Early Stage Inflammation and Cancer as Reflected in the Gastrointestinal Mucus Composition

Implications for Diagnosis, Prognosis and Pathogenesis

Karolina Sjöberg Jabbar

Department of Medical Biochemistry and Cell Biology
Institute of Biomedicine
Sahlgrenska Academy, University of Gothenburg

UNIVERSITY OF GOTHENBURG

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Cover illustration: *Brachyspira* bacteria lining the epithelial cell surface of the large intestine in a patient with irritable bowel syndrome.

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karolina.sjoberg@medkem.gu.se

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Never express yourself more clearly than you are able to think.

Niels Bohr

To my Mother

- Abstract -

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Karolina Sjöberg Jabbar
Department of Medical Biochemistry, Institute of Biomedicine
Sahlgrenska Academy, University of Gothenburg, Gothenburg, Sweden

Mucus covers our inner interfaces towards the environment, providing protection while enabling vital interaction with the outside world. The mucus is built around mucin proteins, which are important for our defences against infection and inflammation, but may also contribute to carcinogenesis and tumour progression. These divergent aspects of mucin biology are exemplified in the different studies in this thesis, which are all based on mass spectrometry. The topics covered range from mucin biomarkers for the early diagnosis of pancreatic cancer, to the discovery of a link between a mucus-associated bacterial genus and irritable bowel syndrome.

Pancreatic cancer is a relatively rare tumour form, but is postulated to become the second leading cause of cancer deaths in the United States by 2030. The poor prognosis is largely explained by late detection. With advances in imaging, cystic precursors of pancreatic cancer are detected with increasing frequency, offering an unprecedented opportunity for prevention and cure. Unfortunately, available diagnostic tools are not sufficiently robust to enable targeted intervention against high-risk cystic lesions. Here, we describe the development and evaluation of a mass spectrometry based method for quantification of mucins and other biomarkers in pancreatic cyst fluid samples. In a prospective validation cohort, the analysis identified cystic precursors of pancreatic cancer with 97% accuracy. This result represented a significant improvement upon state-of-the-art diagnostic methods. Thus, clinical implementation of the analysis may facilitate early detection of pancreatic cancer, which is a prerequisite for increasing survival rates.

Ulcerative colitis is an inflammatory bowel disease with a chronic and relapsing course. According to the current view, ulcerative colitis results from inappropriate interactions between colonic microbiota and host immunity, against a background of genetic susceptibility. Normally, the abundant luminal microbes are segregated from the colonic epithelium, through an impervious mucus barrier. Here, abnormalities in mucus protein composition were detected in ulcerative colitis patients, also in samples from non-inflamed areas. This implies that structural weakening of the colonic mucus barrier could be important for the development of the disease.

The natural history of ulcerative colitis varies considerably between patients. Prognostic markers for the disease course could reduce relapses and colectomies, as well as the unnecessary use of medication. Herein, we identified a ratio of two proteins in colonic mucus as a powerful predictor of the requirement for intensified medication or surgery during a five year period. Interestingly, these two proteins differentially regulate the sensing of specific microbial ligands. An altered equilibrium of these proteins was tentatively associated with infiltration of bacterial endospores in the colonic lamina propria. Thus, intermittent activation of endospores may conceivably contribute to relapses in patients with severe ulcerative colitis.

Brachyspira is a bacterial genus that includes several pathogenic species. In this thesis, *Brachyspira* colonization of the colonic epithelial surface and inner mucus layer was detected in one third of IBS patients, but rarely observed in healthy individuals. Furthermore, *Brachyspira* colonization was associated with a distinctive symptom profile and mucosal immune response. This suggests that targeted antibiotic therapy of this patient group may reduce the morbidity burden of IBS. However, in our investigation antibiotic treatment paradoxically resulted in *Brachyspira* invasion of goblet cell mucus granules. This observation may represent a novel bacterial strategy to evade and survive antibiotic treatment, with potential, broad implications for our understanding of therapy resistant infections and pathogen persistence in the intestinal reservoir.

Keywords: Mucus, mass spectrometry, MUC2, MUC5AC, IPMN, pancreatic cysts, ulcerative colitis, IBS, Brachyspira

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SAMMANFATTNING PÅ SVENSKA

Medan utsidan av vår kropp är täckt av hud, skyddas insidan av våra luftvägar, tarmar och urinvägar av ett slemlager. Detta lager är uppbyggt av särskilda proteiner som kallas muciner. Muciner är viktiga för vårt försvar mot infektion och inflammation, men kan också i vissa fall bidra till uppkomsten av cancer. I denna avhandling beskrivs hur muciner och andra proteiner i slemlagret påverkar olika aspekter av hälsa och sjukdom i magtarm-kanalen. Fynden omfattar bland annat hur muciner kan fungera som biomarkörer för tidig bukspottskörtelcancer, hur ett försvagat slemlager kan bidra till tarmsjukdomen ulcerös kolit, och en ny koppling mellan förekomsten av ett särskilt bakteriesläkte i slemlagret och IBS (irritable bowel syndrome).

Cancer i bukspottskörteln diagnosticeras ofta i ett sent stadium, och överlevnaden är därför mycket låg. Cystiska (vätskefyllda) förstadier till bukspottskörtelcancer kan upptäckas med skiktröntgen och magnetkameraundersökning, men är svåra att skilja från godartade cystor. I avhandlingen presenteras en ny metod för att identifiera cystiska förstadier till bukspottskörtelcancer. Analysen utförs på cystvätskeprover. Dessa tas vid en endoskopisk undersökning som används rutinmässigt i sjukvården. Metoden innebär att nivåerna av vissa muciner och andra proteiner mäts i cystvätskan med hjälp av masspektrometri. Med denna teknik kunde cystiska förstadier till bukspottskörtelcancer upptäckas med 97 % säkerhet, vilket var signifikant bättre än resultaten för de diagnostiska metoder som används idag. Sammantaget skulle införandet av analysen i sjukvården potentiellt kunna leda till fler fall av tidig upptäckt av bukspottskörtelcancer, vilket är centralt för att förbättra överlevnaden.

Ulcerös kolit är en kronisk inflammatorisk tarmsjukdom, med omväxlande skov av aktivitet och symptomfria perioder. Orsaken till sjukdomen är okänd, men tros bero på ett avvikande immunsvar mot den normala bakteriefloran i tarmen. Normalt sett skyddas insidan av tarmen av ett tjockt, ogenomträngligt slemlager. Här visar vi att försvagning av detta slemlager föregår inflammationen, och därigenom skulle kunna bidra till sjukdomens uppkomst. Förloppet av ulcerös kolit varierar påtagligt mellan individer. Metoder för att förutspå sjukdomsaktiviteten skulle kunna förbättra behandlingen för den enskilda patienten. I avhandlingen presenteras två biomarkörer som kunde förutsäga behovet av ökad medicinering och kirurgi under en femårsperiod med nästan 90 % säkerhet. Dessa två proteiner reglerar slemhinnans immunsvar mot vissa bakterier, varför vi också studerade bakterieförekomsten i vävnaden. Vi noterade då en tänkbar koppling mellan täta och svåra skov och fynd av bakteriella sporer i slemhinnan. Genom sporbildning kan bakterier överleva under lång tid och svåra förhållanden. Re-aktivering av sporer i tarmvävnaden skulle potentiellt kunna utlösa skov av ulcerös kolit, även om detta måste studeras vidare.

Var femte till var tionde person är drabbad av IBS. I denna avhandling påvisas en koppling mellan IBS och Brachyspira – ett bakteriellt släkte som innehåller flera sjukdomsalstrande arter. Brachyspira koloniserade tarmcellernas yta hos en femtedel av alla IBS-patienter som undersöktes i studien. Motsvarande fynd sågs inte hos några friska personer. Växt av Brachyspira på tarmcellsytan var kopplat till diarré, snabb tarmpassage, inflammation och aktivering av mastceller. Det har tidigare visats att mastceller utsöndrar substanser som påverkar tarmens smärtkänslighet vid IBS. Behandling med antibiotika ledde till förbättring av symptomen, men utrotade inte infektionen. Istället noterades att Brachyspira hittat en ny nisch inuti tarmens bägarceller. Sammantaget visar våra fynd på en potentiellt botbar orsak hos en hög andel individer med IBS. Invasionen av bägarceller efter antibiotika motsvarar en ny mekanism för bakterier att överleva antibiotikabehandling. Detta fynd skulle kunna ha en övergripande betydelse för vår förståelse av svårbehandlade och återkommande infektioner.

LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. Jabbar KS, Verbeke C, Hyltander AG, Sjövall H, Hansson GC, Sadik R. **Proteomic mucin profiling for the identification of cystic precursors of pancreatic cancer**. J Natl Cancer Inst. 2014; 106(2): djt439.

II. Jabbar KS, Arike L, Verbeke CS, Sadik R, Hansson GC. **Highly accurate identification of cystic precursor lesions of pancreatic cancer through targeted mass spectrometry: a phase IIc diagnostic study**. J Clin Oncol. 2018; 36(4): 367–375.

III. van der Post S*, Jabbar KS*, Birchenough GMH, Arike L, Akhtar N, Sjövall H, Johansson MEV, Hansson GC. **Structural weakening of the colonic mucus barrier is an early event in ulcerative colitis pathogenesis.** *Submitted.*

IV. Jabbar KS, van der Post S, Johansson MEV, Hansson GC. **The protein composition of the colonic mucus barrier predicts disease course in ulcerative colitis patients.** *Manuscript.*

V. Jabbar KS, Dolan B, Eklund L, Wising C, Ermund A, Johansson Å, Törnblom H, Simrén M*, Hansson GC*. **The presence of *Brachyspira* species in the colonic epithelium and inner mucus layer may be linked to disease development in up to a third of IBS patients.** *Manuscript.*

* Joint first/senior authorship

CONTENT

ABBREVIATIONS

BCAP31	B-cell receptor-associated protein 31
CEA	Carcinoembryonic antigen
CLCA1	Calcium-activated chloride channel regulator 1
CT	Computed tomography
DMBT1	Deleted in malignant brain tumors 1 protein
ESI	Electrospray ionization
ER	Endoplasmic reticulum
EUS	Endoscopic ultrasound
FASP	Filter aided sample preparation
FCGBP	IgGFc-binding protein
FLII	Protein flightless-1 homolog
H&E	Hematoxylin-eosin
HGD	High-grade dysplasia
IBS	Irritable bowel syndrome
IBS-SSS	IBS Severity Scoring System
IL	Interleukin
IPMN	Intraductal papillary mucinous neoplasm
LC	Liquid chromatography
LPS	Lipopolysaccharides
LRRFIP1	Leucine-rich repeat flightless-interacting protein 1
MCN	Mucinous cystic neoplasm
MRI	Magnetic resonance imaging
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
MUC1	Mucin-1
MUC2	Mucin-2
MUC5AC	Mucin-5AC
MUC6	Mucin-6

MYD88	Myeloid differentiation primary response protein MyD88
m/z	Mass-to-charge ratio
PanIN	Pancreatic intraepithelial neoplasia
PAS	Periodic acid-Schiff
PCR	Polymerase chain reaction
PRM	Parallel reaction monitoring
PSCA	Prostate stem cell antigen
RAB10	Ras-related protein Rab-10
SCFA	Short chain fatty acids
SDS-PAGE	Sodium dodecyl sulfate–polyacrylamide gel electrophoresis
SLC26A3	Chloride anion exchanger
Th	T helper
TLR	Toll-like receptor
UC	Ulcerative colitis
ZG16	Zymogen granule membrane protein 16

1 INTRODUCTION

This thesis encompasses three disorders of the gastrointestinal tract: pancreatic cystic tumours, inflammatory bowel disease and irritable bowel syndrome. Not only do these conditions affect two different organs, they also represent three traditionally distinctive categories of gastrointestinal diseases: neoplastic, inflammatory and functional. Pancreatic cystic tumours and irritable bowel syndrome are about equally common, but differ fundamentally in other ways. One affects the elderly, the other the young. One is associated with findings, the other with symptoms. Finally, pancreatic cancer has the highest and irritable bowel syndrome the lowest mortality among the major gastrointestinal diseases. However, all three conditions share one frustrating and compelling characteristic: they are poorly understood.

Hence, the studies in this thesis may appear very disparate. Nevertheless, while the organs and disorders may be different, the cells and molecules of interest are much more similar. This investigation is based on mass spectrometry analyses of mucus-resident proteins, hopefully demonstrating some of the versatility of this technique.

Mucus and its integral components, mucins, are produced by specialized cells. Mucins are important in infection, inflammation and cancer, both to our benefit and our detriment. Aberrant expression of the MUC1 mucin is postulated to contribute to nearly two thirds of cancer cases in the United States. On the other hand, the MUC2 mucin protects the inside of our intestines from inflammation and colon cancer development.

The abundant gut microbiota influences our gastrointestinal, metabolic and even mental health. Importantly, mucus is the arena where the intestinal microbiota and the host meet, and proteins the main functional tools through which they interact. Thus, further studies of the gastrointestinal mucus proteins, both human and microbial, could potentially contribute to our understanding of other disorders –that may appear more unrelated than those investigated in this thesis.

1.1 PROTEOMICS AND MASS SPECTROMETRY

Proteomics is the study of the *proteome*; a synthesis of the words "protein" and "genome".[1] The proteome refers to the full set of proteins identified in a system, such as a tissue or a biological fluid. However, in contrast to the genome, the proteome is dynamic and alters over time, and with varying requirements of the cell, tissue or organism. The properties of proteins may also be influenced by various post-translational modifications, such as glycosylation and phosphorylation of particular

amino acids. Thus, the proteome represents an ideal source of disease-specific biomarkers.

Although proteomics was originally a generic term for all studies of protein expression, including for example antibody-based approaches, nowadays it usually refers to the analysis of proteins by mass spectrometry (MS). While it is possible to analyse full proteins by mass spectrometry (top-down proteomics), this technique is more time-consuming and complicated than the analysis of a mixture of peptides (bottom-up proteomics).[2] Hence, enzymatic digestion of proteins is generally part of the sample preparation for MS.

1.1.1 Sample preparation

The first step in the work-flow for proteomic analysis has traditionally been to reduce sample complexity, in order to aid the subsequent identification of proteins. Separation of proteins by their molecular mass, e.g. through a one-dimensional gel electrophoresis, is still a common strategy.

In order for proteases (i.e., "protein-cleaving" enzymes) to gain access to the protein backbone, proteins must first be solubilized and denatured. This is achieved through the addition of a chaotropic agent such as urea or guanidine hydrochloride. Disulphide bonds (between cysteine residues) that are stabilizing the protein, are typically broken by reduction (e.g. through dithiothreitol) and prevented from reforming by alkylation (e.g. through iodoacetamide). The break-down of the three-dimensional conformation of the protein allows proteases to digest it. The most commonly used protease in proteomic studies is the pancreatic enzyme trypsin. It is very important that the protease used is specific, i.e., cleaves the amino acid chain in a predictable manner. Trypsin, for instance, cleaves the peptide backbone at the carboxyl side of amino acids arginine (R) and lysine (K), unless they are followed by a proline (P). Multiple libraries of peptides that can theoretically be produced through the digestion of a particular protein with a particular protease have been generated, and are used for peptide and protein identification.

1.1.2 Liquid chromatography

In order to enhance the sensitivity of the MS analysis, prior separation of peptides through liquid chromatography (LC) is a common practice. There are various LC methods, suited to different sample types and downstream applications. However, the fundamental principle involves the separation of peptides according to their chemical or physical properties, based on their passage through a system. Typically, this system consists of a column packed with an adsorbent material, known as the "stationary phase".[3] The interaction of the sample with the stationary phase is affected by a third, liquid component: the "mobile phase". The composition of this liquid may be altered in

a controlled manner during the course of the experiment. Commonly, the mobile phase consists of a mixture of polar and non-polar components, where the concentration of the organic solvent is gradually increased or decreased. Thus, peptides will elute from the column at different time points, based on their hydrophobicity. Online LC-MS systems, with direct coupling between the LC unit and the mass analyser are nowadays frequent.

1.1.3 Mass spectrometry

Briefly, a mass spectrometer consists of three parts: an ionizer, a mass analyser and a detector. First, the peptides are ionized, which is prerequisite for mass analysis and detection. For proteomics, "soft" ionization techniques like electrospray ionization (ESI) are typically favoured. ESI, which was used for the studies included in this thesis, is capable of the generation of ions with multiple charges. This allows for the acquisition of mass to charge (m/z) spectra also from relatively large molecules. Analysis in positive or negative ion mode is possible, although the former is more common.[4] During ESI, the peptide ions are contained in minuscule droplets. In positive ion mode analysis, the liquid of these droplets is acidic. This means that the basic amino acids of a peptide, as well as its N-terminus, will be protonated. Trypsin cleaves the peptide backbone after arginine and lysine, which are both basic amino acids. Therefore, each tryptic fragment will have a charge of at least 2+. Non-peptide compounds in the sample can be distinguished from the actual peptides, since they are usually singly charged.

Following ionization, the sample enters the mass analyser. The mucin profiling of pancreatic cyst fluid in Paper I, was done with a Fourier-transform ion cyclotron resonance mass analyser, wherein the movement of ions in a magnetic field is used to deduce their m/z ratio.[5] In the Orbitrap mass analyser used in papers II-V of this thesis, ions oscillate around a central, spindle-shaped electrode and between two outer cup-shaped electrodes, along a defined axis.[6] Again, their oscillation frequency is directly dependent on their mass. In tandem mass spectrometry (MS/MS), following analysis of the full peptide, fragments are also generated. Usually, fragmentation is achieved through collision of the peptides with neutral atoms/molecules (e.g. nitrogen) in gas phase. The peptide fragments will then again enter a mass analyser, where their individual m/z values will be determined. The rationale for this procedure is to facilitate peptide sequence identification. The role of the detector is to register the oscillation frequency and intensity of the ions. Simply put, the intensity is determined by how frequently the signal from a particular ion is registered by the detector, over an observation period. For both examples of mass analysers, the recorded image current is translated into a spectrum of mass to charge ratio (x-axis) and intensity (y-axis) values, through a Fourier transform function.

1.1.4 Identification of peptides and proteins

The basis for peptide and protein identification is the comparison of masses and MS/MS fragmentation spectra from the experiment, with theoretical results from "in silico" digested proteins. Specialized software and databases, nowadays available for many different organisms, are used for this procedure. In order to estimate the frequency of false positive results (the false discovery rate), a parallel search is performed against a decoy database. The decoy database is generally constructed by reversing the amino acid sequences from the original database. A threshold of a minimum of one unique peptide at a false discovery rate of 1%, is typically applied for protein identification.[7] However, in certain situations more stringent criteria may be appropriate.

1.1.5 Relative and absolute protein quantification

One major drawback of proteomic analysis by MS is that the technique is not inherently quantitative. The intensity measured for a peptide does not directly reflect its abundance. It is also dependent on its chemical properties, which will, for instance, affect ionization efficiency. Relative quantification may be achieved through chemical labelling (isotopic/isobaric) or through so-called label-free quantification.[8]The latter involves the comparison of protein abundances between samples through the frequency with which a certain peptide is detected, as well as the intensity of the same peptide, as recorded by the detector. With label-free quantification, variations in sample processing and analysis must be kept to a minimum so as not to introduce bias. Absolute quantification is possible through the selection of a peptide mass or a peptide-fragment-ion pair for analysis through e.g. Selected/Single Ion Monitoring (SIM) or Selected Reaction Monitoring (SRM).[9,10] Instrument parameters can then be set to exclude other m/z values. The introduction of an internal standard, in the form of a known quantity of a labelled custom-designed synthetic peptide, corresponding to the target of interest, makes it possible to determine the exact concentration of a protein in a sample.

1.1.6 Metaproteomics

Metagenomics, the study of the entire repertoire of genes found in environmental samples, has revolutionized the study of microbial communities. Unlike culture-based approaches, metagenomics enables high-throughput profiling of microbes in their natural habitat. By contrast, in metaproteomic studies, MS is used to study the full set of proteins from different organisms within a complex sample. Proteins, the functional product of genes, perform the majority of essential tasks in cells and organisms. Even transcriptomic data do not correlate perfectly with protein expression.[12] This means that meta-proteomic studies could potentially provide a more accurate and comprehensive functional characterization of microbial communities. In summary,

genomic data informs us about which microbes are present in a sample, and what they are theoretically able to do. By contrast, metaproteomics could tell us what they are actually doing in that environment- for instance inside the human body. Within the last few years, metaproteomic studies have investigated the microbial communities of the oral cavity, the vagina and the gut.[15-17] A few studies have also begun to address metaproteomic alterations associated with particular disorders, including inflammatory bowel disease.[18,19]

While the sample preparation workflow is largely similar to standard mass spectrometry-based proteomics, the bioinformatics analysis poses formidable challenges. As an example, the human intestine could contain up to a thousand different species.[20] Each of these express thousands of different proteins. Protein homology between species, horizontal gene transfer and strain variation add to the complexity of the analysis.[21] Thus, the identification and taxonomic annotation of peptides during metaproteomic analysis places a great strain on both hardware and software. Moreover, only a small minority of the global microbial community has been sequenced.[22] As of 2014, 30,000 bacterial genome sequences were publically available - while the number of bacterial species on Earth has been estimated to exceed one trillion.[22]

1.2 MUCINS AND CANCER

Mucins are a family of high-molecular mass, heavily glycosylated glycoproteins characterized by typical domains rich in the amino acids proline, threonine and serine. The serine and threonine residues are frequently O-glycosylated, giving the protein a "bottle-brush-like" appearance. As a result, over half of the mass of the protein is accounted for by glycans.[23,24] Mucins may be transmembrane (such as MUC1 and MUC4) or secreted (such as MUC2, MUC5AC and MUC6). Both forms are important for the protection of the apical surface of epithelial cells.[23,24] Secreted mucins form a gel that lubricates the epithelium and shields it from environmental hazards such as HCl in the ventricle and bacteria in the colon. Transmembrane mucins also provide physical protection. In addition, they are likely important for the cell´s sensing of the external environment.[23,24]

1.2.1 Role in cancer

Both transmembrane and secreted mucins have been reported to be overexpressed, or aberrantly expressed, in malignancies. The role of mucin-1 (MUC1) in epithelial cancers has been particularly well elucidated. In fact, 64% of the cancers diagnosed in the United States each year are estimated to exhibit overexpression or *de novo* expression of MUC1.[25]

Loss of polarity in cancer leads to expression of transmembrane mucins on the basolateral side of the cells, enabling their interaction with e.g. receptor tyrosine kinases.[23-24] This phenomenon has been shown to activate signalling pathways associated with cell proliferation and survival. For instance, studies indicate that MUC1 promotes EGFR signaling in cancer cells.[23-26] Aberrant expression of secreted mucins may favour tumour progression by "hiding" the neoplastic cells from innate immunity.[27,28] In pancreatic ductal adenocarcinoma, *de novo* expression of the gel-forming MUC5AC is an early event, occurring in both micro- and macroscopic precursor lesions.[28-30]

1.3 PANCREATIC CYSTIC TUMOURS

The incidence of pancreatic cystic tumours has increased dramatically during the last three decades.[31,32] This is likely due to a combination of two factors: increased detection, as a result of advances in the quality and availability of imaging techniques, and greater awareness and understanding of these conditions. In fact, the most common form of pancreatic cystic tumour, intraductal papillary mucinous neoplasm (IPMN) was recognized as a clinical entity less than 40 years ago.[33]

Pancreatic cystic lesions are very common incidental findings on imaging, identifiable on 2.6% of abdominal computed tomography (CT) scans.[34] Using magnetic resonance imaging (MRI), a prevalence of nearly 50% was established in a population-based study of mostly middle-aged participants (mean age 55).[35] Until relatively recently, over 90% of incidentally detected cystic lesions were believed to be inflammatory pseudocysts. However, several studies indicate that the majority are actually cystic tumours. Furthermore, most of these tumours are now considered to have malignant potential.[31,36]

Pancreatic cystic neoplasms form a heterogenous group with regard to clinical presentation and prognosis. The most common pancreatic cystic tumours are serous cystic neoplasms, mucinous cystic neoplasms (MCN), intraductal papillary mucinous neoplasms (IPMN) and cyst-like ductal adenocarcinomas, while solid pseudopapillary neoplasms and cystic endocrine tumors are rare entities. Of all these tumour types, only serous cystic neoplasms are considered completely benign. At the other end of the spectrum, cyst-like ductal adenocarcinomas have the same dismal prognosis as solid exocrine pancreatic cancer.[31,37,38] IPMN, and MCN have malignant potential.[31,37,38] However, only a minority are malignant at the time of diagnosis and malignant progression is thought to occur over several years, perhaps even decades.[39,40] Nevertheless, these tumours are nowadays recognized as precursors to ductal adenocarcinoma.[29,41] Thus, they can be considered macroscopic counterparts of pancreatic intraepithelial neoplasia (PanIN), from which all exocrine pancreatic

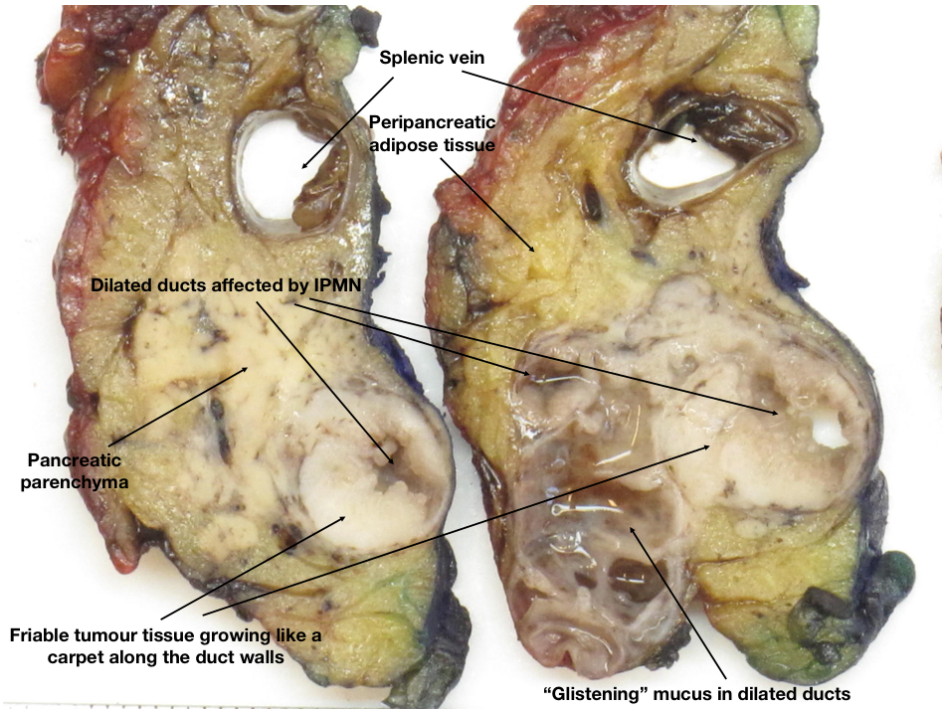

***Figure 1**. Macroscopic images of a resected IPMN in the head of the pancreas. Image courtesy of Professor Caroline Verbeke, University of Oslo*

cancers were previously thought to derive. This insight has offered an unprecedented opportunity for early, or even preventive, intervention against pancreatic cancer. Early diagnosis is pivotal in order to improve the outcome of pancreatic cancer, which at present is almost always discovered at a stage where it has already metastasized to other organs. Consequently, the prognosis has remained very poor, with 5-year survival rates lingering around 8%.[42]

From their respective prevalence figures, it is obvious that most pancreatic cystic lesions will never progress to pancreatic cancer. Resection of a pancreatic cystic lesion, particularly in the head of the pancreas, involves major surgery with a relatively high risk of complications.[32] Thus, accurate diagnosis, and a careful selection of surgical candidates is of paramount importance. Consensus guidelines for the management of different pancreatic cystic tumour types are available.[37,38,43] However, at present a definitive diagnosis is rarely possible before surgery.[44,45]

1.3.1 Pathophysiology

The pathogenesis of pancreatic cystic neoplasms varies with tumour type, but is generally relatively poorly understood.

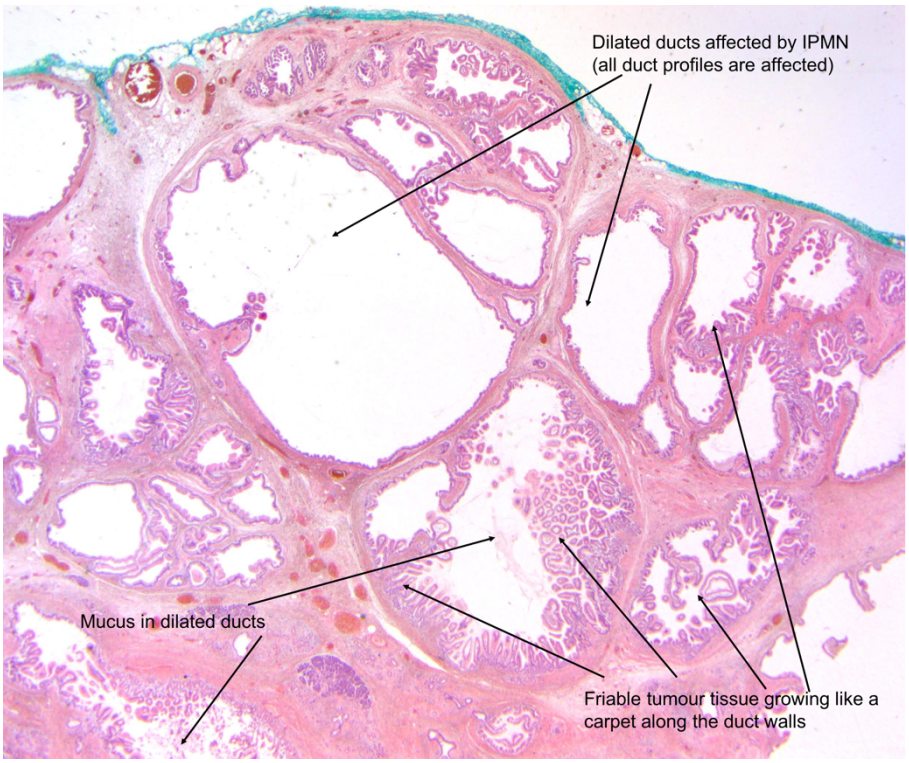

Figure 2. *Microscopic image of an IPMN. Courtesy of Professor Caroline Verbeke, University of Oslo.*

Serous cystic neoplasms affect predominantly females, typically present as a conglomerate of small cystic lesions, and are frequently detected in patients with Von Hippel Lindau syndrome.[31,46] Von Hippel Lindau´s disease is caused by mutation of the VHL gene, which encodes a protein that negatively regulates the expression of Hypoxia Inducible Factor 1 alpha (HIF1α). In the absence of a functional VHL protein, HIF1α will activate the transcription of a set of genes, including vascular endothelial growth factor, erythropoietin and several genes associated with the uptake and metabolism of glucose. These aberrations are consistent with the morphology of serous cystic neoplasms, which are characterized by glycogen accumulation and hypervascularization.[47]

Mucinous cystic neoplasms (MCN) resemble serous cystic neoplasms in that they affect almost exclusively women (>95%), while they share mucinous content and malignant potential with IPMN.[47,48] Morphologically, MCN are macrocystic or unilocular lesions, usually located in the tail of the pancreas.[31,37,38] Unlike IPMN, they have no connection with the pancreatic ducts. Histologically, MCN consist of two components: a columnar cell mucin-producing epithelium and an “ovarian-like” stroma with estrogen and progesterone receptors.[47] Taken together, the predilection of MCN

for females, the fact that most cases occur in the "perimenopausal" period (mean age 48) and the presence of ovarian-type stroma, strongly suggest that hormonal factors are involved in the pathogenesis of the tumour.[31,47] Studies indicate that KRAS is frequently activated in MCN with intermediate or high grade dysplasia. Moreover, mutations resemble the spectrum observed in ductal adenocarcinoma.[49] However, the exact mechanisms underlying the development of an MCN have remained enigmatic.

IPMN per definition involve the pancreatic ductal system; the main duct, the branch ducts or both. Tumours that are limited to the branch ducts have a better prognosis than those that involve the main pancreatic duct.[31,37,38] IPMN can also be categorized based on their epithelium and mucin expression, with prognostic implications.[50,51] However, classification of IPMN according to the latter features has rarely been possible before surgery.

Like ductal adenocarcinomas and MCN, IPMN often harbour activating *KRAS* mutations.[49,52] By contrast, *GNAS* mutations appear to be characteristic of IPMN, although present in only two thirds of cases.[52] Activation of GNAS results in cAMP production and activation of protein kinase A.[53] *GNAS* mutations may also affect mucin expression.[54] Furthermore, in a whole-exome sequencing study, inactivating mutations of the gene encoding for an E3 ubiquitin ligase, RNF43, were found to be common in IPMN.[55] The role of this protein is to "select" other proteins for proteosomal degradation. The full spectrum of proteins ubiquitinated by RNF43 is not known. However, according to the current model, RNF43 acts as a tumor suppressor through the antagonism of Wnt signalling. Most likely, RNF4 inhibits oncogenic Wnt signaling by targeting its receptor, Frizzled, for degradation.[56]

The microscopic PanIN and the macroscopic IPMN are the most common precursors of pancreatic cancer. The possibility of a common origin for these two precancerous conditions is a matter of debate. In fact, both lesions are fundamentally defined by size: PanIN are <5mm; IPMN >10mm.[57] Thus, a large group of lesions falls between these two categories, and may be an interesting topic for future studies. Furthermore, patients with IPMN have an increased risk of developing pancreatic cancer from synchronous or metachronous microscopic precursors. This points to a "field defect" of acquired mutations or epigenetic alterations, which contribute to cancer development through both macroscopic and microscopic forerunners.[31,58]

1.3.2 Diagnosis

Pancreatic pseudocysts are usually observed in a setting of either acute or chronic pancreatitis. However, pancreatic cystic tumours, particularly IPMN, may also cause acute pancreatitis. Furthermore, main-duct IPMN can morphologically mimic chronic pancreatitis, since dilation of the main duct is a feature of both conditions.[31]

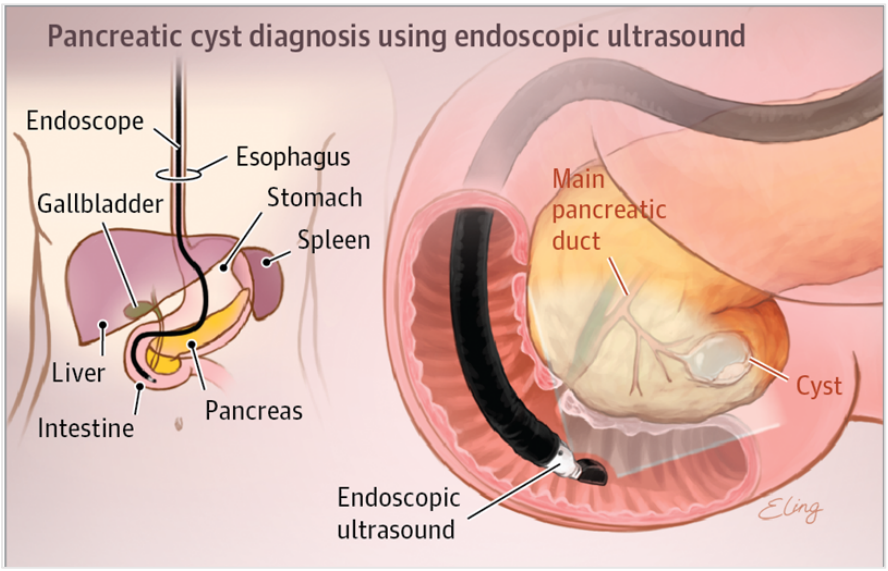

Figure 3. *Schematic representation of the endoscopic ultrasound examination. Fine - needle aspiration of pancreatic cyst fluid may be performed during the diagnostic procedure. Image adapted from Nassour I, Choti MA. JAMA. 2016; 316(12):1326., with the permission of the publisher.*

Commonly available imaging methods like computed tomography (CT) and magnetic resonance imaging (MRI) detect pancreatic cystic lesions with increasing frequency. However, they are usually not sufficient for their differential diagnosis.[38,59-61] Studies indicate an overall diagnostic accuracy ranging from 40 to 80% for CT and MRI for the assessment of pancreatic cystic lesions, with no clear advantage for either method.[38,59,60] Interestingly, even for cases where the radiologist reported feeling highly confident about the diagnosis, the accuracy was not substantially higher.[59]

Endoscopic ultrasound (EUS) is a method for the visualization of inner organs adjacent to the esophagus, ventricle and duodenum. With this approach, interferences from the abdominal wall and intestinal gas (that limit the utility of external ultrasound for the assessment of pancreatic pathology) are avoided. Using an ultrasound device attached to a modified endoscope, the organ can be visualized with high resolution, superior to that of CT and MRI. Another advantage of the technique is that it allows for fine-needle aspiration of cyst fluid for cytology, and quantification of the tumour marker CEA (carcinoembryonic antigen). Drawbacks are the facts that it is invasive, and highly dependent on the skills and the experience of the observer.

Despite the high resolution, EUS accuracy for the differential diagnosis of pancreatic cystic lesions can still not be considered satisfactory, even when complemented with fine-needle aspiration.[62] Cytology may result in a diagnosis, but the yield is often

scant.[63-65] Atypia in cystic lesions is typically focal, and may easily be missed.[47,63] Furthermore, cystic tumours often contain areas of denuded epithelium which could lead to a false diagnosis of a pseudocyst, even in the histological examination.[47]

Cyst fluid CEA is widely considered as a state-of-the art method for the identification of cystic precursors of pancreatic cancer (MCN and IPMN) with a reported diagnostic accuracy of 79%. However, in spite of more than 15 years of clinical use, there is still no established consensus on threshold levels for malignant potential. CEA levels also do not correlate with the degree of dysplasia and are not considered useful for the detection of malignant progression.[38,62,65]

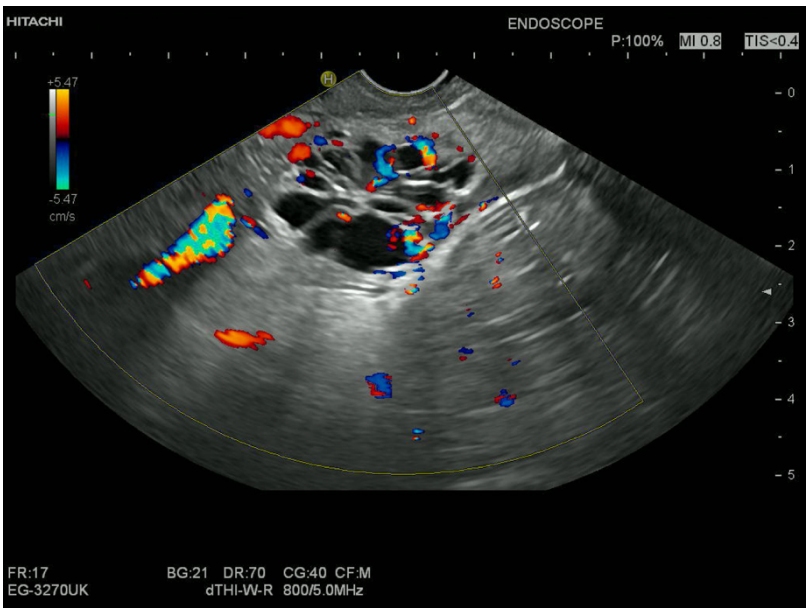

Figure 4. *Image of a serous cystic neoplasm acquired during endoscopic ultrasound examination. Multiple, dark-appearing cysts can be observed. The coloured segments represent blood vessels. Courtesy of Dr. Riadh Sadik.*

Molecular analysis of cyst fluid, targeting genomic alterations such as KRAS mutations, has been performed and evaluated in several studies.[49,66] So far, these methods have not been conclusively shown to improve upon available diagnostic tools.[66] However, recently, targeted next generation sequencing of KRAS/GNAS and TP53/PIK3CA/PTEN in cyst fluid was demonstrated to have high accuracy for the detection of premalignant and malignant pancreatic lesions, respectively.[67] Still, MCN were frequently missed.[67] One problem for genomic analysis in this context is that the cellular yield from cyst fluid samples tends to be very low.[63-65]

There is also a growing number of proteomic and glycomic biomarker studies using pancreatic cyst fluid.[68-71] However, the potential clinical utility of these markers remains uncertain, since these studies involved a laborious and time-consuming work-flow, and/or reported on a small number of patients. Other novel approaches that await further validation include protease activity profiling, microRNA panels, DNA methylation, and metabolomic biomarkers.[72-76] Interestingly, metabolomics identified cyst fluid glucose levels as an indicator of premalignant (mucinous) cystic lesions.[76,77] Such a test would have obvious appeal from a clinical viewpoint, due to the low costs and possibility of instant results.

1.4 STRUCTURE AND FUNCTION OF THE COLONIC MUCUS LAYERS

The colon extends from the caecum to the rectum, and is the site of fluid reabsorption, as well as microbial harvesting of energy from indigestible food components. Its inner (luminal) surface is very large in size, due to the presence of invaginations, referred to as crypts or glands. Further increasing the surface area, the luminal border of the colonocytes forms dense, finger-like projections, called microvilli. By contrast, the thickness of the epithelium corresponds to merely a single layer of columnar cells. This can be compared with the multi-layered, keratinized epithelium covering the external surface of our bodies. However, just like the skin, the colonic epithelium is constantly exposed to xenobiotics and other harmful substances. The colon is also much more densely colonized by microbes.

Intestinal defence mechanisms against external threats include epithelial cell barrier integrity, primarily maintained through the intercellular tight junctions.[78] The intestinal epithelial cells also express traditional innate immune recognition proteins, like Toll-like receptors (TLRs) and Nod-like receptors (NLRs). This means that, like "professional" immune cells, they are able to recognize and respond to microbial ligands and motifs.[79]Another important protective factor is rapid cell turnover, where the colonic epithelium is largely renewed every few days.

Nevertheless, these defences are not by themselves sufficient to cope with the formidable abundance and diversity of the colonic microbiota. In addition, a secreted, filter-like network built around the MUC2 mucin, shields the host from luminal bacteria, while still allowing us to benefit fully from their metabolic activities.[80-83] Importantly, the strict spatial segregation between the abundant colonic microbiota and the host also forestalls inappropriate immune reactions towards commensals and beneficial bacteria, which would otherwise result in chronic inflammation. The clinical significance of mucus protection has been demonstrated in Muc2-/- mice. These animals spontaneously develop severe colitis, and subsequently colon cancer.[84,85]

Similar findings have been observed in mice deficient in predominant Muc2 core *O*-glycans.[86,87]

MUC2 is produced by a distinctive cell type, known as goblet cells. Following translation and folding of the protein in the endoplasmic reticulum (ER), extensive *O*-linked glycosylation occurs in the Golgi apparatus.[88] The glycans are typically anionic, i.e., negatively charged. The rich glycosylation of secreted mucins is essential for their water-binding, and hence gel-forming, properties.[83,88]

Subsequently, MUC2 is packed into goblet cell secretory granules in oligomeric form, having undergone C-terminal dimerization and N-terminal trimerization in the ER and Golgi, respectively.[89,90] High concentrations of calcium and hydrogen ions (corresponding to a pH of about 5.2) inside granules favour a compact configuration of MUC2 oligomers.[91] Following its release into the intestinal lumen, the MUC2 network expands about a 1000-fold. Concurrent bicarbonate secretion accomplishes the dual purposes of raising pH and precipitating calcium, enabling rapid and efficient unpacking of MUC2.[91] Upon secretion, the MUC2 polymers organize into stratified sheets of hexagonal, ring-like structures.[91] These hexagons represent "pores" in the mucus filter, which are further constricted through the formation of isopeptide bonds.[92,93] Their size and configuration serve to restrict bacterial passage, vastly reducing epithelial exposure to microbes.

The colonic mucus layer is in fact a two-tiered structure.[80,83,88] The inner, adherent part is converted into a loose, permeable, outer layer at a distance from the epithelium, by endogenous proteases.[80,83,88,94] The outer layer is densely colonized by microbes, which feed on the mucin glycans.[80] The complex repertoire of the glycans attached to the MUC2 molecule suggest that these carbohydrates may have evolved to select for beneficial bacteria.[83,88,95] Further illustrating the versatile roles of the mucus system in innate immunity, intestinal goblet cells are able to sample luminal microbial antigens or (in colon) even whole bacteria. These are then delivered to antigen-presenting cells in the underlying tissue.[96,97]

Conversely, there is ample evidence that the gut microbiota regulates and influences the mucus system. Germ-free mice and rats have a more penetrable colonic inner mucus layer than their wild-type counterparts.[98,99] However, long-term microbial colonization restored the imperviousness of the mucus barrier.[99] Not only the presence, but also the composition of the colonic microbiota appears to influence mucus barrier properties. For instance, a high abundance of *Proteobacteria* was associated with increased penetrability of the inner mucus layer in mice.[100] In humans, goblet cell numbers and mucus secretion are augmented during the Th2-mediated response to helminthic (worm) infections.[101] However, microbiota can also directly induce goblet cell secretion, even in homeostatic conditions.[102] Colonic upper crypt goblet cells

respond to microbial TLR ligands with a coordinated expulsion of mucus, orchestrated by a specialized cell population denoted as sentinel goblet cells.[102] Thereby, microbes that have broken the host-microbial contract of mutualism and ventured too close are flushed away.

1.5 GUT MICROBIOTA

The human intestine is home to a complex ecological system of viruses, prokaryotes and micro-eukaryotes. Collectively, these organisms are referred to as the gut microbiota. Within an individual, the gut microbiota tends to outnumber the total amount of human cells, and accounts for a biomass of up to 1.5 kg.[103] Over a thousand species have been identified from human intestinal samples, although the average colon is estimated to contain about 160 different species.[20,104] Thus, there is considerable inter-individual variation, which is related to for example genetic, temporal, geographical and nutritional factors, as well as associated with health status and medication.[105-108] Nevertheless, metagenomic studies have indicated that our intestines share a stable functional repertoire of microbial metabolic pathways.[20,109]

The vast majority of the gut microbiota probably consists of bacteria. These can be subdivided into different phylogenetic, metabolic and functional groups. In addition, they are categorized in a practical way from a host perspective, into pathogens, commensals and beneficial bacteria.[110] With the latter, we exist in a mutualistic relationship, profiting from their presence in various ways.[111-113] The gut microbiota acts as a bioreactor, contributing to the degradation of nutrients ingested by the host, and producing a wide array of metabolites.[112] Microbial products include vitamins like thiamin (B1), riboflavin (B2), pyrodoxin (B6), biotin (B7) and the vitamin K group.[111] Other important metabolites for the host are short chain fatty acids (SCFA). The most common SCFA are acetate, butyrate and propionate. These compounds provide up to 10% of our caloric requirements, constitute the main energy source of the colonocytes, modulate intestinal motility and host inflammatory responses, and regulate proliferation, turnover and apoptosis of the colonic epithelial cells.[112,114-118] The effects of microbial metabolite signaling are not limited to the intestines. Bacterial products have also been shown to influence distant organs, including adipose tissue, and the cardiovascular and central nervous systems.[112,113,119-121]

Commensals are defined as microbes that “eat at the same table” as their host, implying that they don´t provide either substantial benefit or harm. However, commensals may in fact exert positive effects, by contributing to pathogen colonization resistance.[122] This could occur through nutrient competition or modulation of the environment such that the expansion of pathogens is prevented.[122-124] In addition, several species are able to secrete antibiotic substances (bacteriocins) that help

maintain intestinal microbial homeostasis.[122,125] Moreover, beneficial and commensal bacteria are crucial for the development and education of the immune system.[113,126-128]

According to the taxonomic terminology originating from the Linneaean system, bacteria are hierarchically subdivided into kingdom, phylum, class, order, family, genus and species. Five phyla predominate in the human intestine: *Firmicutes, Bacteroidetes, Actinobacteria*, *Proteobacteria and Verrucomicrobia*.[112] The two first are by far the most abundant.[20,112,113,129] This division has traditionally been based on properties such as morphology, Gram staining and metabolism. However, the advent of molecular, culture independent, methods for bacterial identification has led to re-classification of several species, as well as an upsurge in species detection.[130] Recently, the introduction of high-throughput, low-cost methods for shotgun sequencing of the metagenome has enabled large-scale functional characterization of the gut microbiota in different conditions and disorders.

However, in addition to inter-personal variation, there are large biogeographical differences within individuals. These render sampling issues critical. There is a longitudinal gradient, with microbial abundance and diversity increasing towards the distal (final) part of the intestinal tract.[113,131] The richness and composition of the microbiota in different anatomical locations of the intestines is affected by transit time, pH, redox potential, as well as the presence of antimicrobial proteins and bile acids.[131] Moreover, there are cross-sectional differences, where luminal (faecal) bacteria differ from their mucus-associated counterparts.[129,131,132] The outer, loose, mucus layer is richly colonized by bacterial species, some of which have the capacity to degrade the mucin glycans.[80,,81,131,132] Other species simply possess the ability to scavenge and metabolize mucin sugar units, cleaved off by the former.[133,134]

The colonic inner mucus layer is considered to be largely devoid of bacteria, and constitutes a physical barrier between the microbiota and the host.[80,81] However, a small number of species have actually adapted to colonizing this niche. Previous studies have indicated the existence of a crypt-specific microbiome, largely composed of aerobes, such as *Acinetobacter* and *Pseudomonas.*[135] Some bacteria are also able to directly colonize the epithelium, including segmented filamentous bacteria (SFB) in rodents, and *Brachyspira* in humans.[136,137]

1.5.1 Brachyspira and intestinal spirochetosis

Brachyspira belong to the Spirochaetes phylum, along with more illustrious pathogenic members, such as *Borrelia* (the causative agent of Lyme´s disease) and *Treponema* (syphilis). Spirochetes share the common characteristic of periplasmic flagella.[138-140] These are located not at the exterior of the bacterial cell, but between the inner and

outer membrane. The periplasmic flagella enable the elongated, spiral-shaped bacteria to propel themselves forward with a cork screw like motion.[138-140]

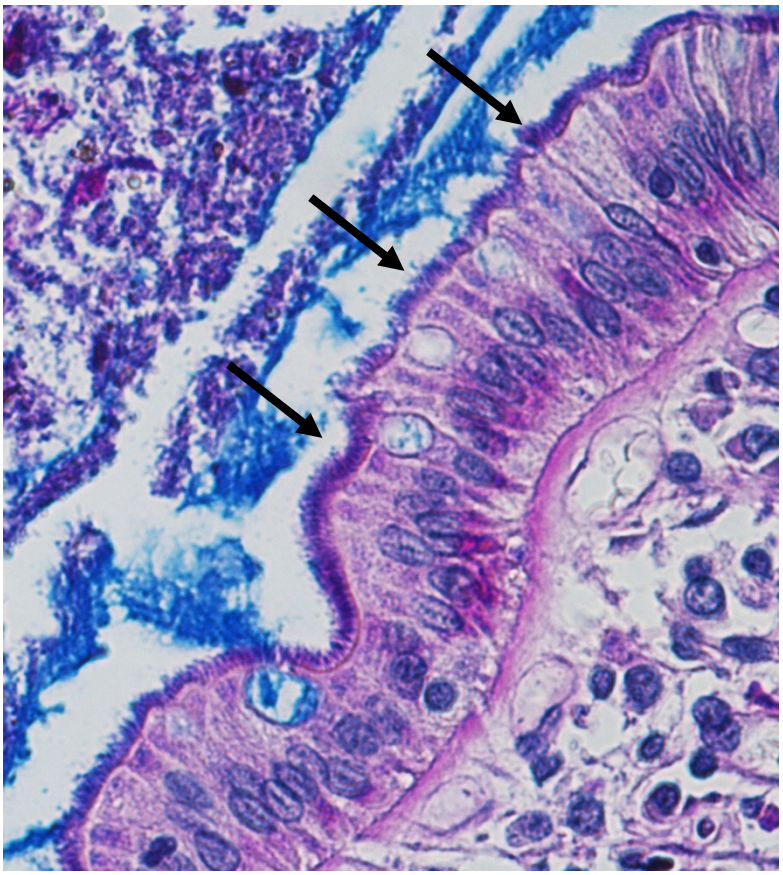

Figure 5. *Intestinal spirochetosis in an IBS patient. Arrows indicate Brachyspira bacteria which form a characteristic, dense "fringe" on the epithelial surface. Image from a sigmoid colon section, stained by Alcian blue/PAS.*

Spirochetes, as well as bacteria in general and *Brachyspira* in particular, were first observed by Dutch microscopy pioneer Antonie van Leeuwenhook in the 17th century.[140] He also appears to have been the first to recognize the pathogenic potential of *Brachyspira*, since he allegedly discovered them in his own diarrhoeal stool.[141]

Unusually, to this day, microscopy has remained the standard way to diagnose *Brachyspira* infections (intestinal spirochetosis) in humans.[137-140] The *Brachyspira* are highly fastidious, slow-growing anaerobes, which typically do not form colonies.[138,139] Thus, they are notoriously difficult to culture.[138,139] However, in intestinal

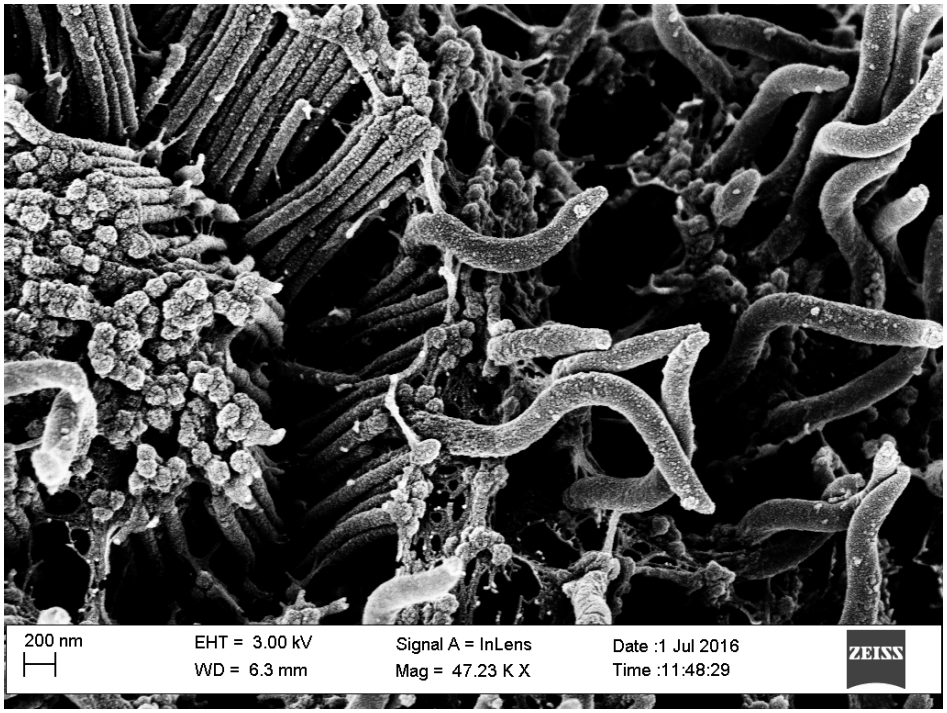

Figure 6. *Scanning electron micrograph of the colonic epithelial surface (top view). Brachyspira can be observed extending through the shorter, straight microvilli that protrude from the surface of the colonocytes. Image courtesy of Dr. Anna Ermund.*

spirochetosis, they can be found in great density at the intestinal epithelial border, where they attach to the membrane in a perpendicular way.[137-141] This produces a characteristic histological image, with a fringe of spirochetes on the epithelium, sometimes referred to as a "false brush border".[137-141]Human-pathogenic *Brachyspira* can reach a length of 10 µm, and thus extend far beyond the microvilli (approximately 1 µm long) into the intestinal lumen.[138]

There are nine known *Brachyspira* species.[138,140] All but one (aptly named *B. innocens*) of these, have pathogenic potential, although only two are postulated to cause disease in humans.[137,138,140] *Brachyspira* are well-known pathogens in veterinary medicine, as the causative agents of swine dysentery and avian intestinal spirochetosis.[138] *B. pilosicoli* is capable of infecting both humans and other species, including pigs, chicken and dogs.[138] Thus, zoonotic transmission could potentially underlie a proportion of intestinal spirochetosis cases in humans. This may be particularly true for rural/peri-urban settings in developing countries, where high prevalences of *Brachyspira* colonization have been reported.[142,143] By contrast, the second known human pathogen from the *Brachyspira* genus, *B. aalborgi*, is believed to be restricted

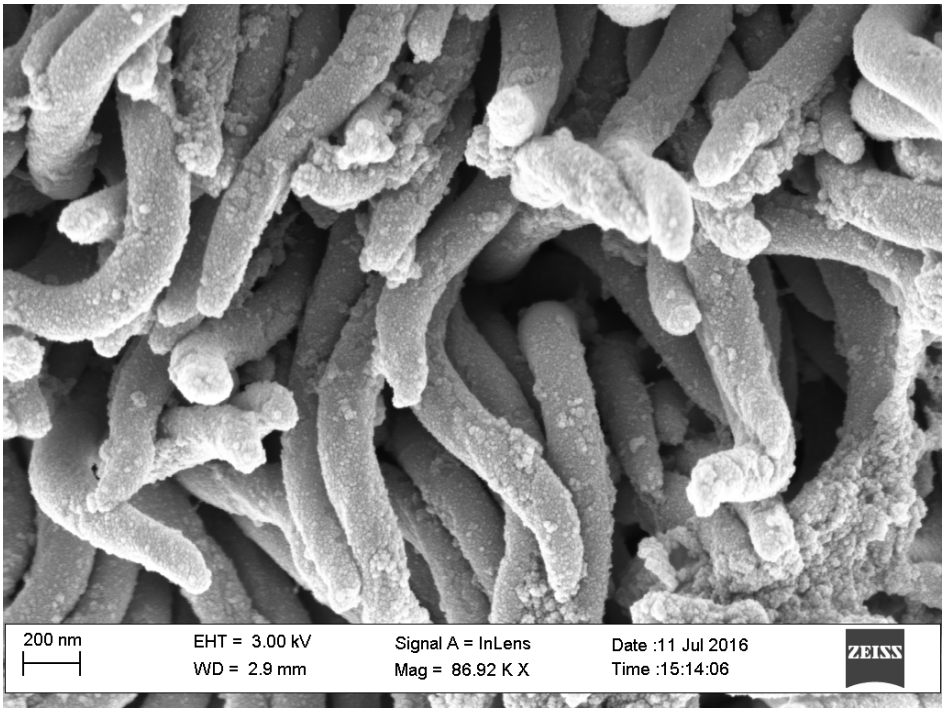

Figure 7. *Scanning electron micrograph showing a massive amount of Brachyspira extending from the colonic epithelial surface in an IBS patient.*

to primates.[138-140] The existence of a third species causing intestinal spirochetosis has been suggested, but awaits further confirmation.[137,144]

Although the concept of *Brachyspira* as a human pathogen dates back 400 years, it has remained controversial. On the one hand, there are numerous case reports linking *Brachyspira* to symptoms, including diarrhoea, abdominal pain, hemorrhagic stools, and even sepsis.[145-149] Especially severe symptoms have been reported in pediatric disease.[148,150,151] On the other hand, some observations argue against the importance of *Brachyspira* in human disease. In two studies, prevalence rates of *Brachyspira* in populations with gastroenteritis and chronic diarrhoea were reported to not differ from healthy controls.[145,152] In contrast to segmented filamentous bacteria, *Brachyspira* infection does not typically elicit a strong local or systemic immune response.[136, 138-140] Furthermore, antibiotic treatment does not always led to full recovery, even though improvement of symptoms has been commonly described.[145,146,150]

Overall prevalence rates of *Brachyspira* ranging between 0.5% and 3% have been reported from Europe, East Asia and Australia.[145,151-155] However, studies of tissue specimens were generally performed in populations undergoing colonoscopy (or even colorectal resection) for clinical reasons. In a Swedish study, *Brachyspira* was detected

in 2.2 % of individuals from a random population sample who accepted an offer of colonoscopy screening.[154] Interestingly, 35% of persons diagnosed with intestinal spirochetosis in that study, also fulfilled criteria for irritable bowel syndrome (IBS).[154] This observation hints at an increased prevalence of spirochetosis in IBS patients. However, a potential association between *Brachyspira* and IBS has not been systematically investigated.

1.6 IRRITABLE BOWEL SYNDROME

The term "irritable" colon, later irritable bowel syndrome (IBS), was coined already in 1915, replacing the earlier name "mucous colitis" (1892).[156,157] However, the definition of this condition has continued to be revised and updated.[158] According to the most recent designation, the Rome IV criteria of 2016, abdominal pain for at least one day per week during the last three months is a prerequisite for an IBS diagnosis.[158] Furthermore, two out of three additional criteria, indicative of a link between the pain and bowel habits, need to be fulfilled. The pain must either be directly related to defecation or associated with a change in stool frequency and/or consistency.[158] Thus, abdominal pain is the unifying symptom in IBS patients who may have either predominant diarrhoea (IBS-D), constipation (IBS-C), or both (mixed-type IBS; IBS-M), or neither (unsubtyped IBS; IBS-U).[158,159]

Since IBS encompasses a wide range of abdominal symptoms, it is unlikely to be explained by one, sole mechanistic model. Moreover, IBS may, to a varying degree, be associated with a plethora of extra-colonic comorbidities.[160] These include urogenital, musculoskeletal, psychological, and not least upper GI symptoms.[160] Considered as a single entity, IBS may be the most common medical disorder in the world.[161] Reported prevalence rates vary between 1% and 30-50%, without any obvious geographical discrepancies.[161] Although figures are not available from all countries and regions of the world, global prevalence is estimated at 5-15%.[161] In the United States, 12% of primary care visits, and 28% of gastroenterology practice are accounted for by IBS.[162,163]

IBS is not associated with mortality, but is typically a chronic disorder.[158,160,162,163] As such, it has a significant economic impact for both the individual and the society, in terms of costs of medical investigations, medication, work absence and decreased productivity.[164] IBS was reported to incur expenditures increasing 1 billion USD per annum in the United States already in 2004.[165] Importantly, the disorder is also associated with a pronounced reduction in quality of life.[164,166] In one large survey, seventy percent of patients reported that they missed at least 10 social activities or events during a three month period, due to IBS.[166]

Prominent risk factors for IBS include female gender, young age, and a recent episode of gastroenteritis.[160-162,167,168] Following a severe enteric infection, the risk of developing IBS is six-fold increased, corresponding to an incidence of about 10%.[168] Conversely, 10% of IBS is estimated to be post-infectious.[168] The association between gastroenteritis and IBS has raised questions about the role of microbial imbalance in this poorly understood disorder.

1.6.1 Pathophysiology

IBS has traditionally been understood as a collection of symptoms from the intestinal tract, without any biological correlate. However, recently, advances in molecular analysis have demonstrated different abnormalities in subsets of IBS patients. These include intestinal dysbiosis, immune activation and even genetic factors.[167,169-172] Nevertheless, no unifying underlying mechanism has been identified. Diagnostic tests to identify subpopulations of IBS patients, which could benefit from targeted therapeutic interventions, are also lacking.[167]

At present, the most distinctive subgroup is post-infectious IBS. The risk of developing IBS after a gastroenteritis episode appears to be partly dependent on the severity and causative agent of the infection.[167,168] However, it is also related to host susceptibility.[167,168] The relationship between IBS and enteric infections provides compelling evidence for microbial imbalance as a causative or contributing factor, at least in some patients. In addition, results from therapeutic trials support a pathogenetic role for the microbiota.[173-176] Reduced intake of certain bacterial metabolic substrates, as well as pre-, pro- and antibiotic treatment have all been associated with symptomatic relief in subsets of IBS patients.[173-176]

Nevertheless, studies of the faecal microbiota composition of IBS patients have yielded inconsistent and even contradictory, results.[169] Still, there is some evidence for decreased microbial richness in a subgroup of IBS patients.[169,177] The *Proteobacteria* phylum appears to be increased, and the *Bifidobacterium* and *Faecalibacterium* genera decreased, in abundance in IBS.[169,177-180] In addition, one study indicated that the mycobiome could be of importance for IBS-related visceral hypersensitivity. [181] Relatively few investigations have addressed alterations of the mucus-associated microbiota in IBS.[177,179] However, microbes inhabiting this niche would be more likely to be able to elicit immune responses, influence the enteric nervous system and cause damage to the mucus and epithelial barriers.

Traditionally, IBS has been understood as a psychosomatic disorder. There does appear to be an association with psychiatric comorbidities, which cannot be fully explained by referral bias.[160,162,167] The term brain-gut axis has long been used to refer to the relationship between the emotional state and intestinal symptoms. This connection is

likely an experience shared by most humans. Still, in recent years, the understanding of the brain-gut axis has broadened and deepened considerably. Its definition includes the central and enteric nervous system and their inter-connection through the sympathetic and parasympathetic autonomous nervous systems, but the signaling pathways are not confined to the neurons.[182,183] The hypothalamic-pituitary-adrenal axis, enteroendocrine cells and the immune system have also been shown to be important for the communication between these two organs.[182,183] The current understanding is that the pathway should be seen as triangular, since it also involves the gut microbiota as a third "multi-organism" organ.[167,182,183]

IBS has also been conceptualized as a primary disorder of the immune system. There is some evidence of a general increase in mucosal immune cell infiltration in subsets of IBS patients, occasionally overlapping with observations in microscopic colitis.[170, 184,185] Studies have indicated normal levels of circulating T and B lymphocytes, but increased gut homing.[170] Augmented local and systemic levels of various pro-inflammatory cytokines have also been reported.[186,187] However, cytokine profiles are divergent and have not been linked to a certain T helper cell response category. Furthermore, there may be elevated levels of circulating antibodies against both self and non-self antigens, including bacterial flagellin and neuronal components.[188,189] As for the innate immune response, there is one prime suspect: the mast cell. Several studies indicate that numbers of total and activated mucosal mast cells are increased in IBS.[190,191] Mast cell products, including histamine and proteases, have been shown to promote visceral hypersensitivity through the sensitization of afferent neurons.[192,193]

Interestingly, the prevalence of IBS seems to be increased in ulcerative colitis (UC) patients in remission, as well as in coeliac disease patients on gluten free diet.[194, 195]This has prompted the hypothesis that IBS may be the clinical manifestation of subtle, chronic inflammation. According to this view, IBS and IBD, i.e., inflammatory bowel disease, may be more alike than not, as reflected in their acronyms.

1.7 ULCERATIVE COLITIS

UC is a chronic inflammatory disease of the colon and rectum. In contrast to the other main form of IBD, Crohn´s disease, the inflammation is limited to the mucosa. Characteristically, bouts of active inflammation alternate with periods of disease remission. The inflammation is continuous, and usually starts from the rectum and distal colon. It may (or may not) progress in a proximal direction to affect the entire colon. Typical symptoms are frequent and urgent defecations, and rectal bleeding. Loose and voluminous stools signal involvement of the more proximal parts of the colon. Severe inflammation may be life-threatening if untreated, as there is a risk of colon perforation. Sometimes colectomy (acute or elective) may be necessary. UC is

frequently associated with extra-intestinal complications affecting the bile ducts, joints, skin and eyes. There is also an increased risk of colon cancer, which is proportional to the time since diagnosis, and the extent of colon involvement, but not to the number and severity of relapses.[196] The life-time risk of colon cancer in ulcerative colitis patients is estimated at 3-5%.[197,198]

The incidence of UC is dependent on age and geography, but not gender.[197,199,200] Reports on prevalence vary greatly between approximately 8-250 cases per 100 000 people. [197,199,200] There are two age-peaks in its incidence, where the first, and highest, occurs in early adulthood.[200] There is another more modest incidence increase around 50-70 years of age.[200] UC is most common in North America and Europe.[197,199] However, reports indicate that the incidence of the disease is rising in other parts of the world.[199] Studies have also shown that migrants to Europe and the US have a higher risk of UC than the population from which they originate.[201] Taken together, this suggests that diet and life-style factors may be of importance for the development of the disease.

1.7.1 Pathophysiology

Despite decades of research, the aetiology and pathogenesis of ulcerative colitis remain insufficiently understood. This indicates that the underlying mechanisms are multifactorial, and probably vary between individuals. The current paradigm is that UC is caused by inappropriate host-microbial interaction in the large intestine.[197, 202] This phenomenon likely results from multiple risk factors, both on the host and the microbial side, which reinforce each other in a complicated, synergistic interplay.

Having a first degree relative with UC is associated with a life-time risk of developing the disease of about 2%.[203] Genome-wide association studies have identified numerous loci associated with ulcerative colitis, some of which are shared with Crohn´s disease.[197,204] However, the concordance in monozygotic twins is less than 20%.[205] This indicates that genetic factors explain only part of UC pathogenesis. Moreover, it is estimated that established risk loci account for only a fraction of the genetic component in UC.[204,206,207]

Nevertheless, functional categorization of the genes that have been associated with UC in unbiased, genome-wide analyses could shed light on the pathogenetic mechanisms. Genes that are likely to have a causative association with UC are linked to innate immunity (e.g. *CARD9*), ER stress (e.g. *XBP1*, *ORMDL1*) epithelial barrier integrity (e.g. *CADH1, HNF4A*), suppression of inflammation (e.g. *IL10* and its receptor, as well as *IL1R2*), and adaptive immunity.[208-213] In the latter category, variants associated with the Th17 pathway (e.g. *JAK2, STAT3* and *IL23R*) are particularly noteworthy.[208,209,212,214] However, interpretation of genome-wide association (GWA)

data is difficult. Most variants are not coding (i.e., do not directly influence the amino acid sequence of a protein), but regulatory. Only for some of these, a direct link with gene expression has been demonstrated. These are known as expression quantitative trait loci (eQTL).

Studies in genetically modified animals can provide biological support for genetic variants, and suggest mechanistic explanations. There is a plethora of mouse models for colitis. One early example is the Il10-/- model.[215] However, genetic models for spontaneous colitis are typically protected from inflammation in germ-free conditions, or during antibiotic treatment.[216] This provides compelling evidence for a role for the gut microbiota. Furthermore, although antibiotics have not shown effects in UC, there are several studies suggesting a therapeutic potential for probiotics.[217,218] UC has been associated with a decrease in microbial diversity, particularly affecting the *Firmicutes* phylum.[219,220] Species reduced in abundance in colitis include butyrate-producers, such as *Faecalibacterium Prausnitzii*.[221] This observation can be understood in the context of the well-known anti-inflammatory effects of butyrate, including the induction of regulatory T-cells.[222,223]

Crohn´s disease is linked to a Th1-dominated adaptive immune response, the key effectors of which are macrophages, IgG-producing B-cells and cytotoxic T-cells. By contrast, UC has traditionally been associated with a Th2 response, which is linked to the activation of eosinophils, basophils and mast cells, through effector cytokines including IL4, IL5 and IL13.[224] Whereas the Th1 response is also triggered by intracellular pathogens, the Th2 profile is induced by extracellular pathogens, including helminths, as well as in allergic reactions. However, UC is also associated with a Th17 response.[225] This is likely to be important for the recruitment of neutrophils, which is a hallmark of the disease.

The intestinal mucus is at the interface between the microbiota and the host. Thus it is hardly surprising that UC has also been associated with abnormalities of the mucus system. Patients sometimes report visible mucus in their stools, although this is not pathognomonic for UC. So-called goblet cell depletion is a well-known histopathological feature of the disease, although this observation likely reflects emptying of goblet cells, rather than an actual reduction in their numbers. Previous studies have demonstrated a more permeable mucus layer in UC patients with ongoing active inflammation.[226] Moreover, mucolytic bacteria such as *Ruminococcus torques* and *gnavus* may be more abundant in UC.[227]

The high production rate and intricate folding of MUC2 exert great demands on the ER of the goblet cell. Mutations in genes involved in ER stress response, have been linked to UC.[210] Interestingly, MUC2 mutations, causing its retention in ER, also induced colitis in mice, with evidence of ER stress.[228] The potential causal relationship between

ER stress and mucus layer abnormalities in UC is unclear, and could well be bidirectional.

The study of mucus has been impeded by its transparency and high degree of hydration, which make it difficult to visualize and preserve. Therefore, the protein composition of the colonic mucus in humans has not previously been determined. Several investigations have focused on mucus-associated microbiota in the gut.[131,132] However, there have been few systematic efforts to stratify the microbiota according to their specific niches and compartments. The microbiota of the inner mucus layer and crypts is likely to be very restricted, both in diversity and abundance. Nevertheless, these select few are in a position to be very powerful regulators of intestinal homeostasis, and immune responses.

2 SPECIFIC AIMS

1. Develop a clinically feasible proteomic cyst fluid analysis that can identify malignant potential and malignant progression in pancreatic cystic lesions.
2. Define and describe the protein composition of the human colonic mucus.
3. Characterize alterations of the colon mucus proteome in ulcerative colitis patients with and without active inflammation.
4. Identify potential associations between mucus protein composition and patient outcome in ulcerative colitis.
5. Investigate differences in the metaproteomic composition of the colonic mucus between IBS patients and healthy individuals

3 METHODOLOGICAL CONSIDERATIONS

An overview of proteomics and mass spectrometry is provided in the Introduction section, and detailed descriptions of the methods used in each study are included in Papers I-V. Hence, this discussion will be limited to selected methodological aspects that merit further consideration.

3.1 RECRUITMENT OF STUDY PARTICIPANTS

For the studies reported in papers I-IV, all participants were recruited among individuals referred for endoscopy for clinical reasons (Papers I-II: EUS; Papers III-IV: colonoscopy). The IBS patients in paper V were informed about the study during scheduled visits to a gastroenterology outpatient unit. The recruitment of the patient populations we were investigating was largely uncomplicated. By contrast, the selection of appropriate controls generally necessitates careful consideration.

For a study of pancreatic cyst fluid biomarkers, "healthy" controls are neither feasible, nor desirable. In contrast to renal and hepatic cysts, pancreatic cystic lesions are almost always a manifestation of an underlying inflammatory or neoplastic disease, although patients are usually asymptomatic.[31,37,38,47] Thus, inflammatory pseudocysts and cystic tumours without malignant potential (serous cystic neoplasms) served as a "control" population. Cystic lesions without malignant potential could be identified through 1) radiological confirmation of their resolution during an observation period, 2) clinical evaluation based on investigation according to consensus guidelines, discussion at a multidisciplinary board, and repeat imaging as necessary. In addition, surgery was performed for constitutively benign lesions in several instances. This highlights the inexact assessment provided by current diagnostic tools, which, moreover, have not improved since these studies were initiated ten years ago

In papers III and IV, controls were selected from patients that underwent colonoscopy, but where macro- and microscopic evaluation did not indicate colonic disease. Patients with known IBD, other than UC, were excluded. The main cause of referral for the control population was iron deficiency anaemia with/without occult or visible blood in the stool. Some of these patients did have minor polyps, but with at most low-grade dysplasia. Another, perhaps more problematic, category was "controls" with diarrhoea and/or altered bowel habits in general. While microscopic colitis was excluded through step-wise, random biopsies in patients with diarrhoeal symptoms, we did not attempt to rule out IBS. Thus, the prevalence of IBS may have been higher in participants of the control group, than in the general population. Nevertheless, studies indicate that IBS may also be more common in patients with inflammatory bowel disease, although reports are somewhat contradictory.[195] Thus, the controls and UC patients may in fact

have been well matched in this respect. Furthermore, the control patients tended to be older than the UC patients with active disease. This likely reflects the fact that altered bowel habits after age 50 is considered a "red flag" symptom, due to the age-dependent increase in the incidence of colon cancer. We did not observe any age-related alterations of the core mucus proteins. Nevertheless, the age difference between patients and controls cannot be dismissed as irrelevant, since both the incidence and activity of UC appear to abate with advancing age.[229-231] Future, larger studies should investigate if there are demographic variations in mucus composition and properties.

In study V, the control population did indeed consist of healthy individuals, as far as can be known. These were recruited through an advertisement and screened by questionnaires, as well as an interview, to exclude major gastrointestinal complaints. The age and gender distribution between cases and controls did not differ significantly.

3.2 MASS SPECTROMETRY

3.2.1 Sample collection and storage

For the proteomic studies of pancreatic cyst fluid biomarkers (Papers I and II), cyst fluid obtained at EUS-guided fine-needle aspiration, was directly frozen and stored at -80°C until analysis. Total aspirate volumes varied from a few µl to several ml, depending on cyst size. Very small volumes, down to 0.5-5 µl – i.e., a hundred to a thousand times less than required for CEA quantification – were sufficient for mass spectrometry analysis. Thus, diagnostic yield was not a limiting factor for the method, in contrast to standard analyses

However, sample contamination could pose more of a problem. Blood contamination is inherent to the sampling procedure, and could be more pronounced in serous cystic neoplasms as well as malignant tumours, which tend to be highly vascularized.[47] High-abundant blood proteins such as albumin and haemoglobin can obscure the proteins of interest in the analysis as these are typically present in much lower concentrations. This problem is partly circumvented by the use of targeted analysis methods. Nevertheless, in Paper II, six samples (corresponding to 4% of patients who fulfilled participation criteria) had to be excluded since they were grossly haemorrhagic.

A more concerning problem, would be contamination by mucus itself. EUS-guided aspiration of cyst fluid is performed either *via* the gastric or duodenal route, depending on the location of the lesion. The epithelium of both these organs is coated by secreted mucins: MUC5AC and MUC6 in the stomach, MUC2 in the duodenum.[23,24,80,232] However, in these studies, MUC5AC in conjunction with MUC2 produced the best peptide biomarker panel for malignant potential. Thus, the procedure-related introduction of these mucins into the sample could potentially result in false positive

results. The quantitative analysis in Paper II was partly designed to mitigate this concern. Our results indicate that contamination of extra-pancreatic mucins did not result in concentrations near the threshold levels for our biomarkers. However, these studies were performed at a single centre, and, overwhelmingly, by a single endosonographist. Thus, multicentre studies will be required to evaluate whether our results can be replicated at different settings.

The collection of colon mucus samples for proteomics was less straightforward, since there are no established ways to sample intestinal mucus *in vivo*. Sigmoid biopsies were obtained at routine colonoscopy. However, outside the body the mucus immediately collapses due to dehydration. Its transparency also makes it hard to see. Hence, it cannot be studied directly. After transport to the laboratory in an oxygenated Krebs solution with physiological pH, biopsies were mounted in a horizontal Ussing-type chamber.[233] Solutions containing nutrients and oxygen were supplied from both the serosal and apical side of the biopsy, and temperature kept at 37°C. Through the addition of charcoal to the top of the biopsy, the mucus could be visualized. Typically, a thin layer can be observed directly after mounting, likely representing the adherent inner mucus. The outer mucus layer is loose, can easily be pipetted away, and thus should be lost during sampling and transport.[233] The biopsies were left to secrete for one hour. Mucus was then collected by gentle scraping and stored together with protease inhibitors at -80°C.

One important aspect for the sampling of colonic mucus, which differed between Papers III/IV and V, is bowel preparation by laxatives. In Paper V, bowel preparation was not performed. When viewed by light microscopy, the mucus appeared less transparent and may have retained more shed cells, which could be a disadvantage for proteomic analysis. On the other hand, bacterial proteins were not identified in any of the 111 samples in Papers III and IV, but were detected in 28% of mucus samples in Paper V. This indicates that the metaproteomic composition of colonic mucus samples is best studied without prior laxation.

3.2.2 Sample preparation

For the studies included in this thesis, MS analyses of two different biological fluids, pancreatic cyst fluid and colonic mucus, have been performed. For optimal results, the protocols for the preparation must be adapted to the nature of the sample. For instance, mucus is more efficiently solubilized by guanidine hydrochloride than by the more common denaturant, urea.[234] In studies III-V, steps had to be added to ensure the removal of the charcoal particles used to visualize the mucus in the ex vivo chamber.[233]

Sampling of an exact volume of mucus can be difficult, given its viscous nature. Nevertheless, this could be achieved for the cyst fluid samples. By contrast, for the

targeted quantification of MUC2 in mucus from colonic biopsies, a different approach had to be adopted. The ex vivo chamber enables sampling of mucus from a fixed tissue area. However, mucus thickness varies between individuals and samples. Thus, we measured the thickness of the mucus as previously described; using this value to calculate the sample volume.[233] Subsequently, the mucus concentration of MUC2 molecules for each patient could be calculated. For the relative quantification studies, the intensity for each peptide/protein was normalised to the summed intensities of all peptides/proteins in that sample. Thus, the normalised intensity values used to compare protein abundances between patients represent *proportions* of total intensity. This approach largely neutralizes the effect of sample volume on protein quantification.

For the proteomic profiling of cyst fluid mucins (Paper I) traditional SDS-PAGE one-dimensional gel electrophoresis was performed, to separate the cyst fluid proteins by mass. The high mass area corresponding to the known molecular weights of the mucins was then cut out and prepared for mass spectrometry. As this project was extended to develop the method into a targeted, quantitative test (Paper II), the gel-based approach was replaced by filter-aided sample preparation (FASP).[235] This method has numerous advantages over in-gel digestion: it provides access to the full (more or less) mass range of the proteome, is more reproducible, and has a shorter work-flow. Thus, it is more compatible with high throughput, and potential clinical use. Furthermore, it also allows for downstream absolute protein quantification. Hence, the FASP technique was used for the other studies in this thesis (Papers II to V).

3.2.3 Targeted protein quantification by Parallel Reaction Monitoring (Papers II-III)

Traditional discovery phase proteomics provides a broad overview of the proteome of a biofluid or tissue during physiological or pathological conditions, and is therefore ideal for the identification of biomarker candidates. However, the broadness of the approach also results in a lower sensitivity and reproducibility. Furthermore, it is not inherently quantitative. The relative abundance of a protein in a sample can be estimated through the intensity values of its peptide ions in the MS analysis. The intensity for a peptide corresponds to how often its particular mass-dependent oscillation frequency signal is registered by a detector. Still, this does not provide information as to the actual concentration of the biomarker in a sample. Absolute quantification can be achieved through targeted MS analysis, using internal standards to determine the exact concentration of a biomarker in a sample. In targeted MS, the analysis is limited to a panel of proteins of interest, often putative biomarkers. Prior to the targeted experiment, a selection list of proteins (based on research interest) and peptides (based on theoretical properties or observations from explorative studies) is constructed. Before analysis, a retention time window and a mass/charge selection filter,

corresponding to the chosen peptide/s is applied. Thereby, the analysis is restricted to the peptides of interest, greatly enhancing the sensitivity for their detection.[8,9] Modern mass spectrometers allow for the selection of multiple, narrow mass windows, such that several peptides can be analysed in one run. In Paper II we employed a technique known as Parallel Reaction Monitoring (PRM).[10] There, after the selection of a precursor peptide, fragmentation is performed, followed by quantification of all the product ions (fragments). The analysis was performed on a Q Exactive instrument, which is a hybrid of two units: a quadrupole, which can be used as a mass filter, and an Orbitrap mass analyser.[236] Simply put, the quadrupole consists of four parallel metal "rods" which creates a very narrow trajectory for the ions. This allows for a precise, mass/charge-based selection of peptides for analysis. Following fragmentation in the collision cell, the product ions are analysed in the Orbitrap unit.

The internal standards used in MS-based absolute quantification typically represent synthetic, custom-ordered stable-isotope labelled variants of the peptides of interest. This means that the synthetic peptides will have almost the same m/z-values as their "native" counterparts, but can be distinguished from these through a slight mass shift. Since the concentrations of the heavy peptides are known, the corresponding concentrations of the (light) peptides of interest in the original biological sample can be deduced.

The proper selection of peptides to quantify is essential for the success of the experiment, but can be quite challenging. The inclusion of amino acids that are frequently modified (for instance methionine, which contains a sulphur group that can be oxidized), chemically unstable sequences and missed cleavage sites, means that results may not be reproducible and reliable. Mucins contain long, repetitive sequences that are heavily glycosylated, and not possible to detect in the MS analysis.[23,24] Nevertheless, since they are very large proteins, they still offered a good choice of possible biomarker peptides.

3.3 HISTOLOGY AND IMMUNOHISTOCHEMISTRY (PAPERS IV-V)

In Papers IV and V, the proteomic investigation was complemented by a number of histological and immunohistochemical analyses. Carbol fuchsin and malachite green were used to visualize endospores which cannot be readily observed by common bacterial staining methods. In malachite green staining, heat is used to "force" the dye into the thick spore wall, where it is retained. The *Brachyspira* "fringe" characteristic of intestinal spirochetosis, was easily detectable by an Alcian blue/Periodic acid-Schiff (PAS) stain, as illustrated on the cover of this thesis. Hematoxylin-eosin (H&E) stained slides were used for the quantification of immune cells (except mast cells) which were counted in five high power fields (magnification x 600). Mast cells were counted in

toluidine blue stained sections, where they are stained pink-purple by the metachromatic dye. Toluidine blue was also useful for the visualization of bacteria inside crypts, probably due to its staining of acidic components, including nucleic acids. Gram stain, in conjunction with immunohistochemical staining for lipoteichoic acid, was used to visualize the presence of other bacteria inside crypts in patients with intestinal spirochetosis. Lipoteichoic acid is a component of Gram positive bacteria, whereas *Brachyspira* are Gram negative.

3.4 STATISTICS

Given the complicated sampling techniques, and the relatively advanced and costly analysis methods used, the study populations were not very large for any of the studies in this thesis. However, the total number of participants exceeded one hundred in Papers II-IV. Still, distinct discovery and validation cohorts, as well as stratification of patients based on their clinical characteristics (symptoms, disease activity, histology findings, prognosis, etc.) meant that many comparisons involved smaller groups. Therefore, non-parametric analyses (Fisher Exact test, Mann-Whitney U-test or Kruskal Wallis test, depending on the nature of data and the number of groups) were generally used. The Bonferroni or Holm-Bonferroni methods were applied to adjust the threshold of significance in the event of multiple comparisons.

The novel methods or sample types also meant that power analyses could not be performed prior to the investigation. In Papers I, II and V, results from a discovery cohort could be used to estimate the required sample size for a validation cohort. Paper III should be regarded as an explorative study, and Paper IV as a pilot study.

4 RESULTS

4.1 PROTEOMIC IDENTIFICATION OF CYSTIC PANCREATIC CANCER PRECURSORS -- PAPERS I AND II

4.1.1 Main observations

- Mass spectrometric quantification of mucins MUC5AC and MUC2 in pancreatic cyst fluid identified cystic cancer precursors with 97% accuracy.
- The accuracy for the detection of cancer/high-grade dysplasia in a cystic lesion was 94%, based on a panel of peptides from proteins MUC5AC and PSCA.
- The performance of the analysis represented a significant improvement upon state-of-the-art diagnostic tools.
- The method is compatible with high-throughput analysis of clinical samples and offers several advantages, including very low sample requirements and simultaneous quantification of multiple biomarkers. Thus, the approach described in Paper II could facilitate the development and analysis of biomarker panels for other neoplastic/non-neoplastic conditions.

4.1.2 Rationale

Reported prevalence rates of pancreatic cystic lesions have surged in the last decades, due to advances in imaging quality, as well as in awareness of the underlying conditions.[31,32,34,35] A significant proportion of this increase is thought to be accounted for by mucinous neoplasms – MCN and IPMN – which are forerunners of pancreatic cancer.[31,36] Unfortunately, the lack of robust diagnostic tools hampers the identification of high-risk cystic lesions.[31,37,38,59-66] Hence, the improved detection of macroscopic precursors has not yet translated into a reduction in pancreatic cancer mortality.

Aberrant expression of secreted mucins is a well-known feature of MCN and IPMN, which also explains their cystic nature.[47,48] Moreover, their mucin profiles have been suggested to correlate with prognosis.[50,51] Previous studies on mucin expression in pancreatic tumours overwhelmingly used antibody-based approaches.[47,48,237] However, due to the extensive and variable glycosylation of mucin proteins this method is not ideal for their detection.[23,24] In particular, cancer-related alterations of the glycans could result in failure of the antibody to recognize its epitope.[23] Thus, we wanted to exploit our access to front-end expertise in interventional EUS, mucin biology and MS-

based proteomics, to identify clinically feasible cyst fluid biomarkers for pancreatic cancer precursors.

4.1.3 Proteomic mucin profiling of pancreatic cystic lesions

In contrast to other mucins, MUC6 is known to be expressed by normal pancreatic epithelium (ductal and acinar), as well as by the intrinsically benign serous cystic neoplasms.[232,238] Therefore, we hypothesized that detection of any mucin *except* MUC6 in cyst fluid, should be indicative of an, at best, premalignant lesion. Given the known role of MUC1 in both pancreatic and extra-pancreatic malignancies, we additionally conjectured that cyst fluid MUC1 content is indicative of malignancy/high-grade dysplasia (HGD).[23,24]

Briefly, cyst fluid aspirates were prospectively obtained from 78 (discovery cohort: 28, validation cohort: 50) patients referred for endoscopic ultrasound (EUS)-guided fine-needle aspiration of pancreatic cystic lesions. Following SDS-PAGE gel electrophoresis, high mass areas (corresponding to the molecular weight of mucins) were excised, and analysed by nano-LC MS/MS. The mucins detected in cystic tumours were, in order of the frequency of their identification: MUC5AC, MUC1, MUC6, MUC5B, MUC2 and MUC16. MUC5AC was detected in 31/37 (84%) of cystic lesions with malignant potential, MUC1 in 23 lesions (62%) and MUC2 in only 9 lesions (24%). However, certain pre-/malignant tumors were uniquely identified by MUC2.

According to histology or clinical follow-up, 37 out of 79 (47%) lesions analysed had malignant potential. Proteomic mucin profiling identified these tumours with 97% accuracy, which compared favourably to cytology (71%), and cyst fluid CEA (78%; $p<0.001$ for both comparisons). Moreover, cyst fluid MUC1 content correctly classified 26/29 lesions with available histology, with regard to the presence or absence of cancer/HGD. Thus, mucin profiling demonstrated an overall accuracy of 90% for the detection of malignant progression, as compared to 52%, 57% and 59% for cytology, cyst fluid CEA, and EUS morphology, respectively. In particular, mucin profiling detected 14/16 malignant lesions, corresponding to a sensitivity of 87%. By contrast, a combination of traditional methods achieved a sensitivity of merely 50%. There were no substantial differences in the performance of the proteomic analysis between the cohorts; in fact, the accuracy was higher in the validation set, both with regard to the detection of malignant potential and cancer/HGD.

In conclusion, the proteomic mucin profiling method demonstrated accuracy results significantly superior to traditional state-of-the-art analyses, for the identification of cystic pancreatic cancers and precursor lesions. But did the method fulfil our objective

of clinical feasibility? At that time, with a relatively modest number of referrals of pancreatic cystic lesions for EUS, and within our institution, the answer would probably be affirmative. However, the subjective and non-quantitative elements of the method were a concern for the reproducibility of the analysis. These included the cutting of gel bands and the data-dependent shot-gun mass spectrometry analysis, where proteins low in concentration may not be selected for fragmentation and analysis if obscured by abundant ones, such as blood components. Moreover, cross-contamination of mucins from the stomach or duodenum during sampling, could potentially compromise the specificity of the analysis.[23,24,80,232] With increasingly sensitive mass spectrometers, we anticipated that these contaminants may sooner or later end up on the "wrong" side of the detection threshold. We reasoned that these problems could best be mitigated through the development of a targeted, quantitative analysis.

4.1.4 Identification of cyst fluid biomarker candidates through an explorative analysis

For initial explorative evaluation, aspirates from 24 well-characterized lesions were selected. These included six inflammatory pseudocysts and three samples from of each of the most common forms of cystic tumours, including IPMN histological subtypes. In total, there were nine benign, five premalignant and ten malignant lesions. With the exception of one highly typical serous cystic neoplasm, detailed histological assessment was available for all tumours in the study.

Briefly, cyst fluid samples were prepared using a modified version of the FASP (filter aided sample preparation) method, and analysed by nano-LC MS/MS.[235] The explorative analysis identified a total of 1385 unique proteins in pancreatic cyst fluid (unpublished data). When single occurrences were excluded, 541 proteins were found to be unique to lesions with malignant potential while 273 proteins were exclusively observed in malignant lesions. Thus, there were major differences between patient categories already in terms of protein identifications, creating a large pool of putative biomarkers.

As discussed in the Methodology section, in mass spectrometry it is usually not proteins, but peptides that are analysed. Therefore, in the selection of markers for targeted quantification, not only the performance of the protein biomarker in the explorative analysis was considered. The choice of peptides that could be stably and reproducibly detected in the patient samples also influenced biomarker selection. In addition, only secreted or plasma membrane proteins were considered to be biologically relevant biomarkers. Filtering according to these criteria resulted in a final list of 30 peptides from eight biomarker proteins. These included the proteins MUC1,

MUC2, MUC5AC, CLCA1, FCGBP, DMBT1, MSLN and PSCA.[82] Between one and ten peptides from each marker were selected for quantification.

The epithelial/histological sub-type of an IPMN has been shown to be an important predictor of clinical outcome. Pancreatobiliary-type IPMN have the lowest survival rates, while gastric-type IPMN are generally indolent, and intestinal-type IPMN have an intermediate prognosis.[50,51] Therefore, in addition to biomarker candidates for malignant potential and cancer/HGD, putative markers for intestinal-type IPMN (MUC2), and pancreatobiliary-type IPMN (PSCA) were also included.

4.1.5 Detection and assessment of cystic precursors of pancreatic cancer by targeted mass spectrometry

Targeted mass spectrometry analysis by Parallel Reaction Monitoring was used to quantify biomarker candidates identified in the explorative phase.[10] As for the previous study, pancreatic cyst fluid samples were obtained by EUS-guided fine-needle aspiration. In a training cohort of 80 individuals, biomarkers were tested, receiver-operating characteristic (ROC) curves generated, and optimal cut-offs determined, using the Youden index. The biomarkers were then evaluated in a validation set of 68 pancreatic cystic lesions. Prevalence rates of malignant potential and HGD/cancer in the cohorts were, respectively, 45% and 19% in the training set, and 71% and 20% in the validation set, based on our endpoints. Briefly, in addition to surgery, results of clinical follow-up were accepted as a diagnostic standard. To exclude HGD/cancer, an observation period of at least three years was mandated, unless the lesion was obviously benign (for instance resolved spontaneously during follow-up).

Based on results from the training cohort as well as previous knowledge of the biology of pancreatic cystic tumours, the best biomarkers for malignant potential were found to be MUC5AC and MUC2. Cyst fluid levels of these proteins were determined based on quantification of a combined panel of 15 peptides. In the validation cohort, this panel could distinguish pre-/malignant tumours from intrinsically benign, with an accuracy of 97%. Corresponding results for CEA and cytology were, respectively, 61% and 84%. Full performance characteristics of targeted MS and standard cyst fluid analyses for the identification of cystic lesions with malignant potential in the validation cohort are shown in Figure 8A.

For the detection of HGD/malignancy, the best-performing panel was MUC5AC and PSCA, with an accuracy of 94% in the validation set. In particular, the sensitivity of the analysis at 95% (whole study population) compared favourably to results for CEA and cytology, which were 54% and 56%, respectively ($p=0.008$ and $p=0.007$).

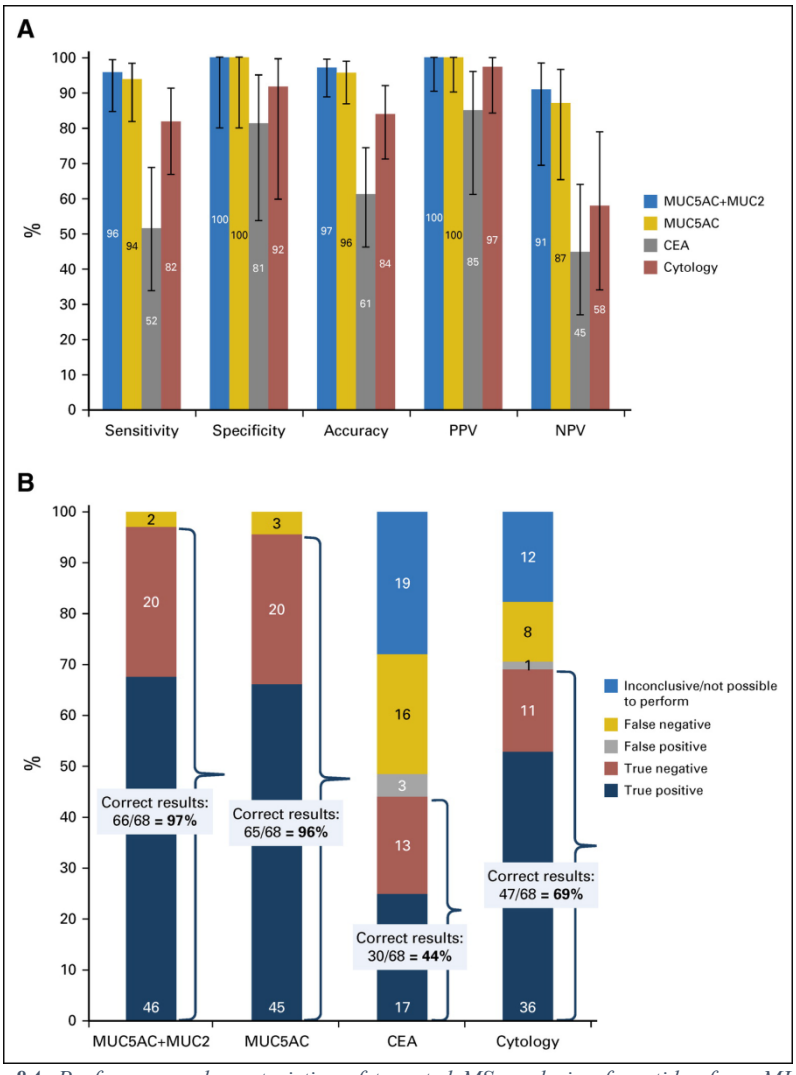

Figure 8A. *Performance characteristics of targeted MS analysis of peptides from MUC5AC and MUC2, and MUC5AC alone, for the detection of pre-/malignant pancreatic cystic lesions. Results for standard methods (cyst fluid CEA and cytology) are also provided.*

Figure 8B*. Full diagnostic outcomes for the different cyst fluid analyses for the detection of pancreatic cystic lesions with malignant potential. Originally published by the American Society of Clinical Oncology. Jabbar KS, et al: JCO 2018; 36(4):367–375.*

Current clinical guidelines are based on three parameters: cyst size, morphology and pancreas-related symptoms.[37,38,43] The size of the cyst, as well as whether it was suspicious for malignancy, was prospectively recorded in the EUS examination. Patients filled out a questionnaire about their symptoms before, or soon after, EUS. In

our study population, the sensitivity and specificity results of cyst size (cutoff ≥ 3 cm), EUS morphology and pancreas-related symptoms for HGD/malignancy were, respectively, 55 and 39%, 50 and 94%, and 85 and 31%.

As predicted, MUC2 levels were elevated in intestinal-type IPMN, as compared to other sub-types (p=0.002). PSCA concentrations were significantly higher in pancreato-biliary type IPMN (p=0.007). Thus, these two biomarkers could potentially identify the vast majority of high-risk IPMN and – given the predominance of this tumour type— high-risk cystic neoplasms in general.[50,51] However, due to the relatively small number of resected IPMN (n=21) in this study, these results will require further validation.

Low cyst fluid and cellular yield, respectively, can be a limitation for biomarker quantification and cytological assessments.[64] Thus, in a separate analysis, all results for each method in Paper II (including inconclusive and missing data) were taken into account in an "intention to diagnose" approach. This analysis was restricted to lesions where aspiration was successful. Grossly haemorrhagic samples, not considered to be representative of cyst fluid, were excluded. Figure 8B shows the full diagnostic outcomes for targeted MS and standard methods, for the detection of pre-/malignant lesions in the validation cohort. With this mode of analysis, targeted MS correctly classified 66/68 (97%) patients with regard to the malignant potential of the lesion. By contrast CEA returned correct results in 30/68 (44%) and cytology in 47/68 (69%) patients ($p<0.001$ for the comparison with targeted MS, in both cases). Furthermore, the MS analysis was able to detect 95% of lesions with HGD/cancer in patients where EUS-FNA was successful, as compared to 35% for CEA and 50% for cytology ($p<0.001$ and $p=0.003$).

4.1.6 Discussion and clinical implications

Taken together, targeted MS analysis of just three cyst fluid biomarkers could identify cystic precursors of pancreatic cancer and detect malignant progression within these lesions, with an overall accuracy around 95%. The diagnostic performance of the MS analysis was significantly superior to current state-of-the-art analyses. Moreover the same biomarker panel showed promise for the prediction of high-risk lesions.

Apart from the performance of the biomarkers, the main strength of the targeted MS analysis is the low cyst fluid requirements, as highlighted in the previous section. In both Paper I and Paper II, premalignant cystic tumours tended to be quite small, with a median diameter around 2 cm. Consequently, CEA could not be analysed for about 40% of lesions with malignant potential, in both studies. This is in keeping with results from previous reports.[64] Similarly, cytological assessments are often hampered by the low cellularity of pancreatic cyst fluid.[62-65] In both studies, a cytopathology technician

was present on-site during sampling, for instant assessment of cellular yield. Nevertheless, there were some inconclusive results. By contrast, for the targeted MS analysis very small volumes of cyst fluid were utilized, corresponding to one hundredth to one thousandth of the volume requirement for CEA quantification. Moreover, the selected biomarkers in both studies were either secreted or transmembrane (with the potential to be shed) proteins. This means that cellular yield is not a limiting factor for the method, which also represents an advantage over genomic analyses.

Thus, diagnostic performance and cyst fluid requirements are factors in favour of the clinical feasibility of the method. By contrast, lack of equipment and expertise could constitute a bottleneck for its clinical introduction. However, MS-based methods have in fact long been used in health care in a small scale, such as for the diagnosis of inborn errors of metabolism.[239] Unlike explorative MS, the targeted method focuses on a limited number of proteins and peptides, which could allow for semi-automated, high-throughput analysis.[10] Furthermore, targeted MS has general advantages over standard, antibody-based methods for diagnostic evaluation, including the ability to analyse large panels of biomarkers in parallel.[8-10] Therefore, it is reasonable to assume that it could become a common diagnostic modality in the future.

The cost of the MS-based analysis of a patient sample is hard to properly assess at present. Importantly, it must also be viewed in the broader context of the current situation and options for this patient group. Presently, the decision whether to resect a lesion is based on consensus documents.[37,38,43] The American (AGA) and international (Fukuoka) guidelines have been evaluated by a recent study.[37,43,240] By adhering to these guidelines, 25% and 19% of cancers were missed respectively.[240] By contrast, for 75% of resected lesions malignant progression could not be verified.[240] Surgery may still have been appropriate for some of these cases. However, previous reports and our own experiences indicate that histology frequently does not match the preoperative assessment that resulted in resection. This is concerning, since pancreatic surgery is still associated with non-negligible mortality, and considerable, sometimes life-long, morbidity.[32,38]

Current guidelines recommend regular follow-up of pancreatic cystic lesions where malignant potential cannot be excluded, up to every 6 months.[37,38,43,241] As reviewed in the Introduction, such lesions are common in the general population.[31,35,38] The psychological effects of this stringent follow-up programme have not been comprehensively evaluated. Still, in the age of information-seeking, it must be expected to cause considerable anxiety, although the actual risk of malignant progression is often low. Importantly, with better diagnostic tools, follow-up could likely be reduced or avoided in many individuals.

One limitation of the MS-based approach is related to the "field defect" phenomenon. This means that IPMN not only have malignant potential, but also imply a broader risk of pancreatic cancer developing synchronously or metachronously from distinct macro- or microscopic precursors.[58] However, cyst fluid analysis can only assess the malignancy status of the sampled lesion.

Finally, an important factor to consider is the changing spectrum of cystic lesions referred for EUS analysis.[32] This shift is obvious from the differences in the distribution of diagnoses between the discovery and validation cohorts in Paper II. The most apparent change is the decrease in the proportion of pseudocysts and increase in IPMN over time. This upsurge in incidentally detected cancer precursor lesions will place great demands on accurate diagnostic tools that allow for detailed and individualized risk predictions.

4.2 COLONIC MUCUS PROTEIN COMPOSITION IN RELATION TO ULCERATIVE COLITIS PROGNOSIS AND PATHOGENESIS -- PAPERS III AND IV

4.2.1 Main observations

- The function of the colonic mucus barrier is maintained by less than thirty protein constituents
- Core structural mucus components are reduced in active ulcerative colitis
- Structural weakening of the mucus barrier occurs independent of inflammation, and may be important for the development of the disease
- The ratio of interacting proteins LRRFIP1 and FLII, which differentially regulate the sensing of microbes, predicts natural history in ulcerative colitis

4.2.2 The protein composition of the colonic mucus barrier

UC pathogenesis is currently understood as an inappropriate host response to intestinal microbiota, against a background of genetic susceptibility.[197,202,206] However, colonic microbes are normally segregated from the host immune system by a filter-like, secreted mucus layer.[80,81] Mucus barrier failure has been demonstrated in a high proportion of patients with active UC.[226] Still, the underlying mechanisms and their causal relationship with the inflammation, have not been established. The investigation

of the potential role of mucus compositional abnormalities in UC has been impeded by a lack of characterization of the baseline state.

Therefore, in a study of mucus sampled *ex vivo* from sigmoid colon biopsies from 47 individuals without colonic disease, we determined the protein constituents of the mucus barrier. About half (47%) of these individuals were female, and the age range was 23-87 years. As discussed in the Methodology section, these participants were not healthy volunteers, but patients that had been referred for colonoscopy due to various symptoms. The most common cause of referral was anaemia. However, none of these individuals had known inflammatory bowel disease, or observable macro-/ microscopic colon pathology.

To define the protein components of the mucus barrier, the proteomic dataset was systematically filtered. Intestinal epithelial cells have a high turnover, and are constantly shed into the mucus. Thus, intracellular proteins were ubiquitous in the samples. Nevertheless, these proteins are unlikely to have a functional role in the mucus. Proteins destined for the secretory pathway (i.e., bound for the membrane, ER, Golgi apparatus, or the extracellular space) typically possess a particular signal sequence at their N-terminus. This feature was used to select for secreted and transmembrane proteins. Subsequently, proteins with an ER retention sequence motif were excluded. Predicted plasma proteins, likely deriving from the biopsy sampling, were also removed from the list.[242] Interestingly, out of a median of 955 protein identifications in the mucus samples, after systematic filtering and manual curation only 29 were found to potentially constitute "true" mucus components.

The shortlist of mucus proteins included homologues of known constituents in mice such as FCGBP, CLCA1 and ZG16, but also non-goblet cell derived proteins such as immunoglobulins.[82] Equally interesting, perhaps, are predicted mucus constituents *not* identified by the analysis. One example is trefoil factors, the small size and amino acid sequence of which likely hamper the generation of unique tryptic peptides that can be identified by MS.[243] Classical Paneth cell-derived antimicrobial proteins/peptides were also absent from the colonic samples, although abundant in ileal mucus.[244,245]

4.2.3 Structural weakening of the colonic mucus barrier precedes inflammation in ulcerative colitis

Having investigated the composition of the colonic mucus barrier under normal conditions, we proceeded to determine whether it is affected in UC. To this end, mucus was collected from sigmoid colon biopsies from 64 UC patients, and analysed by nano-LC MS/MS. Based on a synthesis of symptoms, overall endoscopy findings and histology, 36 patients were found to have active disease, while 28 patients were in remission. Patients were also separately stratified according to the histology of the

sampled segment. Using this definition, only 15 mucus samples were representative of active inflammation. Samples obtained from 47 individuals without colonic disease in the same way and during the same period, as previously described, served as controls. The analyses were focused towards the core mucus proteome.

Label-free, relative quantification of mucus proteins revealed a pronounced reduction in MUC2 in UC patients with ongoing disease activity. This was verified through MS-based absolute quantification in a separate, smaller cohort. Other core components including FCGBP, CLCA and ZG16 were also lower in abundance in patients with clinically active UC, according to relative quantification. By contrast, no differences were observed in UC in remission, except for higher levels of two eosinophil-derived proteins.

Strikingly, when the levels of mucus proteins were compared between active UC patients with (n=15) and without (n=18) inflammation of the sampled segment, core constituents MUC2 and FCGBP were found to be equally reduced in both groups. This phenomenon was observed even in mucus from tissue scored as Sandborn 0; i.e., normal histology, with no evidence of immune cell infiltration.[246] Thus, structural weakening of the mucus barrier occurs independent of, and likely prior to, overt inflammation.

Classical immune cell derived proteins, such as the calprotectin dimer, were only elevated in mucus samples from inflamed tissue. By contrast, an increase in the mucus levels of pro-inflammatory cytokine IL18 was observed in all samples from patients with clinical disease activity, regardless of the inflammation status of the sampled segment. Analysis on the peptide level revealed a selective increase of the bioactive part of IL18. By contrast, the abundance of the N-terminal fragment, which is cleaved off during activation, did not differ from controls. This argues for increased secretion of activated IL18, rather than cellular contamination, as the most likely explanation for our observations. IL18 is activated through caspase 1-mediated cleavage during inflammasome assembly in epithelial or hematopoietic cells, and thereupon secreted to exert its pro-inflammatory effects.[247] The lack of significant immune cell recruitment in the majority of patients with elevated mucus IL18 in this study, suggests that the secreted IL18 at least partly derived from the epithelium. Thus, epithelial inflammasome activation may be an early event in UC pathogenesis.

Since the lower levels of core mucus components in active disease could not be explained by inflammation and tissue damage, aberrations of mucus secretion were evaluated as an alternative mechanism. Goblet cells in the surface epithelium constitutively secrete mucus through the merging of single granules with the membrane.[248] By contrast, upper crypt goblet cells are capable of releasing their entire mucus content in a coordinated burst, known as compound exocytosis.[249] A specialized

goblet cell sub-type, the sentinel goblet cell, has previously been shown to orchestrate this response. These cells sense ligands of TLR2, 4 and 5 (including microbial components such as LPS), and mount a secretory response mediated by NLRP6 inflammasome activation.[102]

Using apical administration of a synthetic ligand of TLR2, the compound exocytosis response was evaluated in *ex vivo* biopsies from patients with active UC, patients in remission and individuals without colonic disease. While stimulation with the ligand resulted in increased mucus growth both in controls and UC patients in remission, this response was abrogated in active UC.

As previously discussed, NLRP6 inflammasome activation is involved in the induction of sentinel goblet cell dependent mucus secretion.[102] Thus, our results suggest that epithelial inflammasome assembly, resulting in activation of the upper crypt compound exocytosis response, could be an early event during the development of colitis. However, frequent or constant stimulation of this mechanism may result in insufficient time for mucus production, lower level of structural components and, ultimately, weakening of the mucus barrier.

4.2.4 Compositional alterations of penetrable mucus samples

To further test our explanatory model, we examined the composition of penetrable mucus samples. Briefly, mucus penetrability was assessed in parallel with sample collection for MS analysis in 49/111 patients (including 25 with active UC, 15 with UC in remission, and 9 controls) using fluorescent beads as surrogate markers for bacteria.[226] Based on this analysis, nine (36%) patients with active UC, three (20%) with UC in remission, and one control (11%) were found to have mucus barrier failure.

As expected, there was a strong tendency towards a reduction of MUC2 in penetrable mucus samples. However, neither this difference, nor other alterations of the core mucus proteins, was statistically significant. Based on the observation that the goblet cell compound exocytosis response to a TLR2 ligand was abrogated in active UC, we sought to identify markers for this type of secretion. In contrast to constitutive secretion, compound exocytosis involves the release of whole mucus granules, rather than just their contents. Thus we identified a group of mucus proteins that 1) are known mucus granule components, but lack signal peptides for secretion, indicating that they form part of the granule membrane 2) exhibited a correlation with MUC2 levels in the MS analysis, and 3) were identified in the majority of mucus samples.[250] Among these proteins, BCAP31 and RAB10 showed the strongest correlation with MUC2. However, interestingly, this correlation was restricted to UC patients. Given that they are likely to be granule wall components, BCAP31 and RAB10 should be released only through

compound exocytosis, making them potential markers for this secretory mechanism.[102, 249,250] Both BCAP31 and RAB10 were significantly reduced in penetrable mucus samples. Taken together, this implies that the goblet cell compound exocytosis response to microbial components is abrogated in active UC, likely due to exhaustion. Furthermore, this secretion mechanism may be important for the maintenance of an impervious mucus barrier.

This model of the role of the mucus system in the development of UC does not include the initial events, leading to inflammasome assembly. However, these are likely to involve increased host-bacterial contact. The mucus penetrability observed in a subset of UC patients in remission could potentially indicate an inherent weakness of the mucus barrier. This patient group, along with some individuals with penetrable mucus despite very mild inflammation, was characterized by lower mucus levels of apical chloride-bicarbonate exchanger SLC26A3/DRA.[251] As discussed in the following, SLC26A3 may be important for mucus barrier formation.

4.2.5 Mucus protein alterations associated with poor disease outcome

Ulcerative colitis is regarded a chronic disease, but affects individuals very differently.[197,229-231] While some patients will require repeated corticosteroid regimes and even colectomy, others will have only one flare in their lifetime. Currently, there are no useful predictive biomarkers for disease activity.[231] As described in the previous sections, mucus from sigmoid colon biopsies was sampled in 64 UC patients, and analysed by proteomics. Sixty-one of these patients (95%) could be prospectively followed for 3 years, and 54 patients (84%) for 5 years; allowing us to correlate mucus protein composition with clinical outcome. High disease activity during the prospective observation period was defined as minimum one relapse per year, or requirement for additional maintenance therapy (e.g. thiopurines or TNF alpha inhibitors), hospitalization or surgery. Eleven (18%) and 14 (26%) patients, respectively, fulfilled this criterion based on 3-year and 5-year follow-up.

The most promising protein biomarker candidates for poor clinical outcome according to our evaluation were LRRFIP1 and FLII. Intriguingly, these proteins are direct binding partners that have been reported to differentially modulate TLR4-MYD88 dependent signaling, and thus the innate response to specific microbial ligands.[252-256]

A ratio of LRRFIP1/FLII ≤ 1 was found to be a significant predictor of high disease activity during three years, with an odds ratio of 37. The prognostic value of the LRRFIP1/FLII ratio was independent of disease activity at baseline. Furthermore, the LRRFIP1/FLII ratio predicted the requirement for intensified medication or surgery during a five year period, with a sensitivity of 90% and a specificity of 89%.

To better understand the underlying mechanisms behind these observations, the localization of LRRFIP1 and FLII was evaluated through immunostaining. Both proteins were detected in the colonic epithelium. Furthermore, the staining intensity inside epithelial cells appeared to correlate with relative quantification levels in mucus. Thus, mucus abundances of these proteins were deemed likely to reflect intracellular expression and function.

Altered TLR4-MYD88 signalling can be expected to result in impaired microbial sensing.[257] Thus, using histology, the presence of bacteria in the epithelium and lamina propria was evaluated. In individuals with a low LRRFIP1/FLII ratio and poor clinical outcome, bacterial endospores were frequently detected in the lamina propria, as confirmed by two independent staining methods.

4.2.6 Discussion and clinical implications

In Papers III and IV, the protein composition of the colonic mucus barrier was studied, in homeostatic conditions as well as in UC patients with and without active inflammation. In total, mucus samples collected *ex vivo* from sigmoid biopsies from over a hundred individuals were analysed. Moreover, the association between mucus composition and the natural history of UC was investigated.

Remarkably, the core proteome of the mucus was found to consist of less than thirty secreted and transmembrane proteins. These included homologues of known mucus constituents in mice, such as FCGBP, CLCA1 and ZG16, but also non-goblet cell derived proteins such as immunoglobulins, eosinophil granule proteins and enterendocrine cell product chromogranin A.[82] The latter protein has been reported to generate several antimicrobial peptides that may be of importance for mucus-associated innate defences.[258,259] However, classical Paneth-cell derived antimicrobial compounds were absent from colonic mucus, although abundant in the ileum. There is consensus that these proteins fulfill an important function in the loose mucus of the small intestine, where they protect us from pathogen colonization and invasion.[244,245] By contrast, the colon is the site of large-scale microbial harvesting of energy, to the benefit of the host.[103,112,113] Thus, in this context, physical protection of the epithelium through a dense mucus barrier may be preferable to the secretion of microbicidal substances.

Given previous reports on mucus abnormalities in UC patients, we next wanted to investigate compositional alterations in UC. We were primarily interested in early events, with a potential causative role in the development of colitis. Thus, mildly affected or normal-appearing segments were preferentially selected for sampling. Core mucus structural components MUC2 and FCGBP were found to be reduced in samples from patients with clinically active UC. Intriguingly, this reduction was equally

pronounced in mucus collected from non-inflamed segments. This indicates that structural weakening of the mucus barrier in UC occurs independent of inflammation and tissue damage, implying that it could be important for pathogenesis. Samples from non-inflamed segments in patients with active colitis may not fully reflect the early stages of the disease. Still, they probably constitute the best "human model" available for studying the initiating events behind UC inflammation.

Since the reduction in mucus core structural components observed in active UC did not appear to be caused by an overt mucosal immune response, we proceeded to further dissect the underlying mechanisms. Screening the proteomic dataset for markers of early inflammation, we detected increased levels of the bioactive form of IL18 in mucus sampled from non-inflamed segments in active UC patients. Thus, we surmised that epithelial inflammasome activation may be an early event in UC pathogenesis. NLRP6 inflammasome assembly mediates the sentinel goblet cell dependent secretory response to microbes.[102] Hence, persistent epithelial inflammasome activation may result in repeated stimulation of this secretion mechanism. *Ex vivo* investigation using a synthetic TLR2 ligand to stimulate goblet cell secretion from biopsies, as well as proteomic studies of the composition of penetrable mucus samples, indicated that the compound exocytosis response to microbes may be exhausted in active UC. Moreover, this phenomenon is likely to be important for the increased penetrability of the inner mucus layer observed in colitis.

Mucus penetrability was not limited to UC patients with clinical activity, but also observed in a subgroup of patients in remission, in line with a previous report.[226] Thus, it is conceivable that a minority of UC patients have intrinsic abnormalities of the mucus barrier, resulting in increased susceptibility to colitis. In this study, mucus levels of chloride-bicarbonate exchanger SLC26A3 were found to be specifically reduced in UC patients who had penetrable mucus in spite of no, or very mild, active inflammation. The biological rationale of this finding is underpinned by several, independent observations. The importance of bicarbonate secretion for proper assembly of the MUC2 protective network following its release into the colonic lumen is reviewed in the Introduction.[91] Moreover, deficiency of Slc26a3 has been linked to mucus barrier failure in mice.[251] Finally, the *SLC26A3* gene is a known risk locus for UC, but not Crohn´s disease.[260] Our observations provide a possible biological explanation for this association.

UC patients have a highly heterogeneous disease course.[197,229-231] Individualized predictions of disease activity could potentially forestall severe relapses and emergency colectomies, as well as minimize unnecessary use of medication. In Paper IV, the ratio of proteins LRRFIP1 and FLII, which antagonistically modulate TLR4-MYD88 signalling, was found to be highly predictive of frequent relapses and requirement for additional medication and surgery.[255] Interestingly, a shift in the balance of these

proteins correlated with extensive infiltration of bacterial endospores in the lamina propria. To the best of our knowledge, this phenomenon has not previously been linked to UC. Spore-forming species encompass several pathogens, including *Clostridium difficile*.[261] Interestingly, the TLR4-MYD88 signalling pathway has previously been shown to be required for a successful innate immune response during *C. difficile* infection.[262] However, there is evidence that more than 50% of the colonic microbiota possesses the capacity to sporulate.[263] Thus, the identities of the spore-forming species observed in UC patients with poor clinical outcome in this study, remain to be determined.

General limitations of the analyses include the composition of the control group, which consisted of patients referred for colonoscopy due to clinical reasons. A screening programme for colorectal cancer has not yet been implemented in Sweden, but will potentially facilitate future studies of the healthy human colon. Furthermore, the biopsy and mucus sampling techniques may incur variable contamination by blood components and intracellular proteins. Still, there were no discernible, systematic differences in contamination between patient categories.

The characterization of the colonic core mucus proteome in homeostatic conditions as well as in colitis, provide a platform for further studies. Functional abnormalities of the mucus system have previously been described in UC. However, in the present work their underlying mechanisms and temporal association with inflammation were comprehensively investigated. Although preliminary, the association between severe UC and tissue infiltration of bacterial endospores is compatible with biological and clinical characteristics of the disease. Intermittent germination of endospores could potentially explain the frequent relapses and poor therapeutic response that characterize a subset of UC patients. Taken together, our observations may inform the future development of treatment options to strengthen mucus protection and minimize host-bacterial contact – potentially including probiotic compounds.

4.3 ASSOCIATION BETWEEN THE *BRACHYSPIRA* GENUS AND IBS -- PAPER V

4.3.1 Main observations

- *Brachyspira* colonization was detected in 34% of IBS patients but only 4% of healthy individuals.
- Attachment of *Brachyspira* to the colonocyte brush border was seen in every fifth IBS patient and associated with diarrhoea and accelerated transit

- IBS patients with *Brachyspira* had mild mucosal inflammation, eosinophilia and mast cell activation
- Antibiotic treatment resulted in long-term clinical improvement, but promoted relocation of the *Brachyspira* into crypts and goblet cell mucus granules.

4.3.2 Rationale

Irritable bowel syndrome (IBS) encompasses a wide range of intestinal symptoms, and is unlikely to be explained by a single mechanistic model. Thus, research efforts have been directed towards identification of subgroups that could be responsive to targeted intervention. The increased incidence of IBS after a gastroenteritis episode, as well as positive effects of pre-, pro- and antibiotics in certain individuals, suggest microbial imbalance as an underlying factor in a subset of patients.[168,174-176] However, studies of faecal, and mucosal samples, have not demonstrated consistent alterations of the microbiota that could be linked to patient characteristics.[169] The colonic inner mucus layer segregates the vast majority of bacteria from the host.[80,81] Nevertheless, studies indicate that this compartment is not sterile, but rather inhabited by a very sparse and select flora.[135] Although low in abundance, the strategic position of these microbes would enable them to influence host physiology and immune responses in a unique fashion.

Therefore, we decided to perform a meta-proteomic study of inner mucus layer samples from well-characterized IBS patients and healthy individuals. To investigate their pathogenic potential, observations were correlated with clinical parameters and investigation of mucosal immune responses.

4.3.3 Metaproteomic analysis of colonic mucus samples identified a potential association between *Brachyspira* and IBS

Twenty-two IBS patients and 14 healthy volunteers underwent sigmoidoscopy with biopsy collection. Biopsies were transported in oxygenated Krebs and mounted in a perfusion chamber. Subsequently, mucus was “harvested” through aspiration and gentle scraping, as described in the Methodology section.[233] Importantly, the outer, loose, mucus layer is easily dislodged and should be lost during biopsy sampling and transport in the buffer. In support of this, a thin mucus layer, corresponding to the previously described thickness of the human adherent, inner mucus layer, could be observed directly after mounting of the biopsy in the chamber.[233]

Mucus samples were analysed by nano-LC MS/MS as described in Paper V. Searches were performed against reviewed human and eubacteria sequences of the Uniprot

database. At least three protein identifications at the 99% significance level were required to validate the presence of a bacterial family/genus in the mucus.

In explorative MS analysis, proteins present in low concentrations tend to be obscured by high-abundant ones; i.e., not selected for fragmentation and identification. Moreover, the bacterial content of the inner mucus layer is expected to be very low, as previously discussed. Therefore, it is hardly surprising that bacterial peptides were not detected in most samples. On the family level, the most frequent identification was *Pseudomonadaceae*, which was over-represented in IBS patients (p=0.06). The second most common family was *Brachyspiraceae* (genus *Brachyspira*), detected in 3/22 (14%) IBS patients, but not in any healthy individuals.

Brachyspiraceae species encompass well-established pathogens of domestic animals.[138,140] *Brachyspira aalborgi* and *Brachyspira pilosicoli* are associated with a condition known as intestinal spirochetosis in humans. Therein, the elongated *Brachyspira* attach to the colonic epithelium in a perpendicular manner, creating the impression of a prominent brush border upon histological examination.[137-141] In this study, the characteristic, continuous spirochete "fringe" could be clearly visualized in Alcian-blue/PAS-stained sections in the three IBS patients with proteomic Brachyspira identifications in mucus. The presence of the spirochetes at the epithelial border was further confirmed through immunofluorescent staining with *Brachyspira* antiserum.[264] Transmission electron micrographs showed a dense colonization of the epithelial surface, with *Brachyspira* attaching to the apical membrane between microvilli. However, there were no obvious signs of damage to the epithelial cell, suggesting that *Brachyspira* is well adapted to long-term colonization of its human host.

4.3.4 *Brachyspira* colonization of the colonic epithelium and inner mucus layer is significantly more common in IBS patients

Having confirmed our initial observations, we wanted to see if we could detect further cases among IBS patients or healthy individuals. To enable rapid screening of biopsy material as well as species detection, we set up a targeted real time PCR analysis of 16S rDNA based on melt curve discrimination, as described by Westerman et al.[144] Using this analysis, in conjunction with immunofluorescent staining, the prevalence of *Brachyspira* colonization in IBS patients was found to be as high as 50% (11/22 patients), as compared to 7% (1/14 individuals) in healthy controls (p=0.01). For the validation of our findings, we had access to a separate cohort of prospectively collected Methacarn-fixed, paraffin-embedded biopsies from 40 IBS patients and 10 healthy controls. In the validation cohort, *Brachyspira* prevalence rates were 25% in IBS patients and 0% in controls. Again, prevalence rates were based on two independent methods: PCR analysis of 16S rDNA extracted from paraffin- embedded biopsies, and

immunohistochemistry of tissue sections. These diagnostic methods showed good agreement, with a Cohen´s kappa of 0.84. In total, *Brachyspira* was identified in 21/62 (34%) of IBS patients, and 1/24 (4%) healthy individuals (p=0.005). Spirochetosis was most common among patients with IBS with diarrhoea (IBS-D). In this subgroup, *Brachyspira* was detected in 44% of patients, with similar prevalence results between the discovery (50%) and validation (40%) cohorts.

Immuno-histochemical analysis could be performed in 56 IBS patients and 21 controls. Classical, membrane-associated spirochetosis, with attachment of the *Brachyspira* to the colonocyte brush border, was observed in 6/19 (32%) IBS patients from the discovery cohort and 5/37 (14%) IBS patients from the validation cohort. Thus, membrane-associated spirochetosis was detected in a total of 11/56 (20%) IBS patients. By contrast, this phenomenon was not observed in any healthy control (p=0.03). In individuals where *Brachyspira* was identified by PCR, but where no involvement of the apical border was seen, spirochetes could typically be detected in the mucus. Invasion of the inner layer was also frequently observed. Thus, this condition was denoted as mucus-associated spirochetosis.

There are two known *Brachyspira* species associated with intestinal spirochetosis in humans: *B. aalborgi* and *B. pilosicoli*. The existence of a third species colonizing humans, denoted as *B. hominis*, has been suggested.[137-140] In this study, using targeted real-time PCR with melting curve discrimination, *B. aalborgi/hominis* was found to be involved in 78% of cases with membrane-associated spirochetosis, whereas *B. pilosicoli* was responsible for 89% of mucus-associated spirochetosis. Thus, these species appear to be associated with distinctive colonization patterns, although with some niche overlap.

4.3.5 Membrane-associated spirochetosis was linked to a distinctive symptom profile and mucosal immune response

The severity of IBS and its individual symptoms (pain, bloating, bowel habit dissatisfaction and general life interference) is traditionally assessed using the IBS Symptoms Severity Score (IBS-SSS).[265] A second module of this score assesses ten extracolonic symptoms (including early satiety, flatulence, nausea, heartburn, headache, fatigue, back pain, pain of the thighs, muscles and joints, and urinary urgency), which are common in IBS patients. IBS patients with and without spirochetosis did not differ with regard to the overall score or the individual items of the IBS-SSS. However, patients with membrane-associated spirochetosis had significantly less extracolonic symptoms.

As part of the study, patients kept a structured diary of their bowel habits for 14 days. Based on this data, patients with membrane-associated spirochetosis were found to have higher average stool frequency ($p=0.04$) and looser average stool consistency ($p=0.02$) than IBS patients without *Brachyspira*. Corroborating these observations, they also had accelerated oro-anal transit ($p=0.02$) as compared to IBS patients without spirochetosis. By contrast, individuals with mucus-associated spirochetosis did not differ from other IBS patients with regard to these parameters.

Next, we wanted to assess mucosal immune responses in *Brachyspira* patients. Using H&E-stained sections, differential counting of lamina propria and sub-/intraepithelial immune cells was performed, as detailed in Paper V. Patients with membrane-associated spirochetosis had increased lamina propria infiltration of leukocytes, ($p<0.001$), particularly plasma cells ($p<0.001$), as compared to IBS patients without *Brachyspira*. Corresponding results for individuals with mucus-associated spirochetosis were variable, with some having elevated and others paradoxically low immune cell counts. Increased numbers of sub-/intraepithelial eosinophils were also observed in patients with classical intestinal spirochetosis, as previously described.[266]

Mast cells are not readily identifiable on H&E stained sections, but have been implicated in IBS pathogenesis, particularly with regard to visceral hypersensitivity.[190-193] Furthermore, clusters of mast cells had been observed in spirochetosis patients in this study, using transmission electron microscopy. Therefore, to visualize mast cells, biopsy sections were also stained by toluidine blue. Based on histology, numbers of both total and activated mast cells were significantly augmented in spirochetosis patients. In addition, mast cell products chymase, tryptase and beta-hexosaminidase were more frequently detected in mucus from patients with *Brachyspira*, according to MS. The proportion of activated sub-epithelial mast cells strongly correlated with rectal sensitivity in IBS patients with spirochetosis, as well as in healthy individuals. By contrast, this correlation was absent in IBS patients without spirochetosis.

The inner mucus layer represents the first line of innate defence against pathogens – although obviously not effective against *Brachyspira*. Using bacteria-sized fluorescent beads, the inner mucus layer was found to be abnormally penetrable in both membrane- and mucus-associated spirochetosis. Similar findings are observed in active UC.[226] However, in contrast to UC patients, individuals with spirochetosis actually had increased MUC2 levels in the mucus, indicating elevated mucin production and secretion. Moreover, invasion of bacteria into crypts and even goblet cells was seen in toluidine-blue stained sections. This finding had not been observed in sections stained by *Brachyspira* antiserum, leading us to hypothesize that these bacteria belong to different genera. This was confirmed by Gram staining, and immunostaining with an antibody against lipoteichoic acid; both of which demonstrated that some of the invading bacteria were Gram positive.

4.3.6 Metronidazole treatment resulted in long-term clinical improvement but also induced a more invasive *Brachyspira* phenotype

Available evidence suggests that intestinal spirochetosis should be treated if symptoms are present.[140,145,146,150] Metronidazole is the best studied antibiotic in this context. However, mindful of previous studies which described only partial improvement, it was decided to perform the treatment protocol as a pilot study.[140,145,146,150] Thus, four patients with membrane-associated spirochetosis were treated with metronidazole. These individuals were followed for over a year after antibiotic therapy, with careful assessment with regard to clearance of the infection, as well as the clinical and histological response.

Traditionally, symptoms and treatment responses in IBS are evaluated using the IBS-SSS questionnaire.[265] The full score ranges from 0-500, with higher values indicating more severe symptoms. Thus, the four patients who received metronidazole treatment handed in IBS-SSS questionnaires, before and at regular intervals up to 15 months after treatment. Three out of four individuals reported clinical improvement after treatment. By consensus, a reduction of IBS-SSS exceeding 50 points is considered as a significant response in IBS treatment studies.[265] The three responders fulfilled this criterion at the final follow-up visit (with a mean and median reduction of 186 and 171 points, respectively) whereas in the fourth patients symptoms were neither better nor worse than before antibiotic therapy. However, only one patient was potentially "cured" of IBS (defined as IBS-SSS <75).[265] Thus, as previously described, the effect of treatment appeared to be favourable, but partial.[140,145,146,150] The most substantial improvement was observed for pain and general life interference.

The patients underwent sigmoidoscopy six weeks after the completion of treatment, to confirm eradication of the infection. Histology demonstrated clearance of the spirochetes from the apical membrane, which is how treatment success was established in most previous studies. Targeted real-time PCR of biopsy material confirmed a dramatic decrease in the abundance of *Brachyspira.* Immunohistochemistry, on the other hand, told a quite different story. Although the spirochetes were largely gone from the epithelial surface in the responders (and reduced in abundance in the non-responder), the *Brachyspira* had relocated into crypts and goblet cell mucus granules.

4.3.7 Discussion and clinical implications

In this study, *Brachyspira* species were shown to be significantly more common in colonic biopsies and mucus from IBS patients. Direct attachment of *Brachyspira* to the epithelial surface was confirmed in every fifth IBS patient, and significantly associated with diarrhoea and accelerated gastrointestinal transit. A nearly equal number of IBS

patients had *Brachyspira* colonization restricted to mucus, with no evidence of epithelial involvement. By contrast, these individuals did not have diarrhoeal symptoms. Taken together, this suggests that the spirochetes cause diarrhoea by physically occupying much of the colonic surface where fluid reabsorption should have taken place, and possibly inducing structural alterations. Accordingly, membrane-associated *Brachyspira* was observed in nearly every third IBS patient with diarrhoea (IBS-D), but not in any IBS patient with constipation, nor in any healthy individual.

However, diarrhoea is not in itself sufficient for an IBS diagnosis, the mainstay of which is abdominal pain.[158] Furthermore, mucus-associated spirochetosis, which was not associated with diarrhoeal symptoms, also seemed to be overrepresented among IBS patients. This indicates that *Brachyspira* also contributes to IBS symptomatology through other mechanisms. Host responses observed in this study included mild mucosal inflammation with increases in eosinophils and mast cells, as well as mucin hypersecretion. This is reminiscent of the Th2 type immunity induced by helminthic infections and allergic reactions, where the augmented mucus production is mediated by effector cytokine IL13.[267,268] Thus, our findings tentatively suggest that the immune response elicited by *Brachyspira* might be Th2 dependent.

In the context of IBS, our observations of mast cell activation in spirochetosis patients may have the greatest immediate relevance. Mast cells have been strongly implicated in IBS pathogenesis, particularly with regard to pain and visceral hypersensitivity.[190-193] Numbers of mucosal, activated mast cells are increased in the IBS population, according to several studies.[190-192] Moreover, mast cell compounds, such as histamine and tryptase, are capable of sensitizing and exciting submucosal afferent neurons *ex vivo*.[192] In this study, visceral sensitivity correlated with mast cell activation in IBS patients with spirochetosis, and healthy controls. However, this correlation was absent in IBS patients without *Brachyspira*. Taken together, our observations tentatively suggest that the previously observed association between mast cell activation and IBS symptoms may at least partly be explained by concurrent intestinal spirochetosis.

Brachyspira is well adapted to its unique niche. Although anaerobic, its expression of the NADH oxidase (nox) gene allows it to tolerate the oxygen diffusion near the epithelium.[269] Thanks to its periplasmic flagella, *Brachyspira* is highly motile.[138,140,270] Furthermore, its swimming speed actually increases in viscous surroundings, like the colonic mucus.[270] Previous investigations also indicate that *Brachyspira* expresses proteases at its outer membrane which potentially assist its passage through the mucus.[271] In this study, we observed that IBS patients with spirochetosis had abnormal penetrability of the mucus barrier. Although the causal relationship is unclear, it is probable that *Brachyspira* affects barrier properties. Apart from direct, protease-dependent effects on the mucus network, the massive *Brachyspira* colonization likely overwhelms and exhausts sentinel goblet cell mediated secretory responses.[102]

As reviewed in the Introduction, the pathogenic relevance of *Brachyspira* is debated. Koch´s classical postulates for the association between microbes and disease, state that it should be possible to introduce a culture of the microorganism into a healthy organism, and thereby cause illness. However, *B. aalborgi*, the species most commonly associated with symptoms in humans, is almost impossible to culture.[139] Moreover, since it is thought to only colonize/infect primates, experimental models are sorely lacking.[140] Thus, the pathogenic relevance of intestinal spirochetosis has mainly been assessed through treatment studies in humans, often limited to few cases.[140,145,146,150] While antibiotic therapy led to improvement in some patients, it did not always produce a full recovery. Similarly, among the four spirochetosis patients treated with metronidazole in our study, only one was potentially "cured" of IBS, although another two showed significant clinical improvement. This is of course a much too small population to draw conclusions from. However, while the placebo response can be prominent in IBS patients, it is typically temporary, whereas the patients in this study experienced a gradual relief in symptoms over many months.

Histology and PCR-based quantification six weeks after completion of treatment indicated successful eradication of the *Brachyspira.* Immunohistochemistry alone revealed persistence of the infection. Although the spirochetes had largely been cleared from the membrane in all three responders, there was evidence of relocation of the *Brachyspira* into goblet cells and crypt lumina. Antibiotic concentrations can be expected to be considerably lower in the tightly packed mucus inside the goblet cells. Thus, the treatment may have selectively induced the expansion of *Brachyspira* strains with a tropism for colonizing this environment. Moreover, *Brachyspira* were found to penetrate into the crypt base, with the potential of direct interaction with the stem cell niche.

Limitations of the analysis include the low number of control subjects, and the fact that patients were recruited from a secondary/tertiary care unit, which means that they may not be representative of the IBS population at large. Among the strengths, the rigorous clinical characterization of the IBS patients – through validated questionnaires, bowel habit diaries, testing of rectal sensitivity and oro-anal transit time – should be mentioned. Equally importantly, the use of multiple complementary diagnostic methods produced novel insights. The initial observations were made using an explorative metaproteomic analysis. However, established techniques such as routine histology and 16s rDNA sequencing of stool samples failed to detect *Brachyspira* in the same individuals.

Taken together, our observations indicate that *Brachyspira*-associated IBS is linked to a distinctive symptom profile and mucosal response, and accounts for up to 20-30% of all IBS cases in a Swedish population. Thus, targeted antibiotic treatment may conceivably have substantial effects on the morbidity burden of this disorder.

However, metronidazole therapy paradoxically induced a more invasive *Brachyspira* phenotype, with the capacity of penetrating down into the crypt base. Hence, our findings argue against indiscriminate antibiotic therapy of IBS patients, and underline the importance of careful follow-up.

Importantly, our observations represent a novel microbial strategy to survive antibiotic treatment that is unlikely to be restricted to the *Brachyspira* genus. Bacterial persistence during antibiotic treatment, e.g. through biofilm formation or colonization of macrophages has been previously described.[272,273] Our findings highlight an alternative mechanism for evasion of antibiotic action. The large intestine acts as a reservoir for multiple pathogens and pathobionts.[274] These are not only capable of causing gastroenteritis but also conditions ranging from recurrent cystitis to life-threatening nosocomial infections.[274,275] Thus our findings could potentially have broad implications for the understanding of bacterial persistence and chronic or relapsing infectious diseases.

5 CONCLUSIONS AND FUTURE PERSPECTIVES

The studies in this thesis not only encompass three different disorders, but also the full translational spectrum from bench to bedside. A direct example of a potential clinical application of our observations is the mass spectrometry based analysis of pancreatic cyst fluid devised and evaluated in this work. This method detected cystic precursors of pancreatic cancer with significantly higher accuracy than state of the art diagnostic analyses. Clinical evaluation is currently underway. It will be essential to confirm the utility of the method through randomized controlled trials, preferably with patient enrolment at different centres. If the analysis is successfully validated, the next challenge will be a seamless transition from the university to the hospital laboratories.

UC is a well described, but poorly understood condition. Better comprehension of the underlying mechanisms may enable targeted, individualized treatment that could improve patient outcomes. In this thesis, structural weakening of the colonic mucus barrier was shown to occur independent of – and likely prior to – inflammation in UC, possibly resulting in increased host-bacterial contact and immune cell recruitment. These findings may contribute to our understanding of UC pathogenesis, and should be explored by further studies.

Another result that merits follow-up is the potential association between primary mucus barrier failure and a reduction in apical chloride-bicarbonate exchanger SLC26A3. Our observations provide a possible biological explanation for the previous identification of the *SLC26A3* gene as a UC risk locus. One way to further investigate this association would be to determine whether persistent mucus penetrability is linked to altered pH near the epithelium. A more acidic pH, due to reduced bicarbonate secretion, would be consistent with defective mucus barrier formation. If confirmed, this observation could represent a causative mechanism and putative therapeutic target for a subgroup of UC patients.

While the diagnosis of UC poses relatively few challenges, the natural course is heterogeneous, and difficult to prognosticate. This may result in overmedication, as well as severe relapses that could potentially have been prevented with more active therapeutic measures. Here, we identified the ratio of interacting proteins LRRFIP1 and FLII as a powerful predictor of frequent relapses and intractable inflammation. Still, the prognostic value of these biomarkers will require confirmation by a high-throughput analysis, using biopsy material or mucus aspirated at colonoscopy.

Interestingly, LRRFIP1 and FLII were previously reported to differentially regulate TLR4-MYD88 signalling, which is required for the sensing of certain microbial ligands. A balance shift in the relative abundances of these proteins, as observed in severe colitis, was associated with extensive, mucosal infiltration of bacterial endospores. Intuitively, intermittent activation of tissue endospores is compatible with the frequent relapses and poor response to immune-modulators observed in this subgroup of UC patients. Additional studies are required to validate these findings, delineate the mechanisms leading to invasion of spore-forming bacteria, and determine the species involved.

Finally, intestinal spirochetosis was shown to be significantly more common in IBS patients, and linked to specific symptoms and mucosal immune responses. This implies that *Brachyspira*-associated IBS represents a large and clinically distinctive subgroup that could potentially be responsive to antibiotic therapy. However, the treatment regime evaluated in this work did not result in eradication of the infection, instead promoting *Brachyspira* invasion into goblet cells.

While further treatment studies of IBS patients with concomitant intestinal spirochetosis may be clinically motivated at this point, many fundamental issues also need to be addressed. One such question concerns the epidemiology of *Brachyspira* colonization. Since the *B pilosocoli* species also infects several domestic animals, zoonotic transmission could be plausible in some cases. However, host susceptibility may also contribute to colonization, in ways not yet known. Other important future research directions include how *Brachyspira* penetrates the mucus and attaches to the epithelial cells, and the specific type of immune response elicited. Strikingly, the relocation of *Brachyspira* into goblet cell mucus granules appears to represent a novel bacterial strategy of persistence during antibiotic therapy. Future studies may identify other contexts where this mechanism contributes to recurrent or chronic infections, both inside and outside the gut.

In summary, the results from this thesis may inform the design of clinical trials, as well as broad mechanistic investigations. Hopefully, the continued pursuit of these research questions will benefit patients with gastrointestinal diseases, both in the short and long term

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