	0.13

B)

TJ protein	**CLD-1**		**ZO-1**	
Region	**SE**	**crypts**	**SE**	**crypts**
Effect size (d)	0.55	0.58	0.86*	0.47
Statistical power	0.48	0.52	0.81*	0.39

Table 5.9 Statistical power analysis at the α=0.05 level for **A)** JAM-A expression in a cohort of IBS, IBD and controls, **B)** CLD-1 and ZO-1 proteins in a cohort of IBS and controls. Effect size was measured using Cohen's d. *P<0.05 Mann-Whitney U test. SE, surface epithelium.

This study was performed on the previously recruited patient cohort. Post hoc power analysis was performed in order to determine the statistical power (α error of 0.05) of the study (Table 5.9). For JAM-A in a cohort of IBS patients and controls, statistical power was slightly below 0.8 and in order to achieve the minimum recommended statistical power of 0.8 (427, 428) to detect a large effect size (surface epithelium d=0.82; crypt epithelium d=0.83) I would need to recruit 2 additional controls assuming that the effect size in the sample is equal to the effect size in the population. For JAM-A expression in a cohort of IBD patients

and controls, the effect size was large, however the power of the study was below 0.8, which is mainly due to the small sample size. In order to achieve a statistical power of 0.8, 8 patients more would be required for JAM-A analysis in the surface epithelium, while for JAM-A analysis in crypts 16 patients more would have to be recruited to the study. The statistical power was greater than 0.8 for ZO-1 expression in the surface epithelium to detect the difference between IBS patients and controls. For CLD-1 the statistical power was approximately 0.5 and to detect a small effect size of 0.55 for the surface epithelium and of 0.58 for crypts I would need significantly more IBS patients and controls, i.e. a total number of 84.

5.3. Discussion

The pathophysiology of IBS is multifactorial, but mounting evidence suggests that increased permeability could contribute to the generation of IBS symptoms. Indeed, increased permeability as measured with lactulose/mannitol tests has been documented in 12-50% of IBS patients (148). The increase in permeability appears to be diffused along the gastrointestinal tract in IBS patients and was detected in the small intestine, colon and rectum (148). Data presented in this study demonstrate alterations in epithelial junctional complexes in the caecal mucosa of IBS patients which may be relevant to the mechanisms of increased permeability. JAM-A was assessed for the first time in IBS patients and was significantly down-regulated in the caecal mucosa of IBS patients relative to controls and lower expression was associated with more severe and longstanding IBS symptoms. Furthermore, lower levels of ZO-1 and CLD-1 were demonstrated in the surface epithelium of the caecal mucosal tissue of IBS patients confirming previously published data (213, 285).

Following up of the *in vitro* findings reported in chapters 3 and 4, this study investigated JAM-A expression in IBS patients and showed significantly reduced expression in the surface epithelium of the caecal mucosa compared to controls. This protein was consistently reduced in both IBS-A and IBS-D subtypes. Since this is the first reporting of JAM-A in IBS, there were no published findings to directly compare with. In IBD, however, lower JAM-A protein expression has been reported (53, 429) in agreement with these findings. And in this study JAM-A levels in IBS and IBD did not differ significantly. Furthermore, this study shows lower expression of ZO-1 in the surface epithelium of the caecal mucosa of IBS patients and a consistent reduction both in IBS-A and IBS-D subtypes. This is in agreement with previous reports of ZO-1 down-regulation in colonic (213) and jejunal tissue from IBS patients (285). This study reported lower CLD-1 levels in

the surface epithelium of the caecal mucosa in IBS patients compared to controls, however, the difference did not reach statistical significance and was noted only in the IBS-D subgroup. This finding fits with the study by Bertiaux-Vandaële *et al.* (213) showing a trend for lower CLD-1 levels in the colonic mucosa in IBS patients, but a more recent study found no difference in jejunal expression of CLD-1 between IBS-D patients and controls (344).

Since the JAM-A finding was novel, it was interesting to investigate its potential clinical relevance in IBS. Importantly, this study demonstrates that the reduction in JAM-A expression was significantly associated with more severe abdominal pain in patients with IBS-A. Others have linked changes in junctional proteins to IBS symptoms. Bertiaux-Vandaële *et al.* (213) showed a negative association between colonic CLD-1 protein expression and severity of abdominal pain in IBS-D patients. Similarly, the authors observed a negative association between occludin and ZO-1 mRNA expression and abdominal pain in IBS patients (213). Piche *et al.* (212) reported a significant correlation between severity of abdominal pain and paracellular permeability in colonic biopsy tissue of IBS patients. Moreover, colonic supernatants from IBS patients reduced ZO-1 mRNA expression and increased paracellular permeability of Caco-2 cells, and the degree of this increase was significantly associated with abdominal pain severity (212). However, in the present study no association was found between abdominal pain severity and the lower levels of the other TJ proteins, namely CLD-1 or ZO-1. Martínez *et al.* (285) also found no association between ZO-1, ZO-2 and ZO-3 mRNA expression in the jejunal mucosa of IBS patients and the severity and frequency of abdominal pain. These discrepancies might reflect heterogeneity of IBS, biopsy sites, and studied proteins and methodology. Perhaps employing more detailed symptom questionnaires (e.g. Severity Scoring System, IBS-SSS) (430, 431) may shed further light into symptom associations.

More recently, the ultrastructural alterations found by TEM, i.e. a higher proportion of dilated junctions and an increase in intercellular distance in jejunal mucosa of IBS patients, was significantly associated with IBS symptoms including abdominal pain severity, bowel movements and stool consistency (344). Severity of diarrhoea was not significantly associated with JAM-A, ZO-1 or CLD-1 in my study. In contrast, Martínez *et al.* (285, 344) found a significant negative correlation between the number of bowel movements or stool consistency and ZO-1 mRNA, and ZO-3 mRNA (285). Nonetheless, more detailed data on stool frequency and consistency (285), over and above our assessment, would be required to better understand associations between stool parameters and junctional protein expression in the caecal mucosa in IBS. Of note, the association between ZO-1 and bowel habit reported by Martínez *et al.* (285) was for ZO-1 mRNA, while this study investigated ZO-1 protein expression at the surface epithelium in the caecal mucosa in IBS.

In this study IBS-A patients with lower JAM-A expression reported more severe abdominal pain, and furthermore appeared to have more longstanding disease. JAM-A levels in the IBS-A subtype appeared to be high in patients up to 1 year after symptom onset and its expression consistently decreased with duration of IBS symptoms. While Bertiaux-Vandaële *et al.* (213) did not investigate JAM-A, the authors, in contrast to this finding, reported the lowest levels of TJ proteins in IBS colonic tissue (occludin and CLD-1 protein and ZO-1 mRNA) within the first 2 years after the onset of IBS, suggesting that altered expression of TJ proteins may be implicated in the initiation stage of IBS. In this study, however, no association between duration of IBS symptoms and the expression of either CLD-1 or ZO-1 was detected. Present data suggest the involvement of JAM-A in more sustained and painful IBS-A, possibly secondary to other pathological mechanisms such as sustained immune activation. These differences indicate that

mechanisms underlying intestinal epithelial barrier disruption differ between IBS subgroups and reflect the variability of this disease.

The mechanisms behind the alterations in TJ protein expression in IBS are not known. As demonstrated in the previous cell culture chapters, JAM-A, CLD-1 and ZO-1 expression was altered following incubation with either tryptase alone or compound 48/80-stimulated mast cells. Also, in the tryptase inhibition study, JAM-A was shown to be down-regulated by tryptase released from stimulated mast cells. However, unstimulated mast cells did not affect the expression of JAM-A, CLD-1 or ZO-1. The present study explored mast cell count data but no association was found between mucosal mast cell numbers and the expression of TJ proteins. An increase in mast cells in the caecal mucosa of IBS patients has been reported by my laboratory (86) and others (90, 118). Also, several studies have reported an increased numbers of degranulating mast cells in IBS, as evidenced by the ultrastructural features of degranulation (118, 144, 175) and/or by the enhanced tryptase release from IBS tissue compared to controls (112, 144, 175, 252, 269). Unfortunately, the data on mast cell activation was not available in the present patient cohorts and alterations in TJ proteins may be expected to result from mast cell activation rather than their numbers. Previously, Martínez *et al.* (285) demonstrated that ZO-1 and ZO-3 mRNA expression correlated with tryptase mRNA, which might be indicative of mast cell activation, whereas no correlation was found with mast cell numbers. Noteworthy, as described in chapter 4, TJ disassembly as evidenced by ultrastructural changes of intercellular junctions, i.e. open TJs, was observed in Caco-2/HMC-1 monolayers following mast cell stimulation, whereas unstimulated mast cells did not affect epithelial integrity in the co-culture model. Therefore, the assessment of the status of mast cell activation would be required to better understand the association between mast cells and TJs. Mast cell distribution within biopsy tissue is also unknown. These data might be informative, especially if the mast cell density was increased

in close proximity to the epithelial lining. Previously, mast cells were reported to be localised significantly closer to nerve fibres in the colonic mucosa of IBS patients compared to controls, suggesting enhanced interaction between immune and nervous system (144). Importantly, there are data suggesting that the abrogation of the epithelial barrier function can induce inflammatory processes, either locally or remotely (25) and therefore associations between mast cells and impaired epithelial barrier might be particularly difficult to identify in some scenarios.

The present study was not designed to analyse comprehensively JAM-A expression in IBD patients. The rationale of including IBD group was to enable comparison of IBS cohort with IBD in the absence of published data on IBS. Though it was not the core aim of the study the interesting findings in JAM-A expression were demonstrated in IBD patients. JAM-A expression appeared to be reduced in the surface epithelium of the caecal mucosa of IBD patients compared to controls but did not differ when compared to IBS patients. JAM-A was significantly reduced in active IBD, whereas in inactive IBD there was a trend towards reduction. This is in agreement with previous findings where lower JAM-A expression was found in colonic tissue of active IBD compared to controls (53). JAM-A was reduced in CD patients whereas in patients with UC its expression was comparable to controls. In the previous study by Vetrano *et al.* (53), however, no difference was found between CD and UC and the reduction in JAM-A was found in both. Noteworthy, this analysis was limited to a small number of IBD patients, but the preliminary data are interesting and merit further analysis in a larger sample size to confirm these findings.

Analysis of the secondary endpoint, expression in the crypt epithelium, further supported the key JAM-A finding of this study. In the crypt epithelium JAM-A was significantly reduced in both IBS and IBD patients compared to controls. In

IBD, comparably to the surface epithelium finding, JAM-A was significantly reduced in CD when compared to controls, but not in UC. Unexpectedly, looking at the disease activity status, JAM-A expression was significantly reduced in inactive IBD when compared to controls but not in active IBD. This however might be due to slightly higher predominance of CD patients in the group of inactive IBD than in the group of active IBD. These differences might also be associated with disease duration, which appeared to be longer for inactive IBD patients compared with active IBD (P=0.051). Longer disease duration significantly associated with lower JAM-A expression in crypts but not in the surface epithelium in IBD patients (r_s=-0.85, P=0.007). Possibly, low-grade and longstanding inflammation, might contribute to barrier dysfunction in crypts of inactive IBD. Vivinus-Nébot *et al.* (432) suggested that a persistent increase in TNF-α in the colonic mucosa of inactive IBD may determine epithelial barrier defects. TNF-α plays a role in inflammation through stimulation of intracellular nuclear factor κB (NFκB). NFκB regulates inflammation such as in IBD, by controlling transcription of pro-inflammatory cytokines including TNF-α, IL-1 and IL-6 (433, 434). These mechanisms, however have not been studies here, and should be addressed in a future analysis.

In contrast to JAM-A findings, no detectable change was found for either CLD-1 or ZO-1 protein expression in crypts, suggesting that different mechanisms are involved in the regulation of these junctional proteins. ZO-1 protein expression positively associated with CLD-1 expression both in the crypt and surface epithelium; however, no relationship was found between JAM-A and either ZO-1 or CLD-1 expression (data not shown). Furthermore, lower JAM-A levels in the surface epithelium positively associated with lower expression in crypts (data not shown) suggesting the global reduction in JAM-A in the caecal mucosa in subsets of IBS patients.

Since lower levels of both JAM-A and ZO-1 were consistently found in the surface epithelium of the caecal mucosa in both IBS-D and IBS-A subgroups, the reduced JAM-A and ZO-1 expression seems to be a feature of IBS which is unrelated to IBS bowel habit. This is in agreement with the previous evidence of altered TJ protein expression (CLD-1, ZO-1, occludin) in the colonic mucosa of each IBS subtype (IBS-D, IBS-A, IBS-C) though the alterations were particularly marked in IBS-D patients and to a lesser extent in IBS-C and IBS-A (213). This is also in line with the studies of intestinal permeability in IBS showing that increased colonic (212) and small intestinal (310) permeability was independent of IBS subtypes. Furthermore, the supernatants of colonic biopsy tissue of IBS patients incubated 2 days with intestinal epithelial cells induced a comparable increase in permeability irrespective to IBS subtype (212). On the other hand, Gecse *et al.* (307) demonstrated that only faecal supernatants of patients with IBS-D could increase paracellular permeability but not these from IBS-C patients.

In this study immunofluorescence staining was assessed semiquantatively in terms of intensity and specificity of staining. Biopsy tissues from control patients were used as internal sample controls to monitor the degree of staining intensity heterogeneity, which typically appeared to be comparable in each batch of experiment. Percentage positivity, another validated semi-quantitative scoring method (435, 436) was not employed in this study. The measurement of intensity and specificity of protein expression appeared to be more relevant in this study since protein staining, if identified in each biopsy tissue, was typically present throughout a whole biopsy tissue and its intensity was heterogeneous. Occasionally, no protein staining was also observed, particularly for ZO-1 protein, and lack of staining tended to be relatively consistent throughout a biopsy tissue. Ideally, the analysis should have included the measurement of both the intensity of the staining and percentage positivity and this should be taken into account in a future analysis.

While this study reports strong novel findings on JAM-A and symptoms severity, the link with mast cells in IBS requires further consideration. Additional studies are needed to better understand the mechanism underlying reduced expression of TJ proteins, including association with activated mast cells that might be determined by the measurement of tryptase release from biopsy tissue or/and the ultrastructural features of degranulation in TEMs. Noteworthy, this study assessed alterations in the expression of TJ proteins that might be considered as an indirect measurement of intestinal epithelial permeability (213). However, further assessment of intestinal permeability *in vivo*, for example with ^{51}Cr-EDTA urinary recovery test, would strengthen these findings (25).

In summary, the results of the present study provide the evidence for the alterations of the expression of TJ proteins in IBS patients and their clinical relevance. JAM-A expression was documented for the first time in IBS patients and appeared to be significantly lower in the caecal mucosa when compared to controls. The reduction in JAM-A expression was associated with more severe abdominal pain and longer duration of symptoms in IBS-A. Furthermore, lower levels of ZO-1 and CLD-1 were found in the surface epithelium of the caecal mucosa of IBS patients compared to controls, suggesting a novel organic background in the pathogenesis of IBS and further challenging the classical concept of IBS as a functional disorder. Taken together, this study shows that altered expression of TJ proteins is linked to IBS symptoms and might provide disease biomarkers.

Key findings

1. JAM-A and ZO-1 expression was significantly lower in the surface epithelium of caecal tissue from IBS patients compared to controls. This reduction in JAM-A and ZO-1 was demonstrated in both IBS-D and IBS-A subtypes.

2. There was a trend for reduced expression of CLD-1 in the surface epithelium of the caecal mucosa of IBS patients.

3. In IBS-A, surface JAM-A expression was significantly negatively associated with severity of abdominal pain.

4. In IBS-A, surface JAM-A expression was significantly negatively associated with the duration of IBS symptoms.

Supplementary material

Gender/Age and TJ protein expression						
TJ protein	**JAM-A**		**CLD-1**		**ZO-1**	
Region	**SE**	**crypts**	**SE**	**crypts**	**SE**	**crypts**
Gender	P=0.64	P=0.97	P=0.89	P=0.78	P=0.16	P=0.09
Age	P=0.55	P=0.47	P=0.60	P=0.82	P=0.57	P=0.60

Supplementary Table 5.1 Age and gender effects on TJ protein expression in the caecal mucosa controlled for in the analysis using a general linear model in the disease group (control, IBS, IBD-JAM-A). SE, surface epithelial cells.

Age and TJ protein expression					
TJ protein	**Region**	**Spearman correlation**		**J-T test**	
		r_S value	P value	r_J value	P value
JAM-A	**SE**	-0.33	0.3	-0.28	0.27
	crypts	-0.37	0.24	-0.25	0.30
CLD-1	**SE**	0.04	0.92	0.04	0.88
	crypts	0.01	0.97	0.00	1.00
ZO-1	**SE**	-0.10	0.76	-0.80	0.75
	crypts	-0.37	0.24	-0.06	0.82

Supplementary Table 5.2 Expression of TJ proteins in the caecal mucosa in the control group according to age (years) of patients at the time of biopsy. Statistical analysis was performed by using Spearman correlation and J-T test. SE, surface epithelial cells.

TJ protein	Region	Gender		Age at the time of biopsy (years)	
		Males (n=7)	Females (n=5)		
		Median (range), mean±SEM		26±2.7	51.6±4.4
JAM-A	SE	4.0 (3-4), 3.6±0.2	4.0 (1-4), 3.4±0.6	4.0 (3-4), 3.7±0.2	4.0 (1-4), 3.4±0.4
		P=0.63		P=0.61	
	crypts	3.0 (1-4), 2.9±0.4	3 (2-4), 2.8±0.4	3.5 (3-4), 3.5±0.3	2.5 (1-4), 2.5±0.3
		P=0.80		P=0.7	
CLD-1	SE	4.0 (2-4), 3.4±0.3	3 (2-4), 3.0±0.4	3.0 (2-4), 3.0±0.6	3.5 (2-4), 3.4±0.3
		P=0.43		P=0.58	
	crypts	3.0 (2-4), 3.0±0.2	2 (2-3), 2.4±0.2	2.5 (2-3), 2.5±0.3	3.0 (2-4), 2.9±0.2
		P=0.10		P=0.33	
ZO-1	SE	2.0 (1-3), 2.1±0.3	2 (2-3), 2.4±0.2	2.5 (2-3), 2.2±0.2	3.0 (1-3), 2.2±0.2
		P=0.86		P=0.92	
	crypts	3.0 (2-4), 3.1±0.3	3 (2-4), 3.2±0.4	2.0 (3-4), 3.5±0.3	2.0 (2-4), 3.0±0.3
		P=0.96		P=0.27	

Supplementary Table 5.3 Analysis of TJ proteins the caecal mucosa in the control group according to gender and age (years) of patients at the time of biopsy < 32 years (the average age of IBS) and ≥ 32 years and resultant TJ protein expression (grade). The expression was assessed semiquantitatively using a 4-grade scale. Data were analysed with Mann-Whitney U test. SE, surface epithelium.

TJ protein expression in crypts							
TJ protein	**Group**	**Abdominal pain severity (scale1-4)**		**Diarrhoea (No. of liquid stools/week)**		**Duration of symptoms (years)**	
		r_s value	P value	r_s value	P value	r_s value	P value
JAM-A	IBS	-0.06	0.75	-0.34	0.073	0.15	0.38
	IBS-D	0.13	0.56	-0.34	0.14	-0.03	0.9
	IBS-A	-0.47	0.14	0.2	0.6	0.29	0.36
CLD-1	IBS	0.06	0.74	0.17	0.38	-0.01	0.96
	IBS-D	0.14	0.54	0.19	0.43	-0.07	0.76
	IBS-A	-0.16	0.63	0.11	0.77	-0.18	0.57
ZO-1	IBS	-0.28	0.11	-0.20	0.29	-0.10	0.57
	IBS-D	-0.27	0.23	-0.28	0.23	-0.03	0.88
	IBS-A	-0.37	0.26	0.18	0.65	-0.41	0.18

Supplementary Table 5.4 Correlation between TJ protein expression in the crypt epithelium of the caecal mucosa of IBS and IBS symptoms/duration of IBS symptoms. Data were correlated by using Spearman correlation.

TJ protein expression in crypts							
TJ protein	**Group**	**Abdominal pain severity (scale1-4)**		**Diarrhoea (No. of liquid stools/week)**		**Duration of symptoms (years)**	
		r_J value	P value	r_J value	P value	r_J value	P value
JAM-A	IBS	0.05	0.75	-0.29	0.05	0.13	0.34
	IBS-D	0.11	0.55	-0.30	0.11	-0.02	0.93
	IBS-A	-0.08	0.78	0.19	0.54	0.24	0.33
CLD-1	IBS	0.05	0.75	0.13	0.41	0.01	0.96
	IBS-D	0.12	0.53	0.13	0.46	-0.05	0.76
	IBS-A	-0.16	0.60	0.11	0.75	-0.09	0.74
ZO-1	IBS	-0.25	0.10	-0.17	0.26	-0.08	0.54
	IBS-D	-0.24	0.21	-0.23	0.21	-0.04	0.81
	IBS-A	-0.33	0.25	0.16	0.61	-0.35	0.17

Supplementary Table 5.5 Association between TJ protein expression in the crypt epithelium of the caecal mucosa of IBS and IBS symptoms/duration of IBS symptoms. Statistical analysis was performed by using J-T test.

Group	Mast cell numbers/mm² lamina propria mean±SEM (range)
Control	40.0±7.5 (13-111)
IBS	59.5±6.0 (5-152)
v control	**P=0.04**
IBS-D	60.0±8.5 (5-152)
v control	P=0.13
IBS-A	58.0±7.5 (26-77)
v control	**P=0.02**
v IBS-D	P=0.59

Supplementary Table 5.6 Mast cells numbers in the lamina propria of the caecal mucosa of controls and IBS patients. IBS patients are also shown according to subtypes: IBS-D and IBS-A. Intergroup comparisons were performed by using Mann-Whitney U test.

Statistical test	Group	JAM-A		CLD-1		ZO-1	
Spearman correl.	IBS	r_s=0.18	P=0.32	r_s=0.14	P=0.41	r_s=0.21	P=0.24
	IBS-D	r_s=0.37	P=0.09	r_s=0.19	P=0.40	r_s=0.26	P=0.23
	IBS-A	r_s=-0.28	P=0.38	r_s=-0.02	P=0.94	r_s=-0.11	P=0.72
J-T test	IBS	r_J=0.14	P=0.28	r_J= 0.12	P=0.38	r_J=0.16	P=0.23
	IBS-D	r_J= 0.30	P=0.09	r_J=0.16	P=0.36	r_J=0.19	P=0.26
	IBS-A	r_J=-0.19	P=0.42	r_J=-0.02	P=0.93	r_J=-0.07	P=0.76

Supplementary Table 5.7 Association between the expression of TJ proteins in the crypt epithelium of the caecal mucosa of IBS patients and mucosal mast cell numbers in the lamina propria. IBS patients are also shown according to subtypes: IBS-D, IBS-A. Data were analysed with Spearman correlation and J-T test.

Group	Mast cell numbers/mm² lamina propria mean±SEM (range)	
	Males	Females
Control	36.5±4.25 (21-55)	46±18 (13-111)
	P=0.93	

Supplementary Table 5.8 Mast cell numbers in the lamina propria of the caecal mucosa according to gender in the control group. The expression was assessed semiquantitatively using a 4-grade scale. Intergroup comparisons were performed by using Mann-Whitney U test.

CHAPTER 6

THE EXPRESSION OF THE AJ PROTEIN E–CADHERIN IS REDUCED IN THE CAECAL MUCOSA OF IBS PATIENTS AND IS ASSOCIATED WITH IBS SYMPTOMS

6.1. Introduction

6.1.1. Rationale

Paracellular permeability is primarily under the control of TJs; however, defects in AJ integrity have been also associated with intestinal barrier dysfunction (66, 437). E-cadherin is a principal component of AJs where it plays a major role in the maintenance of cell-cell contacts (438). E-cadherin is also involved in the retention of effector and memory T-cells in the epithelial compartment through interaction with $\alpha E\beta 7$ integrin, expressed on IELs (439). β7integrin is implicated in the pathogenesis of IBD and in disease pathogenesis in mouse models of colitis, intestinal infections and ileitis (440). E-cadherin was also reported to be expressed on dendritic cells associated with T-cell-mediated colitis in mice (441). The loss of E-cadherin was associated with dissociation of the AJs in mice and produced intestinal inflammation similar to IBD (66). Furthermore, a number of studies have reported reduced E-cadherin expression at sites of active inflammation in IBD (442-444). In IBS, however, the expression of E-cadherin remains unknown and its role in the barrier function largely unexplored.

As demonstrated in the cell culture studies (chapters 3 and 4), the expression of epithelial E-cadherin was unaltered in response to mast cell stimulation with compound 48/80 or tryptase. Although possibly not influenced by mast cells, E-cadherin remains an interesting target in IBS as recent evidence has implicated a polymorphism in the E-cadherin gene in the aetiology of IBS, specifically PI-IBS (211). Genetic variants of the E-cadherin gene might have functional effects on E-cadherin protein levels and susceptible individuals might be more prone to develop intestinal barrier dysfunction upon challenge with bacterial pathogens. Several pathogens such as *Bacteroides fragilis* and *Candida albicans* have evolved

mechanisms for the disruption of AJs through the cleavage of E-cadherin (445, 446).

Based on this evidence, it might be postulated that E-cadherin function is altered in IBS. Therefore, this chapter investigated whether its expression is lower in caecal IBS tissue and if it associates with IBS symptoms. This study may add further insights into the mechanisms underlying impaired barrier function in IBS.

6.1.2. Aims of the study

1. To investigate if E-cadherin expression is lower in caecal biopsy tissue of IBS patients when compared with tissue from controls.

2. To investigate if there is an association between E-cadherin expression and IBS symptoms such as abdominal pain, diarrhoea and duration of symptoms.

3. As a tertiary aim, to explore if potential changes in E-cadherin demonstrated with immunofluorescence could be repeated with immunohistochemistry.

6.1.3. Methods to achieve the aims

Methods are described in detail in chapter 2. In brief, for this study E-cadherin protein was immunostained in formalin fixed, paraffin embedded biopsy tissue from the caecum of patients with Rome II IBS (n=33) and normal controls (n=12). Severity of abdominal pain and diarrhoea were assessed in IBS using a pain rating of 0-4 (0-no pain, 1-mild, 2-relevant, 3-severe, 4-extremely severe) (396) and numbers of liquid stools per week (397), respectively. The patient cohort (IBS patients and controls) was the same as that used for the analysis of TJ proteins in

Chapter 5; however, for E-cadherin analysis, one IBS-A patient was excluded from analysis due to tissue damage during immunohistochemical staining and subsequent comparisons between immunofluorescence and immunohistochemical staining for E-cadherin were based on 33 matched tissue pairs.

Immunofluorescence and immunohistochemical staining was assessed semiquantatively using a 4-grade scale: (1-no specific staining, 2-mild specific staining, 3-moderate specific staining, 4-strong specific staining). Differences in the grade of E-cadherin in the surface epithelium were the primary outcome measure, while these in the crypt epithelium were the secondary one.

E-cadherin expression, as the other junctional proteins, was analysed, according to the study design, with immunofluorescence. In addition, caecal tissue was stained for E-cadherin using immunohistochemistry. The purpose was to explore if important findings detected by immunofluorescence, that provides high-resolution images for protein expression/localisation, could also be detected with immunohistochemistry which is more widely used in clinical settings and may be clinically useful as a marker for IBS.

6.2. Results

6.2.1. Study population

Demographic and clinical characteristics of the study group (n=45) are displayed in Table 6.1. In the IBS group, 22 patients were classified as IBS-D (67%), while 11 as IBS-A (33%). As in chapter 5, the female predominance was larger and the average age was lower in the IBS group compared to controls. Potential age and gender influences on E-cadherin expression were controlled for in the analysis using a general linear model, which confirmed no gender or age effects on E-cadherin expression in the disease group (control, IBS) (Supplementary Table 6.1). Also, the analysis of the control group (213) confirmed that neither gender nor age influenced E-cadherin expression. (Supplementary Tables 6.2, 6.3)

Disease group	IBS (n=33)	Controls (n=12)
Gender		
Male	18% (n=6)	58% (n=7)
Female	82% (n=27)	42% (n=5)
	P*=0.009	
Age (years)		
Mean±SEM (range)	32.2±1.4 (21-54)	43.1±4.7 (18-64)
	P*=0.005	
Abdominal pain severity scale (1-4)		
Median (range)	2 (1-4)	NA
Mild (1)	27% (n=9)	NA
Relevant (2)	48% (n=16)	NA
Severe (3)	12% (n=4)	NA
Extremely severe (4)	9% (n=3)	NA
Diarrhoea severity:		
No. of liquid stools/week	14.9±3.7 (0.2-52.5)	
	(20.7±4.7 IBS-D;	
	1.8±1.1, IBS-A; P=0.001)	
Duration of symptoms (years)		
Mean±SEM (range)	3.3±0.7 (0.3-20)	NA
	<1 year-33% (n=11)	
	1-5 year-52% (n=17)	
	>5 year-15% (n=5)	
	(2.1±0.5, IBS-D;	
	5.3±1.7, IBS-A; P=0.10)	

Table 6.1 Characteristics of IBS patients and controls. Intergroup comparisons were performed by using Pearson Chi-square test (gender) or Student's t-test (age). P*, compared with controls. NA, not applicable.

6.2.2. E-cadherin expression is reduced in the caecal mucosa of IBS patients

In the caecal mucosa of controls, uniform E-cadherin staining was abundant in AJs and was present along the lateral membrane of surface epithelial cells (Figure 6.1, 6.3). The overall E-cadherin expression was significantly lower in the IBS group compared to controls (P=0.034), although the median grade of E-cadherin expression was the same in both groups (median of 3) (Figures 6.1, 6.3; Table 6.2). E-cadherin expression appeared to be reduced to grade 2 in the caecal mucosa of 35% (n=11) IBS patients compared to none (0%) in controls. Furthermore, the strong specific staining was less common in IBS (21%, n=7) compared to controls (42%, n=5). The analysis of E-cadherin expression according to bowel predominance demonstrated that it was significantly lower in patients with the IBS-A subtype when compared to controls (P=0.046) while in IBS-D a trend for lower expression was noted (P=0.067) (Figures 6.1, 6.3; Table 6.2).

Crypt epithelium E-cadherin in crypt epithelial cells (the secondary endpoint) of the caecal mucosa was comparable in IBS patients and controls and was abundantly present along the lateral membrane of epithelial cells in both groups. There were no differences between IBS subtype and controls (Figures 6.2, 6.3; Table 6.2).

In summary, this data shows for the first time evidence of significantly lower E-cadherin expression in the surface epithelium of the caecal mucosa in IBS patients compared to controls. The reduction was found in both IBS-A and IBS-D subtypes, though a statistical difference was only observed in IBS-A.

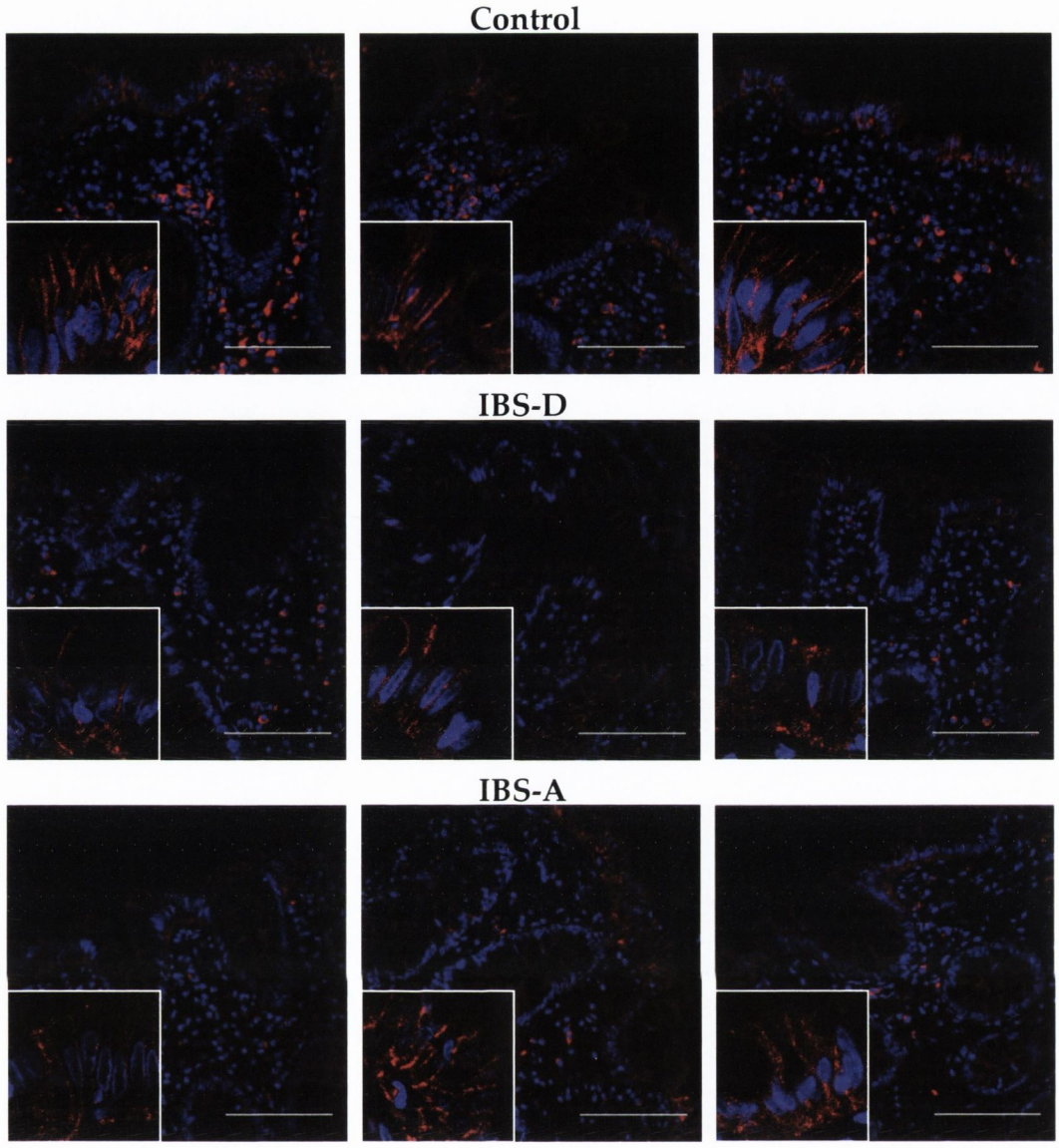


Figure 6.1 Representative photomicrographs showing E-cadherin expression in the surface epithelium of the caecal mucosa of controls and IBS subtypes according to bowel predominance: IBS-D and IBS-A. Immunofluorescence staining for E-cadherin is shown in red, while nuclear staining is blue. Bars=50 μm.

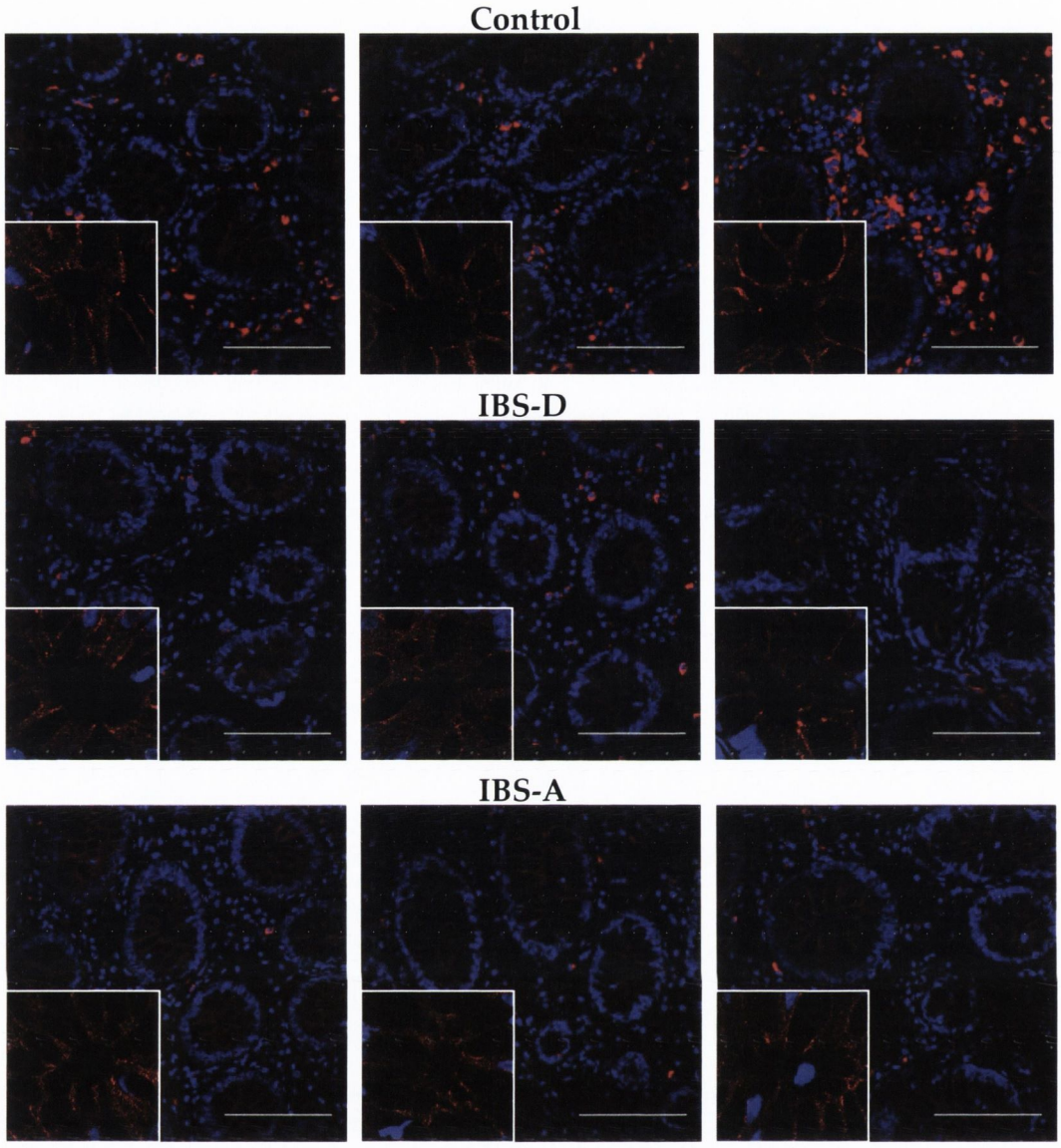
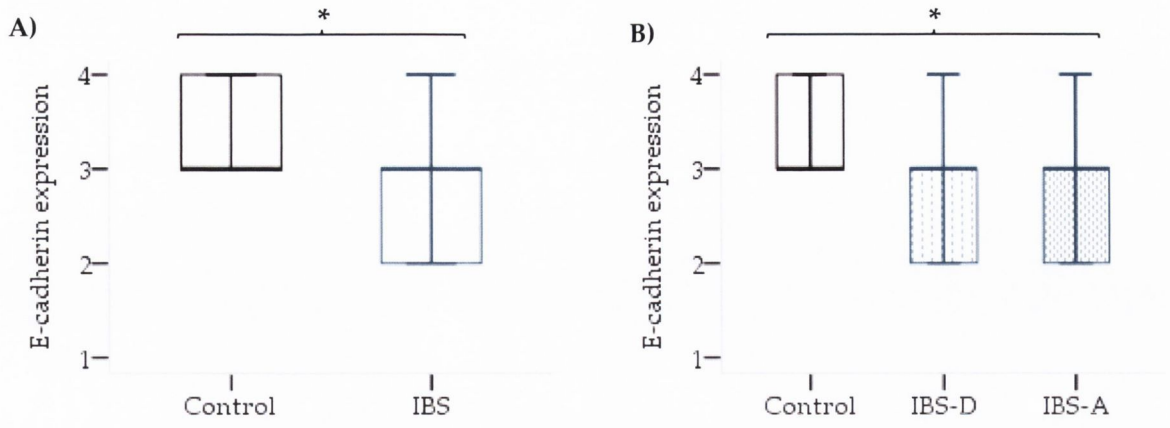


Figure 6.2 Representative photomicrographs showing E-cadherin expression in the crypt epithelium of the caecal mucosa of controls and IBS subtypes according to bowel predominance: IBS-D and IBS-A. Immunofluorescence staining for E-cadherin is shown in red, while nuclear staining is blue. Bars=50 μm.

SURFACE EPITHELIUM



CRYPTS

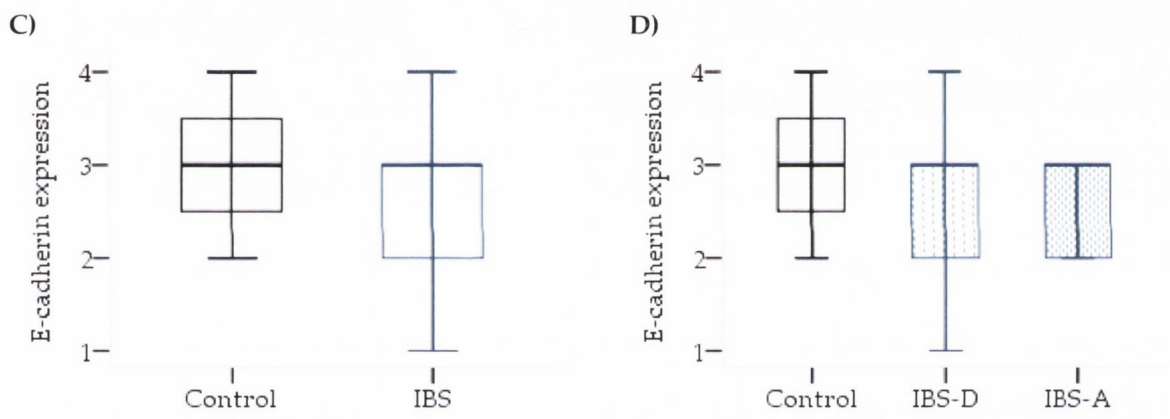


Figure 6.3 E-cadherin expression in **A-B)** the surface epithelium and **C-D)** crypts of the caecal mucosa of controls and IBS patients. **B, D)** IBS patients are also shown according to subtype: IBS-D and IBS-A. Values are represented as a median with interquartile ranges. *P<0.05 Mann-Whitney U test. Kruskal-Wallis P values: **B**, P=0.093; **D**, P=0.45.

E-cadherin		
Region	**Surface epithelium**	**Crypts**
Group	Median (range), mean±SEM	
Control	3.0 (3-4), 3.4±0.1	3.0 (2-4), 3.0±0.2
IBS	3.0 (2-4), 2.9±0.1	3.0 (1-4), 2.7±0.1
v control	**P=0.034**	P=0.22
IBS-D	3.0 (2-4), 2.9±0.2	3.0 (1-4), 2.7±0.2
v control	P=0.067	P=0.32
IBS-A	3.0 (2-4), 2.8±0.2	3.0 (2-3), 2.6±0.1
v control	**P=0.046**	P=0.21
v IBS-D	P=0.64	P=0.81

Table 6.2 E-cadherin expression in the caecal mucosa of controls (n=12) and IBS patients (n=33). IBS patients are also shown according to subtypes: IBS-D (n=22) and IBS-A (n=11). E-cadherin expression was analysed with immunofluorescence and then assessed semiquantitatively using a 4-grade scale. Intergroup comparisons were performed by using Mann-Whitney U test.

6.2.3. E-cadherin expression is significantly associated with IBS symptoms

Abdominal pain: There was a significant negative correlation between E-cadherin expression in surface epithelial cells and abdominal pain severity in IBS patients (r_s=-0.39, P=0.029) (Table 6.3). In addition, abdominal pain severity appeared to increase with reduction in E-cadherin expression in the caecal mucosa of IBS patients using J-T test (r_J=-0.34, P=0.031) (r_J=-0.34, P=0.031, J-T test) (Table 6.4, Figure 6.4).

Duration of IBS symptoms: There was a significant negative correlation between E-cadherin expression in the surface epithelium and duration of IBS symptoms in IBS-A patients (r_s=-0.64, P=0.034) (Table 6.3) suggesting that patients with longer duration of symptoms have a lower expression of E-cadherin in surface epithelial cells. Indeed, the lowest expression of E-cadherin (grade 2) was noted in IBS with the longest duration of symptoms (median of 5 years) (r_J=-0.53, P=0.047, J-T test) (Table 6.4, Figure 6.5).

Diarrhoea (the number of liquid stools per week): There was no association between diarrhoea and E-cadherin expression in the surface epithelium of the caecal mucosa of IBS patients, overall or in subtypes.

IBS symptoms by E-cadherin protein expression in crypts: There was no significant association between E-cadherin expression in the crypt epithelium of caecal mucosa and abdominal pain severity, diarrhoea and duration of IBS symptoms (Supplementary Table 6.4, 6.5).

In summary, reduced E-cadherin expression in the caecal surface epithelium was significantly associated with more severe abdominal pain in IBS patients. Furthermore, reduction in E-cadherin significantly associated with longstanding symptoms in patients with the alternating subtype of IBS. These findings are comparable to the JAM-A findings reported in chapter 5 where reduced JAM-A expression associated with more severe abdominal pain and longer duration of IBS symptoms in patients with IBS-A. However, no correlation between the expression of both proteins was found either for the surface (P=0.20, r_S=0.23) and crypt epithelium (P=0.23, r_S=0.22).

E-cadherin expression in the surface epithelium						
Group	**Abdominal pain severity (scale1-4)**		**Diarrhoea (No. of liquid stools/week)**		**Duration of symptoms (years)**	
	r_s value	P value	r_s value	P value	r_s value	P value
IBS	**-0.39**	**0.029**	-0.10	0.61	-0.29	0.11
IBS-D	-0.37	0.09	-0.28	0.24	-0.10	0.65
IBS-A	-0.44	0.21	0.23	0.55	**-0.64**	**0.034**

Table 6.3 Correlation between E-cadherin expression in surface epithelium of the caecal mucosa of IBS patients and IBS symptoms. Data were correlated by using Spearman correlation.

E-cadherin expression in the surface epithelium						
Group	**Abdominal pain severity (scale1-4)**		**Diarrhoea (No. of liquid stools/week)**		**Duration of symptoms (years)**	
	r_J value	P value	r_J value	P value	r_J value	P value
IBS	**-0.34**	**0.031**	-0.08	0.58	-0.23	0.11
IBS-D	-0.33	0.09	-0.23	0.22	-0.08	0.66
IBS-A	-0.39	0.20	0.20	0.53	**-0.53**	**0.047**

Table 6.4 Association of E-cadherin expression in the surface epithelium of the caecal mucosa of IBS patients with IBS symptoms. Data were analysed with J-T test.

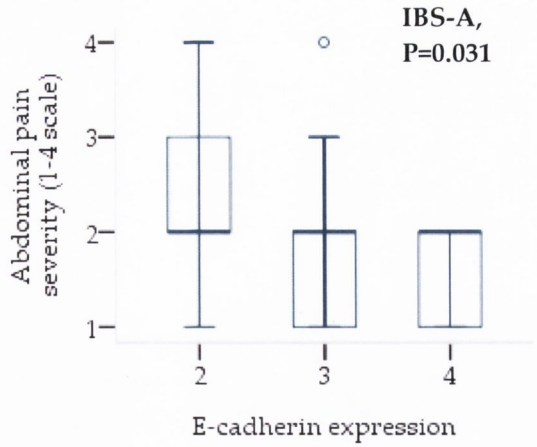


Figure 6.4 Abdominal pain severity as a function of E-cadherin expression in the surface epithelium of the caecal mucosa of IBS patients. Lines represent individual data and medians values. Data were analysed with J-T test.

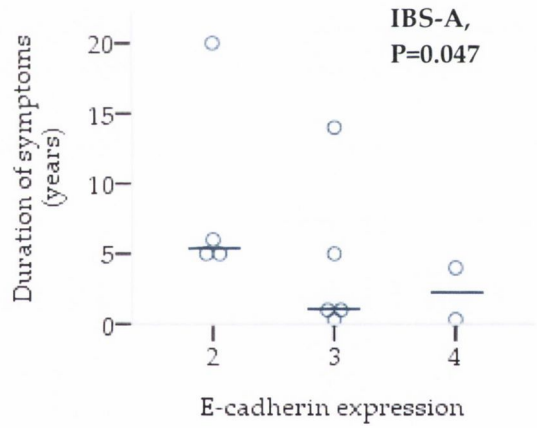


Figure 6.5 Duration of symptoms as a function of E-cadherin expression in the surface epithelium of the caecal mucosa of IBS-A patients. Lines represent individual data and medians values. Data were analysed with J-T test.

6.2.4. E-cadherin expression in the caecal mucosa of IBS patients – a comparison of immunofluorescence with immunohistochemistry

Following up the interesting clinical findings, E-cadherin was further analysed with immunohistochemistry to investigate if the significant changes shown with immunofluorescence might also be detected with this method and, if so, might have potential to be more widely assessed clinically or in research as a biomarker for IBS.

E-cadherin expression determined by immunohistochemistry appeared to be generally reduced in the surface epithelium of the caecal mucosa of IBS patients compared to controls (Figures 6.6, 6.8; Table 6.5), however, in contrast to the immunofluorescence findings, a statistically significant change was not apparent (P=0.072). The immunohistochemical analysis based on IBS subtypes, similarly to immunofluorescence, demonstrated a trend towards reduction in IBS-D patients (P=0.071). However, no reduction was found in the IBS-A subgroup (Figures 6.7, 6.8; Table 6.5) and this was shown with immunofluorescence. Looking at the immunohistochemical staining for E-cadherin expression in crypts, the secondary measurement, there was no detectable difference between IBS patients and controls (Figure 6.7; Table 6.5), in line with the immunofluorescence findings. Predictably, when immunohistochemistry was used no significant association between E-cadherin expression and IBS symptoms was found (data not shown). Thus, the significant changes in E-cadherin levels and the associations with IBS symptoms demonstrated with immunofluorescence were missed with immunohistochemistry.

In summary, in contrast to immunofluorescence analysis, the significant reduction in E-cadherin expression was not detected with immunohistochemistry and consequently no associations with IBS symptoms were found.

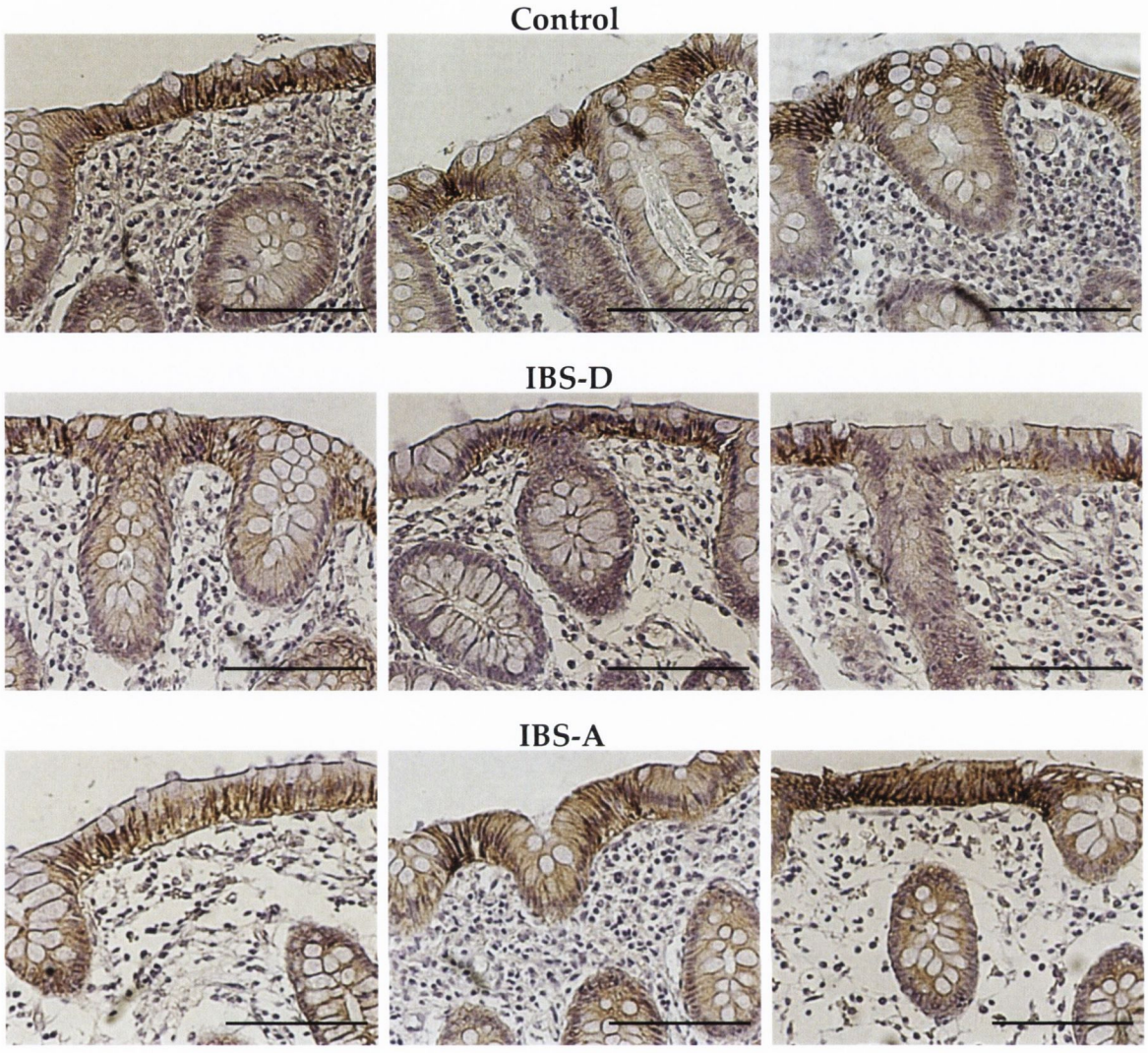


Figure 6.6 Representative photomicrographs showing E-cadherin expression in the surface epithelium of the caecal mucosa of controls, and IBS subtypes according to bowel predominance: IBS-D and IBS-A. Immunohistochemical staining for E-cadherin is shown in brown, while nuclei are stained blue. Bars=50 μm.

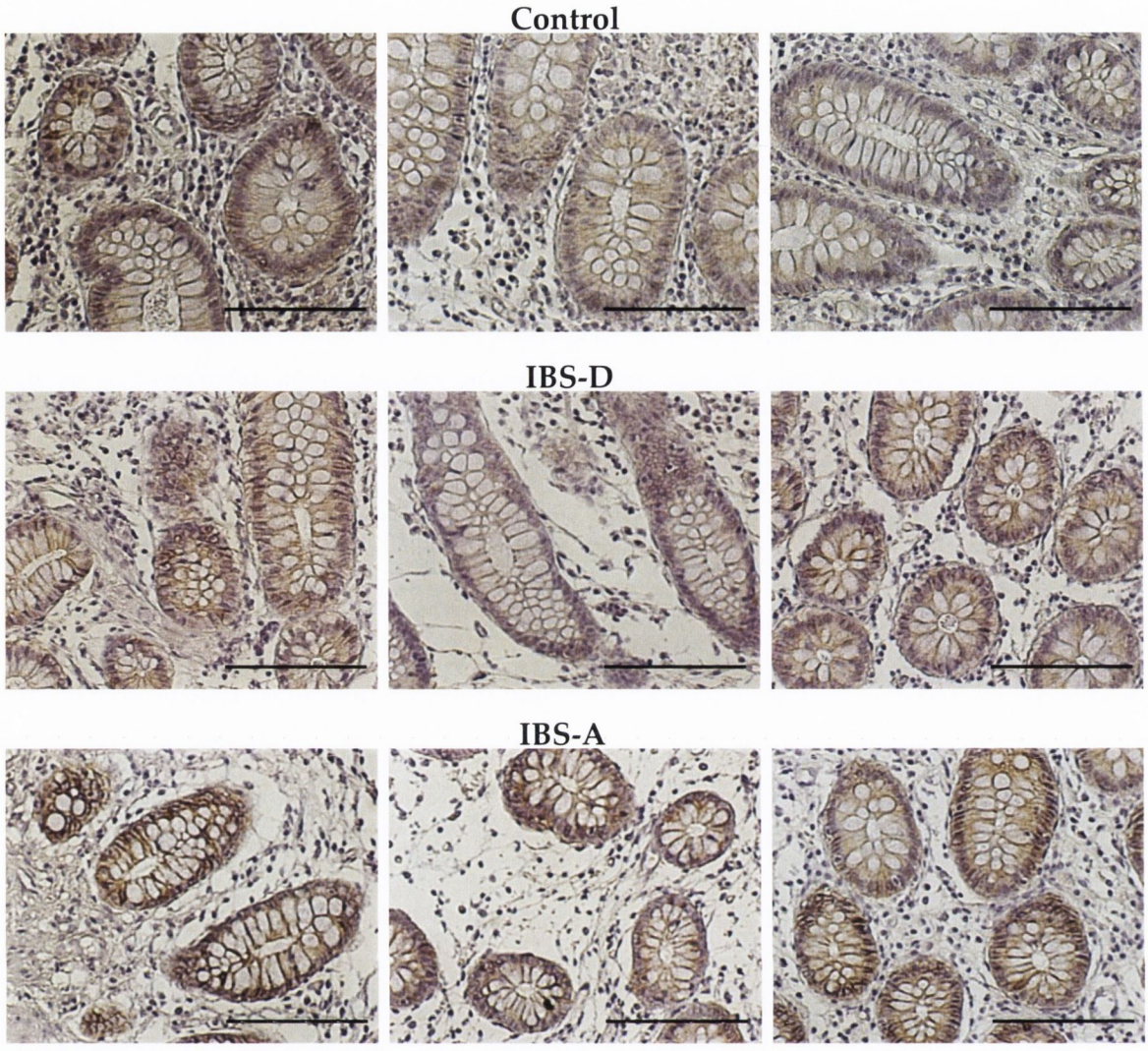


Figure 6.7 Representative photomicrographs showing E-cadherin expression in the crypt epithelium of caecal mucosa of controls, and IBS subtypes according to bowel predominance: IBS-D and IBS-A. Immunohistochemical staining for E-cadherin is shown in brown, while nuclei are stained blue. Bars=50 μm.

SURFACE EPITHELIUM

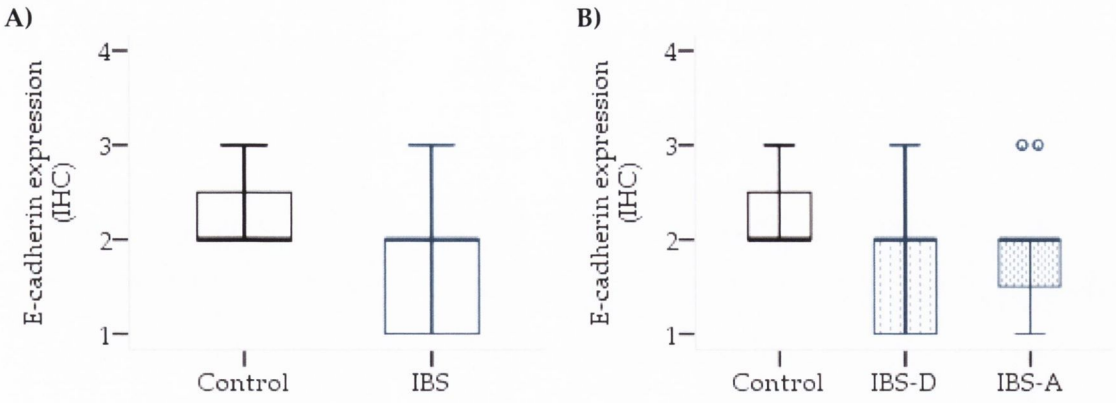


Figure 6.8 E-cadherin expression in the surface epithelium of the caecal mucosa of **A)** controls and IBS patients **B)** controls and IBS patients according to subtype: IBS-D and IBS-A. Values are represented as a median with interquartile ranges. IHC-immunohistochemistry. **B)** Kruskal-Wallis P=0.18.

E-cadherin expression - immunohistochemistry		
Region	**Surface epithelium**	**Crypts**
Group	Median (range), mean±SEM	
Control	2.0 (2-3), 2.2±0.1	2.0 (1-2), 1.8±0.1
IBS	2.0 (1-3), 1.8±0.1	2.0 (1-3), 2.0±0.1
v control	P=0.072	P=0.28
IBS-D	2.0 (1-3), 1.8±0.2	2.0 (1-3), 1.9±0.1
v control	P=0.071	P=0.52
IBS-A	2.0 (1-3), 1.9±0.2	2.0 (2-2), 2.0±0.0
v control	P=0.19	P=0.17
v IBS-D	P=0.71	P=0.31

Table 6.5 E-cadherin expression in the caecal mucosa of controls (n=12) and IBS patients (n=33). IBS patients are also shown according to subtypes: IBS-D (n=22) and IBS-A (n=11). E-cadherin expression was assessed semiquantitatively using a 4-grade scale. Intergroup comparisons were performed by using Mann-Whitney U test.

Statistical power analysis

TJ protein	E-cadherin IFF		E-cadherin IHC	
Region	**SE**	**crypts**	**SE**	**crypts**
Effect size (d)	0.71*	0.4	0.6	0.6
Statistical power	0.67*	0.32	0.54	0.54

Table 6.6 Statistical power analysis at the α=0.05 level for E-cadherin in a cohort of IBS and controls. Effect size was measured using Cohen's d. *P<0.05 Mann-Whitney U test. IFF, immunofluorescence; IHC, immunohistochemistry; SE, surface epithelium.

The statistical power was less than 0.8 (α error of 0.05) for E-cadherin expression in the surface epithelium to detect medium effect size (immunofluorescence d=0.71; immunohistochemistry d=0.6) when comparing differences between IBS patients and controls (Table 6.6). For immunofluorescence, to achieve a statistical power of 0.8, 8 more control patients would need to be recruited assuming that the effect size in the sample is equal to the effect size in the population.

6.3. Discussion

Data presented in this study support the evidence of impaired intestinal barrier in IBS patients and show that alterations in AJC are not only confined to TJs proteins but might also apply to AJs proteins. This study showed for the first time, to my knowledge, significantly lower E-cadherin expression in the caecal mucosa of IBS patients compared to controls and its clinical relevance as determined by associations with IBS symptoms.

The present study provides novel evidence for reduced expression of E-cadherin in the surface epithelium of the caecal mucosa of IBS patients compared to controls. The expression of this protein was significantly reduced in patients with IBS-A whereas in IBS-D patients there was a trend towards reduction. A similar reduction was shown in chapter 5 describing JAM-A and ZO-1, suggesting that the disruption of the epithelial barrier in IBS is irrespective of bowel habit. This is in line with a previous study where alterations in TJ protein expression were observed in the colonic mucosa of IBS patients either in IBS-D, IBS-A or IBS-C subtype (213). Interestingly, this finding demonstrates that the disruption of the epithelial barrier in IBS encompasses not only alterations in TJ proteins, but also significant alteration in AJs. This provides new evidence of potential AJs contribution to the pathogenesis of symptoms in IBS. While there is no published data on IBS to compare with, E-cadherin expression has been widely investigated in other conditions with increased permeability and appeared to be significantly reduced in the surface epithelium of mucosal tissue in patients with IBD (444) and in coeliac disease (447). Furthermore, down-regulation of E-cadherin in other diseases has been implicated in carcinogenesis, including colon (448) and breast cancer (449).

The strongest clinical data that came for E-cadherin in this study were the association of its expression in the caecal surface epithelium with duration of IBS symptoms. The reduction in E-cadherin expression significantly associated with the longer duration of symptoms in the alternating subtype of IBS. This result is in agreement with the data on JAM-A shown in chapter 5 and further supports the hypothesis that the disruption of intestinal epithelial barrier is secondary to the other pathological processes and this might be longstanding immune activation.

Another important finding was the association between E-cadherin expression and abdominal pain severity in IBS patients. The lower E-cadherin levels were found in IBS patients experiencing more severe abdominal pain. This result not only supports the finding of JAM-A association with abdominal pain severity but also extends previously published data linking alterations in TJ proteins with abdominal pain in IBS patients (212, 213). This study shows for the first time the significant association between disruption of AJs, as evidenced by the lower E-cadherin levels, and abdominal pain in IBS. Martínez *et al.* (344) also have shown association between alterations in the expression of junctional proteins and bowel movements or stool consistency; however, in this study no associations were found between E-cadherin expression and diarrhoea.

The mechanisms involved in E-cadherin alteration in IBS remain unclear. In the cell culture chapters E-cadherin expression seemed to be unaffected by mast cell mediators. Therefore, alternate mechanisms are likely to be involved in its down-regulation. A recent study by Villani *et al.* (211) investigated genetic risk factors for PI–IBS in the Walkerton community following outbreak of bacterial acute gastroenteritis and provided evidence that the SNP in the E-cadherin gene, CDH1, might contribute to the development of PI-IBS. This study identified CDH1 variant (rs16260; -C160A) that appeared to be more common in PI-IBS

patients than in controls (31% IBS v 26% controls) (211). Previously this variant was associated with reduced transcriptional efficiency (329). This allele variation might result in lower E-cadherin expression in AJs, promoting increased pathogenicity of invasive bacteria and subsequent barrier disruption and increased intestinal permeability. Importantly, in the following study investigating intestinal permeability, predominantly in the Walkerton residents who developed PI-IBS, significantly higher intestinal permeability was demonstrated 2 years after waterborne outbreak of gastroenteritis compared to controls (337). In the present IBS patient cohort, however, mechanisms other than genetics seem to be involved in E-cadherin down-regulation, since a significant reduction in E-cadherin expression was found only in the surface epithelium but not in crypts in IBS patients, which would be expected in the case of genetic determination.

Though this study was not designed to investigate associations with intestinal permeability, it is possible that reduced E-cadherin underlies mechanisms involved in intestinal barrier dysfunction in IBS. Interestingly, in experimental E-cadherin knockout mice, the complete loss of E-cadherin appeared to be lethal whereas in heterozygous mice (50%) normal TJs were formed as shown in TEMs (450). Therefore, it is likely that other mechanisms are also implicated in increased intestinal permeability in IBS. Villani *et al.* (211), besides variations in E-cadherin gene, identified SNPs belonging to the IL-6 gene and TLR9 gene. Furthermore, altered expression of several TLRs (TLR4, TLR5, TLR7, TLR8) have been recently shown in colonic biopsy tissue of IBS patients (319) Therefore, the mechanisms underlying impaired barrier function are likely more complex and might include immune activation (IL-6) and altered host-microbial interactions (TLRs). Interestingly, epithelial E-cadherin appears to be a target of some intestinal pathogens including *Bacteroides fragilis* (445) and *Candida albicans* (446), which found a way to disrupt AJs through the cleavage of E-cadherin *in vitro* that

associated with the increased permeability of intestinal epithelial cells (446). Furthermore, the invasive *Escherichia coli* strains isolated from IBD tissue replaced E-cadherin from AJC and subsequently disrupted barrier function of intestinal epithelial cells (451). In addition, in a subsequent study, strong correlation was found between deregulation of E-cadherin but also P-cadherin and the progression of the inflammation in the colon of IBD patients (452). Similar mechanisms are possibly involved in barrier disruption in IBS where pathogenic strains might modulate epithelial permeability through alterations of E-cadherin function in IBS. In fact, a recent study by Gecse *et al.* (307) has suggested that enhanced microbial proteolitic activity in faecal supernatants from IBS patients evoked visceral hypersensitivity in mice and barrier dysfunction *in vitro* through cytoskeleton contractions and alterations in TJs (307).

A tertiary aim of the present study was to investigate if significant changes found with immunofluorescence, which is a highly specific method, could be replicated with immunohistochemistry and, if so, could immunohistochemistry be a useful method to explore protein markers in IBS in more routine practice. In this study the significant changes for E-cadherin were found with immunofluorescence whereas routine processing for the caecal mucosa for immunohistochemical staining revealed no significant differences in E-cadherin expression between IBS patients and controls, though a trend towards reduction was shown. Consequently, as expected, no association with IBS symptoms was found. This might be due to higher-resolution microscopy performed with confocal immunofluorescence microscope. Based on this assumption, although immunohistochemistry is a desirable diagnostic method in a variety of diseases (e.g. breast cancer) (449) and is superior to immunofluorescence for tissue structure analysis (453), it might be advisable to perform immunofluorescence labelling, if possible, especially in studies where subtle changes between patients and controls are expected. However, the choice of primary antibodies might have

also been important for differences in E-cadherin expression observed with these two staining methods. Different clones of anti-E-cadherin antibodies were used for immunofluorescence and immunohistochemistry, and the latter was performed in an external commercial laboratory. Clone 36/E-Cadherin, which recognises the cytoplasmic domain of E-cadherin, was used in immunofluorescence analysis while clone HECD-1, which recognises extracellular domain near the amino terminus, was used in immunohistochemistry analysis. Therefore, different results for E-cadherin expression in this study might not truly reflect methodological differences between immunofluorescence and immunohistochemistry in general, but differences due to epitope antibody binding (454) and these points should be addressed in a future analysis.

A growing body of evidence suggests that disrupted intestinal epithelial barrier function might lead to the generation of IBS symptoms. This study supports this hypothesis and demonstrates that E-cadherin might be clinically relevant to the development of IBS, though its role in the maintenance of intestinal barrier integrity and the mechanisms behind its down-regulation in IBS still need to be defined. Based on emerging evidence, it is possible that the lower expression may be determined by genetic factors, the activity of pathogenic bacteria or both. It may be that the genetic variations in CDH1 increase the susceptibility to sustained barrier dysfunction following a gastrointestinal insult such as gastroenteritis. On the other hand, it might be that genetic variations in CDH1 do not affect E-cadherin expression in AJs and other factors such as gastrointestinal infection play a predominant role in surface E-cadherin down-regulation in IBS. In normal colonic epithelium, at the bottom of the crypts stem cells give rise to colonic epithelial cells which migrate towards the surface epithelium, where they are exfoliated to the lumen (455). Given that E-cadherin expression was reduced in the surface epithelium but not in the crypts, it seems that reduction in E-

cadherin occurs when migrating crypts epithelial cells reach the surface epithelium where luminal factors, perhaps infectious bacteria, affect its expression. In this study, however, IBS patients were not PI-IBS and this study was not designed to investigate these mechanisms. Therefore, in order to follow this hypothesis, further studies are needed including the analysis of E-cadherin in PI-IBS cohort in comparison to non-PI-IBS, a genetic association study and the measurement of E-cadherin mRNA levels.

To sum up, the study assessed for the first time E-cadherin expression in IBS patients. Its expression in the caecal mucosa was reduced in both IBS subtypes, but to a larger extent in IBS-A. In addition, reduced E-cadherin expression was associated with longer duration of IBS symptoms and more severe abdominal pain, providing the clinical relevance of these alterations and a potential new target to explore for therapeutic intervention in IBS.

Key findings

1. E-cadherin expression was significantly lower in the surface epithelium of the caecal mucosa of IBS patients compared to controls. A significant reduction of E-cadherin was found in the IBS-A subtype whereas in IBS-D there was a trend towards reduced expression compared to controls.

2. Lower E-cadherin expression was significantly associated with more severe abdominal pain in IBS patients.

3. In the IBS-A subtype, reduced E-cadherin expression was significantly associated with longer duration of IBS symptoms.

4. The significant reduction in E-cadherin expression shown by immunofluorescence and its association with IBS symptoms was not detected by immunohistochemistry.

Supplementary materials

Gender/Age and E-cadherin expression				
Method	**IFF**		**IHC**	
Region	**SE**	**crypts**	**SE**	**crypts**
Gender	P=0.80	P=0.41	P=0.41	P=0.53
Age	P=0.73	P=0.52	P=0.40	P=0.74

Supplementary Table 6.1 Potential age and gender effects on E-cadherin expression in the caecal mucosa controlled for in the analysis using a general linear model in the disease group (control, IBS). SE, surface epithelium; IFF, immunofluorescence; IHC, immunohistochemistry.

Age and E-cadherin expression					
Method	**Region**	**Spearman correlation**		**J-T test**	
		r_S value	P value	r_J value	P value
IFF	**SE**	-0.07	0.82	-0.06	0.81
	crypts	0.14	0.67	0.07	0.76
IHC	**SE**	0.17	0.60	0.14	0.58
	crypts	-0.19	0.54	-0.17	0.52

Supplementary Table 6.2 Expression of E-cadherin in the caecal mucosa in the control group according to age (years) of patients at the time of biopsy. Statistical analysis was performed by using Spearman correlation and J-T test. SE, surface epithelium; IFF, immunofluorescence; IHC, immunohistochemistry.

Region	Gender		Age at the time of biopsy (years)	
	Males (n=7)	Females (n=5)		
	Median (range), mean±SEM		26±2.7	51.6±4.4
SE IFF	4 (3-4), 3.4±0.2	3 (3-4), 3.4±0.2	3.5 (3-4), 3.2±0.2	3.5 (3-4), 3.5±0.2
	P=0.92		P=0.43	
Crypts IFF	3 (2-4), 3.0±0.2	3 (2-4), 3.0±0.4	3.0 (3-3), 3.0±0.0	3.0 (2-4), 3.0±0.3
	P=1.00		P=1.00	
SE IHC	2 (2-3), 2.1±0.1	2 (2-3), 2.4±0.2	2.0 (2-2), 2.0±0.0	2.0 (2-3), 2.4±0.2
	P=0.33		P=0.18	
Crypts IHC	2 (1-2), 1.7±0.2	2 (2-2), 2.0±0.0	2.0 (1-2), 1.7±0.2	2.0 (1-2), 1.9±0.1
	P=0.21		P=0.60	

Supplementary Table 6.3 Analysis of E-cadherin expression in the caecal mucosa in the control according to gender and age (years) of patients at the time of biopsy < 32 years (the average age of IBS) and ≥ 32 years and resultant TJ protein expression (grade). The expression was assessed semiquantitatively using a 4-grade scale. Data were analysed with Mann-Whitney U test. SE, surface epithelium; IFF, immunofluorescence; IHC, immunohistochemistry.

Group	E-cadherin expression in crypts					
	Abdominal pain severity (scale1-4)		Diarrhoea (No. of liquid stools/week)		Duration of symptoms (years)	
	r_s value	P value	r_s value	P value	r_s value	P value
IBS	-0.18	0.31	-0.15	0.44	-0.25	0.17
IBS-D	-0.22	0.33	-0.25	0.29	-0.23	0.31
IBS-A	-0.08	0.82	-0.08	0.82	-0.27	0.42

Supplementary Table 6.4 Correlation between E-cadherin expression in the crypt epithelium of the caecal mucosa of IBS and IBS symptoms/duration of IBS symptoms. Data were correlated by using Spearman correlation.

Group	E-cadherin expression in crypts					
	Abdominal pain severity (scale1-4)		Diarrhoea (No. of liquid stools/week)		Duration of symptoms (years)	
	r_J value	P value	r_s value	P value	r_s value	P value
IBS	-0.16	0.30	-0.12	0.43	-0.19	0.19
IBS-D	-0.19	0.32	-0.20	0.26	-0.16	0.35
IBS-A	-0.08	0.80	-0.09	0.78	-0.24	0.39

Supplementary Table 6.5 Association of E-cadherin expression in the crypt epithelium of the caecal mucosa of IBS with IBS symptoms/duration of IBS symptoms. Statistical analysis was performed by using J-T test.

CHAPTER 7

GENERAL DISCUSSION, FUTURE STUDIES AND CONCLUSION

7.1. General discussion

An increase in mast cell numbers (72, 86, 144, 259, 264, 266) and an increase in intestinal permeability (148, 212, 335, 337, 339) have been independently implicated in the pathogenesis of IBS. There are emerging data that tryptase, released from mast cells, may alter intestinal epithelial permeability (57, 176), however, the mechanisms involved and the potential translation to IBS have not been fully elucidated. Previously, it was shown that mast cell tryptase activates and cleaves PAR-2 receptors (168) which are expressed both on the apical and basolateral membrane of the intestinal epithelial cells (170). The general objective of this thesis was to investigate the mechanisms that underlie increased intestinal epithelial permeability in IBS patients. This study hypothesised that tryptase released from mast cells may disrupt intestinal epithelial integrity and alter the expression of junctional proteins, and that these changes might translate to IBS. To address this hypothesis, the mechanisms underlying epithelial barrier disruption were investigated using Caco-2 and Caco-2/HMC-1 cell models, and the most interesting and novel findings were followed up in the analysis of junctional protein expression in caecal IBS tissue and compared to controls.

The findings from this thesis showed that tryptase and compound 48/80-stimulated mast cells significantly increased intestinal epithelial permeability, disrupted epithelial integrity and decreased the expression of junctional proteins JAM-A, CLD-1 and ZO-1 *in vitro*. Inhibition of mast cell tryptase with NM (391) mitigated the effect of mast cell stimulation on epithelial integrity through reduced expression of JAM-A protein, confirming the role of mast cell tryptase in epithelial barrier dysfunction. Reduced JAM-A was a novel interesting finding and was pursued in tissue from IBS patients. A significant decrease in JAM-A epithelial levels was shown in the caecal mucosa of IBS patients compared with controls; this finding appeared to be clinically relevant as it significantly

associated with abdominal pain severity in the IBS-alternating subgroup. In this thesis, TJ proteins CLD-1 and ZO-1 were also further investigated in IBS tissue. A significant decrease in ZO-1 and a trend for reduced expression of CLD-1 was observed in the surface epithelium of the caecal mucosa of IBS patients as compared with controls. Besides TJ proteins, this study also investigated the expression of the AJ protein E-cadherin in IBS tissue. Though mast cell mediators did not affect its expression *in vitro,* recent evidence has linked a SNP in the E-cadherin gene to the pathogenesis of PI-IBS (211) and, therefore, it seemed interesting to follow up its expression in IBS tissue. E-cadherin expression appeared to be significantly reduced in the surface epithelium of the caecal mucosa of IBS patients compared with controls. Moreover, comparably to JAM-A, the expression of E-cadherin in caecal tissue significantly associated with IBS symptoms, providing evidence of its clinical relevance to IBS. This study is the first reporting of JAM-A and E-cadherin expression in IBS. Given that mast cell mediators decreased JAM-A, ZO-1 and CLD-1 expression *in vitro,* the thesis data suggest that clinical manifestations of a subset of IBS patients may be linked to mast cell-related impairment of caecal TJ function. Furthermore, it might be postulated that JAM-A is important in IBS pathogenesis through mast cell tryptase-dependent mechanisms. In contrast, reduced E-cadherin expression appears to be regulated by non-mast cell mediated mechanisms and, based on the emerging evidence, these might relate to polymorphisms in the E-cadherin gene (211), to pathogen activity (445, 446), or a combination of both (Figure 7.1).

The early studies in this thesis reported in chapter 3 focused on tryptase, an important mast cell protease. Tryptase has been associated with increased intestinal permeability both in intestinal cell lines (57) and in IBS (176). Furthermore, increased levels have been reported in the gut of IBS patients compared to controls (175, 228, 285). The effects of tryptase on epithelial integrity were investigated in a cell culture model of intestinal epithelial cells, Caco-2 cells.

The results, presented in chapter 3, showed that tryptase significantly disrupted the integrity of the epithelial barrier as evidenced by a drop in TER and increased permeability to FITC-dextran within 24 hours. These changes were associated with significantly reduced expression of TJ proteins JAM-A, CLD-1 and ZO-1. Noteworthy, this study is the first to demonstrate that tryptase alters the expression of TJ proteins, and extends previous data showing alteration in TJ protein expression via PAR-2 activation (57). Based on this evidence, tryptase appears to regulate intestinal epithelial integrity through the disintegration of junctional complexes, and it might be postulated that tryptase is a potential regulator of barrier integrity that would translate to IBS.

Mast cells are the major producers of tryptase in the gut (456). In IBS, mast cell numbers have been shown to correlate with mucosal tryptase content (144, 175). Having shown in chapter 3 that tryptase altered intestinal permeability and TJs *in vitro*, the second aim of the thesis, therefore, was to investigate if mast cell mediators, and in particular if mast cell tryptase, affect the integrity of the epithelial barrier. In order to explore the mechanisms of epithelial barrier disruption in response to mast cell tryptase a novel co-culture model of epithelial and mast cells, Caco-2/HMC-1, was developed. Epithelial cells maintained their integrity when co-cultured with underlying mast cells for approximately 3 weeks, as evidenced by the tightness of apical junctional complex when assessed by TEM. However, TJ integrity of the Caco-2 cells was lost when mast cells were stimulated with compound 48/80 and incubated for up to 24 hours following mast cell stimulation. The deteriorating effect of mast cell stimulation on the epithelial barrier was also confirmed by a significant drop in TER and an increase in permeability to FITC-dextran, and the mechanisms involved a reduction in JAM-A, CLD-1 and ZO-1. This study further confirmed the role of mast cell tryptase in the disruption of epithelial integrity by showing that specific mast cell

tryptase inhibition (378, 391) diminished the effect of mast cell stimulation on the epithelial barrier and on JAM-A expression.

While the key consistent finding across the *in vitro* studies was the reduced JAM-A expression and its link with tryptase, initially identified using apically applied tryptase in the Caco-2 model and further confirmed in the more relevant co-culture model and in inhibition studies, the results from other junctional proteins, for example CLD-1 and ZO-1, were also of interest. CLD-1 was consistently significantly decreased at 24 hours, both in Caco-2 monolayers in response to tryptase and in response to stimulated mast cells in the Caco-2/HMC-1 model, and was accompanied by increased permeability. But in contrast to JAM-A, tryptase inhibition did not alter CLD-1 expression in the co-culture model - thus suggesting that mast cell mediators other than tryptase (TNF-α and IFN-γ) may play a predominant role in the maintenance of its lower expression (352, 353). In a study by Jacob *et al.* (57) no apparent change in CLD-1 was observed in responses to PAR-2 agonists in intestinal epithelial cells. The findings for ZO-1 protein show that its levels in Caco-2 cells were significantly decreased after 24 hours of tryptase exposure and were accompanied by an increase in epithelial permeability, in agreement with others (57). These data may fit with reports of down-regulation of ZO-1 mRNA in Caco-2 cells incubated 48 hours with supernatants from colonic biopsy tissue (212). In the Caco-2/HMC-1 model, however, initial decrease in ZO-1 was observed for up to 4 hours after mast cell stimulation with compound 48/80, but this decrease was not apparent at 24 hours. It may be that ZO-1 down-regulation in IBS is maintained by other non-mast cell related factors.

The next step was to investigate if the findings from the cell culture models might be translated to IBS, in particular the reduction in JAM-A protein. The unique finding highlighted in chapter 5 was the reduced expression of JAM-A protein in

the caecal mucosa of IBS patients, and its consistent reduction in both IBS-D and IBS-A subtypes compared with controls. The clinical relevance of reduced JAM-A expression was confirmed by the significant association with abdominal pain severity and longer duration of IBS symptoms in the IBS-A subtype. Furthermore, reduced JAM-A expression in IBS patients was in agreement with the cell culture findings suggesting that mast cell tryptase might be involved in the mechanisms underlying JAM-A down-regulation in IBS patients. Since this study is the first to show JAM-A expression in IBS tissue, there were no published data to directly compare with. In IBD patients, however, lower JAM-A protein expression has been reported (53, 429) in agreement with these findings. In contrast, changes in CLD-1 and ZO-1 have been published in IBS (213, 285) (Section 1.2.5.2). In this study, the expression of both CLD-1 and ZO-1 appeared to be reduced in the surface epithelium of the caecal mucosa of IBS patients compared to controls in agreement with the published data (213, 285). Looking at the IBS subtypes, ZO-1 was significantly reduced both in IBS-D and IBS-A subtypes, whereas for CLD-1 a trend towards reduced levels was found only in IBS-A patients.

The objective of the final result chapter was to investigate the expression of AJ protein E-cadherin in the caecal mucosa of IBS patients. E-cadherin levels, in contrast to JAM-A, CLD-1 and ZO-1, remained unchanged upon tryptase treatment in the Caco-2 cell model or compound 48/80-mediated mast cell stimulation in the co-culture model. Other mechanisms, however, may be implicated in the modulation of E-cadherin expression in IBS, given the recent genetic evidence where a SNP in the E-cadherin gene reported by Villani *et al.* (211) was associated with the susceptibility to develop PI-IBS and where pathogens appeared to disrupt epithelial integrity through alteration in E-cadherin expression in AJs (445, 446, 451) (Figure 7.1). To my knowledge, the expression of E-cadherin in biopsy tissue has not been studied in IBS to date. Therefore, a novel finding of this thesis was to show that E-cadherin was

significantly reduced in the caecal mucosa of IBS patients compared to controls. Also, E-cadherin epithelial levels were lower in the IBS-A subtype. Comparable to JAM-A findings, lower E-cadherin levels were significantly associated with the severity of abdominal pain and longer duration of IBS symptoms. The mechanisms that underlie a reduction in E-cadherin expression in IBS have not been investigated in this study and should be addressed in a future analysis. A previous history of acute gastroenteritis should be recorded on IBS patients to explore the potential association with E-cadherin expression, followed up by a genetic association study and E-cadherin mRNA analysis in the mucosal biopsy tissue. Furthermore, given that both gut enteritis and antibiotic therapy appear to alter gut microbiota (304), the properties of microbiota to protect a host against pathogenic bacteria activity may be impaired and result in uncontrolled growth of gut pathogens in susceptible individuals. Therefore, potential pathogenic strains associated with mucosal tissue of IBS patients should be addressed in a future analysis.

The findings in this study show that the reduction in both JAM-A and E-cadherin appeared to be clinically relevant and significantly associated with the severity of abdominal pain in IBS patients and with longer duration of symptoms, a parameter that may be a marker of chronic symptoms instead of new onset disease. Thus, the reduction in JAM-A and E-cadherin might reflect longstanding and more severe disease. While there are no data on JAM-A or E-cadherin published to compare with, the other junctional proteins (CLD-1, occludin, ZO-1 mRNA) have been associated with the severity of abdominal pain (212, 213) and disease initiation (213). Taken together, this suggests that ZO-1 and CLD-1 are involved in the initiation of symptoms in subsets of IBS patients while JAM-A and E-cadherin may be markers of more severe and long lasting disease. In this study, however, no associations were found for either ZO-1 or CLD-1 proteins but for JAM-A and E-cadherin. Since the expression of both JAM-A and E-

cadherin associated with IBS symptoms, it led to the investigation of a correlation between the expressions of both proteins; however, none was found either for the surface or crypt epithelium. This finding further confirms that the expression of JAM-A and E-cadherin protein is most likely regulated by different mechanisms in IBS.

In addition, while this study predominantly explored associations between the expression of junctional proteins and clinical data, the pathological factors might be more informative for the pathophysiology of IBS. IBS is characterised by the absence of gross organic changes, and caecal biopsy tissue from both IBS patients and controls in this study were confirmed by pathologist to have macroscopically and histologically normal mucosa. Nevertheless, exploration of associations with factors such as volume of intestinal mucosa, whose thickening is associated with inflammatory processes, or density of mucin-producing goblet cells could be considered in a future analysis. Intestinal mucosal thickening is found in IBD (457) and, while these changes are not typically reported for IBS, perhaps some trend of lesser magnitude could be identified. In addition, it appears that alterations in mucin synthesis/secretion or the number of goblet cell might be involved in the pathogenesis of intestinal diseases (92, 458). In IBS, for example, increased expression of genes involved in mucins production was identified (92). These changes could be explored with immunohistochemistry, which is superior to immunofluorescence for tissue structure analysis.

The cell culture studies provided evidence that JAM-A, CLD-1 and ZO-1 expression was reduced in response either to tryptase or to compound 48/80-stimulated mast cells. Furthermore, changes in these TJ proteins were identified in IBS tissue. Data on mucosal mast cell numbers were available in the IBS database and this gave the opportunity to explore the association between mast cell counts and the expression of TJ proteins in caecal IBS tissue. However, no

association was found in this study. Noteworthy, the analysis was confined to mast cell numbers and activation of mast cells, rather than their quantity, may be associated with the expression of TJ proteins, which has been evidenced in a recent study (285). Therefore, determination of the status of mast cell activation as evidenced by the measurement of tryptase content in biopsy tissue (144, 175, 344) or by ultrastructural features of degranulation with TEM (144) may add additional insight into the mechanisms underlying changes in TJ protein expression in IBS.

This thesis investigated the mechanisms underlying the impaired intestinal epithelial barrier in IBS, and focussed mainly on the effects of mast cell mediators on epithelial integrity *in vitro* and on translation of these findings to IBS. In summary, the findings of this thesis extend previous data on impaired intestinal barrier in IBS by demonstrating 1) novel JAM-A and E-cadherin expression in IBS tissue and its significant reduction in the caecal mucosa of IBS patients with severe abdominal pain and longer duration of IBS symptoms 2) lower ZO-1 and CLD-1 expression in the caecal mucosa of IBS tissue in agreement with previous findings in jejunal (285), ileal (346) and colonic (212, 213, 346) IBS tissue 3) that clinical manifestation of the reduced expression of junctional proteins, based on findings from the cell culture models, may be determined by mast cell-related mechanisms (Figure 7.1).

Mechanisms by which lower expression of junctional proteins may contribute to IBS symptoms are unclear. Perhaps, reduced levels of junctional proteins lead to increased intestinal permeability in IBS patients, and subsequently to local gastrointestinal dysfunction and symptoms. Enhanced exposure of mucosa to noxious stimuli might activate mucosal immune system and nerve sensitisation resulting in pain. Piche *et al.* (212) reported a correlation between paracellular permeability and pain scores of IBS patients and suggested the involvement of

soluble mediators released from colonic biopsy tissue. These might include mast cell mediators since histamine, tryptase but also serotonin appeared to sensitise human submucosal neurons (112). Furthermore, severity of abdominal pain associated with the proximity of activated mast cells to colonic nerves in IBS patients (112, 144). The factors that regulate these close contacts between mast cells and nerves are poorly explored, but might include NGF produced by mast cells or neuropeptides produced by nerves (108). In addition, faecal supernatants from IBS-D patients induced visceral hypersensitivity in murine colon and increased intestinal permeability *in vitro* (307). The chemical nature of these supernatants still needs to be elucidated, but possible candidates that mediate these changes include bile acids (369, 459) and serine proteases (307) which were increased in stools of IBS-D patients, and the latter were postulated to be of microbial origin. This is in line with evidence of alteration in gut microflora in IBS patients. Deregulated immune responses to intestinal commensal and pathogenic bacteria are also associated with IBD pathogenesis (460-462). Bacteria might activate immune cells e.g. mast cells through activation of TLRs to promote release of pro-inflammatory cytokines for recruitment and activation of immune cells. During infection cytokines might impact epithelial function and increase exposure of mucosa to bacteria. This might sustain cytokine production and contribute to chronic inflammation resulting in IBS or IBD (402). Damage of intestinal epithelium might also result in loss of containment of commensal bacteria, which is associated with the pathogenesis of IBD (463). The "leaky gut" permits bacteria from the gut lumen to gain access to the submucosal compartments or even systemic circulation, and renders potential risk of sepsis (464). Furthermore, mast cells appear to be involved in intestinal ion secretion (122), and increased ion secretion might lead to diarrhoea (465). It has been reported that mast cell stimulation resulted in ion secretion in human biopsy tissue, which involved mediators such as histamine and eicosanoids, and nerves (122-124). In addition, several mast cell pro-inflammatory cytokines such as IL-1,

IL-3 and TNF-α have been involved in stimulation of intestinal epithelial ion secretion (402). Taken together, the mechanisms underlying barrier dysfunction in IBS and inflammatory related gastrointestinal diseases are complex and while this thesis presents the potential role of mast cell mediators, and particularly tryptase, they are likely to be modified by other mast cell, immune and clinical factors. Also, the mechanisms that may account for mast cell activation remain to be fully characterised but, based on the emerging evidence, the candidates that might be involved include microbial products (13), CRF (466) and IgE (467), and gonadal hormones (261, 262) (Figure 7.1).

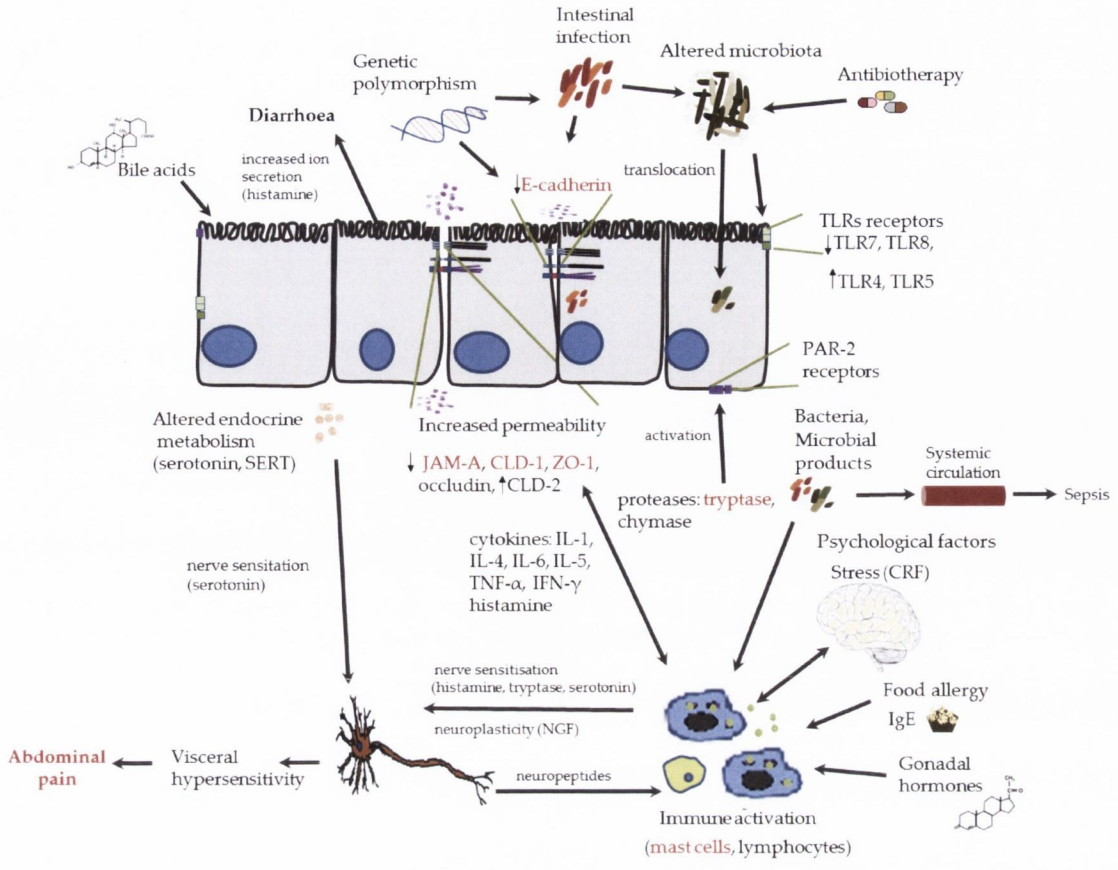


Figure 7.1 Conceptual model for barrier dysfunction in the pathophysiology of IBS. This thesis complements the previous evidence on barrier dysfunction (in red) by showing that the clinical manifestation of IBS symptoms (abdominal pain) may be related to mast cell–dependent (e.g. tryptase activity) enhanced epithelial permeability through reduced expression of TJ proteins JAM-A, ZO-1 and CLD-1. Reduction in E-cadherin levels might relate to a polymorphism in the E-cadherin gene or to pathogen activity, or a combination of both.

Key findings

1. Tryptase alone, or released from compound 48/80-stimulated mast cells, significantly reduced intestinal epithelial integrity and the expression of junctional proteins JAM-A, ZO-1 and CLD-1 *in vitro*.

2. Inhibition of tryptase attenuated the effect of mast cell stimulation with compound 48/80 on epithelial integrity and on JAM-A.

3. This is the first reporting of a significant reduction in JAM-A in response to mast cell tryptase and of significantly lower epithelial JAM-A expression in IBS tissue compared to controls.

4. Epithelial ZO-1 expression was significantly reduced and there was a trend towards reduction for epithelial CLD-1 expression in IBS tissue compared to controls, in agreement with previously published data.

5. This is the first reporting of disrupted AJs in IBS as evidenced by significantly lower epithelial E-cadherin levels in IBS tissue compared to controls.

6. JAM-A and E-cadherin appear to be clinically relevant as their expression in IBS patients was associated with more severe abdominal pain and longstanding symptoms.

7. A better understanding of these mechanisms *in vitro* and *in vivo* may improve understanding of IBS pathogenesis and therapeutic strategies targeted at either mast cells or intestinal permeability.

Critical analysis and discussion of key findings

1. This study reports that tryptase alone, or released from stimulated mast cells, significantly altered expression of TJ proteins JAM-A, ZO-1 and CLD-1 as shown by western blotting and confirmed by immunofluorescence microscopy. These changes in junctional protein expression were associated with disrupted intestinal epithelial integrity. However, whether the changes in the expression of junctional proteins directly contributed to reduced barrier integrity was not confirmed here. It is likely that alterations in AJC are more complex. Laukoetter *et al.* (54) showed that JAM-A$^{-/-}$ mice exhibited increased permeability and increased expression of CLD-10 and CLD-15. The authors suggested that JAM-A mediates changes in claudin expression, which determines epithelial permeability. In line with this, other junctional protein, not studied here, might be involved in the mechanisms underlying reduced barrier integrity. Noteworthy, changes not detectable by either western blotting or immunofluorescence microscopy, including altered protein-protein interactions in AJCs, may still play a role in intestinal epithelial barrier dysfunction in this study.

2. In this study HMC-1 cells were stimulated with compound 48/80 to induce degranulation. Mast cells stimulation disrupted integrity of Caco-2 monolayers; however, mast cell degranulation was not validated in this study. Previously it was reported that HMC-1 exposed to compound 48/80 degranulated as evidenced by measurement of released tryptase (57). In this study, NM was used to inhibit tryptase and thus to elucidate its effects on the intestinal epithelial barrier following mast cell stimulation. NM was used at low concentrations which were previously reported to be tryptase specific; however specificity of NM for tryptase was not investigated in the present study. Others showed that at higher concentrations NM inhibited a number of enzymes including trypsin, thrombin, plasmin and kallikrein, and complement components (378, 392). There might be still other unknown mediators that are inhibited by NM and perhaps when it is

used at concentrations that for now are known to be tryptase specific. For example, to my knowledge, the effects of NM on other mast cell proteases such as chymotrypsin, carboxypeptidase A or cathepsins have not been investigated to date. This needs to be clarified in a future analysis. Of note, while HMC-1 appear to not express chymotrypsin and carboxypeptidase A these should be taken into account in tryptase inhibition studies when using other mast cell lines or primary mast cell cultures.

Furthermore, tryptase might act synergically with other mast cell mediators. For example, synergy was shown between mast cell mediators TNF-α and histamine to stimulate ion secretion in intestinal epithelium (111). Histamine and tryptase, however, did not synergise to increase microvascular permeability in guinea pigs (468). Nevertheless, synergy between tryptase and other mast cell mediators is possible. Therefore, the diminished effects of mast cell stimulation on intestinal integrity observed in the presence of NM might have involved more mediators than tryptase.

3. This study reports reduced expression in TJ proteins JAM-A, ZO-1 and CLD-1 following exposure to tryptase or mast cell stimulation in cell culture models. Reduced expression of TJ proteins was further confirmed in caecal surface epithelium of IBS patients when compared to controls, however the association with mast cells was not identified. This might be due to mast cell staining method and quantification. Data on the status of mast cell was not available in this study; also the level of mast cell heterogeneity in biopsy tissue was not reported. While this thesis focused on the potential role of mast cell mediators in the regulation of junctional integrity in IBS, other immune cells and their mediators might also be involved in the disruption of intestinal epithelial barrier. These cells include T-cells, macrophages, eosinophils and dendritic cells. However, apart from T-cells,

the numbers of these immune cells has not been explored in IBS or available data are inconsistent (4, 72).

Mast cells appear to be one of the main sources of secreted proteases in human body and, while this study focused on the potential role of tryptase in the regulation of intestinal epithelial barrier function in IBS, intestinal microflora might be also a potential source of proteases (469). A study by Gecse *et al.* (307) reported that enhanced protease activity in faecal supernatants from IBS patients was neither of mast cell nor pancreatic origin. This is supported by the fact that bacteria are efficient source of proteases, and protease producing bacteria have been reported to signal to mammalian cells by PAR-2 activation, leading to progression of periodontal disease (470).

4. This study identified alterations in TJ proteins JAM-A, CLD-1 and ZO-1 in response to stimulation of mast cells in the cell culture *in vitro* models, while E-cadherin levels were comparable to controls. E-cadherin expression was further pursued in caecal biopsy tissue of IBS patients, and its levels appeared to be significantly lower in the surface epithelium compared to controls. Cell culture findings suggest that non-mast cell dependent mechanisms are involved in its down-regulation in IBS. However, the cell culture models do not reflect the complexity and chronic nature of IBS. Mast cells might still affect E-cadherin expression, thought the effects are likely to be more complex and perhaps less dramatic than for TJ proteins. Possibly, to identify E-cadherin associations with mast cells, incubation time with stimulated mast cells in the co-culture should be extended from 24 to 48 hours. E-cadherin down-regulation is associated with inflammatory processes and its loss has been observed in many forms of colitis (66, 441, 443). Recently an association between reduced E-cadherin expression and mast cell counts was identified in the duodenal mucosa from patients with functional dyspepsia (471). Furthermore, IFN-γ was reported to induce E-

cadherin redistribution from AJs in colonic epithelial cells (472). Possibly, concentration of IFN-γ, which is released from activated mast cells (110, 473) was not sufficient to alter E-cadherin expression in the given time-frame of 24 hours.

5. In the present study, the association with abdominal pain severity and IBS symptom duration was noted for JAM-A and E-cadherin only and was not demonstrated for either ZO-1 or CLD-1. Interestingly, association between longstanding IBS symptoms and expression of either JAM-A and E-cadherin was consistently identified in the IBS-A subtype only. These proteins may be mechanistically involved in maintaining epithelial barrier disruption over time or be surrogate markers of chronic disease. Interestingly, the disease duration in the IBS-A group was longer than that of IBS-D, but not significantly so, and larger studies would be required to independently tease out the effects of IBS subtypes and symptom duration on junctional proteins. This might be particularly important to understand mechanisms for E-cadherin since a polymorphism in E-cadherin gene was identified in PI-IBS, and this subtype tends to be similar to IBS-D (206) and therefore changes in E-cadherin might be expected to translate to an IBS-D cohort. Possibly, correlation of E-cadherin expression with pathological data such as volume of intestinal mucosa or goblet cell numbers would help to understand differences between IBS subtypes and this should be addressed in a future analysis.

7.2. Future studies

Cell models

Permeability studies in the Caco-2/HMC-1 model: Several techniques have been employed to study intestinal epithelial permeability and mechanisms behind its alteration. In this study a novel co-culture model of epithelial cells and mast cells, Caco-2/HMC-1, was developed to study the mechanisms underlying increased epithelial permeability and its potential translation to IBS; the results that were obtained from this model were interesting and reproducible. Therefore, this model might be a useful tool for mechanistic studies of epithelial and mast cell pathologies. This model extends the study performed by Jacob *et al.* (57) that investigated paracellular permeability of intestinal epithelial cells either exposed to mast cells supernatants for 24 hours or following degranulation of short-term co-cultured mast cells. The co-culture model developed for this study appears to be a more robust model for IBS, since it allows monitoring the interaction between both cell lines throughout the co-culture and thus is more likely to better reflect the biology of IBS. This model may be useful in the provision of new molecular insights in the pathophysiology of IBS complementing other models/techniques.

In IBS, predominantly lactulose/mannitol and ^{51}Cr-EDTA tests have been used (148). However, routine testing for intestinal permeability in IBS may be confounded by other non-disease factors, such as drugs, smoking and alcohol, which alter gut permeability (335, 474, 475). Animal models of IBS, predominantly based on the induction of a numbers of stressors, have provided mechanistic insights on various aspects of IBS pathophysiology (233, 235, 240-242). For example, in the context of gastrointestinal barrier function, high anxiety Wistar-Kyoto rats that experienced crowded stress exhibited increased epithelial permeability associated with mast cell activity (235). Other studies explored the

effects of biopsy extracts or faecal supernatants from IBS patients when applied to Caco-2 monolayers or animal tissue (212, 307). The measurement of intestinal permeability of mucosal tissue in Ussing chambers also provides evidence of increased permeability in IBS (176, 212). However, tissue viability may decrease as early as 1.5 to 4 hours (476-478) in contrast to transformed intestinal epithelial cell lines that offer an opportunity to investigate longer-term effects in controlled conditions and complement human and animal model studies.

This thesis focused predominantly on the role of mast cell tryptase in the regulation of epithelial barrier integrity; however, an array of mediators is released from compound 48/80-stimulated mast cells and research into their potential effects on epithelial barrier function should be addressed in future studies. These mediators include cytokines, such as TNF-α, IFN-γ, 1L-1β and IL-6 (71), whose levels appear to be altered in IBS and whose activity has been associated with epithelial barrier dysfunction *in vitro* (Section 1.2.6.2). Specific inhibition studies could be employed to elucidate their potential effects on the epithelial barrier, similar to the inhibition studies performed in this thesis (Section 2.2.6).

This co-culture model also enables investigation of the mutual interaction between undegranulated mast cells and epithelial cells throughout the co-culture. An interesting observation from this model was that unstimulated mast cells co-cultured for approximately 3 weeks with epithelial cells enhanced epithelial integrity as determined by the measurement of TER and permeability to FITC-dextran (Figure 4.3). Therefore, further studies might attempt to explore the mechanisms that underlie these alterations, such as phosphorylation in the TJ assembly mediated by the selective activity of protein kinases such as PKC (363, 424) (Section 4.3).

Extension of the Caco-2/HMC-1 model to Caco-2/human intestinal mast cell model: HMC-1 cells lack some features of human intestinal mast cells, for example they do not express the surface FcεRI receptor that is activated in allergic reactions (385). Therefore, the Caco-2/HMC-1 model could be further extended to a co-culture of Caco-2 with primary intestinal mucosal mast cells. Primary mast cells might better reflect conditions *in vivo* and contribute to a better understanding of the pathophysiology and other gastrointestinal diseases such as food allergies, and provide further new disease biomarkers and targets for IBS treatment. The culture of primary cells, however, also has its weaknesses. For example, primary mast cells 1) derived from different patients can behave differently in culture conditions depending on the individual characteristics 2) proliferate more slowly than cell line 3) may be contaminated with fibroblasts. Therefore, it might be advisable to extend the newly-defined mechanisms in HMC-1 cells to primary mast cell cultures.

Translational studies

Clinical evaluation: In this study the novel findings from the co-culture model, such as JAM-A down-regulation, were further confirmed in IBS setting. Further experiments are required to confirm the junctional protein alterations in larger patient cohorts, especially to explore differences between IBS subtypes. More detailed clinical data on IBS symptoms and other factors such as psychological status should be collected. Abdominal pain severity and duration could be assessed, for example, using IBS Severity Scoring System (IBS-SSS) (430, 431), whereas levels of anxiety and depression in IBS patients could be determined using the hospital anxiety and depression scale (HADS) (479, 480). With respect to E-cadherin expression, based on the evidence that the SNP in the E-cadherin gene was recognised as a risk factor for the development of PI-IBS (211), in future studies E-cadherin expression studied in IBS patients should be compared to PI-IBS cohort (206).

Further studies exploring the role of intestinal permeability and related mechanisms in IBS: Since the AJC is the determinant of the epithelial barrier, the alterations in the expression of junctional proteins JAM-A, CLD-1, ZO-1 but also E-cadherin might be considered as an indirect marker of intestinal epithelial permeability (213). Therefore, further studies employing the measurement of intestinal permeability *in vivo,* for example with ^{51}Cr-EDTA urinary recovery test, would reinforce these findings (25). It would also be essential to explore associations for junctional proteins with pathological data including volume of intestinal mucosa and goblet cell density (4, 457). Additional work is required to tease out the mechanisms underlying reduced TJ protein expression in IBS tissue, including its association with tryptase in biopsy tissue or with activated mast cells using TEM. Further studies should also be performed to investigate the mechanisms underlying E-cadherin down-regulation in IBS tissue, including a comparative study on E-cadherin expression between PI-IBS patients and IBS without a history of acute gastroenteritis, a genetic association study and the measurement of E-cadherin mRNA levels. Furthermore, given that pathogens appear to disrupt E-cadherin expression in AJs *in vitro* (445, 446), and that invasive strains (*Escherichia coli*) isolated from IBD tissue (451) decreased TER of intestinal epithelial cells through displacement of E-cadherin from AJs, further analysis might also address the role of pathogens in this regulation.

7.3. Conclusion

The findings of this thesis are novel - they provide insights to intestinal permeability, junctional proteins and mast cells in IBS. These data implicate the role of barrier dysfunction in IBS pathogenesis as evidenced by significant alterations in caecal junctional proteins and their association with IBS symptoms. This thesis and future studies on intestinal barrier dysfunction may contribute to a better understanding of IBS pathogenesis and result in the development of disease biomarkers and a better treatment.

CHAPTER 8

APPENDICES

APPENDICE A1 (PRESENTATIONS RELATED TO THIS THESIS)

International meetings

21/1/2013 Oral presentation "Laborbesprechung Institut für Ernährungsmedizin", COST, Short Term Scientific Mission (STSM), University of Hohenheim, Stuttgart, Germany

Title: "Investigations into the Role of Mast Cell Tryptase on Gastrointestinal Epithelial Integrity *in Vitro*: Implications for the Pathogenesis of Irritable Bowel Syndrome (IBS)"

22-26/10/2011 Poster presentation, 19th United European Gastroenterology Week (UEGW) Stockholm, Sweden

Title: "Mast Cell Mediated Effects on Epithelial Integrity are Partially Reversed by Tryptase Inhibition: Implications from a Novel Caco-2/HMC-1 Cells Co-Culture Model for Irritable Bowel Syndrome (IBS)"

7-10/05/2011 Poster presentation, Disease Digestive Week, DDW Chicago IL, US

Title: "Mast Cell Tryptase Reduces Junctional Adhesion Molecule-A (JAM-A) Expression in Human Intestinal Epithelial Cells: Implications for the Mechanisms of Barrier Dysfunction in Irritable Bowel Syndrome (IBS)"

23-27/10/2010 Oral presentation, 18th United European Gastroenterology Week Barcelona, Spain

Title: "Effects of Mast Cells on the Epithelial Barrier: Developing an *in Vitro* Model of Permeability for Irritable Bowel Syndrome (IBS)"

My presentation at UEGW meeting in Barcelona 2010 was highlighted during "BEST OF UEGW EUROPEAN MEETING 2010" at DDW Chicago 2011 meeting

1-5/05/2010 Poster presentation, Disease Digestive Week, DDW New Orleans, US

Title: "Effects of Tryptase and Mast Cells on Intestinal Permeability and Tight Junction Proteins: Implications for the Pathogenesis for Irritable Bowel Syndrome (IBS)".

Local meetings

19/09/2011 Poster presentation, 4th School of Medicine Postgraduate Research Day, Trinity College Dublin, Ireland

Title: "Mast Cell Tryptase Reduces Junctional Adhesion Molecule-A (JAM-A) Expression in Human Intestinal Epithelial Cells: Implications for the Mechanisms of Barrier Dysfunction in Irritable Bowel Syndrome (IBS)".

16/09/2010 Poster presentation, 3rd School of Medicine Postgraduate Research Day, Trinity College Dublin, Ireland

Title: "Effects of Tryptase and Mast Cells on Intestinal Permeability and Tight Junction Proteins: Implications for the Pathogenesis of Irritable Bowel Syndrome (IBS)".

30/03/2010 Oral presentation, 8th Annual Science Research Day, Institute of Technology Tallaght Dublin, Ireland

Title: "Effects of Tryptase and Mast Cells on Intestinal Permeability and Tight Junction Proteins: Implications for the Pathogenesis for Irritable Bowel Syndrome (IBS)".

7/04/2009 Poster presentation, 7th Annual Science Research Day, Institute of Technology Tallaght Dublin, Ireland

Title: "Investigations into the Role of Gut Permeability, Mast Cells and Tight Junction Proteins in Irritable Bowel Syndrome".

APPENDICE A2 (AWARDS/SCHOLARSHIPS RELATED TO THIS THESIS)

29/4-2/5/2013 Training School European Cooperation in Science and Technology (COST) scientific programme on "Mast Cells and Basophils - Targets for Innovative Therapies", COST Action BM1007 Nottingham, University of Nottingham, UK

I was awarded a travel grant of 820 Euro.

7/1-10/2/2013 Short Term Scientific Mission, European COST Action BM1007

I was awarded STSM grant of 2400 Euro to collaborate with lead researchers Professor Stephan C. Bischoff and Dr Axel Lorenz in the Institute of Nutritional Medicine, University of Hohenheim, Stuttgart, Germany.

23-27/10/2010 18th United European Gastroenterology Week Barcelona, Spain

I was awarded UEGW travel grant of 1000 Euro for one of the best submitted basic science abstracts.

16-18/07/2010 United European Gastroenterology Federation (UEGF) Teaching Activity on Basic Science on "Innate Immunity and the Gut", Cambridge, University of Cambridge, UK

I was awarded UEGF travel grant of 550 Euro.

APPENDICE A3 (PUBLICATIONS RELATED TO THIS THESIS)

Peer-reviewed articles:

Wilcz-Villega, E., McClean, S., and O'Sullivan, M. (2013), Reduced E-cadherin expression is associated with abdominal pain and symptom duration in a study of alternating and diarrhea predominant IBS. Neurogastroenterology & Motility. doi: 10.1111/nmo.12262.

Wilcz-Villega EM, McClean S, O'Sullivan MA. Mast Cell Tryptase Reduces Junctional Adhesion Molecule-A (JAM-A) Expression in Intestinal Epithelial Cells: Implications for the Mechanisms of Barrier Dysfunction in Irritable Bowel Syndrome. Am J Gastroenterol. 2013;108(7):1140-51. (PMID: 23588236).
Highlighted in "What's Hot in the Red Journal".

Peer-reviewed abstracts:

E. Wilcz-Villega, S. McClean, M. O'Sullivan. Mast Cell Mediated Effects on Epithelial Integrity are Partially Reversed by Tryptase Inhibition: Implications from a Novel Caco-2/HMC-1 Cells Co-Culture Model for Irritable Bowel Syndrome (IBS). Gut 2011; 60 (Suppl 3) A170.

Wilcz E, McClean S, O'Sullivan M. Mast Cell Tryptase Reduces Junctional Adhesion Molecule-A (JAM-A) Expression in Human Intestinal Epithelial Cells: Implications for the Mechanisms of Barrier Dysfunction in Irritable Bowel Syndrome (IBS). Gastroenterology. 2011;140(5);S-504.

E. Wilcz, C. O'Morain, M. O'Sullivan, S. McClean. Effects of Mast Cells on the Epithelial Barrier: Developing an *in vitro* Model of Permeability for Irritable Bowel Syndrome (IBS). Gut 2010; 59 (Suppl III) A53.

Wilcz E, O'Morain C, McClean S, O'Sullivan M. T2069 Effects of Tryptase and Mast Cells on Intestinal Permeability and Tight Junction Proteins: Implications for the Pathogenesis for Irritable Bowel Syndrome (IBS). Gastroenterology. 2010;138(5);S-625.

Neurogastroenterology & Motility

Neurogastroenterol Motil (2013) doi: 10.1111/nmo.12262

Reduced E-cadherin expression is associated with abdominal pain and symptom duration in a study of alternating and diarrhea predominant IBS

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Key Messages

This study identified reduced E-cadherin expression in the cecal surface epithelium in irritable bowel syndrome (IBS) compared to controls. Lower E-cadherin in the IBS group was associated with more severe abdominal pain, and in the IBS-alternating subtype (IBS-A) with longer duration of symptoms.

- Impaired intestinal barrier function appears to be implicated in the pathophysiology of IBS. While there is evidence of altered tight junction (TJ) proteins in IBS, the role of adherens junctions (AJ) proteins is largely unexplored. The aim of this study, therefore, was to investigate the expression of AJ protein E-cadherin and TJ proteins ZO-1 and CLD-1 and associations with IBS symptoms.
- Protein expression was analysed by immunofluorescence in the cecal mucosa of 34 Rome II IBS patients comprising both IBS-A (n = 12) and IBS-D (n = 24) subtypes and 12 controls, and interpreted in the context of symptom data.
- Altered E-cadherin expression identified in this study may provide novel insights into mechanisms underlying intestinal barrier dysfunction in IBS.

Abstract

Background *Increased intestinal permeability and altered expression of tight junction (TJ) proteins may be implicated in the pathogenesis of irritable bowel syndrome (IBS). This study aimed to investigate the expression of adherens junction (AJ) protein E-cadherin and TJ proteins zonula occludens (ZO)-1 and claudin (CLD)-1 and associations with IBS symptoms.* ***Methods*** *Junctional proteins were immunostained in cecal biopsy tissue of Rome II IBS patients (n = 34) comprising both alternating (IBS-A) and diarrhea predominant (IBS-D) subtypes, and controls (n = 12). IBS symptom duration, abdominal pain severity and stool frequency were assessed for IBS patients. Protein expression was determined by immunofluorescence.* ***Key Results*** *E-cadherin and ZO-1 protein expression was significantly lower ($p = 0.03$ and $p = 0.016$, respectively) in the cecal surface epithelium of the IBS group comprising both IBS-A and IBS-D subtypes. CLD-1 expression was not significantly altered compared with controls. On subtype analysis, ZO-1 expression was significantly reduced in both IBS-A and IBS-D compared with controls, whereas E-cadherin was reduced only in IBS-A. Lower E-cadherin expression was associated with longer symptoms duration specifically in IBS-A patients ($r_s = -0.76$, $p = 0.004$). Reduced E-cadherin associated with abdominal pain severity in the overall IBS group ($r_s = -0.36$, $p = 0.041$), but this association was unrelated to IBS subtype.* ***Conclusions & Inferences*** *E-cadherin protein expression in the cecum was significantly lower in IBS-A compared with*

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Received: 16 July 2013
Accepted for publication: 23 October 2013

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controls and associated with longstanding symptoms. E-cadherin was further associated with abdominal pain severity in the IBS group overall, but unrelated to IBS subtype. Altered E-cadherin expression may provide novel insights into mechanisms underlying intestinal barrier dysfunction in IBS.

Keywords *adherens junction proteins, E-cadherin, intestinal permeability, irritable bowel syndrome (IBS), tight junction proteins, ZO-1.*

INTRODUCTION

Irritable bowel syndrome (IBS) is a common chronic functional bowel disorder[1] characterized by abdominal discomfort, pain and altered bowel habit, and it is associated with poor quality of life and high health care costs.[2] The etiology of IBS is complex and not fully understood and a number of pathophysiological mechanisms have been proposed including visceral hypersensitivity, immune activation and impaired intestinal barrier function.[3]

Intestinal permeability may be increased in an estimated 12–50% of IBS patients.[4–8] Both impaired small bowel[4–8] and colonic permeability[9,10] have been reported in IBS, as measured by the lactulose/mannitol test and urine excretion of orally ingested ^{51}Cr-ethylenediaminetetraacetic acid (^{51}Cr-EDTA), respectively. *In vitro* work, similarly suggests increased intestinal permeability in studies of mucosal biopsies from IBS patients.[11,12] Interestingly, Zhou *et al.*[6] showed that increased intestinal permeability was associated with increased visceral sensitivity in diarrhea predominant IBS (IBS-D).

Paracellular permeability is regulated by the intercellular apical junctional complexes (AJCs) at the intestinal mucosal barrier which are composed of tight junctions (TJs) and adherens junctions (AJs).[13] We have recently shown that expression of a TJ protein, junctional adhesion molecule-A (JAM-A), was significantly lower in the cecal mucosa of IBS patients compared with controls; moreover, this reduced expression was associated with more severe symptoms in patients with alternating IBS (IBS-A).[14] Furthermore, *in vitro* studies to investigate mechanisms demonstrated that the mast cell protease tryptase and degranulated mast cells reduced the expression of JAM-A, zonula occludens (ZO)-1 and claudin (CLD)-1 and increased paracellular permeability.[14] In IBS, other authors have reported lower levels of ZO-1 in the colonic[15] and jejunal mucosa[16] of IBS-D patients compared with controls. In line with this, Piche *et al.*[11] reported decreased ZO-1 mRNA levels and impaired intestinal epithelial cell integrity in response to supernatants of IBS colonic tissue. In terms of potential clinical importance, lower expression of ZO-1 and JAM-A have both been linked with more severe abdominal pain.[14,15] Reduced occludin expression has been shown in IBS colonic mucosa[15,17] whereas CLD-1 expression appears to be comparable to that of controls.[18]

E-cadherin, a principal component of AJs, also regulates intestinal barrier function and maintains cell to cell contacts.[19,20] Reduced expression of E-cadherin, has been shown in inflammatory bowel disease (IBD)[21–23] and more recently in functional dyspepsia[24] although there is a paucity of data in IBS. Villani *et al.*,[25] however, implicated a polymorphism in the E-cadherin gene in the etiology of the post infectious subtype of IBS (PI-IBS).

There is growing evidence of altered TJ proteins in IBS whereas, currently, the role of AJ proteins, such as E-cadherin, is largely unexplored. The aim of this study was to determine the expression of the AJ protein E-cadherin and the TJ proteins ZO-1 and CLD-1 in the cecal mucosa of IBS patients. Furthermore, we aimed to investigate if expression of these proteins was associated with the severity or duration of IBS symptoms.

MATERIALS AND METHODS

Materials and reagents

Reagents were purchased from Sigma-Aldrich (Arklow, Ireland) unless stated otherwise. The mouse anti-ZO-1, mouse anti-CLD-1, and Alexa Fluor® 633-conjugated goat antimouse antibodies were sourced from Zymed Laboratories Inc. (Paisley, UK). The mouse anti-E-cadherin antibody was sourced from BD Biosciences (Oxford, UK).

Patients and clinical data

Irritable bowel syndrome and asymptomatic control participants were recruited consecutively from the Endoscopy Unit in the Department of Gastroenterology of the Adelaide and Meath Hospital, Dublin, Ireland between 2007 and 2008. Ethical approval was received from the Adelaide and Meath Hospital Ethics Committee and informed consent was obtained from participants.

The inclusion criteria for study enrollment were: patients undergoing full colonoscopy and biopsy for clinical reasons, macroscopically and histologically normal colonic mucosa and no evidence of organic bowel disease. Those with current use of the following medications were excluded: non-steroidal anti-inflammatory drugs, corticosteroids, aspirin, mast-cell stabilizers or antibiotics. Biopsy tissue was obtained from the cecum of patients with symptom compatibility with Rome II criteria IBS[26] and from asymptomatic patient controls.

Patients were screened using a checklist of the Rome II criteria[26] to confirm symptom compatibility in IBS and absence in controls. Irritable bowel syndrome patients were sub-classified

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as (i) IBS-D based on the presence of loose/watery stools, urgency and/or more than three bowel movements a day, or as (ii) constipation predominant IBS (IBS-C) based on the presence of hard/lumpy stools, defecation straining and/or less than three bowel movements a week. Patients alternating between IBS-D and IBS-C were sub-classified as the IBS-A subtype.[26] Control patients were undergoing a colonoscopy for clinical reasons (for example, screening for colon cancer, anemia, and hemorrhoids) and were required to be free of bowel symptoms and not fulfill the Rome II criteria for IBS.

Severity of abdominal pain in IBS was assessed according to a previously validated score,[27] employed in published IBS permeability studies.[11,14] This score used a rating of 0–4, where pain was rated as: 0, absent; 1, mild (not influencing usual activities); 2, relevant (diverting from, but not urging modification of, usual activities); 3, severe (influencing usual activities markedly enough to urge modifications); 4, extremely severe (precluding daily activities). As a proxy measure of diarrhea, IBS patients reported the frequency of soft/liquid stools (number per week), based on a component of the Crohn's Disease Activity Index.[28] The duration of symptoms was recorded for IBS as time since symptom onset as reported by the patient.

Assessment of E-cadherin, ZO-1 and CLD-1 expression by immunofluorescence

Biopsy tissue specimens from the cecum were fixed in formalin. For the current study, 3–4 μm sections were cut from the paraffin-embedded blocks and fixed on slides. Tissue sections were dewaxed in xylene (first for 8 and then 5 min) and passed through decreasing concentration of graded alcohol (twice for 5 min in 100%, 5 min in 90%, 5 min in 70%) to water (twice for 5 min) with 10–20 s agitation every 30 s. Antigen retrieval was performed using target retrieval solution (Dako, Ely, UK) at 95–96 °C for 30 min (water bath) and allowed to cool at RT for 20 min. Non-specific binding was blocked with 20% goat serum in phosphate buffered saline (PBS; E-cadherin, ZO-1) or 2% bovine serum albumin (BSA) in PBS (CLD-1) for 1 h at RT. Sections were incubated in 2% BSA in PBS with primary antibodies as follows: anti-E-cadherin, anti-ZO-1 or anti-CLD-1 (2–20 μg/mL) for 1 h at RT. Sections were washed three times with 0.2% in BSA in PBS and incubated with Alexa 633-conjugated goat antimouse antibody (2 μg/mL) for 1 h at RT. Sections were washed in PBS, three times in PBS containing 4',6-diamidino-2-phenylindole (1.43 μM), and mounted in vectashield (Vector Laboratories, Burlingame, CA, USA). Slides were visualized using Olympus (Hamburg, Germany) Fluoview FV100 microscope with *UPlan FLN* 40×/1.30 lenses and FV10-ASW 2.0 Viewer software (Hamburg, Germany). Slides were assessed semiquantitatively blind to the sample identity. Tissue sections for which the primary antibody was omitted were used as negative controls for junctional proteins.

Assessment of E-cadherin by immunohistochemistry

Given that the assessment of E-cadherin using immunohistochemical techniques is routinely available in many laboratories and clinical settings (for example in the assessment of breast cancer[29]), we proposed to determine if immunofluorescence findings could be replicated using this more widely available technique. E-cadherin was immunostained in 3–4 μm formalin fixed paraffin-embedded cecal tissue by immunohistochemistry. Sections were stained using an autostainer with Super Sensitive™ Polymer HRP IHC Detection System (Biogenex, Fremont, CA, USA). Slides were visualized using Nikon (Nikon Instruments Europe BV, Amsterdam, Netherlands) eclipse 80i fluorescence microscope with Plan Apo 20×/0.75 lenses and with ACT-1 software (Micron Optical Ltd., Enniscorthy, Ireland). Slides were assessed semiquantitatively while blind to the sample identity. Breast cancer tissue was used as a positive control for E-cadherin immunohistochemistry staining.

Analysis of immunofluorescence/ immunohistochemical staining

Epithelial expression of E-cadherin, ZO-1, and CLD-1 based on immunofluorescence was graded semiquantitatively using a 4-point scale, as previously described[14] where 1 represents no specific staining, 2 - mild specific staining, 3 - moderate specific staining, 4 - strong specific staining. Protein staining in all fields of view of epithelium (surface and crypt) was assessed and the average staining score was calculated separately for the surface epithelium (primary variable) and crypt epithelium (secondary variable). Immunohistochemical staining for E-cadherin was similarly assessed.

Statistical analysis

Statistical analysis was performed using SPSS software (IBM Corporation, Armonk, NY, USA). The primary outcome variable was differences in surface epithelial junctional protein expression in the cecal mucosa of the IBS group overall, IBS subtypes (IBS-D, IBS-C, IBS-A) and controls. Differences between two groups were compared using two-tailed Student's *t*-test or Mann–Whitney *U*-test for parametric and non-parametric data, respectively. Differences between more than two groups were compared with non-parametric Kruskal–Wallis test followed by Mann–Whitney *U*-test for single comparisons. Associations between protein expression and symptoms were assessed using Spearman's correlation. A general linear model was employed to determine the potential influence of age and gender on junctional protein expression in controls and IBS patients. Results are expressed as mean and SEM or as median and range. $p \leq 0.05$ was considered statistically significant.

RESULTS

Characteristics of the study group

The study comprised 34 Rome II Criteria IBS patients[26] and 12 asymptomatic controls. In the IBS group, the majority (65%, $n = 22$) were classified as IBS-D and 12 as IBS-A (35%). No patient fulfilled criteria for IBS-C.[26]

All IBS patients were newly diagnosed but reported a mean onset of symptoms of 3.2 ± 0.7 years prior to diagnosis (IBS-D: 2.1 ± 0.5 years *vs* IBS-A: 5.3 ± 1.7 years, $p = 0.10$). Duration of IBS symptoms was less than 1 year in 32% ($n = 11$) of patients, from 1 to 5 years in 53% ($n = 18$) of patients and more than 5 years in 15% ($n = 5$). Patients rated abdominal pain[11,14,27] as mild (27%, $n = 9$), relevant (51%, $n = 17$), severe (12%, $n = 4$), or extremely severe (9%, $n = 3$). The reported frequency of soft/liquid stools was

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A

	Gender			Age at the time of biopsy (years)		
	Males (n = 7)	Females (n = 5)	p	26±2.7#	51.6±4.4##	p
E-cadh.	3.5 (2.6-3.9)	3.4 (2.8-4.0)	0.93	3.5 (3.4-3.6)	3.5 (2.6-4.0)	1.00
ZO-1	1.9 (1.5-2.8)	2.6 (1.9-3.3)	0.10	2.1 (1.6-3.3)	1.9 (1.5-2.8)	0.61
CLD-1	3.6 (2.4-4.0)	3.0 (2.5-4.0)	0.68	3.2 (2.4-3.9)	3.3 (2.5-4.0)	0.67

B

Gender/Age and protein expression			
	E-cadh.	ZO-1	CLD-1
Gender	p = 0.53	p = 0.07	p = 0.79
Age	p = 0.72	p = 0.23	p = 0.36

C

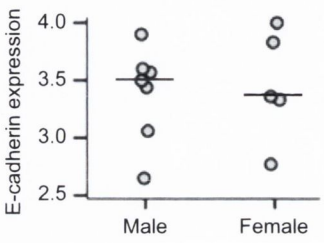

Figure 1 Expression of junctional proteins according to gender and age (years) of patients at the time of biopsy. (A) Analysis of protein expression according to gender and age in the control group. # shows controls of age <32 years (the average age of patients with irritable bowel syndrome, IBS) and ## shows controls of age ≥32 years. Values show resultant protein expression (grade). Data were analyzed with Mann–Whitney U-test. (B) Age and gender effects on junctional proteins controlled for in the analysis using a general linear model in the disease group (control, IBS). (C) E-cadherin expression according to gender in the control group. Values are individual data and medians. Data were analyzed with Mann–Whitney U-test. E-cadh., E-cadherin; ZO-1, zonula occludens-1; CLD-1, claudin-1.

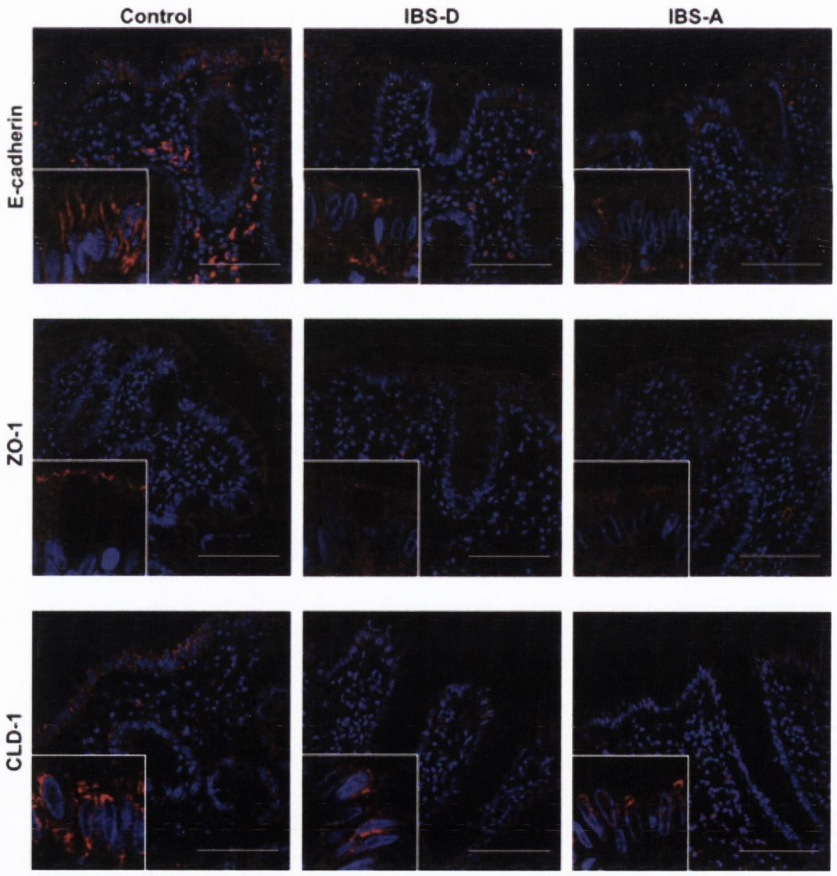

Figure 2 Representative photomicrographs showing the expression of junctional proteins in the surface epithelium of the cecal mucosa of controls and irritable bowel syndrome (IBS) patients. The expression of junctional protein in IBS is shown according to bowel predominance: IBS with diarrhea predominance (IBS-D) and alternating IBS (IBS-A). Immunofluorescence staining for junctional proteins is shown in red, while nuclear staining is blue. ZO-1, zonula occludens-1; CLD-1, claudin-1; bars = 50 μm.

a mean of 14.9 ± 3.7 per week for IBS patients and predictably higher in IBS-D than IBS-A (20.7 ± 4.7 compared with 1.8 ± 1.1, p = 0.001).

The IBS group had a female predominance (82%, n = 28 *vs* 41%, n = 5; p = 0.008) and was younger in mean age (32.1 ± 1.4 *vs* 43.1 ± 4.7 years; p = 0.004)

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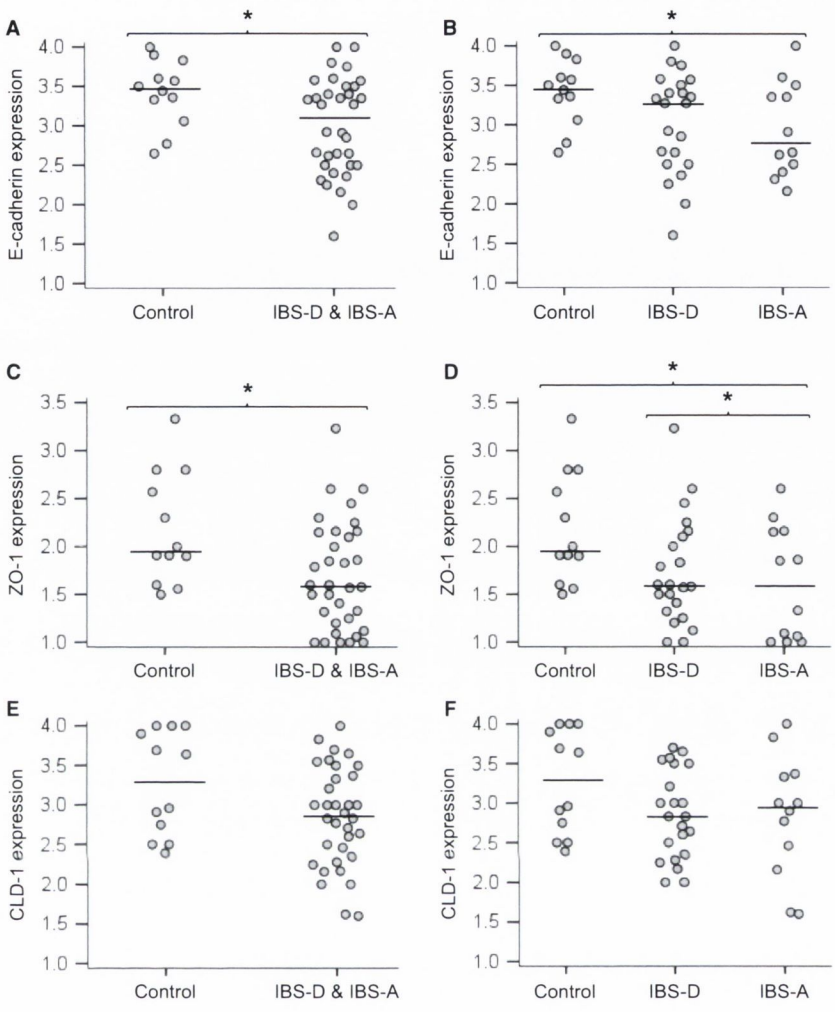

Figure 3 Expression of junctional proteins in the surface epithelium of the cecal mucosa of controls and irritable bowel syndrome (IBS) patients. The expression of junctional proteins shown in (A, C, and E) controls and the IBS patient group overall, or (B, D and F) controls and IBS subtypes according to bowel predominance: IBS with diarrhea predominance (IBS-D) and alternating IBS (IBS-A). The expression of junctional proteins was assessed semiquantitatively using 4-point scale. Values are individual data and medians. Kruskal–Wallis *p*-values (not marked in the graphs): B, $p = 0.086$; D, $p = 0.05$; F, $p = 0.21$. $^*p < 0.05$ Mann–Whitney *U*-test. ZO-1, zonula occludens-1; CLD-1, claudin-1.

compared with controls. Consequently, we conducted detailed age and gender analysis showing that neither gender nor age significantly influenced expression of junctional proteins (E-cadherin, ZO-1, or CLD-1) in the study group (Fig. 1), in line with previous findings.[14]

E-cadherin, ZO-1 and, CLD-1 expression in IBS and controls

E-cadherin In the cecal mucosa from controls, uniform E-cadherin staining was abundant along the lateral membrane of surface epithelial cells (Fig. 2). Overall surface epithelial E-cadherin expression was significantly lower in the IBS group (IBS-D and IBS-A) (median of 3.1 [1.6–4.0]) compared with controls (median of 3.5 [2.6–4.0], $p = 0.03$) shown in Fig. 3A. Further analysis of E-cadherin according to bowel predominance, showed significantly lower expression in patients with IBS-A subtype (median of 2.6 [2.2–4.0]) compared with controls ($p = 0.043$), while in IBS-D differences (median of 3.3 [1.6-4.0]) in expression did not reach statistical significance ($p = 0.063$; Figs 2 and 3B).

ZO-1 In controls, ZO-1 was focally expressed at the apical region of epithelial cells (Fig. 2). ZO-1 was significantly lower in the surface epithelium of the IBS group (median of 1.6 [1.0–3.2]) compared with controls (median of 1.9 [1.5–3.3], $p = 0.016$; Figs 2 and 3C). Reduced ZO-1 expression was a consistent feature of both IBS subtypes, with significantly lower levels documented in IBS-D (median grade of 1.6 [1.0–3.2], $p = 0.029$) and IBS-A patients (median grade of 1.6 [1.0–2.6], $p = 0.04$) compared with controls (Figs 2 and 3D).

CLD-1 CLD-1 was typically abundantly expressed in the surface epithelium of controls and did not localize specifically to the apical region but was expressed basolaterally (Fig. 2). CLD-1 expression in the IBS

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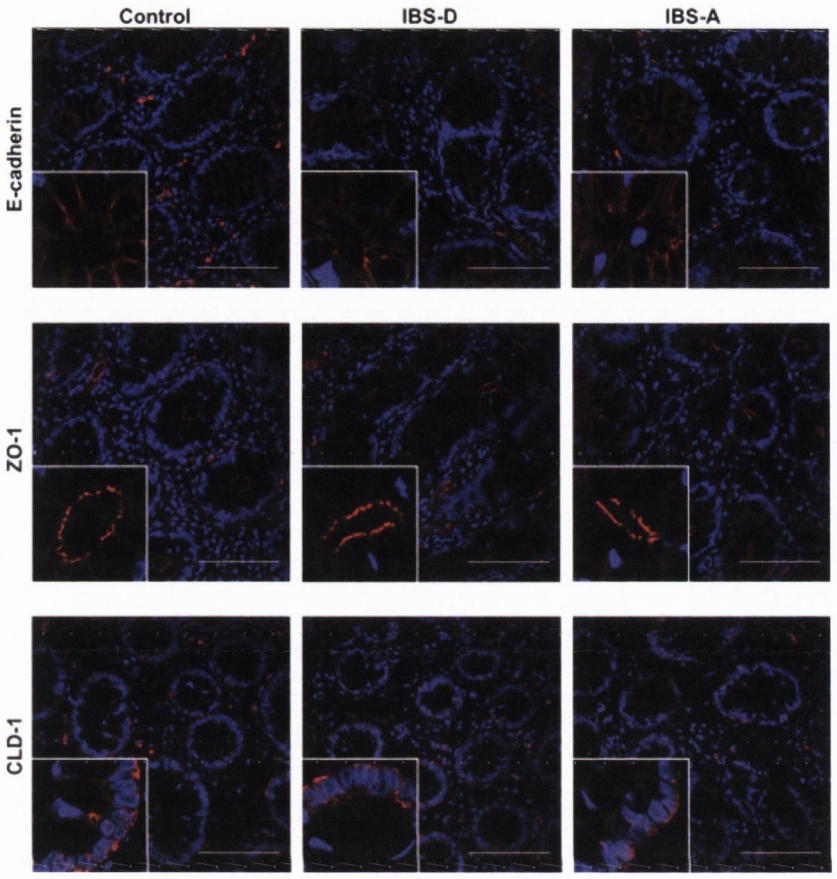

Figure 4 Representative photomicrographs showing the expression of junctional proteins in the crypt epithelium of the cecal mucosa of controls and irritable bowel syndrome (IBS) patients. The expression of junctional protein in IBS is shown according to bowel predominance: IBS with diarrhea predominance (IBS-D) and alternating IBS (IBS-A). Immunofluorescence staining for junctional proteins is shown in red, while nuclear staining is blue. ZO-1, zonula occludens-1; CLD-1, claudin-1; bars = 50 μm.

group overall or in the IBS subtypes (IBS-D and IBS-A), was not significantly different compared with controls. For the IBS group, median CLD-1 grade was 2.9 (1.6–4.0) *vs* grade of 3.3 (2.4–4.0) in controls ($p = 0.077$). A median grade of 2.8 (2.0–3.7) for CLD-1 was noted for IBS-D ($p = 0.071$) and median grade of 2.9 (1.6–4.0) for IBS-A ($p = 0.23$) subtypes and the expression was not significantly altered compared with controls (Figs 2 and 3E and F).

The expression of junctional proteins in IBS and controls was further analyzed by the secondary variable, namely expression in the crypt epithelium, and typical expression is shown in Fig. 4. Analysis of median levels in the IBS group, and according to IBS subtype, showed that the expression of E-cadherin, ZO-1, and CLD-1 in IBS was comparable to controls.

E-cadherin, ZO-1, and CLD-1 expression and associations with IBS symptoms

Abdominal pain There was a significant negative correlation between E-cadherin expression in surface epithelial cells and abdominal pain severity in the overall IBS group (comprising IBS-D and IBS-A; $r_s = -0.36$, $p = 0.041$). The finding, however, was not related to subtype and this association was not found when the analysis was confined either to IBS-D or IBS-A patients (Fig. 5A and B). Abdominal pain severity was not associated with either ZO-1 or CLD-1 expression (Fig. 5A).

Frequency of soft/liquid stools There was no association between the number of soft/liquid stools reported per week and E-cadherin, ZO-1, or CLD-1 expression in the surface epithelium of the IBS group overall or in IBS subtypes (Fig. 5A).

Duration of IBS symptoms A significant negative correlation between E-cadherin expression in the surface epithelium and duration of symptoms was noted specifically in the IBS-A group ($r_s = -0.76$, $p = 0.004$), suggesting that patients with more longstanding symptoms had a lower expression of E-cadherin (Fig. 5A and C). No significant association with duration of IBS symptoms was found for E-cadherin either when analysis was performed in the IBS patient group overall or in the IBS-D subtype. In contrast, duration of IBS symptoms was not associated with either ZO-1 or

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A

		Abdominal pain severity (scale1–4)		No. of liquid/soft stools per week		Duration of symptoms (years)	
		Spearman correl.		Spearman correl.		Spearman correl.	
		r_s	p	r_s	p	r_s	p
E-cadherin	IBS-D & IBS-A	**–0.36**	**0.041**	–0.04	0.82	–0.32	0.06
	IBS-D	–0.30	0.18	–0.26	0.28	–0.08	0.71
	IBS-A	–0.44	0.18	0.24	0.54	**–0.76**	**0.004**
ZO-1	IBS-D & IBS-A	0.29	0.10	0.18	0.35	0.23	0.18
	IBS-D	0.30	0.18	0.12	0.61	0.30	0.18
	IBS-A	0.31	0.35	0.02	0.96	–0.04	0.90
CLD-1	IBS-D & IBS-A	0.11	0.54	0.27	0.16	–0.09	0.60
	IBS-D	0.15	0.50	0.30	0.20	–0.24	0.29
	IBS-A	0.14	0.68	0.18	0.64	–0.04	0.90

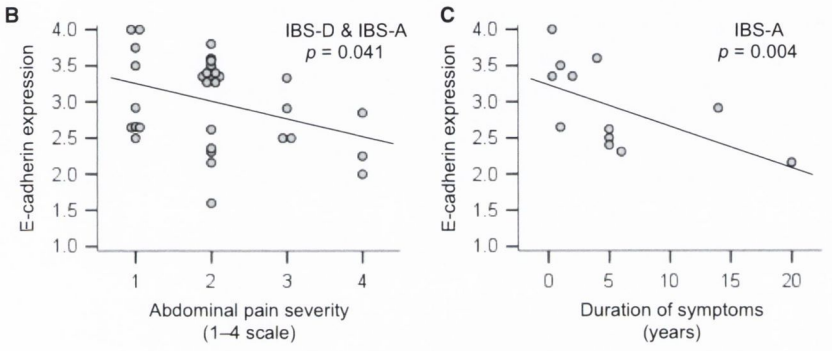

Figure 5 Correlation between junctional protein expression in the surface epithelium of the cecal mucosa with irritable bowel syndrome (IBS) symptoms. (A) Correlations between the expression of junctional proteins and IBS symptoms were determined in the IBS patient group overall and in IBS according to subtypes: IBS with diarrhea predominance (IBS-D) and alternating IBS (IBS-A). Data were analyzed with Spearman correlation. ZO-1, zonula occludens-1; CLD-1, claudin-1. (B) Correlation between abdominal pain severity and E-cadherin expression in the surface epithelium of the cecal mucosa in the IBS group (IBS-D and IBS-A). (C) Correlation between duration of symptoms and E-cadherin expression in the surface epithelium of the cecal mucosa of IBS-A patients.

CLD-1 levels in the surface epithelium of IBS patients (Fig. 5A).

On analysis of crypt epithelium, no associations between expression of junctional proteins and IBS symptom severity or duration were identified (data not shown).

Expression of E-cadherin by immunohistochemistry

In the present study, expression of E-cadherin based on immunohistochemistry appeared to be lower in the surface epithelium in IBS than controls. On analysis, however, compared with controls (median grade of 2.2 [1.7–3.3]), neither expression of E-cadherin in the IBS patient group (median grade of 2.0 [1.0–3.0], $p = 0.085$) nor in IBS-D (median grade of 2.0 [1.0–3.0], $p = 0.071$) or in IBS-A (median grade of 1.9 [1.3–2.7], $p = 0.29$) subtypes were statistically significant. In line with this, associations between IBS symptoms and E-cadherin expression were not detectable (data not shown). The significant reduction in E-cadherin demonstrated by immunofluorescence was not observed using a routine immunohistochemical technique in this study.

For E-cadherin immunohistochemical analysis, one IBS-A patient was excluded from analysis due to tissue damage during the autostaining process and subsequent comparisons between immunofluorescence and immunohistochemical staining for E-cadherin was based on 33 matched tissue pairs (instead of $n = 34$).

DISCUSSION

There is growing evidence that increased intestinal permeability and altered TJ protein expression may be implicated in the pathophysiology of IBS. This study shows for the first time to our knowledge, alterations in AJs proteins, specifically in E-cadherin in IBS. We report significantly lower E-cadherin expression in the surface epithelium of the cecal mucosa in the IBS group compared with controls and corresponding potential clinical importance as determined by associations with IBS symptoms. Furthermore, this study demonstrates significantly reduced ZO-1 expression in the cecum of IBS compared with controls, whereas differences in CLD-1 did not reach statistical significance.

The present study provides novel evidence for lower expression of E-cadherin in IBS compared with controls, although the results show that this reduction appeared to be specific to the IBS-A subtype. E-cadherin was significantly lower in patients with IBS-A, whereas in the IBS-D subtype this finding was not statically significant. There is a lack of published data on E-cadherin expression in IBS, but significantly reduced levels have been reported in the surface epithelium of mucosal tissue in other diseases where increased intestinal permeability is observed, such as IBD[21–23] and celiac disease.[30] In addition, Vanheel *et al.*[24] recently showed reduced E-cadherin expression in duodenal biopsy tissue from patients with functional dyspepsia. Although, the present study is the first

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reported observation of E-cadherin protein loss in IBS tissue, the finding may broadly fit with previous results from Villani *et al.*[25] which identified an association between a single nucleotide polymorphism in the E-cadherin gene and the development of PI-IBS.

This study further identified significantly lower ZO-1 protein expression in the cecal epithelial mucosa of IBS patients, with a consistent reduction both in IBS-A and IBS-D subtypes. This is in agreement with previous reports of ZO-1 down-regulation in colonic[15] and jejunal tissue from IBS patients.[16] In this study CLD-1 expression at the cecum was not significantly altered in IBS compared to controls, although an arguable trend toward reduction in IBS-D may warrant further investigation given that published data for CLD-1 in IBS appear to be inconsistent. Bertiaux-Vandaële *et al.*[15] showed a trend for lower CLD-1 levels in colonic tissue in IBS patients, whereas others did not find differences in the expression of CLD-1 in jejunal tissue in IBS-D patients compared to controls.[18]

An important aspect of this study was to understand if lower junctional protein expression may be relevant to IBS symptoms. The present study reported reduced epithelial expression of E-cadherin in the IBS group and its significant association with more severe abdominal pain. This association, however, was not related to IBS subtype and on further analysis was not identified specifically in either IBS-D or IBS-A patients. Although, there are no published comparable data, to our knowledge, for E-cadherin expression in IBS, associations between abdominal pain and other junctional proteins have been reported. For example, lower expression of CLD-1, occludin and ZO-1 mRNA in IBS tissue has been associated with the severity of abdominal pain.[11,15] Similarly, we recently linked lower JAM-A in the cecal mucosa of IBS-A with more severe abdominal pain.[14] In the present study, the association with abdominal pain severity was noted for E-cadherin only and was not demonstrated for either ZO-1 or CLD-1. Furthermore, we found no association between the frequency of soft/liquid stools in IBS and protein expression for E-cadherin, ZO-1 or CLD-1 in our data. Martínez *et al.*,[16] however, have identified a significant negative correlation between the number of bowel movements or stool consistency and ZO-1 mRNA, and ZO-3 mRNA in the jejunal mucosa. Nonetheless, more detailed data on stool frequency and consistency,[16] over and above our assessment, would be required to better understand associations between stool parameters and junctional protein expression in the cecal mucosa in IBS.

This study shows that E-cadherin expression was reduced in patients with IBS-A and furthermore that this reduction was significantly associated with longer duration of IBS symptoms, a parameter that may be a marker of chronic disease. In line with this finding, we previously showed that JAM-A expression negatively associated with duration of IBS symptoms in IBS-A patients.[14] Bertiaux-Vandaële *et al.*[15] reported that the lowest levels of TJ proteins in colonic IBS tissue (occludin and CLD-1 protein and ZO-1 mRNA) were associated with symptoms initiation. It is plausible that a reduction in expression of other junctional proteins, such as E-cadherin and JAM-A, may be associated with maintenance and progression of IBS, although the underlying mechanisms are not known. While the present study demonstrated reduced expression in ZO-1 in the IBS group; we found no evidence of an association with IBS symptom duration.

Mechanisms underlying E-cadherin down-regulation in IBS were not investigated in this study and remain unclear. Possible mechanisms may involve genetic factors, immune activation including mast cells, altered host-microbial interactions or bacterial infection. Intestinal pathogens have evolved mechanisms for the disruption of AJs through the cleavage of epithelial E-cadherin.[31,32] In addition, the invasive *Escherichia coli* strains isolated from IBD tissue replaced E-cadherin from AJC and subsequently disrupted barrier function of intestinal epithelial cells.[20] Of particular interest, however, is the work by Villani *et al.*,[25] which implicated a polymorphism in the E-cadherin gene (rs16260; -C160A), in the etiology of IBS, specifically for PI-IBS in the Walkerton community. This variant has been associated with reduced transcriptional efficiency of the E-cadherin gene in human prostate cancer cells.[33] This allele variation may result in lower E-cadherin expression in AJs, promoting increased pathogenicity of invasive bacteria, subsequent barrier disruption, and increased intestinal permeability. Importantly, in a follow-up study by Marshall *et al.*[4] intestinal permeability appeared to be increased in those who developed PI-IBS 2 years after the initial outbreak of gastroenteritis compared to controls. The characteristics of PI-IBS tend to be similar to IBS-D[34] and therefore changes in E-cadherin might be expected to translate to an IBS-D cohort. Contrary to this, our findings show that E-cadherin protein expression was significantly lower in IBS-A and associated with longstanding symptoms in this subtype. This protein may be mechanistically involved in maintaining epithelial barrier disruption over time or be a surrogate marker of chronic disease. The disease duration in our IBS-A group was longer than that of IBS-D, but not significantly so, and larger studies would be required to independently tease out the

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effects of IBS subtypes and symptom duration on E-cadherin. Investigating changes in E-cadherin expression in biopsy tissue from IBS patients over time would also be informative, but clinical follow-up studies of this nature can be challenging to conduct.

In IBS, it is not clear if our findings in gastrointestinal barrier integrity may be a primary defect or secondary to factors such as immune activation including increased mast cell numbers.[35,36] Previously, we reported that epithelial ZO-1, CLD-1, and JAM-A expression was reduced in response to the mast cell protease tryptase and to degranulating mast cells *in vitro* and subsequently confirmed JAM-A downregulation in IBS.[14] Martínez *et al.*[16] reported that reduced ZO-1 and ZO-3 mRNA expression was associated with enhanced tryptase mRNA expression in IBS tissue. While these studies show alterations in some TJ proteins in response to activation of mast cells, the implications specifically for E-cadherin are less clear. For example, previously we did not observe changes in E-cadherin protein expression in response to mast cell degranulation *in vitro*.[14]

In the present study, we investigated E-cadherin, ZO-1 and CLD-1 expression and symptoms in IBS, and this is the first report of altered E-cadherin expression in IBS. The findings, however, need to be confirmed in larger studies and that can more fully explore E-cadherin expression according to both IBS subtypes, including IBS-C and PI-IBS, also symptoms. Further studies could also employ measurements of both protein and mRNA expression of junctional proteins, intestinal permeability *in vivo*, and the complex interaction with factors such as immune activation in IBS.

In conclusion, the present study reports for the first time that E-cadherin expression was significantly reduced in this IBS-A and IBS-D patient group, essentially in a cohort comprising non-constipation predominant IBS. On subtype analysis, E-cadherin appeared to be significantly reduced only in IBS-A patients. Furthermore, in the IBS-A group, lower E-cadherin levels were associated with longstanding IBS symptoms duration. Interestingly, reduced E-cadherin expression was associated with abdominal pain severity in the IBS group, but associations with pain were not related to subtype. ZO-1 expression was significantly lower in both IBS-D and IBS-A subtypes, but was not associated with IBS symptoms. These novel findings relating to E-cadherin may improve the understanding of altered intestinal barrier function in IBS and its application to therapeutic strategies targeted at enhancing the intestinal barrier.

ACKNOWLEDGMENTS

The authors wish to thank Mr. James Reilly, Department of Science, Institute of Technology Tallaght, Dublin, Ireland for assistance with statistical analysis.

FUNDING

This research was funded by the Programme for Research in Third Level Institutions (PRTLI) Cycle 4, supported by the European Union Regional Development Plan, the Irish Government National Development Plan 2007–2013 and administered by the Higher Education Authority in Ireland.

DISCLOSURE

We, the authors, have no competing interests.

AUTHOR CONTRIBUTION

Guarantor of the article: Maria O'Sullivan; MOS, SMcC and EW-V contributed to the study concept, design and implementation, the interpretation of data, writing of the paper and approved the final manuscript; EW-V conducted the experiments.

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Mast Cell Tryptase Reduces Junctional Adhesion Molecule-A (JAM-A) Expression in Intestinal Epithelial Cells: Implications for the Mechanisms of Barrier Dysfunction in Irritable Bowel Syndrome

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OBJECTIVES: **The objective of this study was to investigate how mast cell tryptase may influence intestinal permeability and tight junction (TJ) proteins *in vitro* and explore translation to irritable bowel syndrome (IBS).**

METHODS: **We investigated the effect of: (1) tryptase on Caco-2 monolayers, (2) mast cell degranulation in a Caco-2/human mast cell-1 (HMC-1) co-culture model, (3) mast cell degranulation±tryptase inhibition with nafamostat mesilate (NM). Epithelial integrity was assessed by transepithelial resistance (TER), permeability to fluorescein isothiocyanate (FITC)-dextran and transmission electron microscopy (TEM). The expression of junctional proteins zonula occludens-1 (ZO-1), junctional adhesion molecule-A (JAM-A), claudin-1 (CLD-1), CLD-2, CLD-3, occludin and E-cadherin was determined by western blot analysis and immunofluorescence confocal microscopy. Based on the *in vitro* results, we further assessed JAM-A expression in biopsy tissue (cecum) from 34 IBS patients, 12 controls, and 8 inflammatory controls using immunofluorescence confocal microscopy and explored associations between JAM-A and IBS symptoms.**

RESULTS: **Tryptase disrupted epithelial integrity in Caco-2 monolayers as shown by a significant decrease in TER, an increase in permeability to FITC-dextran, and a decrease in the expression of junctional proteins JAM-A, CLD-1, and ZO-1 within 24 h. Correspondingly, in the Caco-2/HMC-1 co-culture model we showed a significant decrease in TER, an increase in permeability to FITC-dextran, and the presence of open TJs (TEM) in response to mast cell degranulation within 24 h. In this co-culture model, mast cell degranulation significantly decreased JAM-A and CLD-1 protein expression at 24 h. Tryptase inhibition (NM) significantly reduced the effect of mast cell degranulation on the junctional protein JAM-A, TER, and FITC-dextran flux. In IBS, epithelial JAM-A protein expression was significantly reduced in IBS tissue compared with controls. Lower JAM-A expression was associated with more severe abdominal pain ($r_s = -0.69$, $P = 0.018$) and longer duration of symptoms ($r_s = -0.7$, $P = 0.012$) in IBS-alternating subtype.**

CONCLUSIONS: **Reduced JAM-A expression *in vitro* appears to contribute to the underlying mechanisms of altered epithelial integrity in response to tryptase released from degranulating mast cells. In IBS, JAM-A expression was significantly reduced in the cecal epithelium and associated with abdominal pain severity. JAM-A may provide new insights into the underlying mechanisms in IBS.**

SUPPLEMENTARY MATERIAL is linked to the online version of the paper at http://www.nature.com/ajg

Am J Gastroenterol advance online publication, 16 April 2013; doi:10.1038/ajg.2013.92

INTRODUCTION

Irritable bowel syndrome (IBS) is a highly prevalent functional bowel disorder characterized by abdominal pain or discomfort and associated with altered bowel habit, abdominal bloating, and disturbed defecation (1). The pathogenesis of IBS remains unclear, but is believed to result from a complex interaction between several

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Received 2 August 2012; accepted 19 February 2013

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factors including visceral hypersensitivity, psychological factors, brain–gut interactions, and immune activation. Emerging factors such as altered intestinal permeability have been added to this list (2–4).

Intestinal epithelial permeability is regulated by a complex protein system comprising tight junction (TJ) and adherens junction proteins (5). These proteins include TJ proteins such as claudins (CLDs), occludin, the zonula occludens (ZO), junctional adhesion molecule (JAM), and the adherens junctional protein E-cadherin. A number of studies have suggested an increase in intestinal permeability (2–4,6) in IBS patients. Recently, a decrease in ZO-1, occludin and claudin-1 (CLD-1) was identified in colonic biopsies from IBS patients (7). Reduced ZO-1 expression was also reported by Piche *et al.* (2) in epithelial cells incubated with tissue supernatants from IBS patients. The role of E-cadherin and JAM in barrier function in IBS remains largely unexplored. E-cadherin may be associated with risk of developing post-infectious IBS (8). Although JAM-A regulates intestinal epithelial permeability (9,10) and is reduced at sites of active inflammation in inflammatory bowel disease (IBD) (11), its role in IBS is unknown.

Increased mast cell numbers in the colonic mucosa of IBS patients (12–17) is generally well documented, as is their interaction with enteric nerves and association with abdominal pain, although not all studies support these findings (18). Beyond brain–gut interactions, the involvement of mast cells in other pathogenic mechanisms, such as intestinal permeability, are incompletely understood. We proposed that mast cells, and specifically the mast cell protease tryptase, may regulate intestinal permeability in IBS. Santos *et al.* (19) have shown that chronic stress resulted in increased mast cell numbers and activation in parallel with epithelial barrier dysfunction in animal models. More recent data suggest that tryptase may increase intestinal permeability (20) and downregulate ZO-1 expression *in vitro* (21).

Our aim was to investigate mechanisms by which the increased mast cell activity documented in IBS may alter intestinal permeability and TJ proteins. Based on emerging data, we hypothesized that tryptase released from mast cells would reduce colonic epithelial integrity and alter the expression of junctional proteins. We developed a human *in vitro* epithelial-mast cell model to investigate the effects of mast cell tryptase on epithelial integrity and on the expression of the junctional proteins JAM-A, ZO-1, CLDs, occludin, and E-cadherin. Finally, we aimed to translate the key *in vitro* junctional protein findings to IBS. To achieve this, we investigated JAM-A protein levels in IBS tissue and its potential clinical relevance as determined by associations with IBS symptoms, namely, abdominal pain, diarrhea, and duration of symptoms.

METHODS

Material and reagents

Reagents were purchased from Sigma-Aldrich, Arklow, Ireland unless stated otherwise. Mouse anti-ZO-1, rabbit anti-CLD-1, rabbit anti-CLD-3, mouse anti-CLD-2, rabbit anti-occludin, rabbit anti-JAM-A, goat anti-rabbit Alexa 633-conjugated antibodies were from Zymed Laboratories (Paisley, UK). Mouse anti-E-cadherin was sourced from BD Biosciences (Oxford, UK) and mouse anti-glyceraldehyde-3-phosphate dehydrogenase from Millipore (Cork, Ireland). Horseradish peroxidase-conjugated anti-mouse and horseradish peroxidase-conjugated anti-rabbit antibodies were purchased from Pierce (Dublin, Ireland) and BD Pharmingen (Oxford, UK), respectively. Fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse and anti-rabbit antibodies were purchased from Jackson ImmunoResearch (West Grove, PA).

Cell culture

Caco-2 cells. Colonic human epithelial cell line, Caco-2, was purchased from ECACC, Salisbury, UK. Cells (P13 to P35) were cultured in Dulbecco's modified Eagle's medium with 2 mM L-glutamine, 1% v/v non-essential amino acids, 10% v/v fetal bovine serum. Cells were cultured in 75 cm^2 tissue culture flasks until confluent, then seeded at a density of 5×10^5 cells/ml on Transwell® polyester filters (0.4 μm pore), and cultured until established differentiated and polarized monolayer, from 21 to 26 days, at 37 °C in a humidified atmosphere with 5% CO_2 with feeding on alternate days.

HMC-1 cells. The human mast cell line, HMC-1, was a gift from J.H. Butterfield, Mayo Clinic, Rochester, MN. HMC-1 cells (P68–P94) were cultured in Dulbecco's modified Eagle's medium with 2 mM L-glutamine, 1% v/v non-essential amino acids, 10% iron supplemented calf serum, 1.2 mM alpha-thioglycerol. Cells were cultivated in 75 cm^2 tissue culture flasks and passaged approximately once per week (37 °C, 5% CO_2).

Co-culture of Caco-2 and HMC-1 cells. Caco-2 cells (P22–P35) were seeded at a density 5×10^5 cells/ml on Transwell® filters. HMC-1 cells (P77–P95, 5×10^5 cells/ml) were added to the basolateral compartment of the Transwells. Cells were co-cultured until Caco-2 established differentiated and polarized monolayer, from 21 to 23 days, in Dulbecco's modified Eagle's medium with 2 mM L-glutamine, 1% v/v non-essential amino acids, 5% fetal bovine serum, 5% calf serum with feeding on alternate days (37 °C, 5% CO_2). In parallel, Caco-2 cells were also cultured without HMC-1 cells as controls for the co-culture.

Integrity and paracellular permeability of Caco-2 monolayers incubated with tryptase

The integrity of Caco-2 monolayers was determined with transepithelial resistance (TER), measured with an EVOM voltohmmeter (World Precision Instruments, Sarasota, FL). Polarized monolayers of Caco-2 cells were washed with pre-warmed Hank's balanced salt solution (HBSS) buffer containing 11 mM glucose and 25 mM HEPES (HBSS/HEPES). Tryptase (3 or 15 mU) was added to the apical compartment of the Transwells and incubated for up to 24 h. TER was measured periodically over 24 h and the results were displayed as a percentage change over the untreated control. To assess epithelial permeability, FITC-dextran (4 kDa, 100 μg/ml) was added to the apical compartment of the Transwells up to 4 h. Basolateral samplings were taken at intervals and were replenished with fresh pre-warmed HBSS/HEPES buffer at each sample timepoint. The apical-to-basolateral flux of FITC-dextran was measured

with a Thermo Fisher Varioskan Flash spectrophotometer (Thermo Fisher Scientific, Loughborough, UK) using an external standard curve. The apparent permeability coefficient (Papp) of each treatment can be calculated according to Papp = dQ/dt ($1/AC_0$), where dQ/dt is the permeability rate derived from the slope of the line, A is the diffusion area and C_0 is the initial donor solution concentration (22).

Development of an epithelial-mast cells co-culture model

Caco-2 cells were seeded on filters and HMC-1 cells were added to the basolateral compartment of the Transwells either at 1st, 15th, or 18th day of culture. The integrity of Caco-2 monolayers was determined with TER on alternate days, the morphology of both cell lines was analyzed with a light microscope, and the viability of HMC-1 was assessed periodically with Trypan blue staining. HMC-1 cells ($5–6\times10^6$ cells/ml) were degranulated with compound 48/80 (5 μg/ml) in Dulbecco's modified Eagle's medium or HBSS/HEPES and TER and FITC-dextran flux were measured over 24 h as outlined previously. The results were displayed as a change over the undegranulated control.

The effect of nafamostat mesilate (NM), a tryptase-specific inhibitor when used at low concentrations ($10^{-10}–10^{-11}$ M) (23,24), on TER and permeability was also examined by incubating cell co-cultures with the NM for up to 24 h together with mast cell degranulation. To examine the ultrastructure of the co-culture model, polarized monolayers of Caco-2 cells or Caco-2/HMC-1 monolayers were rinsed with warm HBSS/HEPES buffer and fixed in HBSS with 4% glutaraldehyde. Cells were processed into resin, ultramicrotomy, and contrasted in preparation for examination by transmission electron microscopy (TEM) with a Tecnai 12 microscope (FEI, Hillsboro, OR).

Expression of TJ/adherens junction proteins by western blotting

Polarized monolayers of Caco-2 cells or Caco-2/HMC-1 co-cultures were lysed in cold RIPA buffer with protease inhibitor cocktail (Roche Applied Science, Burgess Hill, UK) for 20 min on ice and sonicated for 1 min. Protein content was quantitated and equal amounts (20 μg) were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (6–10% acrylamide, depending on the protein) and transferred to nitrocellulose membrane (Whatmann, VWR International, Dublin, Ireland). Membranes were blocked in Tris-buffered saline with either 5% nonfat dry milk, 0.1% bovine serum albumin (BSA), 0.1% Tween 20 (CLD-1, CLD-3, and E-cadherin) or 5% BSA and 0.1% Tween 20 (JAM-A, ZO-1, and CLD-2) or 10% non-fat dry milk, 0.1% BSA, 0.1% Tween 20 (occludin) or 5% nonfat dry milk and 0.1% Tween 20 (glyceraldehyde-3-phosphate dehydrogenase). Blots were incubated with a primary antibody (0.05–1 μg/ml) overnight at 4 °C and were washed three times with Tris-buffered saline containing 1% Tween-20 and incubated with horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit antibodies (1:3,000–50,000) as appropriate for 1 h at room temperature. Proteins were detected by chemiluminescence (Millipore). The density of each individual band was compared with the corresponding control band and normalized against glyceraldehyde-3-phosphate dehydrogenase (loading control protein) by densitometry. ImageJ software was used to analyze western blot signals and to adjust contrast and brightness of the images (http://rsbweb.nih.gov/ij/). The results were expressed as a change relative to the untreated control.

Expression of TJ/adherens junction proteins by immunofluorescence confocal microscopy

Polarized monolayers of Caco-2 cells were rinsed with prewarmed PBS and permeabilized with cold methanol (−20 °C) for 30 min. Nonspecific binding sites were blocked with 1% BSA in PBS for 10 min. Cells were incubated in 1% BSA in PBS with primary antibodies as follows: anti-JAM-A, anti-ZO-1, anti-CLD-1, anti-CLD-3, anti-occludin (5–20 μg/ml) for 1 h at room temperature. Cells were washed three times with 1% BSA in PBS and incubated with FITC-conjugated goat-anti mouse or anti-rabbit antibodies (1:50–100) as appropriate for 1 h at room temperature. Cells were washed three times with 1% BSA in PBS and postfixed with 4% paraformaldehyde for 10 min. Monolayers were mounted on slides with vectashield containing 4,6-diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA), and covered with a coverslip. Specimens were stored at 4 °C before analysis on an Olympus Fluoview confocal FV100 microscope (Hamburg, Germany).

Expression of JAM-A in IBS tissue

JAM-A was immunostained in formalin-fixed, paraffin-embedded biopsy tissue from the cecum of patients with IBS ($n=34$), normal controls ($n=12$), and inflammatory controls with confirmed IBD ($n=8$) (25). Ethical approval was received from the Adelaide and Meath Hospital Ethics Committee and informed consent was obtained from participants. Patients had Rome II criteria IBS (26) macroscopically and histologically normal colonic mucosa and no evidence of organic bowel disease. Control patients were undergoing colonoscopy for clinical reasons (colon cancer screening, hemorrhoids, anemia, and vomiting) and were free from organic bowel disease and IBS. Other exclusion criteria applied to IBS and normal controls were current use of the following medications: non-steroidal anti-inflammatory drugs, corticosteroids, aspirin, anti-inflammatory drugs, mast cell stabilizers, or antibiotics. Severity of abdominal pain was assessed in IBS using a pain rating of 0–4, where patients rated pain as: 0, absent; 1, mild (not influencing usual activities); 2, relevant (diverting from, but not urging modification of, usual activities); 3, severe (influencing usual activities markedly enough to urge modifications); 4, extremely severe (precluding daily activities) (27). Severity of diarrhea was quantified in IBS by the reported number of soft/liquid stools per week, based on a similar variable used in IBD, namely the Crohn's Disease Activity Index (28).

Tissue sections (3–4 μm) were stained using rabbit anti-JAM-A (20 μg/ml) primary antibody (Dako, Ely, UK) according to the manufacturer's instructions. Slides were visualized using confocal microscopy (Olympus Fluoview FV100) and assessed by the researcher blinded to the sample identity. Immunofluorescence surface epithelium staining was graded semiquantatively on a four-point scale: (1—no specific staining, 2—mild specific staining, 3—moderate specific staining, and 4—strong specific staining). JAM-A was graded in all fields of view that contained surface epithelium and the median grade was calculated. Representative

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photomicrographs showing each grade of staining for JAM-A are displayed in **Supplementary Figure 1**.

Statistical analysis

Statistical analysis was performed using SPSS (Armonk, NY). Results are expressed as mean and s.e.m. or as median and range. For cell culture experiments, one-way analysis of variance was used for multiple comparisons followed with the *post hoc* Tukey's test whereas single comparisons were performed with two-tailed Student's *t*-test. For clinical data, differences between two groups were compared using two-tailed Student's *t*-test or Mann–Whitney *U*-test for parametric and non-parametric data, respectively. Differences between more than two groups were compared with non-parametric tests: Kruskal–Wallis or Jonckheere–Terpstra (order of groups is meaningful) and followed by Mann–Whitney *U*-test for single comparisons. Associations with symptoms were assessed using Spearman's correlation. A general linear model was used to determine the potential influence of age and gender on JAM-A expression in controls, IBS and IBD patients. $P \leq 0.05$ was considered statistically significant.

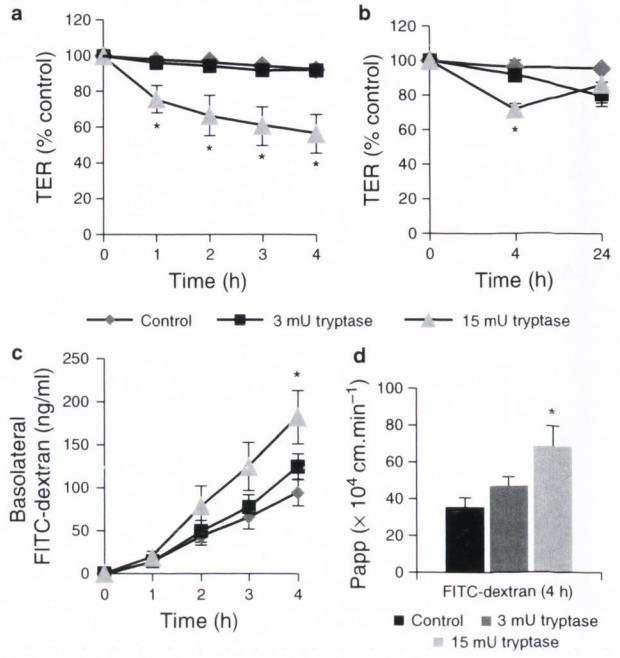

Figure 1. The effects of tryptase (3 or 15 mU) on the epithelial barrier. (**a**, **b**) Transepithelial resistance (TER) of Caco-2 monolayers incubated with tryptase (**a**) for up to 4 h or (**b**) for up to 24 h compared with untreated controls. (**c**) Apical-to-basolateral flux of fluorescein isothiocyanate (FITC)-dextran through Caco-2 monolayers incubated with tryptase for up to 4 h compared with untreated controls. (**d**) Permeability coefficients of FITC-dextran. Results represent the mean±s.e.m. of four independent experiments. *$P<0.05$ (one-way analysis of variance followed by Tukey's *post hoc* test).

RESULTS

Tryptase decreases epithelial integrity and increases paracellular permeability in Caco-2 monolayers

Application of tryptase (15 mU) to Caco-2 cell monolayers had a dramatic effect on epithelial integrity as seen by a significant drop in TER within 1 h of incubation that was maximal at 4 h (36±11%, $P=0.008$) compared with controls (**Figure 1a**). This effect was partially reversible after 24 h of incubation (**Figure 1b**). The drop in TER was associated with a twofold ($P=0.048$) increase in permeability of Caco-2 monolayers to FITC-dextran (**Figure 1c,d**) at 4 h compared with controls. For the lower concentration of tryptase (3 mU), there was a tendency toward the reduction in TER levels compared with untreated controls, however, the difference was not statistically significant. Also 3 mU tryptase did not significantly alter permeability to FITC-dextran (**Figure 1**).

Tryptase decreases the expression of JAM-A, CLD-1, and ZO-1 in Caco-2 monolayers

JAM-A expression was significantly decreased when Caco-2 monolayers were exposed to tryptase (15 mU) for 4 h (0.68±0.12-fold, $P=0.03$) as assessed with western blotting (**Figure 2a,b**), which was concomitant with the drop in TER and increased paracellular permeability. Furthermore, confocal microscopy shown that the intensity of staining for JAM-A in TJs was clearly diminished, and JAM-A was redistributed towards tricellular junctions (**Figure 2c**). The effect of tryptase on JAM-A expression was significantly reversible after prolonged incubation (24 h) and was in line with the observed partially reversible effect on TER. CLD-1 was also significantly reduced (0.68±0.01-fold, $P=0.03$) relative to controls after 24 h of incubation with 15 mU tryptase, although, initially elevated expression (1.97±0.35-fold, $P=0.05$) was observed at 4 h as shown with western blotting (**Figure 2a,b**). CLD-1 intensity within TJs did not change significantly; however, additional CLD-1 staining appeared in punctate cytoplasmic areas in cells exposed to 15 mU tryptase for 4 h, which were not observed in controls. This cytoplasmic CLD-1 was not apparent following prolonged incubation (24 h, 15 mU tryptase) and, consistent with the western blot analysis, overall CLD-1 staining in TJs was lower (**Figure 2c**). The lower concentration of tryptase (3 mU) did not affect JAM-A or CLD-1 expression. The western blot analysis of ZO-1 showed that prolonged exposure (24 h) of Caco-2 monolayers to tryptase (3 or 15 mU) significantly decreased its levels (0.44±0.05-fold, $P=0.0004$ and 0.23±0.02-fold, $P<0.0001$, respectively; **Figure 2a,b**). ZO-1 expression appeared elevated, but not significantly, following 4-h exposure to either 3 or 15 mU tryptase (2.40±1.06-fold, $P=0.22$ and 3.85±1.91-fold, $P=0.17$ respectively). Consistent with western blot findings, the intensity of staining for ZO-1 in TJs increased after 4 h, but decreased after 24 h of incubation with 15 mU tryptase compared with controls (**Figure 2c**).

CLD-3 increased after 4 h of exposure to 15 mU tryptase (2.62±0.40-fold, $P=0.06$), returning to basal levels within 24 h (**Supplementary Figure 2a,b**) and remained unchanged in response to the lower concentration of tryptase (3 mU). Occludin expression increased after 4 h of incubation with tryptase (either 3 mU (1.36±0.05-fold, $P=0.001$) or 15 mU (2.15±0.37-fold, $P=0.02$)) but there was no detectable change after 24 h of incubation (**Supplementary Figure 2a,b**). In line with the western

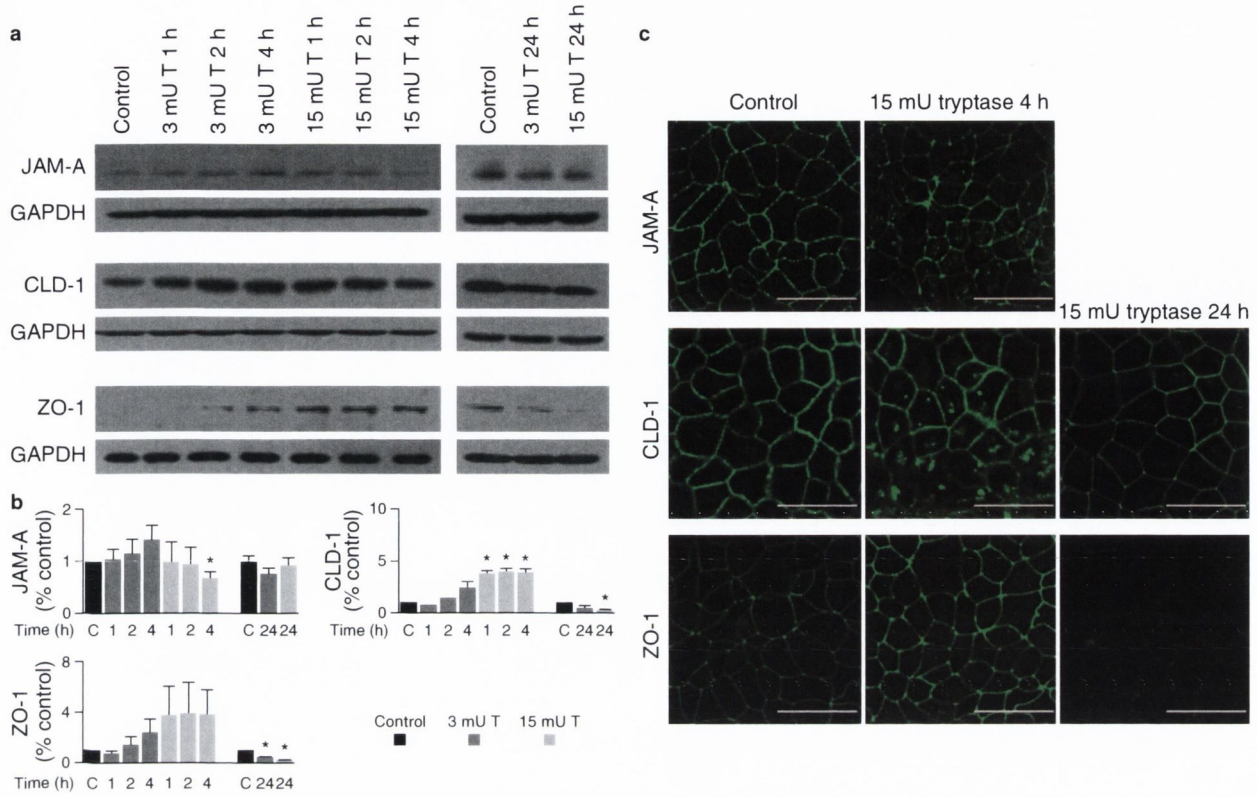

Figure 2. Expression of TJ proteins (JAM-A, CLD-1, and ZO-1) in Caco-2 monolayers incubated with tryptase, T, (3 or 15 mU) for up to 24 h. (**a**) Blots show a representative of a minimum of two independent experiments. (**b**) The results are expressed as a change relative to untreated controls. The density of each individual band was compared with the corresponding control band and normalized against GAPDH by densitometry. $^*P<0.05$ (Student's *t*-test). (**c**) Expression of junctional proteins in the apical region of Caco-2 monolayers compared with untreated controls. Bars = 20 μm. CLD-1, claudin-1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; JAM-A, junctional adhesion molecule-A; TJ, tight junction; ZO-1, zonula occludens.

blot results, the levels of CLD-3 and occludin in TJs increased after 4 h of incubation with tryptase (15 mU) (**Supplementary Figure 2c**). Tryptase did not alter CLD-2 or E-cadherin expression in Caco-2 monolayers (**Supplementary Figure 2**).

Mast cell degranulation decreases epithelial integrity and increases epithelial paracellular permeability in a Caco-2/HMC-1 co-culture model

We developed a co-culture model of Caco-2/HMC-1 cells to more fully investigate the role of mast cells in the disruption of epithelial barrier integrity. Caco-2 cells continuously co-cultured with mast cells formed a tight epithelial barrier as demonstrated by the gradual increase in TER over time, which was maximal at day 20, and was higher when compared with Caco-2 cells alone (day 22, 170%; $P<0.0001$; **Figure 3a**).

When HMC-1 cell were challenged with compound 48/80 to initiate degranulation, Caco-2 cells showed a significant decrease in TER within 2 h of incubation (19±2%, $P<0.0001$)—this effect was maintained at a comparable level up to 24 h (**Figure 3b**). Degranulation of mast cells also induced a significant increase in permeability to FITC-dextran after 6 h of incubation (1.22±0.08-fold, $P=0.03$) with a maximal difference at 24 h (1.68±0.07-fold, $P=0.002$; **Figure 3c–e**) demonstrating that mast cell degranulation disrupted gastrointestinal permeability. TEM analysis of Caco-2/HMC-1 co-cultures confirmed that there was no difference in TJ ultrastructure between Caco-2 monolayers alone and those co-cultured with undegranulated mast cells for 21 days. Mast cell degranulation, however, clearly disrupted epithelial integrity as determined by the presence of open TJs at 6 and 24 h in TEMs (**Figure 3f**), which showed a lack of electronegativity in the TJ complex.

Mast cell degranulation decreases the expression of JAM-A, CLD-1, and ZO-1 in a Caco-2/HMC-1 co-culture model

In the Caco-2/HMC-1 model, we confirmed that JAM-A expression was significantly reduced 6 h after mast cell degranulation (0.67±0.07-fold, $P=0.01$) compared with controls, and the effect was maintained up to 24 h (0.63±0.06-fold, $P=0.0008$; **Figure 4a,b**). This decrease in JAM-A expression following degranulation was confirmed by confocal microscopy (**Figure 4c**). Interestingly, the level of CLD-1 expression in Caco-2/HMC-1 co-cultures up to 6 h after degranulation was comparable to controls. However, 24 h after degranulation, CLD-1 expression appeared significantly reduced (0.57±0.08-fold, $P<0.0001$) as demonstrated by western blotting (**Figure 4a,b**). These findings were also confirmed by confocal microscopy (**Figure 4c**). ZO-1 levels were significantly reduced after mast cell degranulation

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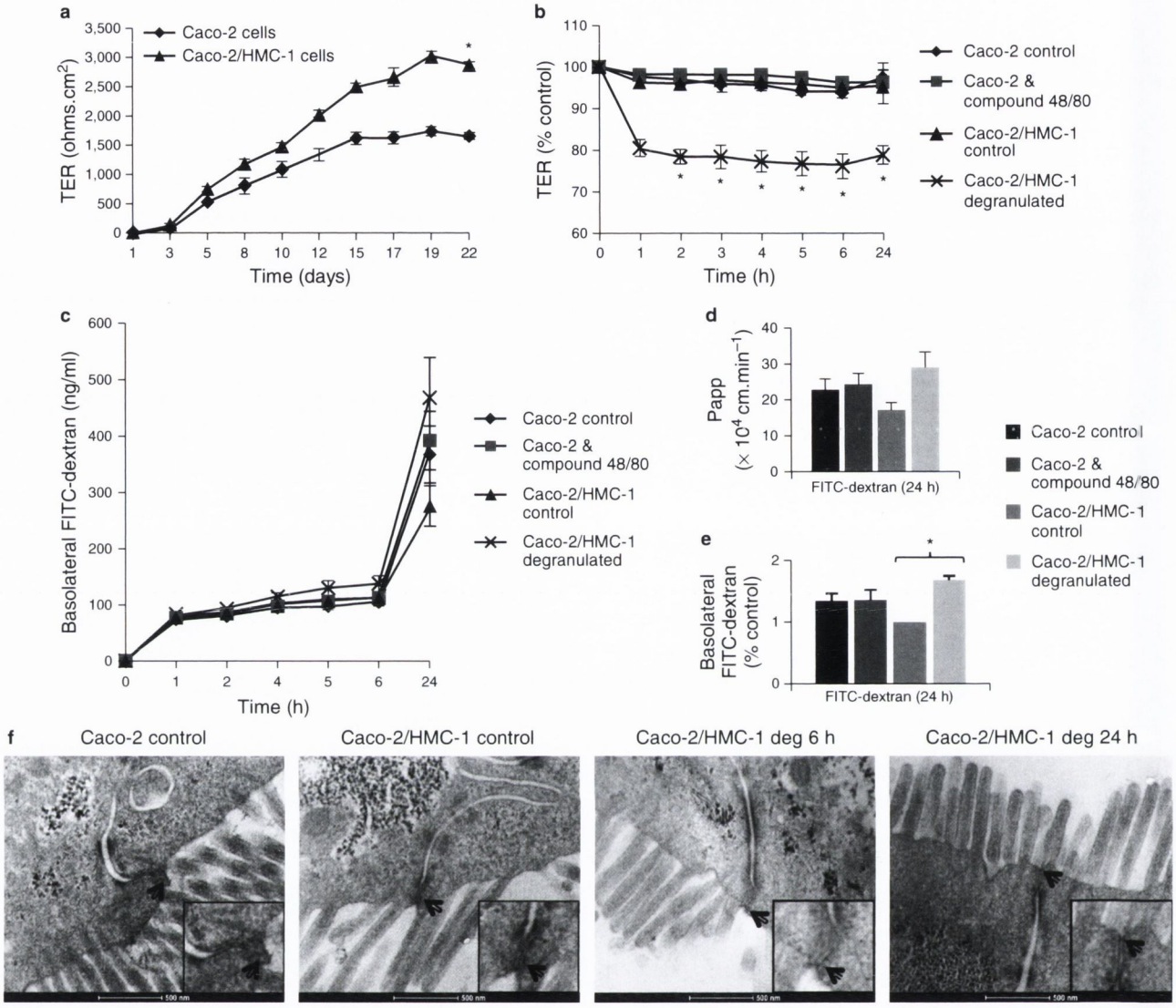

Figure 3. Epithelial barrier function of Caco-2 co-cultured with human mast cell-1 (HMC-1) cells. (**a**) Transepithelial resistance (TER) of Caco-2 cells co-cultured with HMC-1 cells ($n=8$, mean ± s.e.m.) for 22 days compared with Caco-2 cells cultured alone ($n=3$, mean±s.e.m.). (**b**) Effect of HMC-1 degranulation, induced by compound 48/80, on TER of Caco-2 monolayers monitored for up to 24 h and compared with untreated controls (Caco-2/HMC-1 controls). (**c**) Effect of HMC-1 degranulation on paracellular permeability to fluorescein isothiocyanate (FITC)-dextran in Caco-2 monolayers monitored for up to 24 h and compared with untreated controls. (**d**) Permeability coefficients of FITC-dextran. (**e**) Basolateral FITC-dextran content 24 h after HMC-1 degranulation expressed as a change relative to untreated controls. (**f**) Ultrastructural analysis of tight junction (TJ) regions of Caco-2 co-cultured with HMC-1 cell for 21 days either 6 or 24 h after mast cell degranulation. TJs are marked with an arrow. (**b–e**) Results represent the mean±s.e.m. of a minimum of four independent experiments. *$P<0.05$ (one-way analysis of variance followed by Tukey's *post hoc* test).

initially (at 1, 2, and 4 h), but not at 24 h. Both JAM-A and CLD-1, showed a significant decrease at 24 h (**Figure 4**), and, thus, were selected as a focus for follow-up experiments.

Tryptase inhibition diminished the effect of mast cell degranulation on epithelial integrity and paracellular permeability in a Caco-2/HMC-1 co-culture model

To better understand the role of tryptase on epithelial permeability, we investigated the effect of tryptase inhibition (NM) on TER. NM did not have a significant effect on TER of Caco-2/HMC-1 co-cultures for up to 6 h following degranulation compared with controls (**Figure 5a**). After 24 h, however, the reduction in TER following mast cell degranulation was significantly inhibited returning to almost control levels ($P=0.046$). Consistent with this, NM significantly inhibited the effect of mast cell degranulation on paracellular permeability to FITC-dextran over 24 h of incubation (1.15±0.11-fold, $P=0.016$) back to the levels of the undegranulated co-cultures (**Figure 5b–d**).

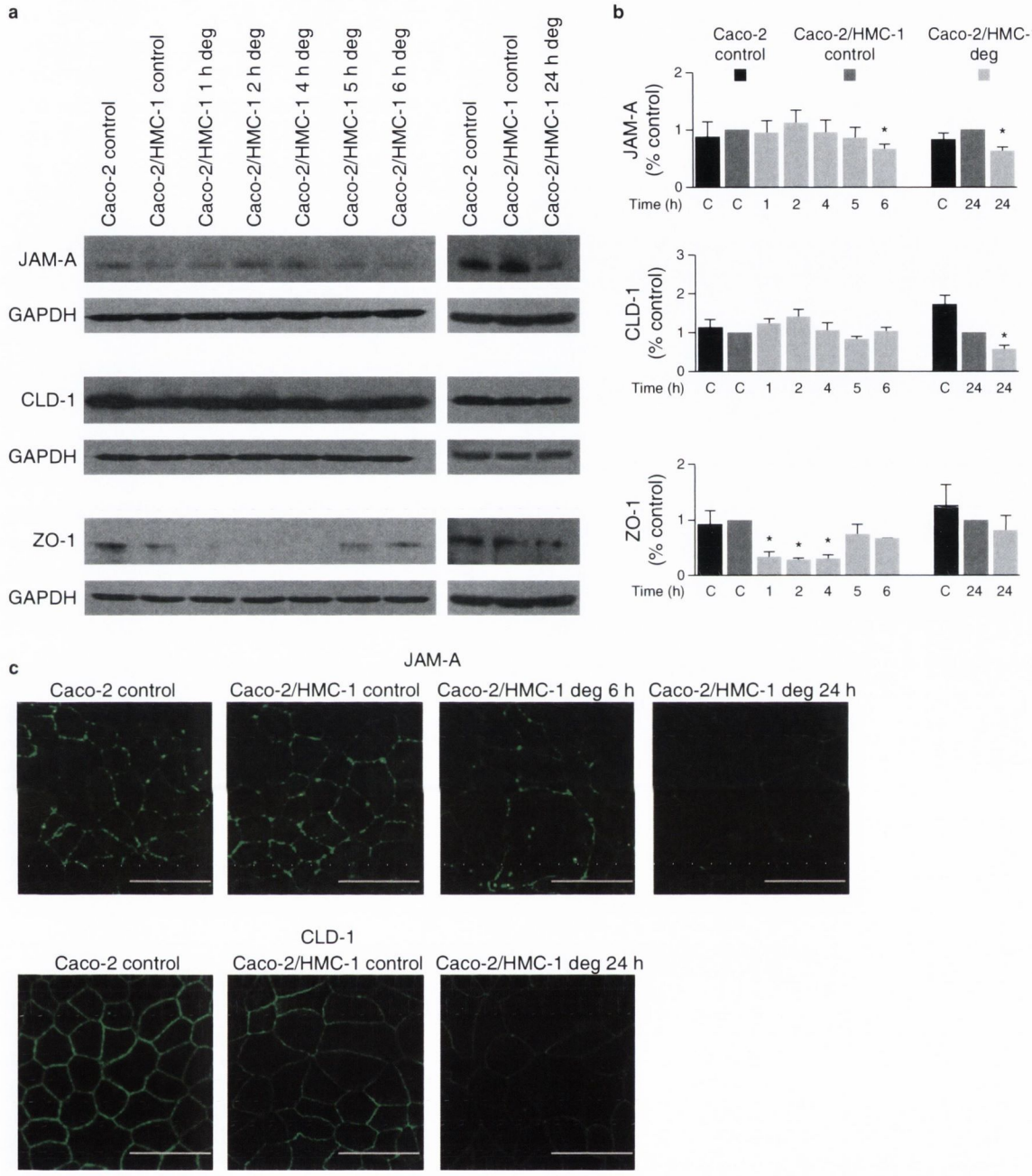

Figure 4. Expression of JAM-A, CLD-1, and ZO-1 in Caco-2 co-cultured with HMC-1 after mast cells degranulation. (**a**) Blots show a representative of a minimum of two independent experiments. (**b**) The results are expressed as a change relative to untreated controls. The density of each individual band was compared with the corresponding control band and normalized against GAPDH by densitometry. $^*P<0.05$ (Student's *t*-test). (**c**) Expression of JAM-A and CLD-1 in Caco-2 monolayers and corresponding controls. Bars = 20 μm. CLD-1, claudin-1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HMC-1, human mast cell-1; JAM-A, junctional adhesion molecule-A; ZO-1, zonula occludens.

Tryptase inhibition diminished the effect of mast cell degranulation on the expression of JAM-A in a Caco-2/HMC-1 co-culture model

We investigated the effect of tryptase inhibition on junctional proteins in the Caco-2/HMC-1 co-culture model. We focused on JAM-A and CLD-1, since in experiments to date, both were consistently reduced at 24 h in response to mast cell degranulation and accompanied by a decrease in TER and an increase in FITC-dextran flux.

Degranulation of mast cells significantly reduced the expression of JAM-A in Caco-2/HMC-1 co-cultures within 24 h (0.69±0.02-fold, $P<0.0001$). Incubation of tryptase with NM significantly reversed the effect of degranulation on JAM-A expression (0.87±0.05-fold, $P=0.02$) to that of undegranulated controls

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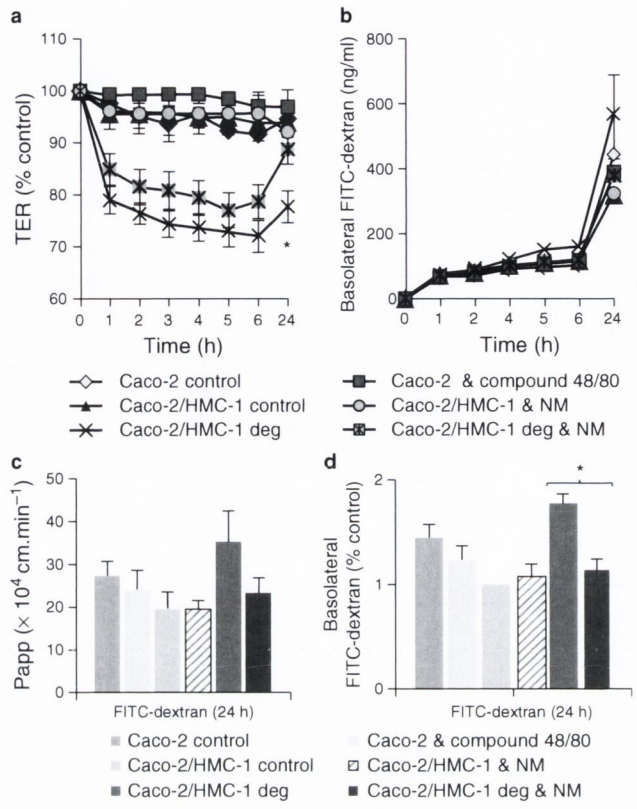

Figure 5. Effect of tryptase inhibition on the epithelial barrier of Caco-2 co-cultured with human mast cell-1 (HMC-1) for up to 24 h after degranulation. Compound 48/80 was used to induce mast cell degranulation. Mast cell tryptase activity was inhibited with nafamostat mesilate, NM. (**a**) Transepithelial resistance (TER) of Caco-2 monolayers. (**b**) Apical-to-basolateral flux of fluorescein isothiocyanate (FITC)-dextran through Caco-2 monolayers. (**c**) Permeability coefficients of FITC-dextran. (**d**) Basolateral FITC-dextran content 24 h after degranulation expressed as a change relative to untreated controls. Results represent the mean±s.e.m. of three independent experiments *$P<0.05$ (one-way analysis of variance followed by Tukey's *post hoc* test).

as shown by western blotting (**Figure 6a,b**) thus supporting that the reduction in JAM-A was due, at least in part, to effects of mast cell tryptase. Confocal microscopy demonstrated that JAM-A levels were reduced in TJs in the co-cultures 24 h after degranulation and that this effect was mitigated by NM treatment (**Figure 6c**). Although CLD-1 expression was significantly reduced 24 h after mast cell degranulation (0.50±0.07-fold, $P<0.0001$; **Figure 6a,b**), it was unaltered by tryptase inhibition in this model.

JAM-A expression is significantly reduced in tissue from IBS patients

To explore the clinical translation, we set out to determine if the novel reduction in JAM-A identified in the *in vitro* studies could be identified in IBS. We studied 34 IBS patients, 22 (65%) were classified as diarrhea predominant (IBS-D) and 12 (35%) as alternating (IBS-A) subtype (26). In the IBD group, three patients had clinically confirmed ulcerative colitis (37%) while five had Crohn's disease (63%) whereas according to disease activity status three patients (37%) were identified with active IBD and had histological evidence of inflammation in the cecum whereas five (63%) with inactive IBD and had no evidence of inflammation in the cecum. All IBS patients were newly diagnosed but had an average reported onset of symptoms of 3.2±0.7 years whereas the average duration of IBD diagnosis was 4.5±1.7 years. IBS patients scored abdominal pain severity as mild ($n=9$, 27%), relevant ($n=17$, 51%), severe ($n=4$, 12%), or extremely severe ($n=3$, 9%). A female predominance ($n=28$, 82% vs. $n=5$, 41%; $P=0.008$) and younger age (32.1±1.4 vs. 43.1±4.7 years; $P=0.004$) was noted in IBS compared with controls. IBD patients and controls were gender matched but IBD patients were younger in age (29.0±2.4 years, $P=0.033$). Potential age and gender effects on JAM-A expression were addressed in further analysis using a general linear model, which confirmed that neither gender ($P=0.64$) nor age ($P=0.55$) significantly influenced JAM-A expression in the disease group (control, IBS, and IBD). In line with this, analysis of the control group, similarly to previously published (7), confirmed no gender or age influences on JAM-A expression in controls (**Supplementary Table 1**).

In controls, JAM-A expression was typically abundant in TJs and was present along lateral membrane of epithelial cells of cecal mucosa. In IBS patients, however, the overall JAM-A expression in the surface epithelium was significantly lower (median: IBS grade 3 vs. controls grade 4, $P=0.012$) compared with controls (**Figure 7a,b**). In 44% ($n=15$) of IBS patients, low JAM-A expression was noted (staining grade 1 or 2) compared with 8% of controls ($n=1$). The reduction in JAM-A was consistent in both IBS-D (median: grade 2.5, $P=0.016$) and IBS-A (median: grade 3, $P=0.041$) compared with controls (**Figure 7a,c**). JAM-A expression in inflammatory controls (IBD) was significantly lower compared with controls (median: inflammatory controls grade 2 vs. controls grade 4, $P=0.01$) in agreement with previously published data (10) (**Figure 7a,b**), but not different to IBS patients.

JAM-A expression is associated with IBS symptoms

JAM-A expression in surface epithelium of cecal mucosa was significantly negatively associated with abdominal pain severity in the IBS-A subgroup ($r_s=-0.69$, $P=0.018$; **Figure 8a**). The lowest JAM-A expression was observed in patients reporting severe abdominal pain (pain severity score 3; JAM-A expression median of 2) and the highest in those reporting mild abdominal pain (pain severity score 1; JAM-A expression median of 4; $P=0.025$, Jonckheere–Terpstra test; **Figure 8a,b**). No association was found with severity of diarrhea in this study (**Figure 8a**). Symptom onset (years) was significantly negatively associated with JAM-A expression ($r_s=-0.7$, $P=0.012$) in IBS-A subtype, with lower expression being associated with longer duration of IBS symptoms ($P=0.017$, Jonckheere–Terpstra test; **Figure 8a,c**). The highest expression of JAM-A was noted in IBS-A patients with relatively short duration of IBS symptoms (up to 1 year).

DISCUSSION

An increase in mast cell numbers (12–17) and an increase in intestinal permeability (2–4,6,29) have been independently implicated

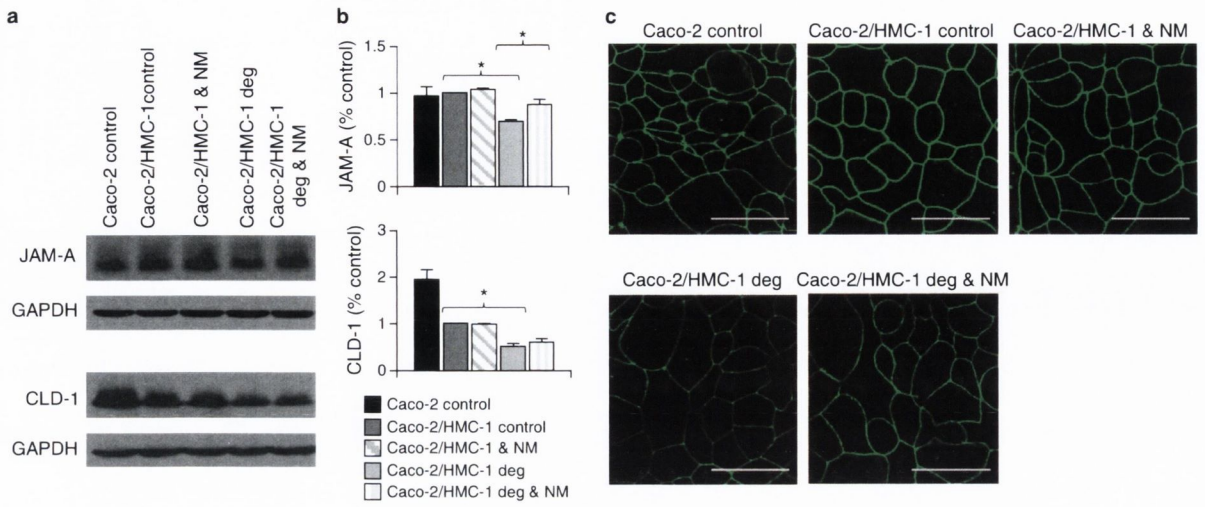

Figure 6. Effect of tryptase inhibition on the expression of junctional adhesion molecule-A (JAM-A) and claudin-1 (CLD-1) in Caco-2 co-cultured with human mast cell-1 (HMC-1) cells up to 24 h after degranulation. Compound 48/80 was used to induce mast cell degranulation. Mast cell tryptase activity was inhibited with nafamostat mesilate, NM. (**a**) Blots show a representative of three independent experiments. (**b**) The results are expressed as a change relative to untreated controls. The density of each individual band was compared with the corresponding control band and normalized against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) by densitometry. $^*P<0.05$ (Student's *t*-test). (**c**) JAM-A expression in Caco-2 monolayers and corresponding controls. Bars = 20 μm.

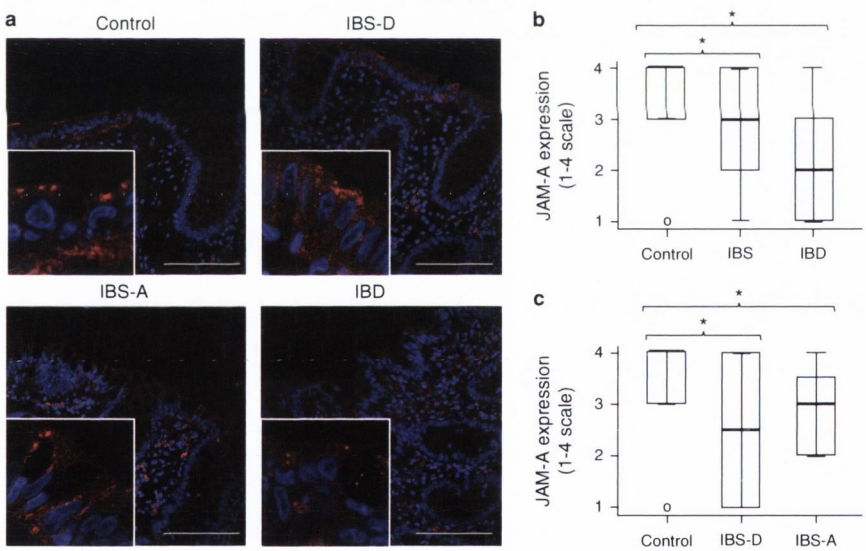

Figure 7. Expression of junctional adhesion molecule-A (JAM-A) protein in surface epithelium of cecal mucosa of controls, irritable bowel syndrome (IBS) subtypes: IBS with diarrhea predominance (IBS-D) and IBS with constipation alternating with diarrhea (IBS-A), and inflammatory bowel disease (IBD) patients. (**a**) Representative micrographs for JAM-A expression. (**b**, **c**) The JAM-A expression was assessed semiquantitatively using a four-grade scale. Values are represented as a median with interquartile ranges. $^*P<0.05$ Mann–Whitney *U*-test. (**b**) Kruskal–Wallis $P=0.012$. (**c**) Kruskal–Wallis $P=0.034$. Bars = 50 μm.

in the pathogenesis of IBS. There are emerging data that tryptase, released from mast cells, may alter intestinal epithelial permeability (20,21), however, the mechanisms involved and the potential translation to IBS have not been fully elucidated. Previously it was shown that mast cell tryptase activates and cleaves protease-activated receptor-2 (30), which is expressed both on the apical and basolateral membrane of the intestinal epithelial cells (31). In this study, we hypothesized that tryptase released from mast cells may reduce colonic epithelial integrity and alter the expression of junctional proteins. We showed that both tryptase and degranulated mast cells significantly increased intestinal epithelial permeability, disrupted epithelial integrity and decreased the expression of junctional proteins JAM-A and CLD-1 *in vitro*. Inhibition of tryptase with NM (23) mitigated the effect of mast cell degranulation on

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a

	Abdominal pain severity (scale 1–4)				Diarrhea (No. of liquid stools/week)				Duration of symptoms (years)			
	Spearman correl.		J-T test		Spearman correl.		J-T test		Spearman correl.		J-T test	
	r_s	P	$r_{Jonckheere}$	P	r_s	P	$r_{Jonckheere}$	P	r_s	P	$r_{Jonckheere}$	P
IBS	0.09	0.63	0.08	0.61	−0.19	0.32	−0.14	0.34	0.09	0.59	−0.07	0.61
IBS-D	0.35	0.11	0.32	0.086	−0.28	0.24	−0.21	0.24	0.19	0.38	0.16	0.36
IBS-A	−0.69	0.018	−0.64	0.025	0.45	0.22	0.42	0.19	−0.7	0.012	−0.6	0.017

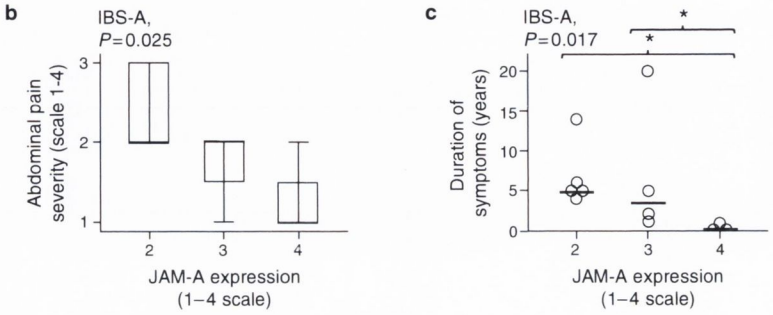

Figure 8. Association between junctional adhesion molecule-A (JAM-A) expression in surface epithelial cells of cecal mucosa of irritable bowel syndrome (IBS) overall and IBS according to subtypes: IBS with diarrhea predominance (IBS-D) and IBS with constipation alternating with diarrhea (IBS-A). (**a**) Data were analyses with Spearman correlation or Jonckheere–Terpstra (J-T) test. (**b**) Abdominal pain severity as a function of JAM-A expression in surface epithelium of cecal mucosa of IBS-A patients. Values are represented as a median with interquartile ranges. J-T test. (**c**) Duration of symptoms as a function of JAM-A expression in surface epithelium of cecal mucosa of IBS-A patients. Values are represented as individual data and medians. J-T test. *$P<0.05$ Mann–Whitney U-test.

epithelial integrity and on JAM-A. This, to our knowledge, is the first reporting of altered JAM-A expression in response to mast cell tryptase. Following up the *in vitro* findings, we showed for the first time, significantly lower JAM-A expression in the cecal mucosa of IBS patients compared with controls, which was associated with more severe abdominal pain and longstanding symptoms in patients with the alternating subtype of IBS.

We have demonstrated that JAM-A levels in Caco-2 monolayers were significantly reduced after exposure to tryptase, which was concomitant with a drop in TER and an increase in paracellular permeability and was reversible at 24 h. In order to explore the mechanism of epithelial barrier disruption in response to mast cell tryptase, we developed a co-culture model of epithelial and mast cells, Caco-2/HMC-1. TEM showed that the presence of intact (undegranulated) mast cells co-cultured with epithelial cells for 3 weeks did not disturb epithelial integrity; TJ integrity of the Caco-2 cells was lost only when the underlying HMC-1 cells were degranulated. We clearly demonstrated that tryptase alone, or when released from degranulating mast cells, was involved in the disruption of the epithelial barrier and that the mechanism involves a reduction in JAM-A. The disruptive effects of tryptase on barrier function in this study are in agreement with findings from *in vitro* studies and in IBS biopsy tissue (20,21). Interestingly, in experimental studies, the absence of JAM-A increases intestinal permeability and cytokine production (9). The involvement of JAM-A, however, is new and this protein has not been investigated either in the context of IBS or in response to tryptase.

Following up on the potential translation of this finding to IBS, we show for the first time, that JAM-A protein was significantly downregulated in IBS patients relative to controls. This protein was consistently reduced in both IBS-A and IBS-D subtypes, suggesting that this alteration might be a feature of IBS independent of bowel habit. Importantly, this reduction in JAM-A was significantly associated with more severe abdominal pain and longer time since symptom onset in IBS-A only. There were no associations between diarrhea and JAM-A expression in this study. As this is the first reporting of JAM-A in IBS, there were no published findings to directly compare with. In IBD, however, lower JAM-A protein expression has been reported (10,11) in agreement with our findings. In addition, in this study levels in IBS and IBD did not differ significantly.

Others have linked changes in junctional proteins in IBS to symptoms. Bertiaux-Vandaële *et al.* (7) showed a negative association between colonic CLD-1 protein expression and severity of abdominal pain in IBS-D. Similarly, the authors noted a negative association between occludin protein and ZO-1 mRNA expression and abdominal pain in IBS (7). Interestingly, Piche *et al.* (2) reported a significant correlation between severity of abdominal pain and paracellular permeability in colonic biopsies of IBS patients; moreover, colonic supernatants from IBS increased paracellular permeability of Caco-2 cells and the degree of this increase was significantly associated with abdominal pain severity (2). More recently, the proportion of dilated junctions in jejunal mucosa was significantly associated with IBS symptoms including abdominal pain severity, bowel movements, and stool consistency (32). However, in this study we identified a negative association between JAM-A and pain only, but not for diarrhea.

We also report an association with duration of symptoms. JAM-A levels in IBS-A appeared to be high in patients up to 1 year after symptom onset and its expression consistently decreased with duration of IBS symptoms. Others, in contrast to our finding, reported the lowest levels of TJs (occludin and CLD-1 protein

and ZO-1 mRNA) within the first 2 years after the onset of IBS, suggesting that altered expression of TJ proteins may be implicated in the initiation stage of IBS (7). Our data suggest the involvement of JAM-A in more sustained and painful IBS, possibly secondary to other pathological mechanisms such as sustained immune activation.

Changes in CLD-1 and ZO-1 have been published in IBS (7,33). In our *in vitro* experiments, CLD-1 was consistently significantly decreased at 24 h both in Caco-2 monolayers in response to tryptase and in response to degranulated mast cells in the Caco-2-HMC-1 model and was accompanied by increased permeability. But in contrast to JAM-A, tryptase inhibition did not alter CLD-1 expression in the co-culture model—thus, suggesting that mast cell mediators other than tryptase may have a role in its regulation. Others (21) have shown no apparent change in CLD-1 in intestinal epithelial cells in responses to protease-activated receptor-2 agonists. In IBS, a trend for lower CLD-1 levels in the colonic mucosa (7) had been reported, but a recent study found no difference in jejunal CLD-1 between IBS-D and controls (32).

We showed that ZO-1 protein levels in Caco-2 cells were significantly decreased after 24 h of tryptase exposure and accompanied by an increase in epithelial permeability, in agreement with others (21). This finding may fit with reports of a downregulation of ZO-1 mRNA in Caco-2 cells incubated with supernatants from colonic biopsies (2) and in ZO-1 downregulation in colonic (7) and jejunal tissue from IBS patients and the significant association between ZO-1 mRNA and tryptase mRNA (33). In the Caco-2/HMC-1 model, however, initial decreases in ZO-1 were observed up to 4 h after mast cell degranulation, but this decrease was not apparent at 24 h. It may be that ZO-1 downregulation in IBS is maintained by other non-mast cell related factors.

This study focused on the effects of mast cell tryptase on epithelial integrity *in vitro* and we sought to translate the novel JAM-A finding to IBS. Further work is required to more fully understand the mechanisms underlying lower JAM-A expression in IBS. Based on the *in vitro* findings, we suggest that this may involve tryptase, however, the mechanisms are likely to be complex and modified by other mast cell, immune, and clinical factors. Although the potential importance of JAM-A and its link with tryptase was initially identified using apically applied tryptase in Caco-2 models, the more relevant co-culture model, inhibition, and clinical studies further confirmed a role for JAM-A. This may fit with findings of increased tryptase in IBS biopsies (33,34). Further studies are required to tease out the mechanisms underlying reduced JAM-A in IBS, including its association with tryptase in biopsy tissue, with activated mast cells using TEM and with intestinal permeability. Importantly, the association between low JAM-A and more severe pain and longer disease duration documented in this study, indicates that this protein may be clinically relevant to IBS pathogenesis or treatment.

In conclusion, this study provides a potential novel mechanism in IBS and demonstrates that tryptase released from mast cells disrupts epithelial integrity via a reduction in JAM-A. Tryptase inhibition significantly reduces the disturbing effect of mast cell degranulation on TER, permeability to FITC-dextran and on the expression of JAM-A protein. We confirmed that JAM-A protein was clinically relevant in IBS, showing significantly lower levels than in controls and an association with more severe abdominal pain and longer disease duration. These findings may offer insights into underlying mechanisms and therapeutic targets for IBS.

ACKNOWLEDGMENTS

We acknowledge Dr Cormac O'Connell and Professor Dimitri Scholz, Conway Institute, University College Dublin, Ireland for preparing the TEM images and Mr James Reilly, Institute of Technology Tallaght, Dublin, Ireland for assistance with statistical analysis. We thank Professor Joseph H. Butterfield, Mayo Clinic, Rochester, MN, for the generous gift of the HMC-1 cell line.

CONFLICT OF INTEREST

Guarantor of the article: Maria A. O'Sullivan, PhD.

Specific author contributions: Maria A. O'Sullivan, Siobhán McClean, and Ewa M. Wilcz-Villega contributed to the study concept, design and implementation, the interpretation of data, writing of the paper and approved the final manuscript. Ewa M. Wilcz-Villega conducted the experiments.

Financial support: This study was funded by the Irish Higher Education Authority Program for Research in Third Level Institutes (PRTLI) Cycle 4 funding for the 'Centre of Applied Sciences for Health' (CASH) consortium. This organization had no role in the study other than funding.

Potential competing interests: None.

Study Highlights

WHAT IS CURRENT KNOWLEDGE

- ✓ Increases in mast cell numbers and in intestinal permeability have been independently linked to the pathogenesis of irritable bowel syndrome (IBS).
- ✓ Emerging data show that tryptase released from mast cells may alter intestinal epithelial permeability.

WHAT IS NEW HERE

- ✓ Tryptase alone, or released from degranulating mast cells, significantly reduced intestinal epithelial permeability and the expression of junctional proteins junctional adhesion molecule-A (JAM-A), zonula occludens-1 (ZO-1) and claudin-1 (CLD-1) *in vitro*.
- ✓ Inhibition of tryptase mitigated the effect of mast cell degranulation on epithelial integrity and on JAM-A.
- ✓ This is the first reporting of a significant reduction in JAM-A in response to tryptase and of significantly lower JAM-A expression in IBS tissue relative to controls.
- ✓ JAM-A appears to be clinically relevant as expression in IBS-A patients associated with more severe abdominal pain and longstanding symptoms.
- ✓ Better understanding of these mechanisms *in vitro* and *in vivo* may improve understanding of IBS pathogenesis and therapeutic strategies targeted at either mast cells or intestinal permeability.

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CHAPTER 9

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