Non-digestible Polysaccharides and Intestinal Barrier Function
Dedicated to my parents Rajan & Cicilia Ganda Mall

"Do, or do not. There is no try"
- Yoda
Non-digestible Polysaccharides and Intestinal Barrier Function
- specific focus on its efficacy in elderly and patients with Crohn’s disease
Abstract


A large number of elderly suffer from gastrointestinal (GI) symptoms such as constipation and diarrhoea. The underlying mechanisms of age-acquired GI symptoms are not well studied but are necessary to clarify in order to recommend the right treatment. Non-digestible polysaccharides (NPS) are dietary fibres that could have beneficial effects on the intestinal immune system and barrier function, although their efficacy needs to be evaluated. Paper I showed that elderly with GI symptoms have significantly higher small intestinal permeability than a general elderly population, along with a stronger association to psychological distress. In Paper II we performed a randomised controlled trial with a general population of elderly that consumed either placebo, the NPS’s arabinoxylan or oat β-glucan for a period of 6 weeks. No protective effects were observed related to indomethacin-induced intestinal hyperpermeability, inflammatory markers, or self-reported health if compared to placebo. Paper III showed that stimulation with a yeast-derived β-glucan significantly attenuated Compound (C) 48/80-induced hyperpermeability in colonic biopsies from elderly with GI symptoms mounted in Ussing chambers, but not in young healthy adults. Arabinoxylan attenuated only C48/80-induced transcellular permeability in elderly but both paracellular and transcellular permeability in young healthy adults. Paper IV showed that the same yeast-derived β-glucan from paper III could cross the epithelium of ileal tissues from patients with Crohn’s disease (CD) and non-CD controls, mounted in Ussing chambers, and attenuate C48/80-induced hyperpermeability. In conclusion, we found that elderly with GI symptoms display a deteriorated barrier function and that administration of selective NPS can have beneficial effect on intestinal permeability in selective populations.

Keywords: non-digestible polysachharides, beta-glucan, arabinoxylan, barrier function, permeability, Ussing chamber, elderly, Crohn’s disease

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Nyckelord: kostfibrer, beta-glukan, arabinoxylan, tarmgenomsläpplighet, Ussingkammare, äldre, magproblem, Crohn’s sjukdom
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I. Are self-reported gastrointestinal symptoms among older adults associated with increased intestinal permeability and psychological distress?
   *BMC Geriatr.* 2018 Mar 20;18(1):75

II. Effects of dietary fibres on indomethacin-induced intestinal permeability in elderly: A randomised placebo controlled parallel clinical trial.
    *Manuscript*

III. Differential effects of dietary fibres on colonic barrier function in elderly individuals with gastrointestinal symptoms.
    John-Peter Ganda Mall, Liza Löfvendahl, Robert J Brummer, Åsa V Keita, Ida Schoultz
    *Manuscript*

IV. A β-glucan-based dietary fiber reduces mast cell-induced hyperpermeability in ileum from patients with Crohn’s disease and control subjects.
    John-Peter Ganda Mall, Maite Casado-Bedmar, Martin E Winberg, Robert J Brummer, Ida Schoultz, Åsa V Keita
    *Inflamm Bowel Dis.* 2017 Dec 19;24(1):166-78
ABBREVIATIONS

AAD  Antibiotic-associated diarrhoea
AD  Alzheimer's disease
AJ  Adherens junctions
ANS  Autonomic nervous system
ATP  Adenosine triphosphate
AX  Arabinopyranose
BDNF  Brain-derived neurotropic factor
C48/80  Compound 48/80
CD  Crohn's disease
CPZ  Chlorpromazine
CR3  Complement receptor 3
CRF  Case-report form
CRH  Corticotrophin releasing hormone
CRP  C-reactive protein
CVD  Cardiovascular disease
DC  Dendritic cell
ENS  Enteric nervous system
FAE  Follicle-associated epithelium
FFQ  Food frequency questionnaire
FGAS  Frändin-Grimby activity scale
FORT  Free oxygen radicals test
GI  Gastrointestinal
GOS  Galacto-oligosaccharides
GSRS  Gastrointestinal symptoms ratings scale
HADS  Hospital anxiety and depression scale
HPA  Hypothalamic-pituitary-adrenal
HPLC  High-performance liquid chromatography
I-FABP  Intestinal fatty acid binding protein
IBD  Inflammatory bowel disease
IFN  Interferon-gamma
IgA  Immunoglobulin A
IQR  Interquartile range
Isc  Short-circuit current
Kd  Kilodalton
LPS  Lipopolysaccharide
M cells  Membranous cells
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<tr>
<td>MβCD</td>
<td>Methyl-β-cyclodextrin</td>
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<td>MC</td>
<td>Mast cell</td>
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<tr>
<td>NGBI</td>
<td>Nutrition-gut-brain interactions research centre</td>
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<td>NPS</td>
<td>Non-digestible polysaccharides</td>
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<tr>
<td>NSAID</td>
<td>Non-steroid anti-inflammatory drugs</td>
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<tr>
<td>PCA</td>
<td>Principal component analysis</td>
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<tr>
<td>PD</td>
<td>Potential difference</td>
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<tr>
<td>PP</td>
<td>Peyer’s patches</td>
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<td>RCT</td>
<td>Randomised controlled trial</td>
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<td>ROS</td>
<td>Reactive oxygen species</td>
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<tr>
<td>SCFA</td>
<td>Short-chain fatty acids</td>
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<tr>
<td>TER</td>
<td>Transepithelial resistance</td>
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<tr>
<td>TJ</td>
<td>Tight junction</td>
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<tr>
<td>TNF</td>
<td>Tumour necrosis factor</td>
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<tr>
<td>VE</td>
<td>Villus epithelium</td>
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<tr>
<td>WP</td>
<td>Work package</td>
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<tr>
<td>ZO</td>
<td>Zonula occludens</td>
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FIBEBIOTICS

Parts of the work presented in this thesis belong to the on-going research within the FibeBiotics consortium. This EU framework 7 programme has the aim to support the development of functional food ingredients and products that are beneficial for the human gut & immune system and therefore of crucial importance for quality of life. The overarching aim of the consortium was to study the effects of specific non-digestible polysaccharides (NPS), also known as prebiotics. Both new and existing NPS were investigated for possible health effects with focus on enhancing the immune defence against pathogens and the reduction of infectious diseases, such as common cold and influenza among elderly, using a wide variety of methodological toolboxes in various scientific disciplines. The results intended to be confirmed by the use of biomarkers supported by the European Food and Safety Administration (EFSA) in helping to understand the underlying mechanisms and providing sufficient evidence for health claims on beneficial effects on the immune function.

Our task within the consortium was to perform in vivo and ex vivo mechanistic studies on the effect of NPS on intestinal barrier function and biomarkers in elderly. All ex vivo experiments were performed using human intestinal samples mounted in Ussing chambers for investigation of the direct effects of specific NPS on intestinal epithelium and permeability. We were also responsible for investigating the effect from oral consumption of different NPS on intestinal permeability and systemic health in a human clinical intervention trial with elderly participants.

Our results would thus provide important knowledge on NPS efficacy that would lay the bridge between the in vitro work performed by Wageningen University in the Netherlands, where specific NPS were investigated for their immunological effect, and the clinical trials performed in Kiel, Germany where the immune boosting effect of NPS against infectious risks were investigated.
Overview of the organisational network of interacting work packages (WP) within the FibeBiotics consortium. Örebro University belonged to WP4 and was responsible for in vivo and ex vivo mechanistic studies and biomarkers. The image is used from the original FibeBiotics study protocol.
INTRODUCTION

An ancient saying goes "With old age comes knowledge and wisdom". However, it is now also known that bowel related problems accompany old age. Along with an increasing elderly population as of today, a growing knowledge of the highly prevalent gastrointestinal (GI) symptoms in elderly is emerging.

The growing ageing population

The human population is growing at an unprecedented rate with a total of 7 billion people inhabiting the world to this date (1, 2). This is in stark contrast to the global population of 600 million people just 400 years ago (3). This 10-fold increase can partly be credited to medical and technological advancements from the industrialisation era, which in the modern time has resulted in an all time-high life expectancy. An epidemiological study in ageing by Ferrucci et al (2008) showed that the survival rate has increased significantly after the second world war (4), resulting in the elderly population living longer than previously. Cohen et al has described a shift in demographics from the 1950’s in which the dominating population consisted of people aged 40-50 and less people at 55-66 years of age (3). Since the year 2000 these demographics started shifting in the opposite direction and by estimations, given the same patterns follows, the elderly population will outnumber the middle-aged populations by the year 2050 (3). This trend is not only exclusive for the developed countries but similar patterns can be observed for developing countries as well. Demographic data from Latin America, Asia and Africa show that persons over 65 years of age will continuously grow over the coming years (4), suggesting a global increase in elderly.

GI symptoms in elderly

Living a long life might be what most of us wish for, but remaining in good health and achieving life-satisfaction are important aspects for living a meaningful life, according to interviewed older adults (5-7). Many elderly suffer from comorbidities, such as cardiovascular diseases, metabolic syndrome and diabetes (8-10), but it is also noteworthy that an increasing number of studies are highlighting GI symptoms as being highly abundant in the elderly population. This outcome demonstrates what a huge impact gut health can have on the elderly's quality of life, wellbeing and life-satisfaction (5-7). Reports show a varying degree of prevalence for elderly
having bowel-related problems (11-13) but some have shown as high as 50-70% (11, 14) of elderly having GI symptoms, with constipation and/or diarrhoea being common amongst the elderly.

The occurrence of constipation varies from 30-50% (15) but has been found in over 70% of elderly in geriatric hospital, 60% of elderly living in care houses and approximately in 40% of elderly living in their own homes (12). Elderly women are more frequently observed to suffer from constipation in the western world with reports suggesting 2-3 times higher prevalence than men (16-18). For instance, women were shown to more frequently report problems with constipation and use of laxatives in community-dwelling elderly in Australia (19). Researchers in China are reporting similar trends appearing with high rate of constipation, especially in women (20). Similar results were also found in Swedish women of varying age as constipation was reported to a higher degree in women compared to men (21). In addition, twice as many elderly women suffered from constipation compared to elderly men (21). Albeit not a life-threatening condition, constipation does clearly affect both mental health and the quality of life to the same extent as severe inflammatory conditions like inflammatory bowel diseases (IBD) (22). Leaving constipation untreated could result in faecal impaction, e.g. the formation of an unmovable solid bulk of stool, which would require immediate medical attention (23).

Diarrhoea is a different form of GI dysfunction being highly prevalent in the elderly population. Chronic diarrhoea has been seen to vary from 4-14% (18, 24, 25), this variation could be explained by different criteria’s used for the definition of diarrhoea between the studies (25). Antibiotic-associated diarrhoea (AAD) is a common side effect from antibiotic treatment against infection. Around 5-39% of elderly undergoing antibiotic treatment develops diarrhoea, most likely caused by changes in the intestinal microflora that could also subsequently increase the risk of GI infections (25-27). Due to the fact that elderly commonly suffer from multimorbidity, a wide repertoire of medicinal drugs are usually prescribed as treatment to various conditions (28). More than 700 drugs (not including antibiotics) have been reported to commonly induce diarrhoea, a trait accounting for 7% of all drug-adverse effects (25). A high prevalence of the elderly suffering from drug-induced diarrhoea (11%) take 3-5 medications that are typically associated to gut-regulation and/or mental health issues (25, 29). Similarly has constipation also been more frequently reported in elderly taking multiple medications (30). Despite the lower prevalence of diarrhoea in elderly compared to constipation its consequences
can be quite severe, for instance leading to other morbidities (31), involuntary defecation (faecal incontinence) (32), considerably decreased functional state and quality of life (33) and sometimes even leading to mortality due to dehydration (34).

Other GI symptoms affecting elderly involves abdominal pain (18), reflux diseases (35) and dyspepsia, among others (36, 37). These observations point to GI symptoms being highly prevalent in the growing population of elderly people. Estimations and observations based on the growing elderly populating and the commonly prevalent multimorbidities all point towards a significant economical burden on the health care systems worldwide. Some reports predicted a substantial amount of the collective health-care resources in need of being allocated for the elderly health care in the coming future (3, 15, 38). This proves that finding therapeutic and preventive options to alleviate GI symptoms in elderly will benefit several stakeholders, both on an individual and societal level.

**GI anatomy**

Understanding the physiology and anatomy of the GI tract is essential to understand the widespread GI problems in elderly. The GI tract can very simply be seen as a cylindrical motor highway, to which food components are being driven through and taking various exits at different locations along the gut. This complex highway includes the stomach, small intestine and large intestine and is typically around 7-8 meters. The stomach contains high levels of gastric acid that helps to digest food but also destroy harmful microorganisms, thus preventing them from causing infection. Connecting to the end of the stomach is the small intestine, subdivided into duodenum, jejunum and ileum. The small intestine is approximately 3-5 m long but has a surface area equivalent to half the size of a badminton court (39), a feature important in fulfilling the main task of digestion and absorption of nutrients from food efficiently.
The large intestine consists of caecum, colon, rectum and the anal canal. Colon can be subdivided into ascending, transverse, descending – and sigmoid colon, lastly ending with the rectum. It spans approximately 1-1.5 meters in length and the main functions include the ability to absorb water and electrolytes from the passing faecal matter but also nutrients produced by our resident bacteria. The colon harbours our microbial community, the microbiota, which has numerous health functional properties and is essential for the development of an optimal functioning immune system.

Due to the different functions of the small and large intestines there are also significant differences in microscopic structures. For instance, the lining of the small intestine has a wave-like pattern called plica circularis, which further is made up of several finger-like protrusions termed villi. On the luminal surface of these villi sits several microvilli that further enhance luminal uptake, hence maximising the absorptive capacity for nutrients in the small intestine. Although both parts of the intestine share most types of cells, the large intestine does not contain the villus structure found in the small intestine but instead has crypts enriched by tubular glands.

The whole intestinal wall is made up from several layers of different components and is sectioned into the mucosa (epithelium, lamina propria, muscularis mucosae), submucosa, muscularis propria and serosa. The mucosal section is closest to the gut lumen and entails a closely packed assembly of cells that make up the epithelial barrier, an important component of the intestinal barrier function with the aim to prevent unwanted passage of toxic substances and luminal antigens into the system (described in detail under the intestinal barrier function section). An overview of the anatomical structures of the whole intestine and the structural organisation of the small intestine can be viewed in Figure 1.
Figure 1. Anatomical overview of the gastrointestinal tract with further magnification of the small intestinal organisation. Illustration was generously provided by Dr Anders Carlsson, Ph.D.
GI function in elderly

When normal functions get out of order, various disturbances can occur and manifest as in the case of GI symptoms seen in elderly. Many of the factors that contribute to the pathophysiology of constipation affect gut motility i.e. the ability of the bowel to propagate content forward by synchronised wall movement (peristalsis) along the GI tract. The muscles in the muscularis propria contract in synchronised manners by interplay between the enteric nervous system (ENS) and longitudinal - and circular muscles along the gut axis. The risk of developing constipation is partially related to life-style factors such as physical inactivity, changes in dietary intake (especially decreased dietary fibre intake) and the usage of multiple medications (40, 41). Various age-related functional aberrations of the colon, such as decreased neurons in the ENS (42, 43), have also been proposed. However, the full explanation to why constipation develops is still unknown. Failure to absorb water in the colon leads to diarrhoea, which can be caused by either infections and/or medications. The use of pharmaceutical agents in elderly is high and known to affect GI function by altering the defences against infection, causing damage to the mucosa in both small – and large intestine, and by disturbing the balance in fluid - and electrolyte absorption/secretion (29). The use of antibiotics, in particular broad-spectrum antibiotics, triggers diarrhoea by inducing damage to the commensal microbiota in the gut and thus making way for pathogenic bacteria such as Clostridium difficile to proliferate and cause infection (44, 45).

Intestinal microbiota

This leads us to the importance of the intestinal microbiota, a term involving bacteria, fungi, and viruses (including bacteriophages). About 1.5 kg of our bodyweight consists of microorganisms living in our gut, with the majority of them inhabiting the colon. The bacterial cell count has been estimated to about 1 x 10^{14} per gram of colonic content, which put into perspective would roughly be 10 000 times more than the whole human population fit into 1 gram. Over 1000 different bacterial species make up for the diversity (46) that is mostly stable during the human life span but subject to change, especially in childhood and late age (47, 48). The first colonisation might not happen during birth, as initially thought when the mother’s vaginal and faecal flora comes in contact with the new-born baby (49), but could instead already occur in the placenta (50). The microbiota composition is then changing during childhood and settles at a
stable configuration in adulthood up to old age (47, 48). Older adults from the age 65 years and upwards start to show a change in microbiota composition characterised by decreased diversity and an increase in pathobionts (microbes that are potential drivers of inflammation), compared to younger adults (51, 52).

Health beneficial bacteria like the *Bifidobacterium* and *Lactobacillus* have been found in both reduced total numbers as well as in species diversity (48, 53, 54). Likewise seems the species of *Bacteroides* reduced in elderly. These beneficial bacteria have various important health-promoting actions of which a major characteristic is their ability to ferment dietary fibres (48). A reduced number of *Bacteroides* would lead to a decrease in the production of important metabolites, such as the short-chain fatty acid (SCFA) butyrate, and subsequently lead to a decreased uptake of butyrate by the colonocytes. Butyrate has many health beneficial properties such as playing an important role in the protection against infection by maintaining the epithelial barrier integrity (55). Many bacteria producing this SCFA have been found in decreased amounts in elderly (56, 57), which could be associated to a deteriorated health. This also highlights the mutualistic relationship shared between the host and the microbiota, with diet being one of the great modulators that can shift the composition (48) depending on food content such as dietary fibres and proteins. The intestinal microbiota also provides protection against infection from pathogens by producing antimicrobial peptides and competing for space and nutrients (58). Although many bacterial species of the gut microbiota have health beneficial properties, the intestinal barrier protects the body from having any of the microorganisms entering into the blood circulation. Would such an event occur they should be attacked by an advanced and complex immune system.

**Immunology**

A well-functioning immune system is essential for maintaining a healthy life. A majority of the immune cells in the human body reside in the gut, assembling a force of 10^{12} lymphocytes to form the mucosal defence (59). Not only is the immune system expected to combat antigens and microbes but also generate tolerance to non-harmful food products/substances. Failure to mount and regulate appropriate immune responses can lead to infections, immunopathology and inflammatory conditions.
These inflammatory conditions can originate from an immune system targeted against non-harmful exogenous compounds (allergens), endogenous products or the intestinal microbiota in cases such as allergy (60, 61), autoimmune diseases (62) and IBD (63), respectively.

Like all parts of the body, the immune system is also subject to ageing. Both the innate and adaptive immune system in elderly people is compromised, as summarised in the debated term immunosenescence (weakening of the immune system caused by ageing) (64, 65). This age-associated condition is for instance characterised by an abnormal T-cell ratio between reduced naïve T-cell populations and increased memory T cells, contributing to the increased susceptibility to infection and attenuated vaccination response that is common in elderly (66). The innate immunity is also affected by immunosenescence (67), a few examples would be through decreased chemotactic ability of neutrophils (68) and increased systemic activity of monocytes (69). Mild but significantly increased systemic levels of the pro-inflammatory cytokines TNF-α and IL-6, acute phase reactant C-reactive protein (CRP) and several clotting factors have also been reported in elderly (69-71). These changes may contribute towards a chronic state of low-grade inflammation that is associated with ageing, appropriately called inflammageing.

This low-grade inflammation, associated with life-style changes (diet, physical activity) (72), could play a major pathological role as its been identified in several diseases and conditions that are commonly found in elderly, such as type-2 diabetes, metabolic syndrome and cardiovascular diseases (CVD) (64, 73, 74). It is however difficult to assess whether the inflammation is the cause or the result of an unknown trauma or pathophysiological events. For instance, myocardial infarction has been seen to result in systemic inflammation in elderly (75) but a chronic low-grade inflammation is also deemed a risk factor for triggering myocardial infarctions and CVD (76).

The pathogenesis of low-grade inflammation is unknown but an increased level of microbial translocation has been seen associated with elevated levels of CRP in elderly (77). An elevated passage of microbial antigens from the gut lumen into the systemic circulation could be a driver of the chronic low-grade inflammation, implying that the intestinal barrier aimed to stop the passage of microbial antigens is dysfunctional.
The intestinal barrier function

The key feature of the intestinal barrier is not only to prevent harmful substances and bacteria from passing through into our blood system but simultaneously also allow absorption of nutrients and fluids (78). These dual properties confer a delicate balance as the situation in the gut is complex; the barrier has to let vital nutrients through the epithelium and at the same time keep bacteria, toxins and dangerous substances out of the system by gatekeeping the paracellular pathway (passage between the cells) and transcellular pathway (passage through the cell). This mucosal barrier is composed of 3 layers with the mucus layer being the first, second being the epithelial layer with its tight junction (TJ) complexes followed by the lamina propria as the third layer (Figure 2).

![Figure 2. The intestinal barrier is composed of three layers 1) The mucus layer consist of a looser outer layer and a thinner but more dense inner layer protecting the epithelium 2) Tight junction proteins (red and blue) form the paracellular barrier between the epithelial cells, protecting against passage of microbes and toxic substances (black arrows) that would otherwise cross the epithelium in case of tight junction disruption (blue arrows) 3) The lamina propria makes up for the final layer and consists of numerous immune cells ready to initiate an immune response upon antigen recognition.](image)
**Mucus layer**

The mucus layer consists of membrane-bound – and secreted mucin proteins. These latter proteins build two sticky layers at the apical side of the epithelium (79). The mucus serves as lubrication for the ingested food but also has an important role for the intestinal barrier function. It covers the epithelium like a shield and protects against physical and chemical damage but also against the large abundance of bacteria in the colon, making it especially important to that part of the intestine (80). This is evident as two layers build the mucus in the colon: one dense layer bordering to the epithelium and one loose layer closest to the lumen (81). This is in contrast to the small intestine, which has a much lower amount of bacteria and only a single porous layer of mucus to more efficiently allow absorption (81).

The secreted mucus layers in colon trap the microbes in its outer dense layer, consequently inhibiting them from reaching the epithelium and rendering the inner layer of mucus generally sterile (80, 82, 83). The outer loose layer is shaped from the continuous conversion of the bottom dense layer due to bacterial and enzymatic degradation in the lumen (82). The inner dense form of mucus has a gel-like consistency and is filled with enzymes, antimicrobial peptides and the immunoglobulin IgA that together act as a close proximity defence to the epithelium (84).

**Epithelium**

The epithelium of the intestine comprises various cell types, among these are the goblet cells which are responsible for production of mucus and antimicrobial peptides (79). These cells constitute up to 16-20% of the colonic epithelium and their proliferation and secretion can be induced by the gut microbiota (79, 85, 86). Goblet cells also have the ability to sample material from the lumen and present it to dendritic cells (DC) in the lamina propria, illustrating the interplay between the different layers of the intestinal barrier (87). In addition to compounds produced by the epithelial cells that aid in the mucosal defence, the ability to fine-tune the paracellular permeability through TJ proteins is one of the hallmarks of the intestinal barrier function. The epithelial cells are held together by an apical junctional complex consisting of TJs and adherens junctions (AJ). Though only TJs regulate paracellular permeability, AJ aid in intercellular communication and provide a strong coupling between the epithelial cells (88, 89).
The multiple protein complexes that are formed by the TJ proteins are divided into transmembrane and cytosolic proteins, which both react to numerous external stimuli and selectively control paracellular permeability to ions, solutes and water (90). The TJ consist of many different types of proteins that can be either barrier – or pore forming, which allows them to maintain this regulatory ability (90-92). There are four known transmembrane proteins; claudin, occludin, junctional adhesion molecules and tricellulin. The extracellular domains of these proteins couple to the reciprocal protein in the adjacent cells, forming either a barrier or a channel for solutes and ions. The intracellular domains are connected to the cytosolic scaffold proteins zonula occludens (ZO-1, ZO-2 and ZO-3) and anchor the transmembrane proteins to the actin cytoskeleton. This interaction between the TJ proteins and the cytoskeletal part of the cell is vital for maintenance of the TJ function, structure and regulation of permeability through cytoskeletal contraction (93-96).

The pathogenic bacterium *Vibrio cholerae* is responsible for inflicting cholera, a life-threatening condition caused by excessive diarrhoea and dehydration. Several toxins produced by *V.cholerae* are involved in the pathogenesis but one of the main culprits is the zonula occludens toxin (ZOT). This toxin is known to break down the intestinal barrier function through delocalisation of occludin and ZO proteins and cytoskeletal deformation (97). Zonulin, a human analogue to ZOT, was first described in year 2000 as a physiological modulator of tight junctions and reversible inducer of paracellular permeability (98). Although the molecular mechanisms remain to be fully understood it is currently thought that zonulin acts in a similar way to ZOT. It has been suggested that zonulin play a major role in developmental regulation of TJ proteins and movement of immune cells, fluids and macromolecules between the intestinal lumen and lamina propria (98).

**Transport routes**

The transfer of macromolecular luminal content, such as nutrients or bacteria, takes place through the cells by the transcellular pathway. The process of endocytosis handles the absorption of molecules that cannot spontaneously cross the hydrophobic cell membrane. Several different mechanisms exist by which the cells can internalise extracellular material (Figure 3). Macropinocytosis is a process by which a liquid drop with extracellular content is engulfed by the formation of a pocket and fusion of the liquid droplet with the cell membrane, forming a macropinosom (99).
A prolonged macropinocytic activity can be stimulated by antigen activa-
tion in colonic enterocytes, membranous (M) cells and DCs (100).

Phagocytosis is a similar process that internalises microbes, components of dead cells and large particles (101) but requires a receptor-mediated uptake (99). Antibodies bound to antigens are recognised by cell-surface receptors (99) and result in the formation of an engulfing extension that swallows the antigen, similar to the classical video game of Pac-Man™. This process ends in the formation of a phagosome, which fuses with lysosomes in the cytoplasm and undergoes destruction. The fragmented components are then presented on the cell surface and acts as trigger markers for the adaptive immune system. Enterocytes, macrophages, monocytes, DCs and neutrophils all possess this ability (102).

One of the most well-studied endocytosis mechanisms is the clathrin-
mediated uptake. This process is also dependent on a receptor-ligand for-
mation between the cargo molecule and receptor-coated pits formed by clathrins. This interaction results in the formation of an endocytic vesicle trapping the molecules of interest inside (103). The clathrin-mediated endocytosis is involved in nutrient uptake and in maintaining cellular homeostasis through internalisation of ion pumps and ion channels. Interestingly, the intestinal barrier dysfunction found in IBD could for instance be associated to an enhanced clathrin-mediated internalisation of TJ proteins (104).

A similar but clathrin-independent mechanism involves lipid-raft for-
mations that fuse the target molecule with an endocytic vesicle (103). These lipid rafts consist of microdomains composed of cholesterol and glyco-
sphingolipids. The process is not fully understood but is an important endocytosis mechanism as pathogens have been observed to use this pathway to gain entry into the body (105).
Lamina propria
The lamina propria is the final layer of the intestinal barrier and is located beneath the epithelium. It comprises loose connective tissue in addition to capillaries and lymphatic vessels that act as support for the overlying epithelium. The lamina propria also includes a vast amount of immune cells. This pool of immune cells belong to both the innate immune system (macrophages, DCs and mast cells) and the adaptive immune system (T-cells and IgA producing B-cells), all collectively contributing to maintain immune homeostasis (106, 107).

Mast cells
The innate immune system has to rapidly act to combat antigens in a non-specific manner. Mast cells (MC) are an important part of this innate immune system due to their versatility (108, 109), rich amount of granular content and tactical placement in areas of high infection risk such as in the skin (110), respiratory system (111) and GI tract (112). The MCs originate from hematopoietic progenitor cells in the bone marrow and continue their maturation in the peripheral tissue (113).
Although all MCs contain histamine (61), their granular content of proteases is used to subdivide the MCs into two different phenotypes: MC_T containing the MC-characteristic protein tryptase and MC_TC containing both tryptase and chymase. The MC_TC are mainly found in the skin while MC_T can be found in the GI tract and respiratory organs (114, 115). The MCs express a wide repertoire of different receptors on their surface, making them highly sensitive to changes in the environment. These receptors can initiate MC degranulation and/or inflammatory responses based on stimulation from different ligands, such as IgE and IgG antibodies (116), complement cascade factors (117), stem cell factors (118), microbial antigens (117, 119), neuropeptides (120-124) and antimicrobial peptides (125). Experimentally, degranulation can also be achieved by the administration of Compound 48/80, a mixture of low-weight polymers that binds to G-coupled receptors and activate MCs (126).

A number of mediators are released in seconds upon MC degranulation, some pre-formed while others are newly synthesised (127). The mediator histamine is generally well known due to its involvement in allergic reactions (128). The protease tryptase is exclusive to MCs and known to influence gut motility and increase paracellular permeability (129, 130). In addition, MCs also release a plethora of cytokines, including TNF-α and IL-6, which have known effect on increasing paracellular permeability by interaction with TJ proteins and cytoskeletal contraction (131-133). Increased transcellular permeability due to MC degranulation has also been observed (123) although the mechanisms behind this phenomenon are unknown. Studies in rodents showed that persistent psychological stress impair the epithelial defence to luminal bacteria via MCs, thereby potentially facilitating the early steps of intestinal inflammation (134, 135). Moreover, increased permeability after stress is absent in MC-deficient rats, and it has been previously shown that MCs and their interaction with nerves is of major importance for the barrier function of human ileum (124, 136).

MCs are known to express receptors for corticotrophin-releasing hormone (CRH) and degranulate upon its binding, resulting in a deteriorated barrier function upon hypothalamic-pituitary-adrenal (HPA)-axis mediated stress reaction (122). This demonstrates how MCs are directly involved in the gut-brain axis and recent studies also show how they might play a significant role in inflammatory conditions like IBD and diarrhoea/constipation in elderly (137-140). Studies on the dextran sodium sulphate and trinitrobenzene sulfonic acid-induced colitis mice models
showed that transgenic C57BL/6 (B6) mice lacking genes for forming β-
tryptase had less signs of colitis, suggesting an important role of MC dur-
ing intestinal inflammation (141). A higher number of activated MCs have
been found in the mucosa of CD patients (137) and similar findings have
been observed for individuals with constipation (138, 139) and diarrhoea
(140). One study showed some improvements in children with ulcerative
colitis when administered the MC stabiliser ketotifen, suggesting blocking
MC degranulation could be of therapeutic use (142).

**Aberrations of intestinal barrier function**

The link between inflammation and intestinal barrier function is well
known as both have been found to affect each other. A deteriorated intesti-
tinal barrier would, for instance, allow microbial antigens such as lipopol-
saccharide (LPS) to pass into the lamina propria and trigger an excessive
inflammatory immune response (143, 144). The pro-inflammatory re-
response has the aim to eradicate the translocated luminal antigens but also
causes collateral damage to the barrier as pro-inflammatory cytokines (e.g.
TNF-α, IFN-γ, IL-1β, IL-8, IL-6 and IL-17) break down or dislocate TJ
proteins (90, 131, 133). TNF-α is known to increase permeability by pro-
moting cytoskeletal contraction (132) and induces overexpression of the
pore-forming TJ protein claudin-2 (145). IL-6 shares a functional overlap
with TNF-α by promoting increased expression of claudin-2 (146). Many
of the pro-inflammatory cytokines attack the fundamental structures of
the TJ assembly and can have synergistic effects, leading to severely in-
creased paracellular permeability (90). Hence, barrier defects seem to be
tightly linked to the pathogenesis of various intestinal and systemic in-
flammatory diseases (90). Such interplay is very evident in IBD, even
though it’s unknown whether an increased permeability precedes the de-
velopment of inflammation or vice versa (147).

**Inflammatory Bowel Disease**

IBD is an umbrella term that includes Crohn’s disease (CD), ulcerative
colitis and microscopic colitis. CD is a chronic condition highly driven by
inflammation that is most commonly residing in the ileum, even though
any part from mouth to anus can be affected (143, 144). The ileum is
characterised by a high abundance of organised lymphoid follicles called
Peyer’s patches (PP) spread throughout the tissue. These PPs are highly
numerous in immune cells from both the innate and adaptive immune
system and are located just below the follicle-associated epithelium (FAE).
FAE is a highly specialised epithelium (found interspersed between villus epithelium in the ileum) and contain approximately 10% M cells with less- and irregular microvilli in comparison to regular villus epithelium (148). The M cells function is to sample luminal antigens and present them to the immune cells in the PPs in order to evoke an immune response (149). CD is characterised by an increased permeability (150), seen in both FAE and VE compared to non-IBD controls (151). Non-inflamed parts of the intestine have also been shown to display increased permeability (152) and similar observations have been made in the first-degree relatives of CD patients (153).

One of the hypotheses about CD pathogenesis is that there is a deteriorated barrier function (“leaky gut”) in CD patients that allows a constant flux of microbial antigens over the epithelium and chronically activates the immune system, leading to the severe inflammation (154). Many of the pro-inflammatory cytokines known to disrupt intestinal barrier function, e.g. TNF-α and IL-6, are increased in the intestine of IBD patients (155-157). Clinical data also points to lower – and delocalised expression of barrier-forming TJ proteins (occluding, claudin-5) with concurrent increase in the pore-forming claudin-2 (158). Biological agents (monoclonal antibodies) targeted against TNF-α have been successful as treatment for IBD by suppressing the inflammation and attenuating the increased permeability found in patients with CD (159, 160). Thus, treatments that strengthen the intestinal barrier and prevent its deterioration could be an important approach to combat inflammatory intestinal diseases.

Low-grade inflammation in elderly

Although several studies have found that many elderly exhibit a low-grade inflammation, reports on the intestinal barrier function in this population are few. One study investigated the small intestinal permeability of 215 healthy older adults, aged between 60 and 80 years, in comparison to healthy young adults, and found no significant differences (161). However, if stratifying for older adults with low-grade inflammation and type-2 diabetes, a significantly higher small intestinal permeability was detected, concluding that both low-grade inflammation and a minor disease challenge is required to inflict damage to the intestinal barrier (161). Another study showed that elderly with low-grade inflammation had an increase in IL-6, accompanied by a compromised small intestinal permeability in ileal biopsies, due to enhanced claudin-2 expression (133).
Intestinal-Fatty Acid Binding Protein (I-FABP), a systemic marker of intestinal permeability, has also been found elevated in elderly (>$75$ years old) along with a low-grade inflammation (69). A recently published study showed that healthy older adults (>70 years old) had significantly increased serum levels of zonulin compared to a young adult population (18-30 years old) (162). The zonulin levels were further found positively correlated to TNF-α and IL-6 and inversely correlated to muscle strength. The authors speculated that a deteriorated barrier function might play a critical role in the development of age-related inflammation and frailty (162). These findings seem to implicate that the intestinal permeability is compromised in conjunction with a low-grade inflammation.

**Gut-brain axis**

The intestinal barrier is also an important mediator in the bi-directional communication route between the gut microbiota and the brain, a pathway known as the gut-brain axis. The neuronal network in the intestine is popularly called the second brain due to the dense neuronal innervation of the intestinal wall. The bidirectional communication between the gut and the brain goes through the autonomic nervous system (ANS) of which ENS is a part of. Another important component that is part of the communication between the gut and the brain is the HPA axis, which is particularly involved in in the response to environmental stress. CRH is a product of HPA axis activation caused by stress and is well known for having a negative effect on the intestinal permeability (123). Higher levels of CRH along with inflammation have also been observed in patients with major depression (163, 164).

The low-grade inflammatory mediators involved in inflmamageing, such as TNF-α, IL-6 and CRP, are known to affect both mood and induce depression-like symptoms (165-167). Studies have shown that depression and anxiety, both linked with increased CRH levels (168-170), are among the most common mental health problems in elderly (171-173). However, the barrier function, mental state and level of inflammation in elderly with GI symptoms are less known.

Reviewing the literature, a majority of studies have generally focused on the small intestine for elucidating the state of the intestinal immune homeostasis and barrier function. One of the larger studies performed on older adults only investigated the small intestinal permeability (161). The colonic permeability has thus not been deeply investigated in this population.
Considering the major habitat of the gut microbiota is in the colon and that bacterial species have the possibility to modulate the intestinal permeability (174, 175), a deeper understanding on the state of colonic inflammation and intestinal barrier function might generate and improve therapeutic options for elderly with GI symptoms. This barrier function is one of the key components of the immune defence in the gut and is a complex web of networking machineries. An overview over the interacting factors both influencing and influenced by the intestinal barrier function can be found in Figure 4.

Figure 4. The intestinal barrier function both influences and is influenced by several interacting components, such as the microbiota, low-grade inflammation, the brain, environment and lifestyle factors (e.g. diet, exercise, medication, stress), all linked together (highlighted by the black ring).
Measurement of intestinal barrier function and permeability

The importance of the intestinal barrier function cannot be underestimated, as exemplified in the previous pages. Still, there has been some confusion in the literature regarding intestinal barrier function and permeability with regards to how to separate these two terms that both describe the same anatomical structure (176). Bischoff et al describe the intestinal barrier as “a functional entity separating the gut lumen from the inner host, and consisting of mechanical elements (mucus, epithelial layer), humoral elements (defensins, IgA), immunological elements (lymphocytes, innate immune cells), muscular and neurological elements” (176). The intestinal permeability on the other hand is a measurable feature of the barrier function that is intimately connected to the luminal microbiota and mucosal immune system (176). A number of techniques have been developed to study the flux of inert markers across the intestinal wall, as described below.

Ussing chamber

One of the earliest methods developed to study epithelial permeability to ions, solutes and molecules was the Ussing chamber. This methodological gold standard was established in the 1950’s by the Danish researcher Hans Ussing and his colleague K. Zerahn (177). In their attempt to study the active transport of sodium across the semi-permeable skin of frogs they also launched the novel field of epithelial ion transport physiology. The Ussing chamber system has since then been simplified (178) and widely spread to many other laboratories in the world. One of the main advantages of the Ussing system is the possibility to hold excised tissue, from both animal models and humans, in a viable state outside of the body for hours and perform ex vivo studies in real-time. The basic physiology of transepithelial transport has been elucidated using the Ussing chambers in tissues from the reproductive tract, respiratory system, exocrine/endocrine ducts, eyes and the GI tract (179). Studies on the intestinal mucosa has uncovered the complex physiological mechanics behind glucose absorption on a molecular level (179) and also helped to understand the pathophysiology in diseases such as cystic fibrosis (179-181). In addition, the Ussing chamber method seems to be a powerful tool to investigate the effect of compounds on tissues and complex structures like the intestinal barrier function (182).
The key design principle of the Ussing chamber involves separation of two half-chambers by a tissue segment or biopsy placed in between (Figure 5). The orientation of the specimen would allow the mucosal side to face one direction while the serosal side is in the opposite. Both half-chambers are filled with a physiological salt solution, commonly a CO2/HCO3- buffered Krebs solution, and kept at the human body temperature of 37°C by water circulation surrounding the chambers. The chambers are further connected to an oxygen/CO2 gas supply that stirs the bathing solution while simultaneously supplying the tissue environment with oxygen.

The chambers are innervated by a complex set of electrodes and salt bridges that together with electronic components perform electrophysiological measurements of the investigated tissue. These electrophysiological parameters measured consist of potential difference (PD), short-circuit current (Isc) and transepithelial resistance (TER). The PD is used as measurement of viability as it reflects the state of active transport, i.e. the ability of the epithelium to transfer ions or nutrients against an electrical/concentration based gradient. However, for PD to accurately reflect active transport the passive movement through the epithelium has to be nullified. Electrodes passing current through the epithelium are necessary to clamp the spontaneous voltage to zero and the strength of this current is presented as the Isc. Using Ohm’s law (voltage = current x resistance) the TER can be calculated from the two parameters PD and Isc. TER is used as a parameter that reflects the free movement of ions and solutes, which is interpreted as the paracellular integrity of the tissue. All these electrophysiological parameters are linked by Ohm’s law and reflect the viability and integrity of investigated tissue.

In order to more accurately study the permeability and integrity of tissues, permeation markers of different sizes are commonly used. These markers are added to the mucosal side and pass through the epithelium into the serosal chamber over time. FITC-dextran 4000 is a fluorescently labelled sugar molecule of 4 kilodalton (kd) and is a well-documented paracellular marker (176, 183, 184). Horseradish peroxidase is a 45kd protein antigen whose paracellular passage is limited by its size and therefore passes through the cells in a transcellular fashion by micropinocytosis. Both of these markers are well known and used in studies of permeability and barrier function.
One of the advantages of this technique may also turn to its major limitation. Due to the tissue specimen or biopsy being removed from its natural setting and placed in a controlled environment, it also fails to give a fully accurate representation of the system as natural blood supply and nerve innervations are left out. This procedure also only allows the tissue to be viable for a maximum of 3-4 hours, leaving only a small window of time for experiments.

Figure 5. One of the two vertical Ussing chamber systems set up during this thesis project. This figure demonstrates the electrical wiring (1), airgas connections (2) and the water circulation (3) needed for performing an experiment. Each Ussing system contains six independent chambers making up for a total of 12 chambers with two systems (one individual chamber highlighted in red). The biopsies are placed in the middle between the two chambers (white arrow) with the mucosal side facing the right side of the chambers and the serosal side facing the left side.

**Non-invasive multi-sugar permeability test**

A different method to study intestinal permeability that circumvents the limitations described above is the use of the *in vivo* multi-sugar permeability test. In this test the participants ingest sugar probes that are differentially absorbed in various parts of the intestine, while not being systemically metabolised, and subsequently found recovered in the urine. A number of studies have used the absorption ratio between sugar probes of different sizes as a measurement of intestinal barrier function (161, 185, 186).
The ratio is used to adjust for possible confounders that would otherwise influence the urinary recovery rate of the sugar probes (187, 188). These confounding factors can be subdivided into pre-mucosal (e.g. gastric dilution, gastric emptying, intestinal degradation, dilution by secretions), mucosal (motility, mucosal area and intestinal permeability) and post-mucosal (systemic distribution, metabolism, renal clearance and urinary collection) (187, 188). As these confounders equally affect both sugar probes through their passage from mouth to urine, the differential sugar recovery more accurately displays the paracellular permeability than the individual urinary sugar recovery (188). An important feature of these sugar probes is the fact that they are metabolically inert and thus not affected during the passage from intestinal uptake to urinary clearance (188).

A monosaccharide such as mannitol or L-rhamnose passes through the transcellular pathway freely while the disaccharide lactulose is less prone to pass through the paracellular pathway due to its large size (189). A greater paracellular flux of lactulose and an increased ratio between lactulose/L-rhamnose would suggest a deteriorated small intestinal barrier function. Due to the degradation of lactulose and rhamnose from colonic bacteria, other sugar probes such as erythritol and sucralose that remain intact through colonic transit have to be used for measurement of colonic permeability (190, 191). The multi-sugar permeability test can include 5 sugar probes (sucrose, lactulose, L-rhamnose, erythritol and sucralose) to cover the permeability of the whole GI tract (Figure 6) and is sometimes used in conjunction with the non-steroid anti-inflammatory drug (NSAID) indomethacin (192-196). This NSAID is the gold standard for inflicting barrier disruption, resulting in the well-documented effect of increased small intestinal permeability (185, 186). Indomethacin is known to inflict damage to the mucosal epithelium through detaching the oxidative phosphorylation between enterocytes, resulting in increased permeability through the loss of cellular energy from adenosine 5'-triphosphate (ATP) depletion (197). This effect is reversed within 48 hours of indomethacin absence and can also be attenuated with the administration of ATP in situ (194). Experiments performed in in vitro settings have also demonstrated that indomethacin decreases the expression of the TJ protein occludin, leading to a deteriorated paracellular integrity (198).
Zonulin as a marker of small intestinal permeability

Plasma levels of zonulin have been found to correlate to the lactulose/mannitol ratio and thus been used as a marker of small intestinal permeability in blood plasma (199). Both gluten (the protein causing the autoimmune reaction in celiac disease patients) and commensal bacteria are known to act as triggers for zonulin release from intact intestinal tissue (200, 201). This is reflected as elevated systemic zonulin levels in inflammatory conditions such as celiac disease, metabolic syndrome and type-1/2 diabetes (199, 202-204). However, zonulin’s credibility as a marker of permeability is under debate due to high fluctuations over short time spans (205).
Dietary influence on intestinal barrier function

A number of studies have shown food compounds, such as polyphenols and vitamins, to exert a positive effect on the intestinal barrier function (90). Curcumin, the main component of the spice turmeric that is commonly used in the delicious Indian cuisine, has been found to improve intestinal barrier function in cell model systems (206). Many other types of polyphenols (derived from plants, fruits and vegetables) have shown similar barrier enhancing results (90, 207, 208). Even though the results from these studies shows that food compounds can have a beneficial effect on intestinal permeability, most of these studies have used in vitro techniques to elucidate their effect which are difficult to translate for the in vivo situation. To study such a wide variety of food compounds was out of the scope for this thesis, as the main focus was to study the effect of prebiotics.

Prebiotics

Dietary fibres cannot be digested by the endogenous enzymes in the human small intestine but are instead fermented by the gut microbiota in the colon. Fermenting dietary fibres such as non-digestible polysaccharides (NPS) leads to the formation of end products in the form of butyrate, propionate and acetate that have different health beneficial outcomes (90), as described below. The fermentation of ingested NPS is an example of the mutualistic relationship we share with our microbiota, as our body reaps the benefits of the metabolites while the involved bacteria gain the energy to proliferate. Hence the term prebiotics was coined and defined as non-digestible selectively fermented dietary fibres that specifically promote the growth of one or more bacterial genera in the GI tract and thus provide health benefit to the host (209, 210). To proliferate the health-beneficial bacteria directly in situ might give some advantages over probiotics (live microorganisms that when administered in adequate amounts confer a health benefit on the host) as the latter have well-documented health-benefits but also demonstrated some viability issues and colonisation resistance in vivo (211, 212).
Many different classes of NPS are under investigation but some of the most common types are inulin-type fructans, galacto-oligosaccharides (GOS) (210), arabininoxylan (AX) and various beta (β)-glucans. Inulin-type fructans and GOS have both been shown to promote the growth of health-beneficial bacteria like *Bifidobacteria* and modulate the immune system (213, 214). AX is an NPS derived from wheat and commonly found in cereal grains (215). The structure of AX is composed of a linear xylose backbone with varying arabinose side-chains (215). Several studies have also found health beneficial effects from NPS supplementation on systemic levels (209, 216). Subjects with impaired glucose tolerance that underwent 6 week of AX supplementation could significantly reduce postprandial levels of serum glucose, insulin and triglycerides to more favourable levels compared to placebo in a cross-over trial (217). A more recent study saw similar beneficial effects along with decreased feeling of hunger, measured in subjects with metabolic syndrome (218). Such effects are not exclusive to AX but have also been elucidated for other NPS, such as β-glucans.

β-glucans are mainly found in yeasts or grain fibres like oat and barley, and consist of glucose molecules chained by β 1-3 or 1-4 linkages (219). Several randomised clinical trials have shown that adding >3g oat β-glucan to the diet can significantly lower low-density lipoprotein and total cholesterol levels while keeping the more protective high-density lipoprotein and triglyceride levels unaffected (220). Both human and animal studies have found AX and β-glucan supplementation to result in increased proportion of health-beneficial bacteria such as *Bifidobacteria*, in addition to elevated butyrate levels (221, 222). Butyrate was previously mentioned as a SCFA important for barrier integrity. This characteristic is possible due to butyrate acting as a source of energy for colonocytes, down regulating inflammatory pathways, attenuating oxidative stress and enhancing the expression of TJ proteins (55). Oat β-glucan intake in pigs reduced both glucose and insulin levels postprandially along with increased butyrate (223, 224). Studies on mice showed that oat β-glucan administration by intragastric gavage increased the populations of *Lactobacillus* and *Bifidobacterium* and decreased *Enterobacteriaceae*, in addition to increased concentrations of SCFA (225).
Low dietary fibre intake has been found associated with increased risk of IBD (226) while it is also known that elderly fail to consume appropriate amounts of dietary fibres, according to nutrition recommendations (227). Elderly have also previously been reported to show reduced numbers of health-beneficial bacteria in the gut, thus NPS could prove to be a useful therapeutic option to re-balance a disturbed microbiota (228). A clinical trial using daily GOS supplementation in 40 elderly participants showed a significant proliferation of the health-beneficial *Bidifobacteria* but also a re-balancing of the disturbed levels of IL-10, IL-8 and IL-1β commonly found in elderly (229). While there are some studies showing beneficial effects from prebiotic supplementation in children with constipation and/or diarrhoea (230), similar studies with prebiotic supplementation in elderly with GI symptoms are lacking, especially with a focus on intestinal barrier function as outcome. Some studies investigating intestinal barrier function *in vivo* after prebiotic supplementation, either alone or in combination with probiotics (symbiotic), failed to show any significant effect against neither drug-induced (indomethacin) hyperpermeability (231) nor elevated baseline permeability (232).
AIM

The overarching objective of this thesis was to elucidate the potential effect of non-digestible polysaccharides (NPS) on the intestinal barrier function, using both pre-clinical and clinical approaches in populations of suspected barrier dysfunction such as elderly with gastrointestinal (GI) symptoms and in patients with inflammatory bowel disease (IBD).

**Paper I:** Investigate how GI symptoms among older adults correlate to small intestinal permeability (measured by plasma zonulin) and psychological distress.

**Paper II:** Investigate whether 6 weeks of oral supplementation with either wheat arabinoxylan or oat β-glucan could protect intestinal barrier function from indomethacin-induced barrier disruption in a randomised controlled trial (RCT), using a general population of elderly. Inflammatory/oxidative status and self-reported health was investigated as secondary outcome parameters.

**Paper III:** Investigate whether stimulation with either a yeast-derived β-glucan or wheat-derived arabinoxylan was able to attenuate mast cell (MC)-induced hyperpermeability in colonic biopsies from elderly with GI symptoms and young healthy controls, mounted in Ussing chambers. The secondary aim was to investigate any potential differences in the baseline colonic permeability and response to an MC-degranulator between the two different study populations.

**Paper IV:** Assess whether a specific yeast-derived β-glucan was able to attenuate MC-induced intestinal hyperpermeability in patients with Crohn’s disease (CD) ileitis and in non-IBD controls, in addition to explore the mechanisms of β-glucan uptake *in vitro* using two different endocytosis inhibitors.
METHODS

Study populations

Elderly with GI symptoms (paper I and III)
All elderly with GI symptoms (≥65 years old) were recruited based on their questionnaire score from the Gastrointestinal Symptoms Rating Scale (GSRS). Advertisements were put out in the local newspapers most commonly read by elderly to maximise viewer count and respondent rate. In addition, advertisements and visits were also distributed to different senior housings in Örebro County. A web-based form was created for potentially interested participants to sign up for one of several announced information meetings. The information meetings were held at Örebro University and included presentation on study design, inclusion/exclusion criteria and aim of respective studies. All interested were thereafter given the possibility to formally partake in the studies. This recruitment took place between 2015-2017. An online version of the GSRS was thereafter used to characterise the GI symptoms of involved participants. This population was of particular interest for this thesis due to their idiopathic GI symptoms and unexplored intestinal barrier function. Using NPS for treatment of elderly with barrier dysfunction is a novel approach that could be of benefit to this population on both gut related- and overall health. The potential benefit also motivates their inclusion for the Ussing experiments while inclusion of elderly without GI symptoms could be viewed as unethical, as this technique would require the invasive and uncomfortable but necessary procedure of biopsy sampling from the colon. A total of 20 elderly with GI symptoms underwent sigmoidoscopy at the Örebro University hospital. Participants from this population were shared between paper I and III.

Older adults representing a general elderly population (paper I-II)
A 5-day advertisement campaign was rolled out in the biggest local newspaper (Nerikes Allehanda, Örebro) with over 100 000 daily readers. This generated a wide interest with over 90 elderly attending information meetings held at Örebro University, which finally lead to the recruitment of 60 elderly participants (≥ 65 years old) in October 2015 for the clinical trial
This population was selected as a sample representing a general population of elderly in Sweden. Elderly with comorbidities and medications that did not have a priori known effect on intestinal permeability were allowed to join the study. The exclusion criteria’s involved known GI diseases like IBD, GI surgery and use of medications such as NSAID’s, all known to affect intestinal permeability. All participants from this population were involved in studies performed at Örebro University. Data from participants of this population with a GSRS diarrhoea/constipation score below 3 was shared with paper I.

Senior orienteering athletes (paper I)
Orienteering is a popular endurance-running sport in Sweden among all age groups, including elderly. Its popularity can be explained by several factors ranging from the vivid light-hearted social atmosphere to the demanding skills in both physical – and cognitive activity. Senior orienteering athletes are an active group of seniors performing orienteering on a regular basis. They have previously been presented as a potential model of healthy ageing based on higher self-reported health status in comparison to free-living elderly (7). Recruitment was carried out through regular visits, e-mails and letters sent to various senior orienteering clubs in Örebro County during 2012-2013. The inclusion of this group springs from the hypothesis that physical - and cognitive activity in combination with high social life could be reflected in measureable levels of biomarkers and self-reported health parameters. This population, consisting of 27 senior orienteering athletes (≥ 65 years old), was compared against both general elderly and elderly with GI symptoms in paper I. All participants from this population were involved in studies performed at Örebro University.

Healthy subjects (paper III)
A self-reported healthy control population between 18-33 years of age was recruited through advertisements in Örebro University’s student portal. Willing students were interviewed over the telephone regarding their health and medical conditions with all demographic information noted in the clinical report form (CRF). All 24 participants were recruited between 2013-2016 and underwent sigmoidoscopy at the Örebro University hospital.
Patients with Crohn’s disease (paper IV)

Tissue specimens from 8 patients with CD, undergoing surgery for removal of the terminal ileum/neo-ileum, were apprehended during 2014-2017 at the Linköping University hospital. The disease activity phase was assessed according to the Montreal classification evaluated by a pathologist. All Ussing experiments were carried out at Linköping University. Parts of the samples collected and data generated from the experiments were sent to Örebro University for analysis.

Non-IBD controls (paper IV)

Tissue specimens from 9 colon cancer patients undergoing surgical removal of the colon and overlapping ileum were apprehended between 2014-2017 at the Linköping University hospital. Samples from each patient were sent to a clinical pathologist who confirmed the lack of tumour formation in the tissue. None of the cancer patients showed signs of generalised disease and none had received pre-operative chemotherapy or radiotherapy. All Ussing experiments were carried out at Linköping University. Parts of the samples collected and data generated from the experiments were sent to Örebro University for analysis.

Non-digestible polysaccharides (paper II-IV)

Three different NPS were investigated in this thesis, wheat-derived arabinoxylan, yeast-derived β-glucan and oat-derived β-glucan. The arabinoxylan used for the Ussing chamber experiments was supplied in a freeze-dried form from our FibeBiotics partner NOFIMA in Norway. The two different concentrations 0.1 mg/ml and 0.05 mg/ml of arabinoxylan were evaluated in 3 Ussing chambers experiments, using biopsies from 3 separate young healthy controls. The concentration of 0.1 mg/ml arabinoxylan showed most promise in attenuating stress-induced hyperpermeability and was selected as the optimal concentration. The yeast-derived β-glucan, both with and without Alexa594 fluorescence labelling, used for the Ussing chamber experiments was delivered suspended in solution or in powder form respectively from the original manufacturer Biothera (USA). Three different concentrations (50, 5 and 0.5 mg/ml) of yeast-derived β-glucan were initially tested and 0.5 mg/ml was selected as the most promising concentration to proceed with. The oral supplementations of arabinoxylan and oat-derived β-glucan in paper II were manufactured and delivered in identical pre-packed sachets by the FibeBiotics partners Bioactor
JOHN-GERARD MALI AND INTESTINAL BARRIER FUNCTION 39 (Netherlands) and Swedish Oat Fibre (Sweden), respectively, in a powder form.

Ex vivo measurement of permeability

Ussing chamber methodology (paper III-IV)

Colonic biopsies (paper III)
All colonic biopsies were taken in the sigmoid colon without prior to any bowel cleansing procedure. An experienced gastroenterologist (main supervisor) took all biopsies with the support from an experienced research nurse, using a biopsy forceps without a central lance. Responsible researcher for carrying out the Ussing chamber experiments attended all sigmoidoscopies and visually screened biopsies for size and shape suitable for experiment. This interdisciplinary approach allowed for the best possible quality on biopsies for the Ussing chamber experiments.

All biopsies were immediately transported to the lab in +4°C oxygenated transport Krebs solution. The biopsies were thereafter mounted in the Ussing chamber systems (Harvard apparatus Inc, Holliston, MA, USA) using inserts modified from x-ray films to expose 1.76 mm² of the biopsy. The chamber facing the serosal side of the biopsy was filled with 1.5 ml cold 10 nM glucose Krebs solution while the opposite chamber facing the mucosal side was filled with 1.5 ml cold 10 nM mannitol Krebs solution. The cold Krebs solutions were replaced 10 min later with the corresponding Krebs solutions but heated to 37°C. A heated water-circulation kept the temperature in the chambers at 37°C while a gas mixture of 95% O₂ and 5% CO₂ was continuously stirring the Krebs solute and providing oxygen to the biopsies. The biopsies were equilibrated for 40 min to reach a steady state in PD variation. The NPS were added to the mucosal sides halfway through the equilibration period, giving the biopsies 20 min of pre-treatment before adding the barrier disrupting stressor. Fresh Krebs solution was added to the chambers before adding the stressor Compound (C) 48/80 (10 ng/ml) to the serosal side, directly followed by the addition of the permeability marker solution containing FITC-dextran (2.5 nM) and HRP (10⁻⁵ M) to the mucosal side. Samples were taken from the serosal side after equilibration (zero sample) followed by sampling at 90 min after the marker solution was added. Replacement buffers were added after each sampling to compensate for the differences in volume.
Ileal specimens (paper IV)
Surgical specimens of terminal ileum were apprehended at Linköpings university hospital. The specimens were transported to the lab in 4°C oxygenated transport Krebs solution. At the lab the surgical specimens were stripped of their external muscle - and nerve layers. Follicle-associated epithelium (FAE) and villus epithelium (VE) were identified under a dissection microscope, carefully cut out and mounted in inserts exposing 4.9 mm² of FAE and VE respectively. The same procedure as for colonic biopsies was followed after mounting the ileal tissues.

Electrophysiology
As previously described (233), a four-electrode system was used to measure the electrophysiological parameters during the Ussing experiment. One pair of Ag/Ag electrodes (VWR, Pennsylvania, US) extended with 3M NaCl/2% agar bridges were used for measurement of voltage and one pair of platinum electrodes for current passage. Transepithelial resistance (TER), potential difference (PD) and short-circuit current (Isc) were measured through this set-up as previously described by Keita et al (233).

The electrodes were connected to an external 6-channel electronic unit with a voltage controlled current source. This system was connected to a computer via an A/D D/A board, which enabled the computer to control the data sampling through specifically designed software in Labview 2011 (National instruments, USA). Direct pulses of 0, 1.5, -1.5, 3 and -3 uA were sent through the mounted biopsies in the Ussing chambers every 30 second and with a duration of 235 ms. The subsequent voltage response was measured 8 times and the result was displayed as a mean of the voltage response recordings. This procedure reduced the possible influences of AC disturbances of 25-100 Hz. A linear least-squares fit was calculated from the current (I)-voltage (U) pair relationship: U = PD+TER x I. The TER was obtained from the slope of the line and the PD from the intersection of the voltage.

When reaching the end of the experiment, 10⁻⁵ cAMP-dependent Cl secretagogue, forskolin (Sigma), was added to both sides of the biopsies and ∆Isc was recorded as a measure of tissue viability. Biopsies not responding to forskolin through changes in Isc, or had a PD >+0.5 mV, were discarded from final analysis.
Methodological considerations

Although the Ussing chamber technique is a powerful tool to study mechanisms of passage over an epithelium, it does have limitations important to bear in mind. The first procedure of sampling the biopsies can be very important for the end result of the experiment. From our experience we noted the importance of responsible performer of Ussing experiments to join the sampling process and provide real-time input. Even though we tried to take as similar biopsies as possible, it is impossible get them exactly the same or from the same precise location. This leads to small but inevitable variation between biopsies that might affect the end result. After sampling is done only a few hours are available to use the viable biopsies for the Ussing experiments. This window of opportunity, around 4 hours, is optimised and prolonged by the initial use of cold +4°C Krebs solution, braking enzymatic processes from deteriorating the cells.

The process of mounting the biopsies/tissue specimens requires delicate handling and it takes time to learn how to work with it without causing damage. Studying an excised tissue in real-time from different populations gives an advantage over cell culture models due to a much more complex and intact systems being studied. However, it is important to remember that the biopsies/tissues have lost their natural habitat with the omission of natural blood supply and nerve innervations. Despite using live tissue/biopsies, only a small piece of a greater puzzle is being studied. Besides its limitations the Ussing chamber offers the possibility to perform *ex vivo* intervention studies in real-time from both animal and human tissue within a controlled environment. For this thesis the Ussing chamber was a necessary technique to study the effect of NPS on both paracellular and transcellular permeability directly in tissues/biopsies from different populations. As different interventions can be used per chamber, we effectively ran several studies in parallel and could investigate the effect of maximum two different NPS from the same study participants, reducing the number of sigmoidoscopy visits for each of them.

*Ex vivo* stressors of permeability (paper III-IV)

A number of agents with documented effect on inducing hyperpermeability in colon were tested before settling with C48/80. Corticotrophin-releasing hormone (CRH) has previously been shown to increase permeability through degranulating mast cells (MC) (123). CRH concentrations of 1 μM, 5 μM and 10 μM were tested in colonic biopsies from healthy subjects mounted in Ussing chambers but failed to show adequate increase.
in permeability. Similar experiments were performed with the MC degranulator carbachol (10 μM) and the NSAID indomethacin (10 μM and 100 μM) but both failed to show significant increases in permeability.

MCs are unique in the aspect that they show responsiveness to antibody-independent mediators such as cationic substances, otherwise also known as basic secretagogues (234). C48/80, one of the earliest basic secretagogues studied, was first described in the 1950’s (235) (around the same time as the Ussing system) and is a well-documented mediator of MC degranulation (120, 236-238). This synthetic compound is based on a mixed polymer of phenylethylamine cross-linked by formaldehyde (239). Its capability to activate MCs comes from its ability to cross the cell wall and bind to the G-protein coupled receptor MRGPRX2, leading to a cascade of events that results in degranulation (126, 234, 239).

We initially tested the two concentrations 10 ng/ml and 5 μg/ml (236) of C48/80 on colonic biopsies from two healthy subjects, mounted in Ussing chambers. These experiments were repeated 2 times in the same subject and each time showed that the concentration of 10 ng/ml gave a 2-3 fold higher paracellular and transcellular permeability compared to untreated biopsies. From there on we used 10 ng/ml C48/80 as the standard concentration for all Ussing experiments (paper III-IV). One of the biggest drawbacks that we found with this stressor was the generally high rate of non-response in colonic biopsies. About 60% of all participants responded with a minimum of 20% increase in permeability, while the remaining 40% did not show any increase compared to untreated biopsies. This varied from person to person but seemed consistent in the way that when we performed Ussing experiments from one C48/80 response-positive person at two different occasions the response towards C48/80 was the same. This might suggest that there is a phenotypic heterogeneity in MCs among individuals were some have MC phenotypes more sensitive to C48/80 than others. A study by Church et al from 1988 showed that MCs from human skin, lung, adenoids, tonsil and colon respond unequally to different basic secretagogues (240). Skin MCs were the only one shown to respond towards C48/80 with increased histamine levels while colonic tissue (n=5) did not show significantly increased histamine levels (240, 241). However, C48/80 was a good and reliable stressor when used in tissue specimens from ileum with 100% response rate (paper IV), suggesting that there might be differences in MCs regionally in the intestine.
Immunofluorescence (paper IV)

Immunofluorescence refers to a technique exploiting the capability of fluorescence-conjugated antibodies to bind to a specific target protein. A primary antibody is used to bind the target of interest, followed by a secondary antibody conjugated to a fluorochrome that targets the primary antibody. This dual coupling can be visualised as the fluorochrome emits light at different wavelengths. This emitted light can be studied in a confocal microscope. In comparison to a conventional microscope, confocal ones offer enhanced optical resolution and contrast by using point illumination and by effectively eliminating out-of-focus light by deploying a spatial pinhole. One main advantage of the confocal microscope is the use of optical sectioning, *i.e.* the process of capturing in-focus images at various depths and reconstructing them into 3D objects by the aid of a computer.

In brief, tissues aimed for microscopic investigation of immune cell interaction with fluorescence-labelled β-glucan were run for 20 min in the Ussing chambers instead of the usual 90 min, thus allowing better histological tissue preservation and avoiding β-glucan to pass too far in the tissue. All tissues were fixated in 4% formaldehyde for 24 hours (h) and afterwards transferred to a sucrose solution for 48h. Tissues were then embedded in OCT mounting medium and cryosectioned at 10 μm. Serial sections of tissues followed a series of washing and blocking steps before being stained with monoclonal antibodies against MC tryptase, dendritic cells (DCs) and macrophages overnight at +4°C. The following day a series of washing steps were performed before and after adding a fluorescence-conjugated secondary antibody that was incubated for 1h in room temperature. A DAPI-based mounting medium was applied as the last step in the protocol followed by fluorescence microscopy to evaluate stained MC tryptase, DCs, macrophages and fluorescence-labelled β-glucan.

In vitro study of transport mechanisms (paper IV)

A specialised co-culture model was set up to mimic FAE tissue in complement to the Ussing chamber experiments. This model has previously been described (242) to form FAE-like epithelia containing cells with a phenotype similar to M-cells. Their formation can be produced by co-culturing B-cells from Peyer’s patches (PP) together with intestinal epithelial cells. Previously published modifications (233, 243, 244) were used by growing a special clone (clone-1) of Caco-2 cells on Matrigel-coated polycarbonate filters (3 μm in pore size) for up to 14-17 days. This cell line was specifically chosen for its ability to convert into M-cell like cells.
When cells grew confluent the epithelia-covered filters were moved to Raji B cell containing wells, this co-culture was maintained for 4-6 days to reach a cell population containing 10% M-cell like cells. The clone-1 of Caco-2 cell grown without the influence of Raji B cells were used as a model for VE and compared against the FAE model. These two different models were used to study the uptake and passage of fluorescence-labelled β-glucan, both with and without the use of endocytosis inhibitors. The two different endocytosis inhibitors methyl-β-cyclodextrin (MβCD) and chlorpromazine (CPZ) were used to investigate whether different endocytosis pathways were responsible for β-glucan uptake. MβCD interferes with clathrin-independent endocytosis mechanisms by inhibiting lipid-raft formation while CPZ hampers clathrin-mediated vesicle formation.

The human mast cell line HMC-1.1 was grown and maintained for approximately 2-3 weeks with the purpose of evaluating any possible effect on MC degranulation from stimulation with yeast-derived β-glucan. HMC-1 cells were pre-stimulated with β-glucan for 20 min prior to addition of C48/80. Various optimisations on C48/80 concentrations were performed as the working concentration in the Ussing experiments failed to degranulate HMC-1 cells. An optimal concentration of 20 µg/ml C48/80 was found to degranulate HMC-1 cells, as measured by the granular release of β-Hexosaminidase (245). Confocal microscopy was used for evaluation and image capturing of the in vitro experiments described in this section.

Working with cell culture models provides the opportunity for very detailed mechanistic studies on cellular functions but not without its share of limitations. Even though it might reduce/replace the use of animal studies for certain aspects, the origins of cell culture models are usually from cancer cells and thus not likely accurately reflect the physiological cellular condition to 100%. The passage of cells also play an important role, as the cell culture models tend to gain and/or lose properties over time. Our purpose was to grow FAE/VE-type cell models and study the mechanistic details surrounding the transcytosis of yeast-derived β-glucan by blocking specific transportation machineries in the cells. The cell culture approach was chosen due to it being a time-effective method in comparison to apprehending ileal specimens for Ussing chamber studies which were rare, took years to collect and as the number of chambers limited us.
In vivo measurement of permeability

Multi-sugar permeability test (paper II)

The non-invasive multi-sugar permeability test was used in paper II for investigation of whole gut permeability. This method includes 5 sugar probes that are absorbed at 4 sites in the gut. Sucrose is a disaccharide that acts as marker for gastroduodenal permeability. Since it is digested by the enzyme sucrase in the duodenum it has to be measured within the first 5h of urine collection after sugar intake (246). The same time frame is used for the disaccharide lactulose (paracellular pathway marker) and the monosaccharide L-rhamnose (transcellular pathway marker) due to their breakdown from commensal bacteria in the colon. The ratio between lactulose and L-rhamnose in 0-5h urine is used as an indicator of small intestinal permeability and adjusts for the effect from confounding factors such as gut motility and kidney function, as previously described. The synthetic disaccharide sucralose (commonly used as a sweetener) and the sugar-alcohol erythritol avoid degradation from colonic bacteria, as such the ratio between these two sugar probes reflects the colonic permeability for urine collected within 5-24h from intake.

The sugar-mixture handed to participants consisted of 1 gram of sucrose, lactulose, sucralose, erythritol and 0.5 g of L-rhamnose. Participants were instructed to mix them with 150-200 ml of water before intake. All participants fasted overnight before ingesting the sugar solution in the morning. Urine collection was fractioned into 5h and 24h with the former being collected under fasting condition and the latter with diet restrictions against caffeine-based products, alcohol, spicy food, drinks or sweets containing the same sugars as in the multi-sugar mix. All urine was collected in special 5 litre jars and by the end of collection period the study participants transferred the urine to 4 ml tubes at home using the jars built-in vacuum mechanism. The samples were stored in -20°C freezers in their homes until delivery within a week to the University. To study the intestinal permeability from NSAID-induced challenge, the same procedure was carried out after the intake of two different dosages of indomethacin. One dose of 75 mg was taken in the evening; while the second dose of 50 mg was taken 9h later early in the morning, followed by drinking the sugar solution one hour later and repeating the urine collection. The same procedure was conducted after 6 weeks of NPS supplementation, thus covering any potential affect on both baseline permeability and NSAID-induced hyperpermeability.
In brief, all collected urine samples were aliquoted and centrifuged at 21,000 RCF for 25 min at +4°C. The supernatant was saved and further sent to our collaborators at Man-Technology-Environment research centre (MTM), Örebro University for analysis of sugar probes. A volume of 50 µL from the saved supernatant was transferred to LC vials to which 5 µL of 13C labelled lactulose and sucrose internal standard (Sigma Aldrich, MO, USA) was added. The urine aliquots were diluted to a volume of 1 mL with a composition of 80:20 acetonitrile:water. All samples were vortexed for 10 seconds and centrifuged for 15 minutes at 8000 RCF before analysis. Analysis was performed on an Acquity UPLC coupled to a Quattro Premier XE UPLC–MS/MS system (Waters Corporation, Milford, USA) with an atmospheric electrospray interface operating in negative ion mode. Analytes were separated on an Acquity BEH Amide column (1.7 µm, 2.1 x 100 mm) (Waters Corporation, Milford, USA). Column temperature was 50°C, injection volume 10 µL, flow rate 0.17 mL/min. An isocratic method was used with a mobile phase of 0.1 % NH₄OH in acetonitrile and water (70:30). Blanks and external standards were frequently injected during the analysis to control for carry over and monitor instrument stability. Mass-analysis was performed in the multiple reaction monitoring (MRM) mode by monitoring two product ions for each sugar analysed. Lactulose and sucrose were quantified using isotope dilution. Sucralose, erythriol, and rhamnose were quantified using 3-point standard addition curves, since no labelled standard were available for these analytes at the time of analysis. This method of standard additions allowed measurable levels to be used for calculation of concentration in the undiluted sample. An inhouse control sample was included in each batch.

Methodological considerations
This method allows for a non-invasive approach for simultaneous study of whole gut permeability over a larger population. It causes less discomfort for the study participants than a sigmoidoscopy, although fasting over a longer time can prove difficult for elderly. Compliance could be an issue since the study participants have the responsibility to follow the protocol on their own. Clear instructions had to be given along with frequent reminders. Since the sugar probes pass into the bloodstream from the gut and undergo renal processing before being excreted into the urine, kidney function (measured by plasma creatinin) has to be taken into consideration.
Analysis of sugar probes in the urine is a laborious task, due to the large number of samples generated, and requires experienced laboratory analytical skills and time. Lactulose in high doses (>5 gram) could possibly increase gut motility and shorten intestinal transit time (186), although new data show that even a dose as high as 10 g did not have any effect on the transit time (247) but could still reduce the sensitivity of the test due to luminal water retention (248). The method presented above is highly sensitive and can detect low concentrations of sugar probes, thus the use of 1 g of lactulose ensures that the effect on gut motility was not a confounding factor. A limitation with the use of indomethacin is that the induced hyperpermeability is in most cases restricted to small intestinal permeability (185, 186, 194-196, 249). The literature is conflicted regarding a similar effect on colonic permeability (249-251), as were our results in paper II.

Randomised controlled trial design (paper II)

A double-blinded randomised placebo-controlled trial was conducted in paper II. This type of design uses random allocation of study participants to one of several clinical interventions with the aim to study the outcome effect on selected parameters. In modern clinical research this is the most powerful way to perform a clinical study. In this thesis, a 9 week long RCT was performed using a general population of elderly assigned by block randomisation to daily oral NPS supplementation of arabinoxylan, oat β-glucan or placebo for 6 weeks. A third party not involved in the study blinded all NPS supplementation packages and monitoring was carried out through a CRF for each participant. The in vivo multi-sugar permeability test, using indomethacin as a stressor, was performed before and after the 6 weeks of oral supplementation in order to study the effect on intestinal barrier function. In addition, health questionnaire data, blood and faecal samples were collected before and after intervention for study on self-reported health, biomarkers of inflammation/oxidative stress and calprotectin/microbiota analysis from faeces. The primary endpoint was intestinal barrier function and since the barrier function is not fully known in a general population of elderly, a sample size of 20 study subjects per intervention arm was set based on previous studies using the in vivo permeability test (196). The recruitment successfully ended with 60 elderly joining the study, however 4 participants dropped out early in the study. The most common reason was that the 9 week engagement in the study would conflict with other scheduled activities.
Due to the relatively large sample size, Ussing chamber experiments on 60 participants would not be a time-efficient approach. The use of the multi-sugar test would allow the study participants to perform the study tasks mostly from the comfort of their home and reduce the number of University visits. The participants were all given unique study codes and material necessary to carry out urine sampling at home, including cups pre-filled with the multi-sugar mix, containers for collecting urine, tubes, indomethacin and NPS supplementation. Questionnaires were filled out at home on their computers and they only came in to the University to donate blood samples and leave collected material. Having 60 study participants coming in at the same time for these tasks would be problematic. As such all 60 persons came in divided in groups of 20 for three days in a row, thus minimizing the load on medical staff for blood sampling while minimizing the waiting time for each participant that were fasting from early morning.

In order to have elderly to participate in these type of studies, its important to minimise the burden as much as possible and increase compliance. The ability to perform most study tasks from home led to a high-throughput of participants fully completing the study, but also led to a reduced control for responsible researchers. All participants were given clear instructions on paper of how to carry out the protocol and also repeatedly reminded over email of all necessary steps. Despite these efforts it’s not possible to be completely sure if everyone followed the protocol as intended. However, a majority of elderly were very meticulous and had the intention to do everything right by the protocols. When accidently deviating from the protocol they noted all missteps and reported back by email or phone. These reported deviations were few and had no impact on the final results.

Systemic & intestinal biomarkers (paper I-III)

Systemic biomarkers
All participants involved in papers I-III underwent overnight fasting before having their blood drawn by experienced medical personnel at Örebro University hospital. Blood plasma was analysed for concentrations of C-reactive protein (CRP), blood glucose and creatinin levels at the clinical chemistry department of Örebro University hospital. CRP is an acute phase plasma protein originating from the liver and commonly used in
medical settings as a diagnostic tool (252, 253). High levels of CRP can be found in response to bacterial or viral infections, trauma or tissue injury and can be interpreted as a marker of inflammation (254). CRP is now frequently used as a sensitive marker of low-grade inflammation and inflammation (255). The analysis of CRP for this thesis was performed using the high-sensitivity immunoturbidimetric assay CardioPhaseTM and analysed on the ADVIA 1800 chemistry system (SIEMENS Healthcare Diagnostics Inc., NY, USA).

Hydrogen peroxide is a reactive oxygen species (ROS) that in blood plasma can be used as an estimation of oxidative stress levels. This oxidative stress is the result of a misbalance between circulating ROS and the body’s capacity to detoxify such harmful agents. It is hypothesised that human ageing is accelerated by an overload of oxidative stress (256), to which hydrogen peroxide might be a major contributor as it is the most abundant and potent ROS to induce senescence (257) in various cell types. Oxidative stress was estimated by the FORT (Free Oxygen Radicals Test) colorimetric assay (Callegari, Parma, Italy) and is described in further detail in paper I. Zonulin has previously been described as a marker of small intestinal permeability and was analysed in blood plasma using a sensitive zonulin ELISA kit described in paper I.

Intestinal biomarkers
All study participants received material for individual collection and storage of faecal samples at home. The faecal samples were stored in the participant’s own -20°C freezers and delivered within a week to Örebro University for long-time storage in -80°C freezers. Faecal samples were sent to the chemistry department at Örebro University hospital for analysis of calprotectin. This protein is known for binding to intracellular calcium and make up about 60% of the total cytosolic protein content in neutrophils. It has been used as a biomarker of intestinal inflammation due to its strong association with neutrophilic infiltration of the intestinal mucosa. Elevated concentrations of calprotectin in faeces have, for example, been described in numerous GI disorders such as irritable bowel syndrome and IBD (258, 259). Elevated faecal calprotectin levels have also been observed in the elderly (260). The calprotectin levels were analysed according to standard operating procedures at the Örebro University hospital, using the CALPRO® (CALPRO AS, Lysaker, Norway). The CALPRO® is an ELISA based assay for assessment of calprotectin levels in faecal samples.
Methodological considerations
Inflammation and oxidative stress are complex conditions with many networking elements and processes that cannot be grasped by studying individual components. Thus the analysis of CRP, hydrogen peroxide and calprotectin cannot reflect all disturbed biological processes. The immunological changes that accompany ageing leads to an increased release of pro-inflammatory mediators from T-cells, resulting in a rise of the inflammatory cytokines TNF-α, IL-6, IFN-γ, IL-2 and IL-4 (261). Even though CRP is used as a marker of systemic inflammation it fails to capture all the ingredients of an inflammatory state. However, CRP has been shown to correlate to IL-6, which is commonly found increased in elderly with low-grade inflammation (262), thus highlighting its relevance for elderly studies. Similar limitations are shared with the FORT method as it only measures the presence of hydrogen peroxide and thus only reflects a limited part of (anti-) oxidative networking. Calprotectin is used as a marker of intestinal inflammation but only gives an indication of neutrophil invasion in the mucosa, thus only providing a limited view of the local inflammation in the intestine.

Questionnaires of self-estimated health (paper I-III)

Gastrointestinal Symptoms Rating Scale (GSRS)
The GSRS evaluates GI symptoms based on the 5 domains diarrhoea, constipation, reflux, indigestion and abdominal pain. The symptoms are assessed with 15 items, ranging in scores 1 to 7 depending on their severity. A score of 1 represents “no problems” and score 7 represents “severe problems”. The severity of symptoms may be defined as no problems (1 point), mild (1-2 points), moderate (2-4 points), and severe (4-7 points). The scores for each domain was calculated as the mean score of each corresponding item while the mean total GSRS score reflects the general severity of GI symptoms. The validity and reliability of the GSRS is widely recognised (263). The GSRS was used in these studies in particular as a tool for both characterisation and selection of elderly with GI symptoms and for the evaluation of the GI symptoms of other study participants. All participants filled out the GSRS individually, either in paper form or an identical online-version at home through their web-browser.
The Hospital Anxiety and Depression Scale (HADS)
HADS was used to evaluate the psychological distress of study participants. This questionnaire consists of 14 items subdivided in two subscales for the assessment of anxiety or depression. The total score is used as a measure of general psychological distress. The measurement of psychological depression via HADS is generally accepted as a valid and reliable tool in both clinical settings and with elderly (264, 265). This questionnaire was used to both characterise the psychological wellbeing of different populations of elderly (paper I) and to study whether NPS supplementation could affect psychological wellbeing through the gut-brain axis (paper II). All participants filled out the HADS individually in either paper form or an identical version online at home through their web-browser.

Perceived Stress Scale (PSS) (paper II)
The perception of stress was evaluated in paper II using the PSS due to its proven validity among elderly (266, 267). The scale consists of 10 items, including a number of direct questions about current levels of experienced stress. The respondent answers how often a certain emotion has been present during the past month. All participants filled out the PSS individually via their web-browser at home.

EuroQol 5D-5L (EQ-5D-5L) (paper II)
EQ-5D-5L provides a standardised measurement of overall health status (268) with proven validation among elderly (269). The tool consists of two parts; 5Q-5D, which includes 5 items related to wellbeing and function (mobility, self-care, usual activities, pain/discomfort and anxiety/depression) and the visual analogue scale, 5Q-5D-VAS, ranging from 0 to 100. All participants filled out the EQ-5D-5L individually in paper form at home.

Frändin-Grimby Activity Scale (paper I)
The Frändin-Grimby Activity Scale (FGAS) was used to evaluate the participants level of physical activity in paper I. The scale states six different scenarios of physical activity and the participants circle the option that best matches their activity level. The scale estimates the habitual activity level over the past year, including a score for both the summer and winter seasons, and has previously been validated among elderly in Sweden (270). All participants filled out the FGAS individually, either in paper form or an identical online-version at home through their web-browser.
Food Frequency Questionnaire (paper II)

The Food Frequency Questionnaire (FFQ) consists of 66 categories of food and estimates the dietary pattern during a year. It has been validated in a Swedish population (271) and was used in paper II to evaluate the fibre consumption of all elderly study participants. All participants filled out the FFQ individually via their web-browser at home.

Statistical methods and considerations

Statistical analyses and graphs were generated in Graphpad Prism version 6 for Mac (Graphpad Software, San Diego California, USA) for paper I-IV, in addition to R v3.3.0 (Vienna, Austria) for paper I-II. The distribution of data was assessed using the Shapiro-Wilks test for normality and further visualised by plotting histograms. The Mann-whitney U-test was used for pair-wise comparisons between non-parametric data (paper I-IV).

In contrast to the published paper IV, the Wilcoxon matched-pairs signed rank test was used in paper III for statistical comparison between the different treatments in the Ussing chamber experiments. This paired statistical test controls for the variation between study participants, as the variation is subject-dependent and would otherwise hide a real (significant) effect within subjects in a non-paired test.

The non-parametric Kruskal-Wallis test was used for analysing the statistical differences between 3 study groups in paper I. Median values and interquartile range (IQR) were used to demonstrate these findings. In order to correct for multiplicity, the Bonferroni-Holm method was used in paper I. Fishers exact test was used for analysing differences in C48/80 response between two different populations in paper III. Data from cell culture studies underwent Log2-transformation and confirmed normally distributed (paper IV).

A two-way ANOVA was used to analyse the results of the cell culture studies. Log-data were modelled from two factors, experiment and treatment/cell type. By this way the method compensates for the influence of experiment and then tests for treatments/cell type effect. When the difference between experiments was not significantly different, the analyses of treatment/cell type effect were run as Students t test. All normally distributed data were presented as mean ± SD. Differences of p<0.05 were considered significant.
Ethical considerations

Ethical approvals for the studies included in **paper I-III** (Dnr: 2012/309, 2013/037 and 2015/357) were granted by the Regional Ethical Committee in Uppsala. The study presented in **paper IV** was approved by the Committee of Human Ethics in Linköping (Dnr 02-154). All research presented in this thesis was performed in accordance with the Declaration of Helsinki, with written informed consent being obtained from all participants before entering each study. Unique study codes were assigned to each study participant in order to protect their personal integrity and identity throughout data collection and processing. The clinical trial in **paper II** was registered on clinicaltrials.gov (NCT03336385).

Public outreach

The Swedish television channel “Kanal 4” hosted the show Vetenskap (translated Science) in March 2013 featuring part of the research presented in this thesis. This channel is part of the standard TV package and can be viewed by anyone with a TV or Internet in Sweden (also available on youtube). We also published an article in the popular science magazine Svensk geriatrik nr 1, 2016 (translated Swedish Geriatrics) regarding the research presented in this thesis. Several advertisements were placed in the local newspapers for recruitment of study participants, gaining wide interest among elderly. Information meetings about the studies presented in this thesis have been held for over 100 individuals from Örebro County. Social media such as Facebook and twitter was furthermore used to promote our research among a younger population.
RESULTS AND DISCUSSIONS

The following section presents a brief overview over the main findings of this thesis (summarised in figure 7) together with a general discussion on each individual paper. More detailed descriptions of the results can be found in papers I-IV.

Figure 7. A schematic overview of the four papers included in this thesis together with summarised aims and results from the individual studies. CD=Crohn's disease, GI=gastrointestinal, IBD=Inflammatory bowel disease, MC=mast cell, NPS=Non-digestible polysaccharides.
Are self-reported gastrointestinal symptoms among older adults associated with increased intestinal permeability and psychological distress? (paper I)

The aim of this paper was to investigate how self-reported gastrointestinal (GI) symptoms correlated to intestinal permeability and psychological distress in 3 different populations of elderly. We hypothesised that the idiopathic GI symptoms commonly found in older adults are linked to an increased intestinal permeability and psychological distress. The findings in this paper showed that small intestinal permeability, measured by plasma zonulin, was significantly higher (p<0.05) in the population of older adults suffering from moderate GI symptoms in comparison to older adults representing a general population with low-to-no GI symptoms (Figure P1-1 & Table P1-4). The small intestinal permeability in the population of senior orienteering athletes was not significantly different from older adults with GI symptoms or general older adults. Similar to these findings, the total score of the Hospital Anxiety and Depression Scale (HADS) was highest in older adults with GI symptoms, p<0.05 (Table P1-4). The subscales of HADS, representing depression and anxiety, were also scored significantly higher (p<0.05) by the older adults with GI symptoms compared to the other populations (Figure P1-2 & Table P1-4). The senior orienteering athletes, previously shown to report low levels of GI problems, reported the lowest scores on HADS out of all three populations.

Figure P1-1. Intestinal permeability as reflected by plasma zonulin levels in the different groups of older adults. The group of older adults with GI symptoms (n=22) had significantly higher plasma zonulin levels than the general older adults (n=21) but not senior orienteering athletes (n=27). No significant difference was observed between senior orienteering athletes and general older adults. **p<0.01, *p<0.05.
Systemic inflammation, measured by plasma levels of C-reactive protein (CRP), was found within normal ranges for all three study populations. Levels of systemic oxidative stress, measured as hydrogen peroxide in plasma, were found at intermediate levels in all three populations. Local intestinal inflammation was assessed by the measurement of faecal calprotectin and was found below detectable levels. Comorbidities and high usage of medical drugs is common in older adults. About 50% of all older adults with GI symptoms in our cohort took medicine for cardiovascular diseases (Table P1-2), with hypertension being the most common disorder. This group also had the highest consumption of antibiotics and gut motility regulating substances amongst the 3 populations. Taking 5 or more different drugs simultaneously (polypharmacy) was also more prevalent in older adults with GI symptoms. However, neither comorbidities nor medications had any confounding effect on zonulin levels and psychological distress. All data were further analysed in an unsupervised principal component analysis (PCA) in order to map the relationship between the investigated parameters and GI symptoms (Figure P1-3). We found that older adults with GI symptoms and senior orienteering athletes displayed the highest separation between the 3 populations with general older adults overlapping between both of them. The GSRS scores on the domains of diarrhoea/constipation could explain this separation. These GI symptoms also displayed an association to both zonulin and psychological distress while CRP, ROS and calprotectin only seemed associated to each other.

**Discussion**

Few studies have investigated the intestinal permeability in older adults and those have shown either an increased permeability or no change compared to younger adults (133, 161). Our study is the first to describe the relation between GI symptoms, intestinal permeability and psychological distress between 3 different populations of older adults. The results demonstrated for the first time that the level of plasma zonulin, a marker of small intestinal permeability, was significantly increased in older adults with GI symptoms compared to general older adults. The zonulin levels of senior orienteering athletes, previously proposed as a model of healthy aging (7), did not significantly differ from older adults with GI symptoms. This group also reported lower scores on both GSRS and HADS, suggesting their active life-style might promote mechanisms that counteract age-mediated GI symptoms, especially constipation due to physical activity.
This is particularly interesting, as runners in general have been shown to suffer from both increased permeability (272) and higher levels of GI symptoms (273). These data further strengthen senior orienteering athletes as a model of healthy ageing.

The PCA revealed an association between plasma zonulin, GI symptoms and psychological distress. Many intestinal disorders, such as inflammatory bowel disease (IBD) and irritable bowel syndrome, are characterised by elevated intestinal permeability (153, 274). The low-grade inflammation reported in older adults has also been linked to increased permeability (133). Although one large study found no difference in small intestinal permeability in healthy older adults compared to young healthy controls (161), it did find that the combination of low-grade inflammation and type-2 diabetes resulted in a significant increase in small intestinal permeability.

![Figure P1-3. Principal component analysis displaying the relationship between all investigated biomarkers, HADS domains and GSRS domains in the 3 study populations. Both zonulin and HADS appear associated with the GSRS domains unlike ROS, CRP and calprotectin. The ellipsoid markings cover 95% of the total populations.](image)
Our study did not show any increase in CRP or calprotectin levels that could indicate a low-grade inflammation, despite the increased permeability in older adults with GI symptoms. This could imply that intestinal inflammation is not affecting the increased permeability observed with moderate GI symptoms. However, our study had a smaller sample size and did not investigate other commonly used markers of low-grade inflammation, such as the cytokines IL-6, TNF-α and IL-1β. Surprisingly, the PCA plot showed CRP, ROS and calprotectin to be less associated with GI symptoms, zonulin and psychological distress. A possible low-grade inflammation accompanying moderate GI symptoms might be too subtle for detection in blood plasma, hence cytokines and ROS levels investigated locally in intestinal biopsies might be a more sensitive and suitable approach for such investigations.

The zonulin levels were associated with psychological distress as shown in the PCA plot. Older adults with GI symptoms showed significantly higher scores for depression-like characteristics than both senior orienteering athletes and general older adults. A significant difference in anxiety score was only found between the senior orienteers and the other two groups. However, older adults with GI symptoms and general older adults showed no statistical difference in anxiety scores between each other. This is particularly interesting since there are reports showing that generalised anxiety disorders are relatively highly prevalent in older adults. A study from France showed that 14.2% of the elderly population (65 years and above) suffered from anxiety disorders (275) and more studies report anxiety disorder to be prevalent among elderly, as reviewed by Wolitzky-Taylor KB et al (276). Interestingly, anxiety has been linked to depression as studies from both Germany and Netherlands have demonstrated that 29% and 13% of elderly with anxiety also met the criteria’s for depression disorders (277, 278). A study performed by Samuelsson G et al followed a Swedish cohort of elderly people (67 years old) for 34 years and reported that 11% of the females and 2% of the males developed anxiety disorders (279).

Our study showed that both depression and anxiety scores of the HADS scale were increased in the group of older adults with GI discomfort, especially in women as our GI discomfort cohort consisted of twice as many females as males. Nevertheless, it is important to acknowledge that the results for GI symptoms and HADS are based on self-reported data, which relied on the respondents’ honesty, accuracy, and interpretation of the question asked. Depression and anxiety has been linked with higher levels
of corticotrophin-releasing hormone (CRH), a stress hormone that is known to increase intestinal permeability (123, 168-173). CRH, nor cortisol awakening response, was measured in our study but should be of consideration in future studies evaluating the intestinal permeability in older adults. These data support the notion that treatments targeting intestinal permeability might also improve mental wellbeing and vice versa.

Some studies have shown positive associations between a preceding state of Alzheimer’s disease (AD) and depression/anxiety-like symptoms (280, 281). Noteworthy is that AD patients have shown increased levels of lipopolysaccharide (LPS), a component in the cell wall of Gram-negative bacteria, in blood plasma, which could result from an increased permeability in the gut (282). Likewise is Parkinson’s disease, the second most common neurodegenerative disease, prevalent in older adults (283) and has been linked to early onset of GI symptoms. Some studies have shown that many older adults experience constipation long before developing Parkinson’s (283, 284). Notably is that AD patients have an increased intestinal permeability linked with elevated LPS in blood, possibly related to elevated oxidative stress (285). In conclusion, our results demonstrate a possible link between increased intestinal permeability, moderate GI symptoms and psychological distress in elderly.

The influence of prebiotic supplementation on intestinal barrier function in elderly: A randomised placebo controlled clinical trial (paper II)

The study in this paper had the aim to investigate the effect of 6 weeks oral supplementation with either arabinoxylan or oat β-glucan on intestinal barrier function against indomethacin-induced hyperpermeability in a randomised controlled trial (RCT), using a general population of elderly. In addition, changes in secondary outcome parameters of inflammatory/oxidative status and self-reported health were also investigated.

Our primary outcome of intestinal permeability, measured by the multi-sugar permeability test, showed that indomethacin could significantly increase gastroduodenal permeability (p<0.05) and small intestinal permeability (p<0.05). Colonic permeability was found significantly increased (p<0.05) only for the group allocated to the arabinoxylan intervention group. None of the intervention groups showed any significant effect compared against placebo based on the primary outcome of this study.
The secondary outcome parameters of inflammation/oxidative status and self-reported health similarly showed no significant effect from the NPS intervention compared to placebo. However, elderly stratified for symptoms of diarrhoea (n=6, GSRS score >2) showed a significant decrease in diarrhoea score after 6 week of arabinoxylan intervention. However, this was only significant when compared within the group and not against placebo. The Food Frequency Questionnaire showed that dietary fibre intake in 85% of the elderly cohort was found low, reaching only 64% of the Nordic Nutrition Recommendations.

**Discussion**

We found that indomethacin significantly increased both gastroduodenal and small intestinal permeability in all intervention groups, in contrast to colonic permeability that was only increased in one of these groups. These findings are consistent with available literature (185) and demonstrate that indomethacin primarily induces hyperpermeability in the small intestine with inconclusive effect on colonic permeability, possibly due to a thinner mucus layer in the small intestine compared to colon (81) and a longer distance for indomethacin to reach the colon before being absorbed and metabolised. However, indomethacin was found to significantly increase colonic permeability when looking in the whole study group (n=49), suggesting that a larger sample size is needed to detected changes in colonic permeability from NSAID administration compared to small intestinal permeability. The various NPS interventions had no significant effect on intestinal permeability, compared to placebo. This could be related to the need of microbial fermentation for the NPS to induce a positive effect on the barrier function. The small intestine is known to harbour significantly less commensal bacteria than the colon and many prebiotic fibres are fermented mainly in the proximal colon while arabinoxylan is known to be continuously processed until reaching the distal part (286, 287). It is thus possible that any barrier-promoting effect might be restricted to the large intestine, which we were unable to measure adequately due to non-coherent effects from indomethacin in this part of the intestine. Similar lack of effects from NPS intervention were found in another study that investigated the effect from 6 weeks of oral arabinoxylan supplementation between lean and obese individuals with regards to their intestinal permeability, measured via the multi-sugar permeability test (232). The same study did however find that mRNA levels of tight junction (TJ) proteins were increased in colonic biopsies following arabinoxylan intervention,
suggesting that local effects might be more subtle and in need of more sensitive methods for detection. Unpublished results within the FibeBiotics consortium have also shown promising effects on immune enhancement using arabinxoylan. No significant changes were found for the secondary outcome parameters compared against placebo, although elderly with diarrhoea displayed an improvement in GSRS score after oral supplementation with arabinxoylan. It is, however, possible that this effect might not be due to any effect on the intestinal permeability but rather due to the characteristic of dietary fibres to absorb excessive water.

Since no significant changes were found from the clinical trial when comparing the NPS interventions against placebo, any significant differences found within the intervention arms do not provide conclusive results. Our study might have been limited by including only 20 participants in each group, thus being underpowered for detection of any subtle effect that the NPS might have. The use of indomethacin might have inflicted too much damage on the small intestinal barrier for the NPS to have any effect on, and possibly too little damage on the colonic barrier function where the NPS might have had an effect. We also collected faecal samples from all study participants, taken before and after NPS intervention for microbiota analysis, however these results did not reach completion in time for this thesis. Since dietary fibre intake was found to be low in this elderly cohort and with previous studies having found a decreased abundance of butyrate-producing bacteria, dietary interventions with NPS could prove health-beneficial by re-balancing the low number of butyrate-producing bacteria in elderly. Although our results demonstrated that neither arabinxoylan nor oat β-glucan were able to counteract indomethacin-induced intestinal permeability, the low dietary fibre intake among elderly emphasises the importance to further investigate the effect of such fibres on gut health, barrier function and microbiota composition in elderly for the development of appropriate dietary guidelines regarding supplementation of dietary fibres.

**Differential effects of dietary fibres on colonic barrier function in elderly individuals with gastrointestinal symptoms (paper III)**

The aim of this paper was to investigate whether stimulation with a yeast-derived β-glucan or wheat-derived arabinxoylan could attenuate MC-induced hyperpermeability in colonic biopsies from elderly with GI symptoms and young healthy controls, mounted in Ussing chambers. Our second aim was to investigate any potential differences in the baseline
colonic permeability and response to an MC-degranulator between the two study populations.

Our results demonstrated a significant increase (p<0.05) in paracellular and transcellular permeability compared to vehicle after exposure to 10 ng/ml C48/80 in colonic biopsies, taken from both elderly with GI symptoms and young healthy controls (Figure P3-1 and 2). Pre-stimulation with 0.5 mg/ml β-glucan prior to administration of C48/80 lead to a significant decrease (p<0.01) in paracellular (Figure P3-1) and transcellular permeability (Figure P3-2) in elderly with GI symptoms, but not in young healthy controls, when compared to biopsies stimulated with C48/80 only. Colonic biopsies stimulated solely with 0.5 mg/ml β-glucan showed a significant increase (p<0.05) in both paracellular – and transcellular permeability in elderly with GI symptoms, but not young healthy controls (Figure P3-1 and 2). Biopsies pre-stimulated with arabinoxylan before the administration of C48/80 showed a significant (p<0.05) attenuation in both paracellular – and transcellular permeability compared to C48/80 stimulated biopsies in young healthy adults (Figure P3-3 and 4), while elderly with GI symptoms only showed a significant attenuation of C48/80-induced transcellular hyperpermeability, p<0.05 (Figure P3-3 and 4). Stimulation with only 0.1 mg/ml arabinoxylan showed no statistically significant difference compared to vehicle in neither study population.

Figure P3-1. Effects of yeast-derived β-glucan on colonic paracellular permeability in biopsies mounted in Ussing chambers.

Figure P3-3. Effects of the wheat-derived arabinoxylan (AX) on colonic paracellular permeability in biopsies mounted in Ussing chambers.
Comparison of baseline levels of transepithelial resistance (TER), FITC-dextran and horseradish peroxidase (HRP) passage revealed a coherent and significant difference between the two study groups. Elderly with GI symptoms showed close to significantly lower (p=0.089) paracellular integrity than the young healthy controls, as demonstrated by lower TER values (Figure P3-5A). Further findings give weight to these results as a significantly higher (p<0.01) FITC-dextran passage was observed in the same group (Figure P3-5B), in addition to the transcellular passage of HRP being significantly higher (Figure P3-5C), p<0.05, when compared to the young healthy controls.

We also investigated whether the hyperpermeability induced by C48/80 differed in intensity between the two different populations. All data were normalised to vehicle and the fold change in hyperpermeability was compared between the two study groups (Figure P3-6). As stated in the manuscript, no significant difference could be observed between the two populations based on C48/80 induced para – and transcellular hyperpermeability. Data from elderly with GI symptoms was also stratified based on either constipation/diarrhoea or both but no significant differences could be detected. In addition, we investigated whether the numbers of participants responding with a ≥20% increase in permeability were different in the two groups. However, no significant difference could be observed in the number of responders between the healthy controls and elderly with GI symptoms, using Fishers exact test (Table P3-3).

Discussion
Our findings demonstrated a discrepancy in barrier protective outcome from pre-stimulation with a yeast-derived β-glucan between elderly with GI symptoms and young healthy controls. These findings could possibly be explained by differences in processing and uptake of the β-glucan by immune cells in the lamina propria, although this possibility was not specifically investigated in this study.

Macrophages have previously been shown to take up β-glucan particles and process them into enhanced fragments with higher bioactive capabilities (288). Differences in the number of intestinal macrophages between elderly with GI symptoms and young healthy adults could possibly explain the differences in response towards MC-induced hyperpermeability. A significantly higher number of macrophages has been found, among others, in patients with slow transit constipation (289). The results from paper IV showed that macrophages and dendritic cells (DC) in Crohn’s dis...
ease (CD) patients internalise β-glucan in a significantly higher amount compared to non-IBD controls. It is possible that this process is facilitated by the highly active inflammatory state present in CD patients.

A low-grade inflammation could potentially display similar features even though there is a lack of knowledge in this area. Many of the cytokines reported to be elevated in low-grade inflammation can be produced by macrophages and DCs (290). One study found a low-grade inflammation (increased levels of IL-6 and CRP) in older adults, in addition to increased levels of Intestinal-Fatty Acid Binding Protein (I-FABP) and the monocyte/macrophage activation marker sCD14 (69). This may suggest that monocytes and macrophages are more activated due to higher microbial translocation in the gut, as the increased I-FABP levels would reflect an increased permeability. Thus, elderly with GI symptoms and/or low-grade inflammation could possess an intestinal environment that promotes enhanced antigen uptake by antigen presenting cells such as macrophages and DCs. Hence, a higher interaction between β-glucan and these immune cells could lead to higher output of fragmented β-glucan particles with more potent effects on barrier function in elderly with GI symptoms.

Elderly with GI symptoms showed only a significantly reduced transcellular hyperpermeability when pre-stimulating biopsies with arabinoxylan before administration of C48/80, against biopsies stimulated with C48/80 alone. However, biopsies from young healthy adults that were pre-stimulated with arabinoxylan significantly attenuated both C48/80 induced paracellular and transcellular hyperpermeability. The biopsies mounted in the Ussing chambers were sampled from an uncleansed bowel, possibly leaving a larger proportion of mucosal-associated microbiota intact as compared to biopsies taken from a cleansed bowel. Previous studies have shown that bowel cleansing has a significant effect on the detectable bacterial flora of the biopsies (291, 292). It is known that there is an inherent difference in microbial composition between populations of different ages (51, 52), which could possibly also result in the different outcomes seen from the Ussing experiments. A lack or decrease of certain mucosal bacteria in elderly could result in fewer metabolites that transfer the epithelium and possibly affect MC degranulation. Our findings in paper IV visualised that yeast-derived β-glucan particles could pass the ileal epithelium and co-localise to MCs, highlighting the possibility of NPS to interact with immune cells of the lamina propria. There is also the possibility that arabinoxylan affects TJ expression as seen in a previous study (232), but this was not specifically investigated here.
The effect on transcellular permeability from arabinoxylan, that we observed, has previously been demonstrated in piglets fed an arabinoxylan-containing diet (293) and would suggest TJ-independent mechanisms also being involved. This could have clinical relevance as some enteric infections in elderly are caused by enteric pathogens known to invade through the transcellular pathway, such as *Salmonella* (294-297).

Our previous results (paper I) showed elderly with GI symptoms having an increased small intestinal permeability, as measured by plasma zonulin. Many of the previously mentioned studies, including the one performed in paper I, has shown increased permeability through the use of surrogate markers in blood plasma that reflect small intestinal permeability. However, our results here demonstrate for the first time that an increased permeability is present in colonic biopsies from elderly with GI symptoms compared to young healthy adults, showing both para – and transcellular permeability being significantly higher in the elderly group.

Our findings from paper I also showed that elderly with GI symptoms reported anxiety and depression-like characteristics to a higher degree. This could potentially lead to a higher release of CRH, a stress hormone known to increase intestinal permeability through MC degranulation (123). The higher numbers of MCs in both constipation and diarrhoea (138-140) could potentially lead to a greater damage on the barrier integrity and hence constitutively higher baseline permeability. We could not find a difference in number of participants responding to C48/80 between the two populations, neither did we observe a difference in the effect from the same concentration of C48/80 stimulation on permeability between the study populations. As discussed in the manuscript, this would suggest that even though a different distribution of MCs might be present in elderly with GI symptoms, their level of reactivity seems similar to young healthy adults based on stimulation with C48/80.

All results were based on comparison against a young and healthy adult population, which could be confounded by the age differences between the populations and not the GI symptoms. However, unpublished data from paper II showed that colonic permeability between a general population of elderly without GI symptoms versus elderly with GI symptoms was not significantly different, as measured by the *in vivo* sucralose/erythritol sugar test. Taking colonic biopsies from elderly does generally impose a higher risk of causing discomfort. Based on ethical considerations, we did not recruit a population of elderly without GI symptoms for this study as to
avoid unnecessary discomfort, but also since elderly with GI symptoms have the higher chance to benefit from the results of our study.

The increased permeability found after stimulation with 0.5 mg/ml β-glucan alone was intriguing, yet not surprising as the yeast-derived β-glucan is a cell wall component of the fungi *Saccharomyces cerevisiae*. It is therefore possible that the 0.5 mg/ml concentration of β-glucan could trigger an immune response upon stimulation subsequently breaking down the barrier integrity, thus finding an optimal concentration would be necessary to strike a balance between wanted/unwanted effects. Our primary aim was to evaluate the effect of a yeast-derived β-glucan on MC-induced hyperpermeability, thus concentration optimisations were aimed to evaluate a potential attenuating effect against C48/80 stimuli. Due to limitations in Ussing chamber numbers and available participants, a similar optimisation was not performed for stimulation with β-glucan only.

In conclusion, the present study demonstrated that the same dietary fibre exerted different effects in elderly with GI symptoms versus young healthy individuals. We provided evidence of a barrier protective effect from both a yeast-derived β-glucan and arabinoxylan against MC-induced hyperpermeability in colonic biopsies from elderly with GI symptoms, while the young healthy control group showed a similar response to arabinoxylan only. In addition, elderly with GI symptoms displayed a higher basal permeability level compared young healthy adults, thus broadening the knowledge on barrier function in elderly.
A β-glucan-based dietary fiber reduces mast cell-induced hyperpermeability in ileum from patients with Crohn’s disease and control subjects (paper IV)

The final paper of this thesis aimed to study the effects of a yeast-derived β-glucan on MC-induced hyperpermeability in patients with CD and non-IBD controls, respectively. Furthermore, we aimed to 1) study the transport mechanisms of β-glucan using in vitro models of follicle-associated epithelium (FAE) and villus epithelium (VE), 2) study the effects of β-glucan in vitro on cultured human MC degranulation/cytokine secretion.

The results of the Ussing experiments demonstrated that pre-stimulation with 0.5 mg/ml β-glucan before the administration of 10 ng/ml C48/80 resulted in a significant attenuation of the 2-3-fold increase in paracellular permeability seen with C48/80 stimulation alone. This pattern was seen in FAE and VE from both patients with CD and non-IBD controls (Figure P4-1). Transepithelial permeability showed a similar pattern for VE in both CD patients and controls, but pre-stimulation with β-glucan had no significant attenuating effect on C48/80-induced transepithelial hyperpermeability in FAE (Figure P4-2B). Baseline paracellular permeability in FAE was higher compared to VE in both CD patients and controls (p<0.05), along with a more pronounced effect of C48/80 and β-glucan (p<0.05).

Supplementary Figure P4-1. Individual effects of compound 48/80 (C48/80) and yeast-derived β-glucan on paracellular permeability in villus epithelium (VE) and follicle-associated epithelium (FAE) in 7 patients with Crohn’s disease (CD) (A-B) and 8 control subjects mounted in Ussing chambers. (C-D).
Immunofluorescence experiments with fluorescence-labelled β-glucan showed uptake in both FAE and VE after 20 min stimulation in the Ussing chambers (Figure P4 3A-3C). The β-glucan uptake was significantly higher (p<0.05) in VE and FAE of CD patients compared to non-IBD controls (Figure P4-3D). Staining for MC tryptase, macrophages and DCs revealed β-glucan co-localised/close proximity to MCs, macrophages (Figure P4-4) and DCs (Figure P4-5). A significantly higher number (p<0.05) of all 3 cell populations were found in VE of CD patients compared to controls while only macrophages were found in higher numbers in FAE (Table P4-1). MCs were found in significantly higher numbers in VE of both CD patients and controls compared to FAE, while macrophages and DCs did not differ between the two epithelial types. A higher percentage of β-glucan co-localised to DCs and macrophages was found in VE of CD patients compared to controls while no difference could be found in FAE (Table P4-1). However, a significantly higher percentage of macrophages were found co-localised with β-glucan in FAE of both controls and CD patients compared to VE. The percentage of co-localised β-glucan to MCs was not significantly different between either tissue epithelial type and study populations (Table P4-1).

The in vitro experiments with the FAE co-culture model and Caco-2-cl1 showed a significantly higher passage (p<0.05) of β-glucan through the FAE model compared to Caco-2-cl1 after 1h incubation (Figure P4-6A). No statistical significant difference in β-glucan internalisation was found between the FAE model versus Caco-2-cl1 after 1h incubation (Figure P4-6B-C). The lipid raft endocytosis inhibitor methyl-β-cyclodextrin (MβCD) significantly attenuated (p<0.05) β-glucan passage through the FAE model while a trend towards significance (p=0.079) was observed in Caco-2-cl1, after 20 min of incubation (Figure P4-6D). However, 1h incubation with MβCD failed to show a statistical significant attenuation of β-glucan passage even though a trend towards significance (p=0.1) was observed in both FAE model and Caco-2-cl1 (Figure P4-6E). The clathrin-dependent endocytosis inhibitor chlorpromazine failed to show any significant effect on β-glucan passage through the FAE model and Caco-2-cl1.

The effect of β-glucan on MC degranulation and cytokine secretion was investigated in vitro in the human MC cell line HMC-1.1. A significant increase in HMC-1.1 degranulation (measured by the release of β-hexosaminidase) was observed after 24h stimulation with C48/80, compared against un-stimulated cells (vehicle), p<0.001 (Figure P4-7A). A trend towards a significant reduction (p=0.15) of β-hexosaminidase levels
was observed after 20 min pre-stimulation with 0.5 mg/ml β-glucan prior to administration of C48/80, when compared to stimulation with C48/80 only (Figure P4-7A). Figure P4-7B illustrates HMC-1.1 and fluorescence labelled β-glucan. Regarding the pro-inflammatory cytokine TNF-α we observed no significant increase after stimulation with C48/80 for 24h. However, a significant (p<0.05) decrease in the levels of TNF-α was found from pre-stimulation with β-glucan prior to administration of C48/80 when compared to C48/80 stimulation only (Figure P4-7C). In contrast, IL-6 was significantly increased after stimulation with C48/80 compared to vehicle (p<0.001), although pre-stimulation with β-glucan prior to C48/80 administration showed no attenuating effect (Figure P4-7D).

Figure P4-4. Fluorescence microscopy of Alexa Fluor 594-conjugated yeast-derived β-glucan particles translocating through the villus epithelium (VE) and follicle-associated epithelium (FAE) from patients with Crohn’s disease (CD) and control subjects. Tissues were stained for mast cell (MC) tryptase or macrophages (CD68)
(A) Staining of MCs (green) in Peyer’s patches from a CD patient showing β-glucan particles (red, arrows) close to the FAE (1), within the adjacent villi to the FAE (2) and co-localizing with MCs (3). (B) Staining of MCs (green) in VE of a CD patient showing β-glucan particles (arrows) close to MCs (1-2). (C) Staining of CD68 in VE from a control patient showing β-glucan particles (red, arrows) close to macrophages (green) (1-2), or in a direct co-localization with macrophages (2, arrowhead). Overview photographs are in 400X magnification and zoomed pictures in 1000X.
Discussion
This paper demonstrated for the first time in an *ex vivo* setting that β-glucan stimulation had protective effects on MC-induced intestinal barrier disruption in ileal tissue from both CD patients and non-IBD controls. Both paracellular and transcellular permeability were affected, however transcellular permeability in FAE did not show a significant attenuation after β-glucan pre-stimulation even though there was a pattern that resembled the effects in VE. As stated in the paper, when looking at the C48/80-effects individually (shown in Supplementary data), it was revealed that approximately 60% of the CD patients and 67% of control subjects displayed a pattern similar in FAE to what was observed for paracellular permeability. Hence a larger study sample could have been needed to reach statistical significance or, as stated in the section ‘Statistical methods and considerations’ of this thesis, using the Wilcoxon matched-pairs signed rank test would have controlled for the variation between the participants and rendered the results significant.

Since these experiments were performed *ex vivo*, a number of limitations are worth acknowledging. Oral supplementation of the β-glucan could undergo several processing steps during small bowel transit, which was not taken into account in this study. Speculatively, the breakdown by commensal bacteria could potentially alter the capacity of the β-glucan to exert the effects found in this study. Noteworthy is though that we used a soluble form of the β-glucan that is already fractioned into particles of different sizes and also, the processing of β-glucan by mucosal-adherent microbiota might influence the effects from C48/80 on intestinal permeability. It is known that microbial fermentation of dietary fibres takes place along the colon while the small intestine has a significantly lower amount of bacteria (81), this would indicate that the results presented in this paper are due to the direct effects of β-glucan on the epithelium. As both paracellular and transcellular permeability were affected in CD patients and non-IBD controls, the mechanisms by which β-glucan exerts its effects in an inflamed condition versus non-inflamed are most likely the same. Although we did not investigate whether β-glucan stimulation had any effect on inflammatory markers in the Ussing chambers, one study showed that the yeast strain *Saccharomyces cerevisiae* (from which the β-glucan originates) and its derivatives prevented colitis induced by adherent invasive *E. coli* in a mouse model mimicking CD (298).

Our results demonstrated a more pronounced effect in attenuating hyperpermeability in FAE from β-glucan stimulation compared to VE in both
CD patients and controls, which could further be explained by the in vitro results from the FAE co-culture model. The significantly higher translocation of β-glucan in the FAE co-culture model could be due to the presence of M-cells, as it has been previously shown that β-glucan translocation into murine Peyer’s patches was partially M cell-dependent (299). Still, the mechanisms surrounding β-glucan uptake are mostly unknown.

Our results on β-glucan transportation after specific inhibition of two different endocytosis mechanisms revealed only a mild but significant reduction in β-glucan passage in FAE model after 20 min of stimulation with the lipid-raft inhibitor MβCD. As stated in the paper, lipid rafts are specialised microdomains enriched in cholesterol and glycosphingolipids, and it is known that pathogens like E. coli exploit lipid rafts in the plasma membrane to gain entry to the cells (105). However, only a trend towards significance was detected after 1 hour in FAE, which would suggest that lipid-raft endocytosis plays a partial role in the early uptake even though other non-investigated mechanisms seems responsible for the majority of its uptake. Trends towards significance were detected for an attenuated uptake of β-glucan using MβCD in Caco-2-c11 for both 20 min and 1h incubation, further suggesting that lipid-rafts might play a minor role in β-glucan uptake in VE. However, additional experiments might have been needed to reach significance, as the graphs visually appear similar to FAE in Figure P4 6C-D. A different study showed that MβCD could significantly reduce the entry of the β-glucan expressing fungi Candida albicans into human monocytes by disrupting lipid raft formation in cell membranes (300). Chloropromazin is a specific endocytosis inhibitor compared to MβCD. It inhibits endocytosis by a reversible translocation of clathrin and its adapter proteins thus hindering clathrin-mediated vesicle formation (301). No significant effect of CPZ on β-glucan uptake was found in this paper, indicating that β-glucan uptake is not clathrin-dependent.

The close proximity of β-glucan to MCs, DCs and macrophages in both FAE and VE was demonstrated using fluorescence microscopy. The co-localisation to DCs and macrophages is interesting, as previous studies have found orally supplemented β-glucan to be taken up by macrophages and released in particles with higher bioactive capabilities (288). Macrophages have also been found to be involved in the transportation of β-glucan to various tissues sites, such as spleen lymph node and bone marrow (302).

Similar to macrophages, a special subset of DCs in FAE has been shown to be able to extract β-glucan from their environment (303). Our findings
align well with these studies and highlight the interaction between yeast-derived β-glucan and immune cells of the lamina propria. A higher number of MCs, DCs and macrophages were found in the mucosa of CD patients in comparison to controls, reflecting an on-going inflammatory activity. The percentage of β-glucan co-localizing/in close proximity to macrophages and DCs were significantly higher in VE of CD patients compared to controls. These findings highlight a more active uptake and enhanced interaction with antigen-presenting immune cells in the mucosa of CD compared to controls. It is unclear whether or how these findings correlate to the attenuating effect from β-glucan stimulation on MC-induced hyperpermeability, as both CD patients and controls showed similar results. These findings do broaden the knowledge on the internalisation capability of both macrophages and DCs for fungi-associated components in CD patients in a relatively unexplored area. One earlier study by Vazeille et al showed that macrophages from CD patients have a higher internalisation rate of E. coli compared to healthy controls, echoing similar findings to our study (304). DCs have previously been shown to internalise β-glucan (299, 303) but here we demonstrated for the first time that the internalisation rate is higher in CD patients compared to controls. Due to our findings of attenuated hyperpermeability from pre-stimulation with β-glucan and its close proximity to MCs, we hypothesised that the yeast-derived β-glucan achieved its effects through inhibition of MC-degranulation, thereby preventing the release of e.g. TNF-α.

However, this hypothesis could not be fully supported by the results generated from the in vitro HMC-1.1 experiments. The degranulation marker β-hexosaminidase was significantly increased upon C48/80 stimulation but pre-stimulation with β-glucan did not reveal a statistically significant (p=0.15) decrease of β-hexosaminidase, although the results appear to have much lower spread and reduced median level compared to stimulation with C48/80 alone (Figure P4-7A). Based only on 9 independent results, we would interpret this as a non-significant trend that might suggest that β-glucan has an attenuating effect on MC degranulation. Similarly, levels of IL-6 were significantly increased following C48/80 administration while pre-stimulation with β-glucan did not result in a statistically significant decrease.

Interestingly, TNF-α showed an opposite pattern with no significant increase after C48/80 stimulation while pre-stimulation with β-glucan before administration of C48/80 resulted in significantly lower levels of TNF-α.
compared to C48/80 only and a trend towards significance when compared to vehicle.

The findings suggest that β-glucan exerts its protective effects through unknown MC-independent mechanisms, possibly by directly working on mediators released by MCs or on tight junction proteins. It is important to emphasise that the *in vitro* setting does not fully mirror the *in vivo* situation. It is also possible that further optimisations on β-glucan concentrations and incubation times could have been required to reach statistical significance on attenuated degranulation/cytokine levels. We also know from previous studies that β-glucan undergoes processing by macrophages (288). Our results displayed co-localisation of β-glucan to both macrophages and DCs, it is therefore possible that processed fragmented particles with different properties compared to their original form are released from these cells, thereby producing more potent effects on MCs. However, this was not investigated in this study. As stated in the discussion of this paper, one human clinical study showed that 4 weeks of daily oral supplementation with 250 mg Baker’s yeast-derived β-glucan decreased total ragweed allergy symptoms and severity (305). These findings did not correlate with changes in IgE levels and could therefore suggest an attenuation of MC-degranulation since allergic reactions are highly dependent on MC-degranulation (306).

We thereby conclude that yeast-derived β-glucan inflict a beneficial effect on ileal barrier function by inhibiting stress effects on the epithelium. β-glucan was able to attenuate paracellular and transcellular hyperpermeability caused by MC-degranulation. *In vitro* studies showed that β-glucan passed through the FAE at a higher rate compared to VE, and the uptake seemed to involve lipid raft formation. Our results provide important and novel knowledge that highlight the possible application of yeast-derived β-glucan in health disorders and diseases characterised by intestinal barrier dysfunction, such as CD.
GENERAL DISCUSSION

The importance of the intestinal barrier function and permeability has been extensively covered in the ‘introduction’ section of this thesis. A disturbed barrier function followed by an increased permeability is often found in diseases of severe inflammation like Crohn’s disease (CD), as shown in paper IV. Both papers I and III demonstrated that elderly with gastrointestinal (GI) symptoms had a significantly increased permeability, using two different methods. Paper I showed elevated levels of plasma zonulin (reflecting an increased small intestinal permeability) in elderly with GI symptoms compared to a general population of elderly. Paper III demonstrated an increased permeability locally in the colon of elderly with GI symptoms compared to young healthy adults. These findings have, to our knowledge, not been shown previously and could highlight an important aberration in the pathogenesis/pathophysiology of the idiopathic GI symptoms commonly found in elderly. The elevated intestinal permeability found in CD patients has been proposed to be of major influence or possibly even the cause of the pathophysiology and GI symptoms behind the disease (150).

There is a rising prevalence of elderly developing inflammatory bowel diseases (IBD), such as CD or ulcerative colitis (307), that seems to be less dependent on genetic inheritance and more influenced by environmental factors due to the late age onset. This topic however requires further understanding of the population and the underlying pathophysiological mechanisms but it is plausible that elderly with IBD also suffer from an increased intestinal permeability, possibly preceding the disease onset. Thus, our results showing an increased permeability in elderly with moderate GI symptoms displays a pathophysiological condition that is necessary to treat and prevent in order to potentially reduce the risk of further complications of inflammatory character.

Our studies in paper III-IV showed that non-digestible polysaccharides (NPS) could attenuate mast cell (MC) – induced hyperpermeability in Ussing chambers, suggesting a possible therapeutic effect from NPS that could be of benefit to individuals with compromised intestinal barrier function. The yeast-derived β-glucan showed promising effects on MC-induced hyperpermeability in colonic biopsies from elderly with moderate GI symptoms, in addition to similar effects in ileal follicle-associated and villus epithelium from both CD patients and non-IBD controls.
This β-glucan is known to act as a ligand to the complement receptor 3 (CR3), further leading to synergistic effects with the complement system towards eradication of tumour cells (302). The CR3 receptors are expressed on MCs (308), suggesting that yeast-derived β-glucan might interact through competitive binding to CR3 on MCs and possibly block or decrease their degranulation. Although our in vitro data from paper IV showed inconclusive results on whether β-glucan could affect degranulation, the percentage of co-localised β-glucan to dendritic cells (DC) and macrophages was significantly higher in CD patients while co-localised β-glucan to MCs was equal between CD patients and non-IBD controls. This could suggest that the inflammatory process in CD patients drive a higher uptake of foreign substances by antigen-presenting cells such as DCs and macrophages, which in turn could release fragmented β-glucan particles with higher bioactive capabilities as previously shown (288). The presence of a possible low-grade inflammation could explain why the yeast-derived β-glucan had a significant effect on the MC-induced hyperpermeability in elderly with GI symptoms but not young healthy adults in paper III. Although we did not investigate for a low-grade inflammation in paper III, other studies have found increased levels of cytokines (133, 162) and monocyte-activation markers (69) in elderly, which could suggest that an intestinal low-grade inflammatory environment facilitates enhanced uptake and effect from β-glucan. Although the β-glucan could attenuate MC-induced hyperpermeability in elderly, it also induced increased permeability by itself.

Studies have found that β-glucan can bind to both CR3 and Dectin-1 (receptor for fungi-associated components) on phagocytic cells (309), although different physical forms of the same β-glucan bind with varying affinities, the outcome consist of an oxidative burst of reactive oxygen species (ROS) that is dose-dependent on β-glucan concentration (309). ROS is known to cause damage to the intestinal barrier function (310-312). Thus, optimising the β-glucan concentration, within the frame of anti-oxidative networking, could be necessary to reduce ROS-induced barrier dysfunction. In addition, recent studies have shown that the particle size of β-glucan matters for its ability to induce a cascade of pro-inflammatory cytokines (313, 314). Larger sized particles of β-glucan were found to bind to Dectin-1 on DCs and induce production of IL-1β and subsequently IL-23 and IL-6 (313), the latter which is known to increase permeability (133). Smaller particles of β-glucan were, in contrast, inter-
nalised and elicited significantly lower amount of pro-inflammatory cytokines (313).

Laminarin, a β-glucan originating from seaweed, has been shown to be either an agonist or antagonist of Dectin-1 depending on size (molecular weight) and form (315). The antagonist version of Lamerin was found to significantly reduce the severity of DSS-induced colitis in mice, demonstrating how a specific form of β-glucan could possibly reduce inflammation (316). As such, different sizes of β-glucan in addition to differences in quantity of phagocytic cells and their β-glucan-binding receptors between elderly with GI symptoms and young healthy adults could reflect the differing results observed in paper III.

The role of the intestinal microbiota has not been taken into account for the studies presented in this thesis. All NPS investigated in these studies have prebiotic capability, *i.e.* stimulate the proliferation of health beneficial bacteria *in situ*. However, it is less likely that the gut microbiota has a major role on the results seen in paper IV as the ileum harbours significantly less bacteria than colon, where most of the fermentation takes place. The yeast-derived β-glucan used in these studies had a soluble form, *i.e.* contained a mixture of fragmented β-glucan particles of varying sizes; thus the soluble β-glucan is perhaps not as dependent on microbial fermentation as whole particles. Although this does not exclude the possibility of microbial influence on the β-glucan particles, it is still possible that this soluble form might prove to be a better way of delivering the NPS in elderly since they are known to accommodate a less diverse commensal flora compared to young adults (51, 52). This might also partially explain the differential effects from the NPS arabinoxylan in attenuating transcellular but not paracellular hyperpermeability in elderly with GI symptoms (paper III), whereas colonic biopsies from young healthy adults were protected from both MC-induced paracellular and transcellular hyperpermeability by pre-stimulation with arabinoxylan in the Ussing chambers. The large polysaccharide structure (95 kDa) of this fibre might need fermentation from the host microbiota in order to turn into metabolites with beneficial effect on the paracellular part of the barrier function. Our unique approach of taking colonic biopsies from uncleansed bowels, as opposed to normal routine practise, has previously shown to keep a larger amount of mucosal-adherent microbiota intact compared to biopsies from cleansed bowels (291, 292). Although the remaining microbiota on the sampled biopsies was not investigated in this thesis, an inherent difference in microbiota composition between the two populations could possibly be mir-
rored in the biopsies despite the artificial setting of the Ussing chambers, suggesting that the microbial influence could play a role in the effect outcome from arabinoxylan stimulation seen in paper III. This also highlights the importance of understanding personal traits, such as genetics and microbiota composition on individual level, in order to apply personalised nutrition for optimal treatment efficacy. A potential reduction in bacteria that specifically ferment arabinoxylan into metabolites in elderly could possibly explain the lack of a positive outcome effect on paracellular permeability. This could perhaps also partly explain why neither arabinoxylan nor oat-derived β-glucan showed any significant changes compared to placebo in the clinical trial of paper II. Preliminary data on faecal microbiota composition analysed from the clinical trial in paper II by our partners in the FibeBiotics consortium showed that both arabinoxylan and oat-derived β-glucan could shift the microbiota composition, although final analysis are not completed. The lack of any significant effect could suggest that the intervention period of 6 weeks might not have been long enough to shift the microbial composition in favour of a microbial environment that could produce metabolites with effects on the barrier function. However, the results from this thesis demonstrate that selective NPS have the ability to prevent MC-induced hyperpermeability in the Ussing chambers. The use of MC degranulation to induce hyperpermeability could partly mimic the in vivo situation in elderly with GI symptoms, as we found them to suffer from increased psychological distress (anxiety/depression) in paper I that could possibly lead to increased levels of stress hormones and subsequent MC degranulation (123, 168-173). It was recently shown (Stevens et al 2017) that plasma zonulin levels correlate to both anxiety/depression and an altered gut microbiome (317), suggesting that novel therapies targeted against the intestinal barrier and gut microbiota could be of great benefit to elderly with GI symptoms and psychological distress. In conclusion, this thesis presents novel findings on the state of the intestinal barrier function in different populations of elderly that broadens the knowledge on the possible pathophysiologial mechanisms behind highly prevalent age-associated GI symptoms. Furthermore we demonstrated the efficacy of selected NPS using a combination of both pre-clinical and clinical studies on intestinal permeability and found that specific NPS could prevent intestinal barrier disruption ex vivo in various populations. These results suggest that NPS could act as novel non-pharmaceutical therapeutic tools and possibly help elderly with GI symp-
toms. However, larger clinical trials are necessary to find an optimal time frame and concentration that display improvements to gut health.

**FUTURE PERSPECTIVE**

The results presented in this thesis are multifaceted and can help to direct future studies in various directions. Personalised nutrition is a growing field that takes into account the host genetics, life-style factors and gut microbiota composition for shaping dietary recommendation to inflict a positive individual health response. Using prebiotics as a treatment option would benefit from knowledge on the microbiota composition in the target population for best possible outcome. Hence, investigating the microbiota composition and correlating the effect outcome from the prebiotic intervention from paper II could reveal interesting patterns of future clinical relevance. Faecal samples were collected and sent to collaborators within the FibeBiotics consortium for microbiota analysis but did not reach completion in time for this thesis.

Larger clinical studies are necessary to elucidate the effect and mechanisms of dietary interventions as the effect of such compounds can be quite subtle. This could explain why some studies fail to or only show minor effects from dietary interventions (232, 318). Further analysis from the clinical trial in paper II will be conducted in the Nutrition-Gut-Brain Interactions (NGBI) research centre at Örebro University, Sweden by correlating the microbial composition against information on dietary intake and responses from the intervention on systemic, gut-related and mental wellbeing. Future studies in elderly might consider using a mixture of probiotics (live bacteria with health beneficial properties) and prebiotics (collectively called synbiotics) to evaluate a synergistic effect more potent than individual administration of either pre – or probiotics.

The Ussing chamber is a powerful technique for investigating the effect of new compounds on intestinal permeability, but in order to study the effect against barrier disruption a stressor is needed to induce hyperpermeability. For future studies using Ussing chambers, different stressors should be investigated and used based on their *modus operandi* (mode of action). Compound 48/80 was found to increase colonic permeability only in ≈60% of all study participants (papers III) while ileal permeability was significantly increased in all cases (paper IV). While the reason for this is unknown and intriguing, a 60% success rate in causing colonic hyperpermeability is problematic with regard to time and cost spending for per-
forming these experiments. Other compounds such as prostaglandin E2, myosin-light chain kinase and deoxycholic acid are currently under investigation by NGBI as potential stressors to be used in future Ussing chamber studies.

The results from paper III could be followed up in the Ussing chambers by investigating the number of DCs, macrophages and MCs in close-proximity to labelled β-glucan, similar to paper IV. Such examination could elucidate whether there is a difference between young healthy adults and elderly with GI symptoms that confers different outcome from β-glucan stimulation. The application of NPS that improve barrier function in elderly with GI symptoms could potentially also benefit patients with neurodegenerative diseases that are common in elderly (319, 320), such as Parkinson’s disease and Alzheimer’s disease (AD). Recent evidence points towards intestinal permeability being increased in Parkinson’s disease patients (285) and possibly also AD patients (282, 321). Interestingly, a large study consisting of 200 patients suffering from Parkinson’s showed that around 50% of the patients initially displayed GI symptoms in the form of constipation prior to developing the disease (284). Thus demonstrating how important it could be to treat elderly with GI symptoms as preventive care. A recent study (2017) revealed that the brain of AD patients to contained abundant lipopolysaccharide (LPS) deposits originating from the intestinal microbiota and driving a pro-inflammatory reaction (322), highlighting an aberration in the gut-brain axis that likely originates from a dysfunctional intestinal barrier.

Our population of senior orienteering athletes provides a unique look into an elderly population that is mostly devoid of GI symptoms and psychological distress (paper I). Due to our findings, we propose these senior orienteers as a model of healthy ageing. Further studies of this population with regards to their cognitive function and more detailed studies of their barrier function could reveal the role of physical activity in maintaining a healthy mind and gut. A recent publication (2017) showed that dancing activities in elderly could revert anatomical signs of an ageing brain (323) and it has previously been shown that exercise can induce neurogenesis in the brain (324-327), demonstrating the health effects on the mind from physical activity. But could an increased intestinal permeability have any beneficial effects also? Strenuous exercise has been demonstrated to increase the intestinal permeability (328) and could also be linked to the GI symptoms often prevalent in what is called “Runner’s gut”.

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However, since there also seems to be a link between physical activity, intestinal permeability and possibly brain function/mental wellbeing, the intestinal barrier function could play a pivotal role in the microbiota-gut-brain axis. The intestinal microbiota produce many different metabolites in the form of neurotransmitters and neuromodulators that could affect the mental health of the host (329). The term psychobiotics has quite recently been coined to describe probiotics that produce factors that have an effect on the mind. Animal studies have, among others, shown that psychobiotics have the ability to modulate brain-derived neurotropic factor (BDNF) (329), which is a protein involved in shaping the neural processing of learning, memory and neurogenesis (330-332). Hence, there might be a level of intestinal permeability that when slightly raised under certain conditions, such as during moderate physical activity, might promote an enhanced flux of neurotropic factors that has beneficial effects on the brain.

In conclusion, the findings in this thesis could lay the foundation for many interesting and relevant future projects that might both broaden and deepen the knowledge of how intestinal permeability could be modulated to the advantage of elderly’s health and wellbeing.
ACKNOWLEDGEMENTS

The completion of my PhD studies would never have been possible without the help I received from the following people and for that I’m forever grateful.

My deepest and sincerest gratitude goes towards my main supervisor Professor Robert Jan Brummer. Thank you for taking me in as your PhD student and introducing me to a new exciting field of research with a heavy emphasis on an interdisciplinary approach. I especially enjoyed our close work on optimising the Ussing experiments and our interaction when sampling the biopsies. I appreciate your humorous, optimistic and inspirational persona, your passion for cars, that you always were quick to provide feedback on the experiments and for always making time for me whenever I needed it.

To my co-supervisor Dr Ida Schoultz, thank you for always giving me 110% of your support and teaching me how to be a good scientist. It has been wonderful working closely with you from start to finish in all the aspects of my PhD. From designing and performing all the clinical trials and Ussing experiments to all the help and teaching I’ve received in writing my papers and my thesis. I greatly appreciate that you were always available and in contact with me despite being on maternal leave.

Thanks a lot to my co-supervisor Dr Åsa Keita from Linköping University for helping me make sense of the Ussing chamber system and the experimental results. Your help has been invaluable for my thesis work and I deeply appreciate you always taking time for me when I had questions and for the nice teamwork. I would also like to thank you for introducing me to your group at Linköping University and for the yearly invites to your research meeting day. I’ve always enjoyed your welcoming and helpful environment during my visits.

I want to thank Professor Elisabeth (Bettan) Hultgren-Hörnquist for accepting me as a bachelor and master student. Your lectures in mucosal immunology and the projects I worked on with you laid the foundation for my continuation in research and the topic of my thesis work, for that I am very grateful.
Warm thanks to Professor Nikolaos Venizelos, as the head of the biomedicine programme your door was always open for us students and you always inspired and encouraged us to study hard. In the same way I would like to acknowledge Professor Allan Sirsjö. Thank you both for your engagement, cheerfulness and presence in my education during my whole time at Örebro University.

I want to express my gratitude to the FibeBiotics consortium for my funding and all the nice meetings and collaborations. I also want to dedicate a special thanks to Ellen, Coen and Svein for help with my projects and to the FibeBiotics coordinator Dr Jurriaan Mes for your support, understanding and help.

A big thanks to all my study participants, young and old, who donated samples, time and engagement but always participated with great curiosity for our work. My thesis would not have been possible without your involvement and I sincerely hope the results will be to your benefit.

Many thanks to our excellent research nurses Anna, Ulla and Ulla-Britt at the gastroenterology department, your patience with me for always being restrictive with my assessment of biopsies was appreciated=)

A special thanks to Professor Dirk Repsilber for your advices, help and critical thinking on data analysis on my papers. I appreciate how you always took time to thoroughly explain different statistical methods for me.

A special thanks to Karin Hardin for your long, fun and important friendship.

A warm thanks to my long-time friend Sezin Günaltay. I've have always enjoyed your company, humour and friendship, especially during our trips to the GIMICum courses ☺

The Ussing experiments started early in the mornings and luckily I didn’t have to suffer alone. Thank you Liza Löfvendahl for sharing the pain of (many) failed experiments and the joy of (some) successful ones.
Thanks to Caroline Kardeby for being a great friend and also sharing my passion for games despite the fact that you were playing on the wrong platforms, although you made up for that by being a good Doksek president ;)

My sincere thanks to Ashok Kumawat & Sanja Farkas for your long support and encouragement throughout my work. Thank you Ashok for all your help from my first time in Bettans lab and to the intense volleyball & innebandy matches on Fridays ☺

Many thanks Lina Tingö for being an awesome colleague and excellent traveling companion! The trip to Washington and New York was the best!

Thanks to Johanna Sundin for always being caring and supportive since my first days in the lab. Our visits to gastrodagarna, Leipzig and New York were great!

A big hug and thanks to all fantastic members NGBI for creating the most lovable environment to work in

Savanne Holster, my Dutch friend, despite us being the complete opposite of each other I’ve always enjoyed our differences. Thanks for always being supportive, funny, cheerful and open for talks about anything!

Julia König, thanks for all your help & support during my PhD student. I particularly enjoyed our trip to Leipzig for the ESPEN conference and our visits to the Gimiicum courses!

Rebecca Wall, thanks for all your scientific advises and support during my PhD but most importantly I’ve enjoyed our discussions on horror movies, especially to the disgust of all others that had to suffer listening to us ☺

Tatiana Marques, a warm thanks to you for being a friend and providing important advices, help and support, for introducing me to your awesome mother Barbara and for all the cinema initiatives
Julia Sabet, your help and involvement with my clinical trials was greatly appreciated and invaluable for the completion of the studies. It was fun going around to different senior organisations and trying to recruit elderly for our studies, and perhaps less fun working with certain samples in the lab... 😊

Frida Fart, thank you for your positive energy, your funny and infectious laughter and most importantly all the help you have provided me with data management and questionnaire analysis. I’ve greatly enjoyed working with you!

Mårten Lindqvist, huge thanks to you for all the help I received on analysing the results for my papers. I always appreciated you taking your time to help me despite being involved in many other projects

Many thanks to all the new PhD students that joined the group in my final years, it was really great working with you all! Frida Gorejja (Italian food is great!), Maria Fernanda Roca Rubio (El Hippopotamo), Julia Rode (revealing me as Busavillig & destroyer of everything) and Mathias Tabat (you still need to teach me Wing-Chun!). I never quite understood why you all looked up to me but never doubt that I used it to my benefit=)

Dr (Caroline) Kremp, thank you for the great fun we had working on the APRO project and for still keeping in touch afterwards 😊

Hanna Edebol-Carlman, many thanks for your support, encouragement and helpfulness. I always found it fun to talk and collaborate with you 😊

Johnny Karlsson, thank you for accompanying me to the FibeBiotics meetings and helping me with all the administrative tasks!

Ignacio Rangel, thank you for the supervision you provided during my bachelor and master thesis and for the nice collaboration during my PhD

Anette Oskarsson, many thanks for all the help with booking tickets and setting up information & recruitment pages for our clinical trials!
Huge thanks to all the amazing people I’ve met at work

Many thanks to Samal Algilani and Dara Rasoal for the nice collaboration on our paper. I would also like to thank Stina, Carren and Cecilia from NUPARC for their support during my PhD work

A big thanks to the group at Linköping University for the fun collaboration and visits, a special thanks to Ylva, Maite, Martin, Lena and Stephanie for your involvement and help in my projects

To the staff at KFL: Elisabet T, Lena, Seta, Elin, Hanna, Anders, Robert, Anita, Sabina, Jessica, Jonna, many thanks for always gladly helping me whenever I needed it. A special thanks to Anna Göthlin-Eremo for helping me out with immunohistochemistry

A special thanks to Bo-Lennart Silfverdahl and Johan Gråsjö for the help with setting up the Ussing chambers and making it work. Thanks also to Anna, Ulrika, Samira and Melli at MTM for your help in setting up the multi-sugar analysis and to Lotta and Kicki at AKP for all help with the clinical trials

Many thanks to Sonja Nodland for the nice collaboration, support and always being quick with feedback and help

Thanks to the organisers and all involved in Gimiicum and Benght Ihre research school for the very nice courses, venues and networking opportunities

Thanks to all people involved in Doksek for organising interesting seminars and fun social activities for all PhD students

My sincere thanks also goes to all the staff at Campus USÖ, Örebro University and Örebro University hospital with a special thanks to Sören Andersson, Magnus Johansson, Nermina Piric, Anne Salmela and Helena Isaksson
Thanks to the Risbergska group for the fun environment and games to end
the week with (by playing volleyball and innebandy at Risbergska skolan
every Friday evening)

Many thanks also to my friends Robin, Viktor, Selim & Anna-Lisa,
Yasha, Hanif, Kayue, Rami

Thanks to all friends of the family who have supported, encouraged and
prayed for me during the years, a special thanks to Ann-Marie Johansson,
Victor & Solevi John & family for your long and continuous support

And finally, my warmest thanks to my family. Without the never-ending
support from my parents Rajan & Cicilia and my sister Priscilla I
wouldn’t be here. Their love and support is reflected in this thesis and
throughout all my years as a student. Many thanks as well to my cousins
Joshua, Elishba, to Jacob and his family Sandra, Isabelle and Gabriel. To
my maternal uncle Walter (Mamo) and aunt Parmila (Mami). To my rela-
tives in India: my aunts Protima (badi phuppo) and Pamela (choti phup-
po), my uncles: Shekhar uncle and Vijay (pehlwan) uncle, my cousins:
Shifu, Nitti, Richi, Sameer and Tinu

In special memory and dedication to my grandparents: Pyari (Dadi) and
Peter (Dada) Ganda Mall, Pyari (Nani) and John (Nana) McLaren
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