MONASH University

INVESTIGATING MARKERS OF INTESTINAL BARRIER DYSFUNCTION IN GASTROINTESTINAL DISEASES

Mary Aida Ajamian
BA, Neuroscience and Behaviour
MS, Human Nutrition

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Department of Gastroenterology
Monash University Central Clinical School
The Alfred Hospital
Melbourne, Australia

To my family – Dad, Mom, Shushi, and Anoush,

,

I am forever grateful for your unconditional love and support from across the Pacific.

AMDG

Love, Mary

ABSTRACT

There have been several proposed circulating candidate markers of intestinal barrier dysfunction, which include proteins associated with structural integrity, enterocyte damage and microbial translocation. However, there is limited knowledge of the application of these biomarkers in the evaluation of gastrointestinal disorders as clinical tools or clues to pathogenesis.

The overall aim of this research was to evaluate current candidate markers of intestinal barrier dysfunction in several human disease or experimental models of intestinal injury and/or barrier dysfunction. Specific aims were to determine the relevance and clinical utility of the markers alone or in combination.

Circulating levels of candidate markers - zonulin, syndecan-1, intestinal fatty-acid binding protein (I-FABP), lipopolysaccharide binding protein (LBP) and soluble CD14 (sCD14) - were measured by ELISA assays in healthy controls, cohorts of patients with gastrointestinal disease - coeliac disease, ulcerative colitis and non-coeliac wheat sensitivity (NCWS) - and in a model of exercise-induced gastrointestinal damage undergoing various dietary and drug interventions.

Assays for zonulin, a putative marker of tight junctional integrity, showed methodological inconsistencies. Two commercial assays detected different proteins, neither of which was zonulin, as demonstrated by spiking experiments and protein staining. Mass spectrometry and SDS-PAGE followed by protein staining revealed complement C3 and haptoglobin as top matches for their potential identity, but both failed to bind to antibodies in the ELISAs. These observations casted doubt on commercial zonulin ELISA assays' ability to detect serum zonulin and whatever was being detected was not informative in the models assessed.

A wide spread of levels of all markers in 49 healthy controls was observed. In specific colonic epithelial injury (severe ulcerative colitis), syndecan-1 (n=12), though not I-FABP (n=20) was elevated while both were elevated in small intestinal injury (untreated coeliac disease, n=36 for syndecan-1 levels; n=32 for I-FABP levels). Trends were only observed in these markers with regard to mucosal healing in coeliac disease, which casts doubt on their utility for tracking mucosal healing over time. Their combination in patients with NCWS revealed a consistent reduction of syndecan-1, but not I-FABP, when treated with a low FODMAP diet, suggesting reduced colonic epithelial injury, a finding consistent with experimental data. Elevation of sCD14 and LBP levels showed evidence of bacterial translocation in the exercise-induced model of intestinal injury (where I-FABP was also elevated) and in severe ulcerative colitis, but not in coeliac disease or NCWS.

In conclusion, commercial assays that claimed to measure zonulin do not detect this protein and therefore have no utility in clinical practice. Syndecan-1 and I-FABP appear useful in combination, not only to detect intestinal epithelial injury, but also in the determination of whether injury is colonic or small intestinal. Their utility in diagnosis is likely to be small, but longitudinal evaluation associated with an intervention was more likely to have clinical and experimental value. Heterogeneity of changes in the pattern of markers may assist in evaluating pathogenic mechanisms associated with modulation of intestinal barrier integrity and function.

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DECLARATION

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

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LIST OF ABBREVIATIONS

Å	Angström
ANOVA	Analysis of variance
APC	Antigen presenting cells
ASCA	Anti-*Saccharomyces cervisiae* antibodies
ASUC	Acute severe ulcerative colitis
ATI	Amylase-trypsin inhibitors
BASE	Be Active Sleep Eat
bFGF	Basic fibroblast growth factor
BSA	Bovine serum albumin
CD138	Syndecan-1; cluster differentiation (CD)138
CDAI	Crohn's Disease Activity Index
CRF	Corticotropin releasing factor
CRP	C-reactive protein
CV	Coefficient of variability
DTT	Dithiothreitol
EGFR	Epidermal growth factor receptor
ELISA	Enzyme-linked immunosorbent assay
EMA	Endomysial antibody
EndoCAb	Endotoxin core protein antibodies
FABP2	Fatty acid-binding protein 2; Intestinal fatty acid-binding protein (I-FABP)
FDR	False discovery rate

FITC	Fluorescein isothiocyanate
FODMAP	Fermentable oligosaccharides, disaccharides, monosaccharides, and polyols
GAG	Glycosaminoglycan
GST	Glutathione S-transferase
H	Kruskal-Wallis statistic
H+L	Heavy + light chains
H_2O	Water
HDAI	Harvey-Bradshaw Index
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HPLC	High performance liquid chromatography
I-FABP	Intestinal fatty acid-binding protein
IBD	Inflammatory bowel disease
IBS	Irritable bowel syndrome
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IL-1β	Interleukin 1β
IQR	Interquartile range
kDa	Kilodalton
L:M	Lactulose to mannitol
LBP	Lipopolysaccharide binding protein
LC-MS/MS	Liquid chromatography-mass spectrometry

LPS	Lipopolysaccharide
mCD14	Membrane-associated cluster differentiation (CD)14
MD-2	Myeloid differentiation factor 2
MMP	Matrix metalloproteinase
MS/MS	Tandem mass spectrometry
NCWS	Non-coeliac wheat sensitivity
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
pANCA	Perinuclear anti-neutrophil cytoplasmic antibodies
PAR_2	Proteinase activated receptor 2
PBMC	Peripheral blood mononuclear cell
PVDF	Polyvinylidene difluoride
ROC	Receiver operating characteristic
SCCAI	Simple Colitis Clinical Activity Index
sCD14	Soluble cluster differentiation (CD)14
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SEM	Standard error of the mean
SIBDQ	Short inflammatory bowel disease questionnaire
TBST	Tris-buffered saline with Tween 20
TEER	Transepithelial electrical resistance
TLR4	Toll-like receptor 4
TNF-α	Tumour necrosis factor alpha

tTG	Tissue transglutaminase
USA	United States of America
VO_{2max}	Maximal oxygen uptake
ZO	Zonula occludens
Zot	Zonula occludens toxin

CHAPTER 1

LITERATURE REVIEW

1.1 Introduction

Impaired intestinal barrier integrity, colloquially known as "leaky gut," has received increasing attention as a purported mechanism of disease pathogenesis. According to this paradigm, dysfunction of the intestinal mucosal barrier leads to microbial translocation, or the increased passage of dietary and microbial products from the external environment into the host, which consequently initiates local and/or systemic inflammation and immune activation. Impaired intestinal barrier integrity is directly implicated in various gastrointestinal disorders, including coeliac disease, inflammatory bowel disease (IBD), and non-coeliac wheat sensitivity (NCWS).[1,2] Due to possible effects of translocated products onward to the lymph nodes and extra-intestinal sites and subsequent immune activation, the concept is increasingly associated with an even broader range of clinical conditions, especially those with immune abnormalities or inflammation (e.g. autoimmune disorders, human immunodeficiency virus [HIV], and diabetes).[3-7]

As scientific research on this concept continues to grow immensely, there is an immediate need to evaluate commonly used circulating markers used to assess intestinal barrier dysfunction. In the basic research as well as clinical setting, obtaining serum or plasma samples to assess intestinal barrier dysfunction is less invasive, easier to acquire and maintain, and more cost-effective than the analysis of biopsy samples. Assessment of circulating markers is particularly important in studies investigating extra-intestinal diseases, where biopsy samples from patients are

usually unavailable. A common, non-invasive method used to assess intestinal permeability through urine analysis in the clinical setting is the lactulose to mannitol (L:M) test. It is highly variable in terms of assay method, specimen collection, and data reporting, and is unlikely to diagnose barrier dysfunction in individual cases.[8] Additional information, particularly with regards to circulating markers, is required to improve diagnostic utility.

Circulating markers related to intestinal structural barrier integrity featured in this review include zonulin, syndecan-1 or CD138, and intestinal fatty acid binding protein (I-FABP) or fatty acid-binding protein 2 (FABP2). Zonulin and syndecan-1 are proteins directly associated with barrier integrity through their roles of maintaining intercellular tight junctions and cell adhesion. I-FABP is a purported measure of enterocyte damage, which may be useful in determining the cellular viability of the intestinal barrier. Featured microbial translocation markers include soluble CD14 (sCD14) and lipopolysaccharide binding protein (LBP). These proteins are associated with the body's immune response to bacterial products, particularly endotoxin or lipopolysaccharide (LPS), that may cross a damaged mucosal barrier.

Usage of these markers is widespread in the clinical and research setting, though some of these markers remain controversial due to methodological shortcomings in detection or investigation with plasma or serum samples from patients that have not been well-characterised clinically. As such, questions remain about the efficacy of certain markers to detect gastrointestinal disease or if these markers are reliable indicators of an impaired intestinal barrier. Evaluation of the efficacy of these markers in well-characterised cohorts of gastrointestinal dysfunction, for which it is certain that the intestinal barrier is damaged, is a necessary step before any markers are associated with extra-intestinal conditions.

This review aims to 1) provide an overview of candidate circulating serological and plasma markers that are directly associated with intestinal structural barrier integrity, enterocyte damage, and microbial translocation in disease and 2) assess the strength of evidence that these candidate markers are efficacious in detecting gastrointestinal dysfunction.

1.2 Methods

A search for relevant literature on PubMed was conducted for this explanatory review. Specific markers were chosen on the basis of what was readily available and suited to plasma and serum samples obtained together with guidance from their inclusion in seminal studies that investigate intestinal barrier dysfunction. Search terms included were "microbial translocation," "bacterial translocation," "zonulin," "prehaptoglobin-2," "intestinal barrier," "fatty acid binding protein 2," "FABP2," "intestinal fatty acid binding protein," "I-FABP," "syndecan-1," "CD138," "soluble CD14," "sCD14," "lipopolysaccharide binding protein," and "LBP." To understand the role and mechanism of each marker in relation to intestinal barrier dysfunction and resulting microbial translocation, primary research articles as well as review articles were examined. To assess the utility of candidate markers, articles must have investigated circulating plasma or serum levels of markers in the context of human intestinal barrier dysfunction. Additional markers of interest were also identified and described if found applicable to intestinal barrier dysfunction and microbial translocation. Articles that related circulating candidate markers to diseases or conditions that were not primarily gastrointestinal in nature (e.g. diabetes, HIV) were evaluated to determine whether any gastrointestinal effects were observed and were included for assessment of efficacy if any such effects were reported. Duplicate as well as non-English publications were

excluded. Both clinical and pre-clinical studies were included in this review. Additional records were identified through the references of relevant articles.

1.3 Results

1.3.1 Overview and Mechanisms of Action Associated with Circulating Intestinal Barrier Dysfunction and Microbial Translocation Markers

Aside from the regulation of solute and fluid exchange as well as absorption of nutrients, the intestinal epithelium has been implicated as a gatekeeper in the passage of environmental antigens and microbial products from the intestinal lumen into the sub-mucosa.[9,10] A breach or dysfunction in this monolayer of cells, the majority of which are enterocytes, may cause increased passage of macromolecules from the external environment into the host, which initiates local and/or systemic inflammation and immune activation. Contributing factors to decreased barrier integrity include dysfunction or damage of the associated structures of the paracellular space between enterocytes and damage to enterocytes themselves.

Transport across the intestinal epithelial barrier may occur by a transcellular pathway through cells or the paracellular pathway between cells. The transcellular pathway involves the action of passive or active transport channels for specific substrates.[11] The paracellular pathway filters by charge and size, and is also an important route of entry for macromolecules.[11,12] Structures in the paracellular space, namely tight junctions and other membrane-associated proteins, are directly involved in maintaining cell adhesion and a concentration gradient important for both transcellular and paracellular transport; as such, these structures are key regulators of epithelial transport.[11]

Zonulin, syndecan-1, and I-FABP are three circulating protein markers that have been used to assess intestinal epithelial integrity. Figure 1 describes the locations of these protein markers in relation to the intestinal barrier as well as those involved in microbial translocation. Zonulin, discovered as prehaptoglobin-2, is a putative modulator of intestinal epithelial tight junctions, thereby affecting the paracellular route.[13,14] Dysregulation in the zonulin pathway has been implicated in both gastrointestinal and extra-intestinal diseases. Increased levels of zonulin in the circulation has been associated with impaired epithelial barrier integrity. The shed form of syndecan-1, a membrane-associated protein, has been implicated in gastrointestinal disease and has an important role in inflammation and cancer.[15] Syndecan-1 plays a role in cell adhesion and acts in synergy with tight junctions, and is thus associated with the paracellular pathway.[16] I-FABP, a cytosolic protein of the enterocyte, is released upon cellular damage and can be detected in the circulation.[17] Epithelial cell damage implicates the dysregulation of both transcellular and paracellular routes, since it diminishes the integrity of cells and their adhesion to one another.

An impaired intestinal barrier promotes the movement of macromolecules and bacteria from the gut lumen into the lamina propria by means of transcellular and/or paracellular pathway dysregulation. Lipopolysaccharide (LPS), an outer-membrane component of gram-negative bacteria, is a common circulating marker of bacterial translocation. It is also a major contaminant in the laboratory and clinical setting, and has a short half-life in the circulation. Instead of measuring LPS directly, endogenous surrogate LPS markers lipopolysaccharide binding protein (LBP) and soluble CD14 (sCD14) have been widely used to determine levels of circulating LPS.

An overview of the gastrointestinal diseases for which mucosal permeability is implicated and an emerging model of intestinal barrier dysfunction studied in the current thesis investigation are included in this review. A detailed discussion of each marker with respect to gastrointestinal

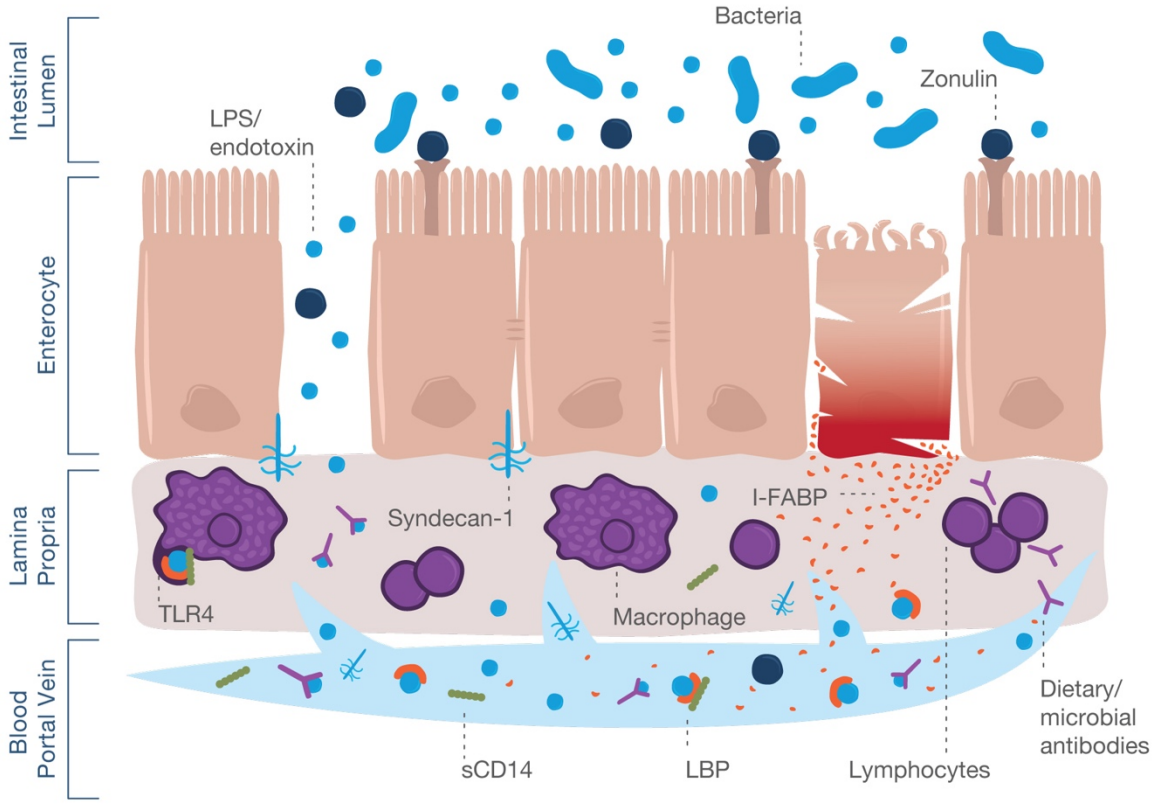

Figure 1. Overview of featured protein markers associated with intestinal barrier dysfunction and microbial translocation. Dietary and microbial products from the intestinal lumen may travel across a compromised intestinal epithelial barrier and initiate cascades of systemic inflammation and immune activation, which contribute to disease pathogenesis. Zonulin, syndecan-1, and I-FABP are endogenous proteins associated with the integrity of intestinal enterocyte and mucosal barrier function. Increased circulating levels of these proteins have been associated with increased intestinal permeability. A compromised intestinal epithelial barrier potentiates the passage of microbial products, such as endotoxins (i.e. LPS), or dietary macromolecules (e.g. gliadin peptides) from the intestinal lumen into the gut lamina propria. Here, exogenous products encounter specialised features of the mucosal immune system, including lymphocytes and proteins associated with innate immunity. In response to the presence of LPS, endogenous proteins LBP and sCD14 are produced, associate with the endotoxin, and aid in cellular response, particularly in conjunction with toll-like receptor 4 (TLR4) and macrophages. The immune response to exogenous products may initiate cascades that induce local and/or systemic inflammation and pathogenic, disease-specific cascades.

disease and an assessment of marker efficacy to detect dysfunction follows. Additional relevant markers of intestinal barrier dysfunction other than those featured in the thesis investigation were also identified, and a brief description of each is included.

1.3.2 Overview of Featured Gastrointestinal Diseases and Syndromes

1.3.2.1 Coeliac Disease

Coeliac disease, an autoimmune disorder characterised by intestinal inflammation, villous atrophy, crypt hyperplasia, and the presence of increased intraepithelial lymphocytes, is triggered by the ingestion of gluten in genetically-susceptible individuals.[18] Once considered a rare disease, coeliac disease is now considered the most prevalent cause of enteropathy and is among the most common genetic disorders in the Western world with prevalence approaching 1% of its population.[18,19] Individuals with coeliac disease carry the human leukocyte antigen (HLA)-DQ2 or -DQ8 genetic polymorphism.[20] Additional diagnostic indicators of disease include increased circulating levels of anti-endomysial (EMA), anti-tissue transglutaminase (tTG), and anti-deamidated gliadin antibodies.[18,20] However, the gold standard for diagnosis has historically involved histological demonstration of villous atrophy in duodenal or jejunal mucosal biopsies with regeneration of the villi after gluten withdrawal.[20] A second biopsy is not needed if serological tests are positive and villous atrophy is present, but is used where the diagnosis is equivocal. In children, the need for biopsy evidence is no longer required when the serology is strongly positive. Symptoms of coeliac disease may include gastrointestinal and extra-intestinal manifestations. Common gastrointestinal symptoms include chronic diarrhoea, abdominal pain, and constipation.[20] Fatigue is the most common extra-intestinal manifestation, but neurological manifestations, including headache, neuropathy and ataxia, are well described.[21] Various

comorbidities may exist, including type 1 diabetes mellitus, Sjögren syndrome, autoimmune thyroid disease and systemic lupus erythematosus.[20] The variety of possible symptoms and comorbidities point to multi-system involvement in the disease.

Gluten, the causative dietary protein, is a protein family found in wheat, barley, and rye. Strict, lifetime avoidance of these foods and adherence to a gluten-free diet is necessary for amelioration of symptoms and mucosal healing in affected individuals, and is currently the only reliable option for treatment. The mechanism of disease action involving the digestion of gluten and the body's response is summarised in Figure 2. In the first step, gluten is subject to digestion by intestinal enzymes into smaller proteins, including gliadins and glutenins, and even smaller peptide fragments. Some peptides also remain resistant to proteolytic activity of intestinal enzymes. Immunogenic peptides cross the intestinal epithelial barrier, which is presumably impaired, and come into contact with tTG. This enzyme initiates the deamidation of glutamine residues.[21] The process of deamidation, which also confers a negative charge to the peptides, further enhances their immunostimulatory abilities.[21] In the next step, the peptides encounter the body's immune cells. Antigen presenting cells (APCs) engulf peptides and present them to HLA-DQ2 or -DQ8 molecules on gluten-specific helper T cells.[21] This interaction leads to the production of pro-inflammatory cytokines, B cell clonal expansion specific for anti-gluten antibody production, and anti-tTG antibody production.[21] Pro-inflammatory cytokines regulate molecules that affect the permeability of the intestinal barrier, including matrix metalloproteinases (MMPs).[21] The candidate markers featured in this review may reflect these resulting intestinal permeability abnormalities in coeliac disease.

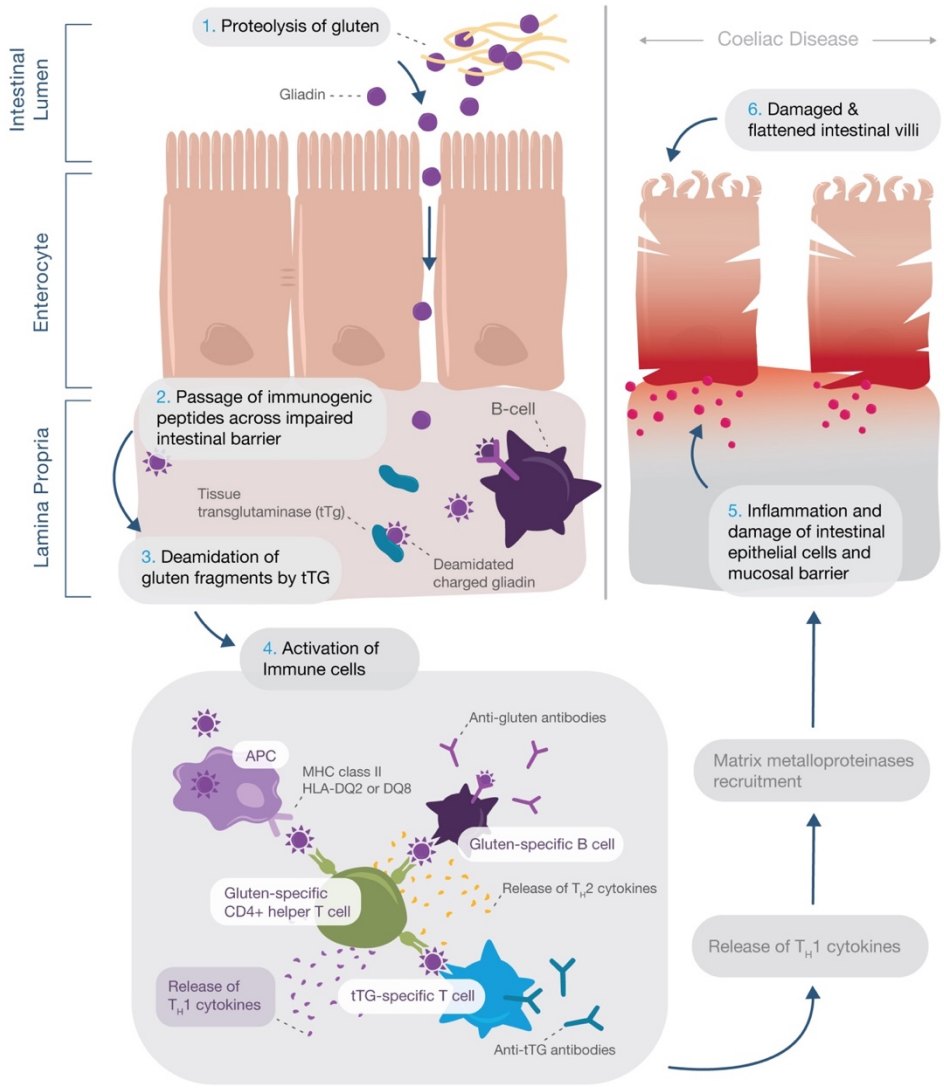

Figure 2. Mechanism of disease action in coeliac disease. 1. The proteolysis of gluten proteins occurs in the intestinal lumen and produces smaller fragments, including gliadin, which are immunogenic in coeliac disease. 2. Immunogenic protein fragments pass through an impaired intestinal barrier into the lamina propria. 3. Deamidation of fragments by tTG enzyme occurs, which creates deamidated, negatively-charged gliadin peptides. 4. Antigen presenting cells (APCs) engulf deamidated gliadin peptides and present them to gluten-specific, CD4-positive(+) helper T cells bearing the HLA-DQ2 or -DQ8 polymorphism, which in turn prime gluten-specific B cells and tTG-specific B cells. These processes contribute to specific antibody and cytokine release, which contributes to matrix metalloproteinase recruitment at the intestinal barrier. 5. Matrix metalloproteinases contribute to inflammation, damage, and diminished intestinal barrier integrity. 6. Morphology of intestinal enterocytes is affected, which includes damaged and flattened intestinal villi.

1.3.2.2 Inflammatory Bowel Disease

Inflammatory bowel disease (IBD) describes chronic inflammatory gastrointestinal conditions of unknown aetiology and is increasing in worldwide incidence and prevalence.[22] Crohn's disease and ulcerative colitis are the two main classifications of IBD. Crohn's disease is characterised by chronic, relapsing inflammation anywhere in the gastrointestinal tract from mouth to anus.[23] The onset of Crohn's disease usually occurs between ages 15 to 30 years, and disease activity is characterised by periods of alternating remission and relapse.[23,24] Manifestations of disease can include gastrointestinal and extra-intestinal symptoms. Gastrointestinal symptoms may be indicative of disease location (e.g. bloody diarrhoea, indicative of colonic disease), though non-specific symptoms such as fever may occur.[23,25] Extra-intestinal manifestations of disease are usually inflammatory in nature; these include arthritis, uveitis, and erythema nodosum.[23] Ulcerative colitis describes mucosal inflammation limited to the colon and rectum. The disease typically presents with rectal bleeding, diarrhoea, and tenesmus.[26] Approximately 20% of individuals with ulcerative colitis experience an acute, severe flare up of their disease, which warrants hospitalisation.[26] Distinguishing ulcerative colitis from Crohn's disease can be difficult, especially with overlapping symptoms, and prodromal periods of Crohn's disease may be lengthy before a diagnosis is established.[27]

Several scoring systems have been developed to assess IBD disease activity in patients. Scoring systems associated with Crohn's disease include the Crohn's Disease Activity Index (CDAI), Harvey-Bradshaw Index (HDAI), short inflammatory bowel disease questionnaire (SIBDQ), van Hees Index, and Lehmann score.[28,29] Of these systems, the CDAI has emerged as the most commonly used index in research trials. Ulcerative colitis scoring systems include the Truelove-Witts Severity Index, Simple Colitis Clinical Activity Index (SCCAI), Mayo Clinical

Score/Disease Activity Index, and Montreal Classification.[29,30] The Truelove-Witts Severity Index is widely used and recommended to assess severe acute disease, whereas the SCCAI and Partial Mayo Clinic Index are recommended to assess mild to moderate disease.[30] These scoring systems solely depend on clinical features of patients to classify disease status and have not been considered fully reliable to detect active disease.[29,31] A need currently exists to create criteria that can accurately classify patients and assess disease activity. Additional indices include circulating inflammatory markers, such as C-reactive protein (CRP), and faecal protein associated with intestinal inflammation, such as calprotectin; these particular markers have emerged to monitor disease activity.[32] Colonoscopy has been considered the gold-standard in assessing disease activity (when disease is within its reach), but cross-sectional imaging, particularly magnetic resonance imaging and intestinal ultrasound, have increasing roles in assessing the state of the disease.

The exact mechanism of IBD pathogenesis remains unknown, though multiple factors associated with environment, genetics, intestinal microbiota and immune dysregulation are suspected contributors.[33] An impaired intestinal epithelial barrier, which may induce mucosal inflammation may lead to an inflammatory state if immune control mechanisms are inefficient, is considered a hallmark of disease and a suspected causative step of immunopathogenesis.[33] Ten percent of first-degree relatives of Crohn's disease patients were observed to have increased intestinal permeability, indicating a potential genetic predisposition of this associated feature of disease.[34] Unfortunately, it is not known whether the increased permeability is cause or effect. Investigation of markers directly related to intestinal barrier function itself and resulting microbial translocation may provide insights into disease classification, activity, and immunopathogenesis.

1.3.2.3 Non-Coeliac Wheat Sensitivity

Non-coeliac wheat sensitivity, or NCWS, is a controversial diagnosis of individuals who lack the genetic, immune, and serologic biomarkers of coeliac disease or wheat protein allergy, yet present with similar symptoms that appear to resolve when following a gluten-free diet. Unlike coeliac disease, biomarkers for NCWS are lacking and the underlying biological mechanisms are poorly understood. Globally accepted, definitive clinical criteria do not currently exist, though the clinical picture most closely resembles irritable bowel syndrome (IBS).[35] Despite a lack of scientific understanding, international awareness of NCWS continues to grow. Many self-diagnose by following a gluten-free diet and subsequently reporting amelioration of symptoms as well as an overall increase in feelings of well-being and positive behavioural health outcomes.

Recent studies have sought to determine whether gluten or other factors may be causative agents of gastrointestinal symptoms in individuals with self-reported NCWS. Most notably, fermentable, oligo-, di-, mono-saccharides and polyols (FODMAPs), which are slowly absorbed or indigestible short-chain carbohydrates and sugar alcohols, have arisen as the primary candidates in these individuals. Fructans, a common type of FODMAP, co-exists with gluten in cereals. Double-blinded, placebo-controlled randomised re-challenge clinical trials have found little convincing evidence of gluten as the causative agent and a recent three-arm study provided evidence that fructans were most likely the culprit in gluten-containing cereals.[36 37] Other non-gluten proteins in wheat, such as amylase-trypsin inhibitors (ATIs), have also been implicated as potential pathogenic triggers that play a role in initiating inflammatory responses, though currently with no direct evidence.[38]

In terms of epithelial barrier dysfunction and microbial translocation, one study reported levels of associated markers, as described by the main featured markers in this review, that were

distinctive from coeliac disease and healthy controls in individuals with NCWS.[2] Symptoms experienced by the NCWS cohort upon the ingestion of gluten appeared during re-introduction of gluten into their diet after dietary withdrawal, and all serological levels of barrier dysfunction, immune activation, and microbial translocation decreased significantly during withdrawal.[2] However, the specificity of the findings to those with NCWS as a control cohort with IBS, though without clear wheat sensitivity, was not studied. A proportion of patients with IBS have intestinal barrier dysfunction and elevated markers of microbial translocation.[39] Further studies are indeed warranted to disentangle the causative factors of disease pathogenesis in individuals with NCWS. Such studies should be conducted with particular care towards patient recruitment and dietary intervention design to rule out potential dietary confounders. In addition, there have been no studies involving individuals with NCWS who experience only extra-intestinal symptom improvement on a gluten-free diet. A better understanding of disease pathogenesis and precise determination of dietary triggers will aid in the clinical management of those with NCWS.

1.3.2.4 Exercise-Induced Gastrointestinal Syndrome

The occurrence of gastrointestinal symptoms has been commonly observed in healthy individuals who undergo strenuous endurance sports such as ultra-marathons, which are increasing in popularity.[40] In particular, gastrointestinal symptoms appear most pronounced in running than in any other form of exercise.[41,42] The causes of gastrointestinal symptoms and gut dysfunction are multifactorial and may be attributed to a circulatory-gastrointestinal pathway as well as a neuroendocrine-gastrointestinal pathway, as described in Figure 3.[40] The circulatory-gastrointestinal pathway mechanism involves the redirection of blood flow to working skeletal muscle, thus creating an environment suitable for splanchnic hypoperfusion in the gastrointestinal

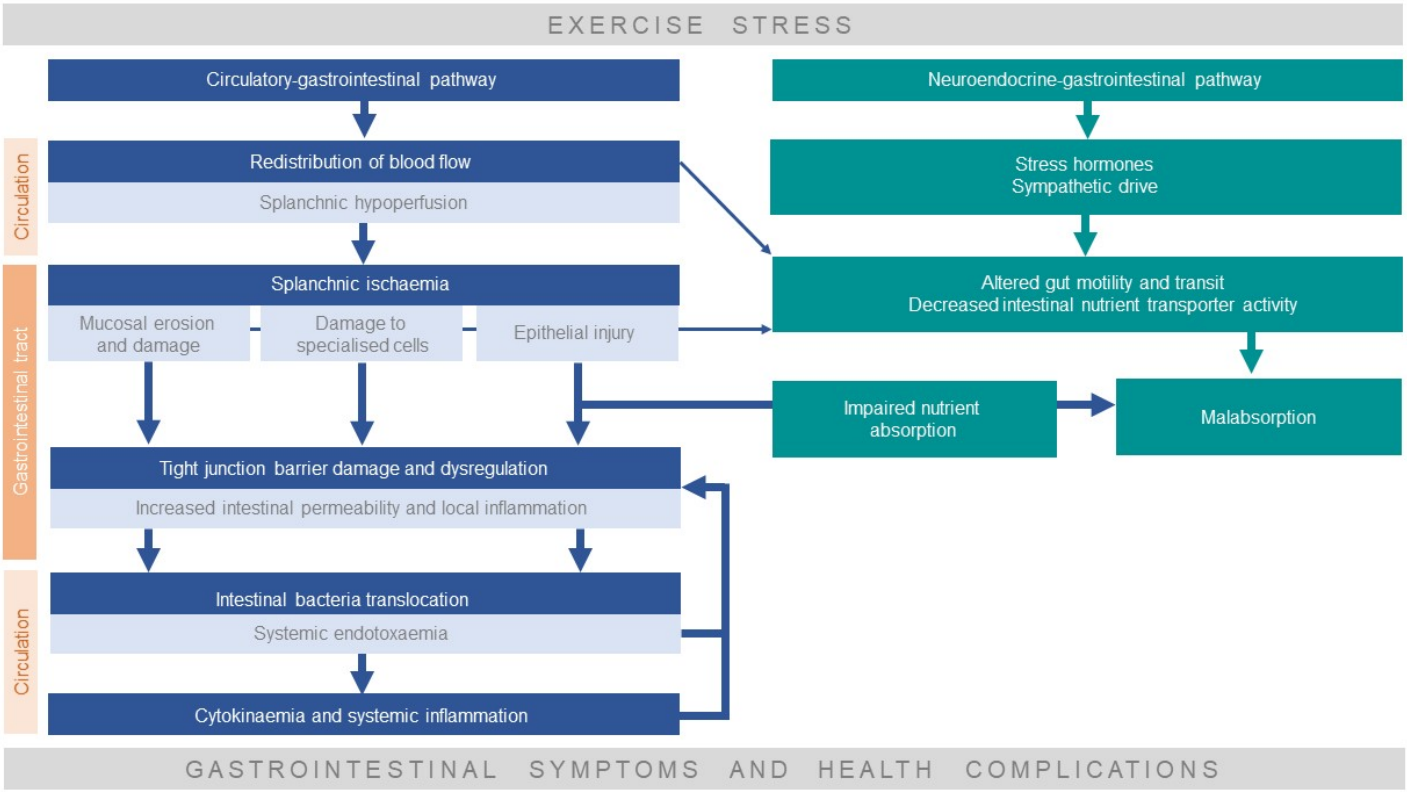

Figure 3. Mechanisms associated with exercise-induced gastrointestinal syndrome. Adapted from Costa et al., 2017.[40] Exercise stress, exacerbated by heat, can contribute to gastrointestinal symptoms and health complications through a circulatory-gastrointestinal pathway or neuroendocrine-gastrointestinal pathway. A redistribution of blood flow from the gut and resulting splanchnic hypoperfusion are key characteristics of the circulatory-gastrointestinal pathway, which lead to an ischaemic state. This contributes to abnormalities of the gastrointestinal tract, which include mucosal erosion and damage, damage to specialised cells, and epithelial injury. These factors, along with the redistribution of blood flow, affect gut motility, lead to decreased intestinal nutrient transport activity, and induce tight junction barrier damage and dysregulation. As a result, increased intestinal permeability, local inflammation, and translocation of intestinal bacteria occur. Systemic endotoxaemia may result from increased microbial products in the circulation, which contributes to increased cytokine levels and systemic inflammation. Increased stress hormones and sympathetic drive are the key characteristics of the neuroendocrine-gastrointestinal pathway that alter gut motility and transit as well as decrease intestinal nutrient transport activity. This contributes to nutrient malabsorption, which is also affected by epithelial injury and resulting impaired nutrient absorption through the redistribution of blood flow associated with the circulatory-gastrointestinal pathway.

tract. This further promotes splanchnic ischaemia, leading to intestinal barrier damage, microbial translocation, and systemic inflammation. The neuroendocrine-gastrointestinal pathway involves sympathetic and enteric nervous system activation, which alters gut motility and transit. This alteration, along with intestinal barrier damage, contributes to impaired nutrient absorption. Gastrointestinal symptoms and health complications may evolve as a result of exercise stress on both pathways. These abnormalities may be transient in nature, though there have been some examples of prolonged, clinically severe manifestations.[40]

Exercise duration, intensity and environmental temperature have been identified as factors that increase the likelihood of exercised-induced gut damage. A recent review has identified a condition of exercise stress at ≥ 2 hours at 60% VO_{2max} as the threshold whereby significant gastrointestinal disturbances manifest, regardless of an individual's physical fitness.[40] Hot ambient temperatures seem to exacerbate stress damage.[40] Significant elevations in markers of intestinal epithelial cell damage, such as I-FABP, and levels of circulating endotoxin, indicative of microbial translocation, have been reported in high intensity exercise to exhaustion at high temperatures.[40]

Although there is a growing amount of literature on healthy individuals with exercise-induced gastrointestinal syndrome, there are few studies that examine this condition in patients with gastrointestinal disease. The current exercise model in healthy individuals, however, offers a unique opportunity to evaluate markers of barrier dysfunction in subjects who have no other physical comorbidity.

1.3.3 Circulating Markers of Impaired Structural Barrier Integrity

1.3.3.1 Zonulin (Prehaptoglobin-2)

The protein, zonulin, is capable of reversible tight junction disassembly and is, therefore, implicated in the regulation of mucosal permeability.[13,14] Zonulin was first discovered as an endogenous human analogue of the bacterial enterotoxin, zonula occludens toxin (Zot), which is produced by the intestinal bacterium *Vibrio cholera*.[14,43] To initiate tight junction disassembly, it is proposed that zonulin activates epidermal growth factor receptor (EGFR) through proteinase activated receptor 2 (PAR_2) as well as G protein-coupled receptor PAR_2, which transactivates EGFR.[13,44] Activation of these two receptors has been shown to decrease transepithelial electrical resistance (TEER), therefore implicating increased intestinal permeability.[13] Zot has been shown to activate intracellular cascades that lead to protein kinase C α-mediated actin polymerization, which suggests that cytoskeleton modulation is involved in enhancing intestinal permeability.[45] As Zot and zonulin are analogues, a similar mechanism of activation associated with zonulin is suspected.

As the precursor to haptoglobin-2, zonulin belongs to the haptoglobin family of proteins. Haptoglobins are acute-phase reaction proteins that have a primary role in haemoglobin scavenging, in which they form a complex with haemoglobin to prevent oxidative damage to the haemoglobin itself and surrounding tissues. Haptoglobins also exert angiogenic and immunomodulatory properties.[46] Three genetic polymorphisms in human haptoglobin expression, Hp1-1, Hp2-1, and Hp2-2, are determined by the *HP1* and *HP2* alleles harboured by chromosome 16q22. As zonulin is the precursor to haptoglobin-2, individuals who bear the heterozygous Hp2-1 or homozygous Hp2-2 polymorphism are zonulin-producers whereas those with the homozygous Hp1-1 polymorphism are unable to produce zonulin. Dimerisation of haptoglobins occurs

cotranslationally and proteolytic cleavage from precursor to active forms takes place while still in the endoplasmic reticulum.[47] As such, the endoplasmic reticulum contains the highest amounts of zonulin as uncleaved, pre-Hp2, yet zonulin has been reported to be measured extracellularly and detectable in human sera.[13]

Dysregulation of the zonulin pathway and subsequent "gut leakiness" due to increased intestinal permeability has been associated with the pathogenesis of gastrointestinal disorders such as coeliac disease, NCWS and IBS, and IBD.[13,48-50] Autoimmune, inflammatory, and neoplastic diseases have also been implicated.[49]

Commercial zonulin assays have been widely used as a convenient method to assess intestinal permeability in a variety of clinical conditions beyond gastrointestinal disease. Upon examination of the literature, 10 publications used a commercial assay by CUSABIO and 61 publications used a commercial assay by Immundiagnostik to detect human plasma or serum levels of zonulin (Supplementary Table 1). Most of these studies investigated zonulin as a marker in extra-intestinal conditions. Of primary significance, one conference proceeding investigated serum zonulin levels in NCWS, IBS, and coeliac disease and found significantly higher levels in all patient groups compared to healthy individuals ($p<0.001$) using a commercial assay manufactured by CUSABIO.[51] However, this conference proceeding as well as most others in the literature did not test study participants for their ability to produce zonulin.

Upon initial investigation, inconsistencies exist in the commercial assays used to measure circulating zonulin levels. Immundiagnostik indicates the epitope GGVLVQPG, a peptide sequence synthetically manufactured as AT-1001, or larazotide acetate, to be the sequence to which assay capture antibodies are raised.[14,52,53] Although the purported zonulin receptor has an affinity for this epitope, it remains unclear whether the generation of captured antibodies raised to

this sequence would bind to actual circulating zonulin. Further study into assay methodology is required to determine whether current commercial assays provide accurate measurement of circulating zonulin levels.

1.3.3.2 Syndecan-1 (CD138)

Syndecan-1, or CD138, is a transmembrane proteoglycan involved in adhesion between cells and extracellular matrixes, which implicates a key role of its maintenance of the intestinal epithelial barrier.[16,54,55] It has additional roles in maintenance of cell structure as well as regulation of immune function and tissue repair.[16,54,56] The extracellular domain of syndecan-1 contains glycosaminoglycan (GAG) side chains, which primarily consist of heparin sulphate, though may be substituted with chondroitin or dermatan sulfate.[54,57] This ectodomain region interacts with a variety of factors, including enzymes, extracellular matrix molecules, and growth factors.[57] The transmembrane region of the protein mediates dimerisation of extracellular domains.[54] The highly conserved C1 and C2 internal cytoplasmic regions help anchor syndecan-1 core protein to the enterocyte's actin cytoskeleton and interact with PDZ domain-containing proteins associated with scaffolding, respectively.[54,57] The variable region between C1 and C2 differentiates syndecan-1 from other proteins of the syndecan family, and is conserved across species.[57]

Syndecan-1 is linked to the maintenance of the intestinal epithelial barrier through the paracellular pathway. The loss of syndecan-1 core protein and ectodomain components has been shown to increase protein efflux into the gut lumen, indicating dysregulation of the intestinal epithelial barrier through intercellular spaces.[55] Syndecan-1 destruction and heparanase overexpression has been shown to aggravate epithelial barrier damage by high glucose concentration in murine models.[58] In contrast, overexpression of syndecan-1 in cell culture

prevented bacterial translocation and promoted maintenance of the intestinal barrier as indicated through TEER and fluorescein isothiocyanate (FITC)-dextran flux measurement.[16] In further support of its protective role, the anchored, unshed form of syndecan-1 significantly lessened expression of inflammatory cytokines and inhibited the production of the chemokine CXCL-1, which depleted neutrophil migration to the intestinal barrier.[59] In a murine model of colitis, the addition of anchored syndecan-1 improved disease activity and histological features.[59]

Studies have also indicated the ability of syndecan-1 to affect bacterial translocation as a result of barrier dysfunction.[60,61] However, due to the basolateral location of syndecan-1 on the enterocyte, direct interaction with bacterial products on the apical side is likely limited. Further investigation links syndecan-1 to act in synergy with tight junction complexes located closer to the apical side through Stat3 signaling.[16] Specifically, syndecan-1 increased the expression of tight junctional proteins, i.e. ZO-1 and occludin, through the binding of Stat3 to promoter regions of *ZO-1* and *occludin* genetic loci.[16,62] As discussed below, studies investigating the interaction of syndecan-1 with growth factors also propose a migration of syndecan-1 to the apical side of the enterocyte.[63,64]

The GAG ectodomains of syndecan-1 are constituently shed in a regulated process by the proteolytic action of sheddases, a specific type of MMP near the plasma membrane of the epithelial cell.[65] These shed domains are soluble and able to migrate into the blood circulation. Pathophysiological events such as infection and inflammation can trigger the release of inflammatory cytokines, including tumour necrosis factor alpha (TNF-α) and interleukin 1β (IL-1β), which have been shown to enhance the activity of sheddases and therefore increase circulating, shed syndecan-1 levels.[66-68] An inflammatory state of the gastrointestinal tract, particularly characteristic to the subepithelial basal lamina as found in IBD, is proposed to increase

widespread shedding of syndecan-1 ectodomains.[69] Increased levels of TNF-α, also characteristic of the inflammatory state associated with IBD, have also been shown to suppress syndecan-1 protein expression in vitro.[70]

It has been proposed that the interaction of syndecan-1 with basic fibroblast growth factor (bFGF), a GAG-binding protein, may potentiate mucosal healing of ulcerative lesions in IBD.[63,64,71,72] In this mechanism, syndecan-1 migrates from the basolateral to apical side of the cell, where bFGF is located.[63,64] The syndecan-1 GAG ectodomains then modulate bFGF receptors and structural morphology, which in turn allows bFGF to bind to epithelial and stromal cells that support the healing process of ulcerations.[71,72] When not activated by syndecan-1, bFGF is proteolytically degraded and thus unable to initiate the tissue repair process.[73] Despite increases of bFGF circulating levels and presence on ulcers in patients with IBD, it is suspected that the beneficial properties of bFGF are not activated in mucosa with downregulated syndecan-1 expression.[72] Exogenous heparin, a TNF-α inhibitor, is purported to protect bFGF binding domains normally covered by syndecan-1.[72]

Further depletion of syndecan-1 in murine models alters cell morphology and organisation at the epithelial barrier. A loss of syndecan-1 in murine epithelial cells yielded abnormal regenerative cell growth.[74] These effects may be of interest in the study of coeliac disease, as its hallmarks are characterised by disorganised tissue morphology; as previously mentioned, crypt hyperplasia, villous atrophy, and leukocyte infiltration from the lamina propria are defining histological features.[75] Villous atrophy, or the stunting of normal finger-like projections of the epithelial cell barrier into the gut lumen, is proposed to be due to cell matrix expansion rather than shortening.[76] According to this mechanism, cell fibril networks and gel matrices grow, which adds to the volume of the intervillous lamina propria; this process can be visualised as a cake rising in

the baking process.[76] Subsequent "flattening" of the villi and decrease of crypt height occurs through matrix "shaping" via cytokine production and the recruitment of inflammatory cells.[76] In support of this theory, increased GAGs in the lamina propria of coeliac disease patients localised with the aggregation of syndecan-1-positive cells in a syncytial manner.[76] This creates an anionic, negatively-charged environment that expands the gel matrix and develops favourable conditions for pathological protein (i.e. albumin) leakage from the lamina propria, across the intestinal barrier, and into the gut lumen.[76] Also consistent with these results is the aggregation of plasma cells in the lamina propria as part of gluten-induced matrix-deposition found in coeliac disease.[77] Shed syndecan-1 ectodomains may also contribute to the innate immune response to gliadin as well as the extracellular release and activation of tTG.[76] To date, the only existing study that characterises circulating syndecan-1 levels in coeliac disease examines paediatric samples.[78] Heightened levels of syndecan-1 correlated to mucosal damage and were significantly different than levels found in children with non-specific abdominal pain.[78] There are currently no existing studies that examine circulating syndecan-1 levels in NCWS.

The evidence of syndecan-1 as a reliable marker of disease remains strongest for IBD, presumably due to the proteoglycan's close association with inflammatory cascades. Two studies have measured marked increase of circulating syndecan-1 levels in IBD patients compared to healthy individuals.[68,79] These studies propose that syndecan-1 may be an effective marker of disease severity. Another study of Crohn's disease patients observed significantly lower mucosal syndecan-1 levels and heightened circulating syndecan-1 levels compared to patients with intestinal tuberculosis, which is also characterised by inflammation.[80] Heightened heparanase activity, which potentiates shedding of syndecan-1 ectodomains, was found in both the circulation and mucosa in Crohn's disease patients.[80] These observations were not found in intestinal

tuberculosis or functional bowel cohorts, and correlated to Crohn's disease activity and injury severity.[80] These results support a specific role of syndecan-1 in IBD pathogenesis.

Syndecan-1 levels have also been measured in response to anti-TNF-α therapy using infliximab and adalimumab in patients with deep remission of Crohn's disease and in patients with ulcerative colitis.[63,64] Compared to a control group with active Crohn's disease, anti-TNF-α treatment yielded lower syndecan-1, TNF-α, and bFGF levels in deep remission patients, which may indicate a significant degree of inflammatory control and mucosal healing.[64] No significant differences in syndecan-1 or bFGF levels were found between infliximab and adalimumab treatments in patients who were in remission.[64] Ulcerative colitis patients also had significantly decreased levels of syndecan-1, TNF-α, and bFGF levels after the administration of infliximab, though TNF-α levels were first observed to decrease, followed by syndecan-1 and bFGF.[64] The authors speculate that differences in results can be attributed to differences in disease mechanisms or a consequence of inflammatory control.[64] Further studies that characterise syndecan-1 levels in response to anti-TNF-α therapy in IBD patients may elucidate disease-related mechanisms and further support its role as a measure of mucosal healing.

Circulating syndecan-1 levels have not been explored in models of exercise-induced gastrointestinal syndrome, though have been investigated in acute hypobaric hypoxia (i.e. environmental atmosphere at 4500 m for 2-4 hours), which is proposed to degrade the endothelial glycocalyx.[81] Plasma concentrations of syndecan-1 were increased compared to normal baseline conditions in an acute hypobaric hypoxic environment.[81] In this respect, heightened syndecan-1 levels were related to the degree of endothelial degradation. A similar mechanism can be inferred in a state of ischaemia as observed in exercise-induced gastrointestinal syndrome, as intestinal

epithelial cells may shed syndecan-1 in response to the hypoxic environment produced by splanchnic hyperperfusion.

1.3.3.3 Intestinal Fatty Acid-Binding Protein (I-FABP)

Intestinal fatty acid-binding protein (I-FABP) or fatty acid-binding protein 2 (FABP2), is expressed throughout the small and large intestines.[82,83] I-FABP belongs to the fatty acid binding protein family, which are proteins that primarily chaperone lipids within cells.[82] Other potential intracellular involvements of this family of proteins include cholesterol regulation and phospholipid metabolism, signalling processes, and enzymatic activity.[82,84,85] Upon damage of the enterocyte, I-FABP is released into the circulation.[17] Due to its small size of 15 kilodaltons (kDa), I-FABP is able to rapidly diffuse from the interstitium into the vascular space.[17]

I-FABP is expressed in the intestinal tract, primarily in the small intestine and to a lesser extent in the large intestine. A study of autopsy samples observed I-FABP tissue concentrations (expressed in μg/g ww) of 2.22 in the duodenum, 4.79 in the jejunum, 1.04 in the ileum, and 0.27 in the proximal colon, and 0.25 in the distal colon.[86] A follow-up study confirmed the highest concentration of I-FABP in the jejunum, lower concentrations in the ileum and duodenum, and trace amounts in the large intestines.[87] As this distribution suggests, circulating I-FABP levels are primarily indicative of small intestinal injury and may theoretically be a valuable marker of gastrointestinal disorders featuring small bowel enteropathy.

The strongest evidence for the use of I-FABP as a circulating marker of disease comes from studies investigating coeliac disease. As previously described, coeliac disease is characterised by small bowel enteropathy, which I-FABP is purported to indicate. Several studies have observed increased circulating I-FABP levels in coeliac disease patients compared to normal healthy

controls, as summarised in Table 1.[2,87-93] As indicated in Table 2, some studies also investigated I-FABP levels in untreated, newly-diagnosed coeliac patients before and after treatment (i.e. adherence to a gluten-free diet); significant differences in I-FABP levels were observed pre- and post-treatment.[87,88,92] Significant correlations of I-FABP levels to other coeliac disease-specific markers were also observed (Tables 1 and 2).[2,88,92] In this regard, I-FABP may have promising utility as an indicator of disease activity and mucosal healing in coeliac disease patients. In one such trial, I-FABP has been used as a marker of responsiveness to gluten in a study involving adult coeliac disease patients undergoing a gluten challenge.[94]

Two studies have also investigated circulating I-FABP levels in relatives of individuals with coeliac disease.[95,96] Relatives of coeliac disease patients may have genetic predisposition (i.e. HLA-DQ2 or -DQ8 genetic polymorphism) to develop gluten intolerance preceding coeliac disease. These studies explore the possibility of circulating I-FABP levels as a predictive indicator of disease, as the marker is purported to reflect early intestinal epithelial cell damage. The first study detected significantly elevated circulating I-FABP levels in first-degree symptomatic relatives compared to controls, though no differences were found between controls and asymptomatic relatives.[95] In symptomatic relatives, anti-tTG antibody levels significantly correlated with I-FABP levels at the beginning of the study. After a period of adherence to gluten-free diet in these subjects, both I-FABP and anti-tTG antibody levels significantly decreased and improvement in symptoms was observed.[95] Biopsy data confirmed normal duodenal histology in the majority of previously symptomatic relatives.[95] The second study observed a relationship between circulating I-FABP levels and the presence of HLA-DQ2 or -DQ8 genetic polymorphism as well as increased intraepithelial lymphocytes in first-degree relatives of coeliac patients.[96] However, no healthy controls for comparison were featured in this study.[96] The results of both

Table 1. Studies investigating I-FABP levels in coeliac disease versus healthy controls.

Study, year	n: untreated coeliac; healthy control	I-FABP level pg/mL: untreated coeliac; healthy control[a]	Significance[b]	Significant coeliac-specific marker correlations with I-FABP
Uhde et al., 2016[2]	40; 40	2600; 1200	p<0.0001	Anti-tTG IgA (r=0.559, p<0.001)
Derikx et al., 2009[87]	13; 26	784.7±145.5;172.7±20.2	p<0.001	ND
Adriaanse et al., 2013[88]	96; 69	691 IQR 447-1266;178 IQR 126-286	p<0.001	Anti-tTG IgA (r=0.403, p<0.01); Marsh grade (r=0.265, p<0.05)
Hoffmanova et al., 2015[89]	43; 41	1700±1400; 800±700	p<0.01	ND
Botasso Arias NM et al., 2015[90]	40; 42	2898.89 range 616-7295; 1356.49 range 256.51-3433.33	p<0.0001	ND
Vreugdenhil AC et al., 2011[91]	68; 19	458; 20	p<0.001	ND
Adriaanse et al., 2017[92]	90; 80	726 IQR 458-1024; 218 IQR 143-323	P<0.001	Anti-tTG IgA (r=0.346, p<0.01)
Oldenburger et al., 2018[93]	95; 161	650 IQR 406-1031; 263 IQR 141-422	p<0.0001	ND

I-FABP, intestinal fatty-acid binding protein; IQR, interquartile range; ND, none detected or investigated.

[a]Mean or median circulating (i.e. plasma or serum) I-FABP concentration from text or tables, or extrapolated from figures.

[b]Significance of differing I-FABP levels between untreated coeliac patients and healthy controls.

Table 2. Studies investigating I-FABP levels in coeliac disease patients pre- and post-treatment with a gluten-free diet.

Study, year	Duration of GFD	n: untreated coeliac disease; after GFD treatment	I-FABP level pg/mL: untreated coeliac; after GFD treatment[a]	Pre-to-post treatment I-FABP level significance[b]	Significant coeliac-specific marker correlations and/or significance with I-FABP
Derikx et al., 2009[87]	1 year	10; 10	725.5±134.4; 266.8, range 41.0-642.6	$p<0.001$	ND
Adriaanse et al., 2013[88]	1 year	96; 69	691; 178	$p<0.001$	Anti-tTG IgA ($r=0.403$, $p<0.23$); Marsh grade ($r=0.405$, $p<0.001$, $n=49$)
Adriaanse et al., 2017[92]	26 weeks	79; 76	726 IQR 458-1024; 231 IQR 185-318	Reached $p<0.05$ significance at 3 weeks	Various significant Marsh stratifications

GFD, gluten-free diet; I-FABP, intestinal fatty-acid binding protein; IQR, interquartile range; ND, none detected or investigated.
[a]Mean or median circulating (i.e. plasma or serum) I-FABP concentration.
[b]Significance of differing I-FABP levels before and after GFD treatment.

studies support the use of I-FABP levels to indicate early intestinal epithelial damage and mucosal barrier healing.

The only study to date that investigates circulating I-FABP levels in a NCWS cohort observed a significant increase in I-FABP levels compared to both healthy controls and coeliac disease patients.[2] The authors speculate that the jejunum (where, as previously mentioned, the highest concentrations of I-FABP produced by enterocytes have been detected) may be a potential site of mucosal damage in NCWS, due to increased I-FABP levels and the lack of abnormal mucosal features in duodenal biopsies of the NCWS cohort.[2] Positive, significant correlations were observed between I-FABP levels and LBP as well as sCD14, with a lesser extent to IgM-class antibody activity towards microbial antigens.[2] Taken together, these results point towards a systemic immune response in the NCWS cohort. Additional studies that integrate biopsy data and I-FABP levels in well-characterised NCWS cohorts are warranted to determine disease location if these individuals are specifically reacting to wheat-related products. As previously mentioned, individuals with suspected NCWS may also have dietary triggers (e.g. FODMAPs) that are not specifically gluten- or wheat-related. Future studies that profile I-FABP levels in suspected individuals, in which alternative triggers have been concluded, may determine whether these triggers might also contribute to epithelial damage of the small intestines.

In contrast to coeliac disease, there is limited evidence to determine whether circulating I-FABP may be a useful biomarker in Crohn's disease. A preliminary study of Crohn's disease patients suggested that I-FABP may be a promising indicator for disease activity.[97] Circulating I-FABP levels were significantly elevated compared to patients in remission and controls, though no significant difference of levels was observed between controls and patients in remission.[97] A positive correlation was also observed between I-FABP levels and CDAI scores as well as CRP

levels.[97] However, a follow-up study featuring IBD patients, which classified Crohn's disease patients by endoscopy and a combined score (i.e. clinical activity index, faecal calprotectin score, and circulating CRP levels), observed conflicting evidence.[98] I-FABP levels in these patients did not differ in active disease compared to remission in classification by endoscopy.[98] Based on the combined score, patients with active disease had higher I-FABP levels than those in remission.[98] Disease location did not significantly affect I-FABP levels.[98] As these two studies utilised different clinical criteria to assess Crohn's disease patients, further study is warranted to integrate the current evidence and clarify whether circulating I-FABP is of value in the assessment of Crohn's disease activity.

As I-FABP is primarily distributed in the small intestines, it may not be a promising circulating marker to indicate disease activity in ulcerative colitis. As previously described, one study classified disease activity, indicated by endoscopy or by a combined clinical score, and I-FABP levels in both Crohn's disease and ulcerative colitis patients.[98] I-FABP levels in ulcerative colitis patients did not differ in active disease compared to remission when classified by endoscopy.[98] Unlike patients with active Crohn's disease, ulcerative colitis patients with active disease, as determined by combined clinical score, had significantly lower I-FABP levels than those in remission.[98] Extent of disease in ulcerative colitis patients did not influence I-FABP levels significantly.[98] In the circumstance of comorbidity of IBD with immune deficiency, one study found that elevations in I-FABP were not observed in HIV patients with ulcerative colitis compared to healthy individuals.[99]

One study acknowledged the lack of utility of circulating I-FABP as an indicator of ulcerative colitis, though found it to be a potential marker for ileitis.[100] Circulating I-FABP concentration was significantly higher in patients with a severe acute form of the disease versus

those in patients with a mild form.[100] In particular, patients with pancolitis had significantly elevated I-FABP levels compared to patients with left-side colitis and healthy controls.[100] Since a previous study reported ileal inflammation in 17% of ulcerative colitis patients, with the majority (94%) of this group experiencing pancolitis, the authors claim that I-FABP may instead be an important indicator of ileal disease in this patient subset and a reliable marker of extended inflammation that correlates well with CRP.[100,101] These observations are consistent with I-FABP levels being primarily indicative of small bowel enteropathy.

Several studies that investigate exercise-induced gastrointestinal syndrome observed increases in I-FABP levels pre- to post-exercise intervention, as outlined in Table 3.[40,102] As biopsy collection is atypical of exercise interventions, I-FABP has arisen as a circulating marker of choice to detect small bowel enteropathy relating to reductions in villous microvascular flow and subsequent ischaemic damage.[40] Heat (above 30°C) and vigorous activity such as running and cycling seem to exacerbate the most significant response in subjects, as the highest concentrations of circulating I-FABP were observed in these conditions.[40]

Table 3. I-FABP levels in exercise-induced gastrointestinal syndrome. Adapted from Costa et al., 2017 with modifications.[40]

Study, year	Population	Exercise protocol	Body temperature	Δ pre- to post-exercise I-FABP concentration (otherwise specified)
Sessions et al., 2016[103]	n = 7 endurance trained male and female participants	60 minutes running at 70% VO_{2max} in 30°C T_{amb} (12% to 20% RH)	T_{re}: 39.5°C	88 pg/mL[d, ns]
van Wijck et al., 2013[104]	n = 12 recreationally trained male participants	30 minutes resistance exercise. T_{amb} not reported	Not measured	90 pg/mL[d]*
van Wijck et al., 2012[105]	n = 9 male cyclists and triathletes	60 minutes cycling at 70% W_{max} · T_{amb} not reported	Not measured	179 pg/mL[d]*
Lis et al., 2015[106]	n = 13 male and female competitive cyclists	45 minutes steady state cycling at 70% W_{max} + 15 minutes time trial. T_{amb} not reported	Not measured	Steady state: 139 pg/mL[d, stat-x] Time trial: 210 pg/mL[d, stat-x]
Morrison et al., Part A, 2014[107]	n = 8 recreationally trained male participants undertaking < 3 exercise sessions per week	15 minutes cycling at 50% HRR + 60 minutes running (30 minutes at 80% HRR + 30 minutes TT) + 15 minutes cycling at 50% HRR in 30°C T_{amb} (50% RH)	T_{oes}: 38.6°C	283 pg/mL[d]*
Barberio et al., 2015[108]	n = 8 endurance trained male participants	Running at 78% VO_{2max} (4 mMol/L blood lactate) until T_c increases 2.0°C or volitional exhaustion (24 minutes) in 40°C T_{amb} (40% RH)	T_{re}: 39.0°C	297 pg/mL[d]*
van Wijck et al., 2011[109]	n = 20 healthy male participants	60 minutes cycling at 70% W_{max} · T_{amb} not reported.	Not measured	306 pg/mL*

Morrison et al., Part B, 2014[107]	n = 7 recreationally trained male participants undertaking > 6 sessions per week	15 minutes cycling at 50% HRR + 60 minutes running (30 minutes at 80% HRR + 30 minutes TT) + 15 minutes cycling at 50% HRR in 30°C T_{amb} (50% RH)	T_{oes}: 38.6°C	806 pg/mL[d]*
Snipe et al., 2018[102]	n = 10 non-heat acclimatised endurance-trained runners	2 hours running at 60% VO_{zmax} in temperate 20°C and hot 35°C T_{amb}	Temperate T_{re}: 37.5-38.25°C Hot T_{re}: 37.5-39.25°C	Temperate: 1230 pg/mL* Hot: 274 pg/mL*

I-FABP, intestinal fatty-acid binding protein; T_{amb}, ambient temperature; RH, relative humidity; T_{re}, post-exercise (or peak) rectal temperature; W_{max}, watt maximum; HRR, heart rate reserve; T_{oes}, post-exercise (or peak) oesophageal temperature.
[a]In order of exercise-associated epithelial injury (i.e. plasma or serum I-FABP concentration), otherwise specified.
[b]Post-exercise (or peak) body temperature of respective measurement technique.
[c]Data (mean or median) from text and tables, or extrapolated from figures to the nearest approximate value (pre-exercise resting to post-exercise peak value difference).
[d]Values of control or placebo group/trial of the respective intervention study.
*Significant pre- (rest) to post-exercise increase, [ns] no significant difference pre- (rest) to post-exercise. [stat-x] no statistical analysis provided or statistical analysis unclear.

1.3.4 Circulating Markers of Microbial and Macromolecule Translocation

1.3.4.1 Soluble CD14 (sCD14) and Lipopolysaccharide Binding Protein (LBP)

Soluble CD14 (sCD14) and lipopolysaccharide binding protein (LBP) are two endogenous proteins commonly studied together to assess levels of circulating bacterial products, namely LPS (i.e. endotoxin) from gram-negative bacteria. Primarily produced in hepatocytes, LBP has a high affinity for circulating LPS and is typically upregulated in response to increased concentrations.[110,111] The presence of LBP is necessary to facilitate the binding of LPS to CD14, a glycoprotein that exists as a membrane-associated form (mCD14) primarily on monocytes/macrophages (considered CD14-positive cells) and as a soluble, extracellular form (sCD14) released from the membrane.[112,113] Both forms of CD14 are upregulated by the presence of LPS and other bacterial wall components.[114] LBP and sCD14 also facilitate the process of transporting LPS to be in close proximity with toll-like receptor 4 (TLR4), a receptor on innate immune cells, and transferring it to the TLR4-myeloid differentiation factor 2 (MD-2) complex for antigen recognition.[115] Activation of TLR4 leads to intracellular cascades that initiate the production of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) transcription factors, thereby promoting the production of pro-inflammatory cytokines that modulate intestinal permeability.[116]

sCD14 can contribute to local or systemic immune effects. On a local, intestinal level, LPS-dependent mast cell activation may be provoked by sCD14 as observed in normal and Crohn's disease tissue.[117] On a systemic level, sCD14 is a potential non-specific marker of monocyte activation in the circulation, as inflammatory cytokines can stimulate peripheral blood mononuclear cells (PBMCs) to release sCD14.[118] LBP is purported to have a regulatory role in the inflammatory cascade according to endotoxin levels. In the case of high circulating endotoxin

levels, LBP is upregulated and enhances the immune response, as previously described.[114] However, levels of LBP may not always be directly reflective of circulating endotoxin levels nor does it always contribute to immune activation. LBP can remain at high levels despite low- to mid-grade endotoxaemia and contribute to an inhibitory effect on the immune response towards endotoxin in these conditions.[31,119,120] Thus, the ratio of LBP to circulating endotoxin is important to consider and may provide insights into the current status of disease, particularly with regards to immune activation.

LBP and sCD14 have been used together recent studies as surrogate markers to identify bacterial translocation. As LPS is a common laboratory contaminant, the direct measurement of the endotoxin, usually by immunoassay, may produce unreliable measurements. LBP and sCD14 also have longer half-lives (24-48 hours) than endotoxin itself (1-3 hours), and may be indicative of long-term endotoxin exposure.[31,121,122] Usage of these markers to indicate microbial translocation across the gut barrier have described intestinal disease activity in HIV patients and recent studies have applied these markers to other conditions, including gastrointestinal cohorts.[2,4] However, there are few studies to date that investigate LBP and sCD14 in coeliac disease and NCWS. As previously described, both LBP and sCD14 had significant, positive correlations with I-FABP in a study of individuals with NCWS.[2] This result suggests a link between intestinal epithelial damage, as measured by I-FABP, and systemic immune activation by microbial translocation as indicated by LBP and sCD14. Another study observed heightened levels of sCD14 along with I-FABP in both treated (i.e. adherence to a gluten-free diet) and newly diagnosed, untreated coeliac disease compared to healthy individuals.[89]

The strongest evidence for LBP and sCD14 as markers of dysfunction in gastrointestinal disease comes from investigations featuring IBD patients who have active disease. One particular

study found heightened mean LBP and sCD14 plasma levels in patients with active Crohn's disease and ulcerative colitis compared to healthy controls.[31] Levels of both markers were also higher in active disease versus inactive disease.[31] The results of both markers were in concordance with each another as well as with circulating endotoxin levels, though levels were slightly elevated in Crohn's disease compared to ulcerative colitis.[31] Normal levels of endotoxin, LBP, and sCD14 were recovered after treatment of active disease, though among various types of medication, immunomodulators in particular appeared to affect LBP levels most significantly.[31] Serum concentrations of endotoxin, LBP, and sCD14 remained similar to those in healthy controls in both inactive Crohn's disease and ulcerative colitis patients.[31] However, a moderate increase in LBP was observed in patients with inactive Crohn's disease with no change in inflammatory effects.[31] This may support an inhibitory role of LBP in low to moderate endotoxaemia. In patients with ulcerative colitis, differences in endotoxin and LBP levels were observed in distal versus fully extended disease.[31] Heightened LBP levels in extensive disease reveals the extent of inflammation in ulcerative colitis; as more mucosa is affected, the greater the potential there may be for LPS leakage across the intestinal barrier.

A follow-up study investigating LPS-associated markers in endotoxaemic patients with Crohn's disease found similar overall results in serum LBP levels, as these were significantly higher in both active and inactive Crohn's disease compared to healthy controls.[123] However, sCD14 levels were significantly lower in both active and inactive disease compared to healthy controls.[123] Longitudinal samples were available from patients who had a relapse of Crohn's disease and subsequent clinical remission; both LBP and sCD14 levels decreased after patients achieved remission.[123] Based on disease activity as indicated by CDAI scores, receiver operating characteristic (ROC) analysis revealed sCD14 and high-sensitivity CRP to be more accurate to

identify patients with active disease than LBP. Levels of LBP in inactive disease were associated with disease behaviour; the highest levels were observed in the penetrating disease pattern, rather than inflammatory or stenosing forms.[123] Among high-sensitivity CRP, LBP, and sCD14 levels, LBP emerged as the best marker for identifying clinical relapse for patients previously in remission in univariate analysis.[123] Kaplan-Meier analysis revealed concentrations of all three markers to be significantly associated with 12-month clinical relapse, and the combination of markers along with a high past relapse frequency as significant determinants for time to clinical relapse.[123] Further Cox-regression analysis demonstrated LBP, sCD14 and a high relapse frequency, though not high-sensitivity CRP, to be independently associated with the probability of clinical relapse.[123]

A study of paediatric IBD patients also detected elevated serum LBP levels in patients compared to those in healthy controls, though, unlike the previous studies discussed, these results were not related to disease activity or location.[124] Thus, LBP levels were significantly increased in both active disease and clinical remission compared to controls and levels also did not vary with disease location or behaviour.[124] However, it should be noted that different criteria were used to categorise clinical disease activity than past studies (e.g. CDAI), as this study used specific paediatric criteria outlined by Griffiths et al. to describe clinical progression in children with Crohn's disease.[124,125] As with the first study discussed, LBP levels were greater in Crohn's disease than in ulcerative colitis. Heightened levels of LBP, along with heightened anti-EndoCAb IgA antibody levels, were associated with growth failure in children with Crohn's disease.[124] These results suggest that endotoxin exposure in the absence of malnutrition may have an effect on growth progression in Crohn's disease patients.[124]

Taken together, circulating LBP and sCD14 levels may provide promising information about disease activity and may serve as a potential indicator of relapse in IBD patients. As observed

in the first study discussed, immunomodulators may have marked effects on LBP levels that are independent of the control of inflammation. Further study is needed to confirm previous results as well as the effects that certain medications may have on circulating levels of these markers in conjunction with inflammatory activity shown by colonoscopy and intestinal ultrasound. In addition, further studies should determine the cross-sectional versus longitudinal utility of these markers to determine disease activity.

1.3.5 Additional Circulating Markers of Intestinal Barrier Dysfunction

1.3.5.1 Antibodies Directed Towards Microbial-Related Antigens

Circulating antibodies directed towards microbial-related antigens have also been considered indicators of translocation across the intestinal epithelial barrier. These antibodies may be directed towards specific (e.g. EndoCAb antibodies produced in response to endotoxin core protein) or nonspecific (e.g. anti-flagellin antibodies produced in response to flagellated bacteria) parts of the microbe.

Antibodies (IgG-, IgM-, and IgA-class) directed towards endotoxin core protein (EndoCAb) have been used to determine exposure and resulting adaptive immune activation to LPS in the acute phase of intestinal barrier damage.[2,126] LPS stimulation of the innate immune response (which involves LBP and sCD14, as previously discussed), under normal conditions or in response to infections, may lead to the evolution of an adaptive immune response, which includes specific B cell production of antibodies targeted towards the core protein of the endotoxin; these antibodies serve to neutralise LPS.[124,127] In IBD patients, EndoCAb levels have been negatively correlated to levels of LPS, which may be indicative of the neutralising role of EndoCAb in LPS clearance.[128] Heightened levels of EndoCAb antibodies have also been associated with chronic inflammatory conditions with persistent endotoxin exposure, such as

cystic fibrosis.[129] In a cohort of NCWS patients, heightened levels of EndoCAb IgM antibodies have been observed, which differed significantly from those in coeliac disease.[2] This may be indicative of a chronic, inflammatory state that may be characteristic of purported NCWS. Taken together, the evidence suggests that EndoCAb may be an indirect marker of mucosal barrier function through its indication of an acute-phase response to endotoxin or as a marker of chronic inflammation.

Microbial-related markers have also been utilised to distinguish disease subtypes. One such example includes anti-*Saccharomyces cervisiae* (ASCA) antibodies, which are directed towards oligomannosidic cellular wall components of the yeast *S. cerevisiae*. These antibodies have been used in conjunction with endogenous perinuclear anti-neutrophil cytoplasmic antibodies (pANCA) as indicators to distinguish between IBD classifications.[130] The ASCA-positive/pANCA-negative phenotype is characteristic of Crohn's disease, whereas the ASCA-negative/pANCA-positive distinguishes ulcerative colitis.[130] The presence of these antibody markers, indicative of an adaptive immune response, suggests accumulated products of microbial translocation in a location-specific manner that may distinguish subtype. One study claims that a panel of anti-ASCA antibodies, in conjunction with other microbial-related antibodies, can predict Crohn's disease behaviour at a median of 6 years before disease diagnosis.[131] Further study to confirm these biomarkers' clinical utility to distinguish subtypes of disease is necessary.

1.3.5.2 Citrulline

Citrulline, a non-essential amino acid, has been proposed as a marker of enterocyte function specific to the upper villi of the small bowel.[132,133] Citrulline is primarily produced from the conversion of glutamine in the enterocyte and released into the circulation. It is then taken up by

the kidneys and converted into arginine. Circulating citrulline concentration is, therefore, assumed to reflect the equilibrium between its synthesis by enterocytes and conversion into arginine.[133] Citrulline concentrations have also been correlated with small bowel length and functional enterocyte mass.[133] It is proposed that a loss of epithelial mass in the small bowel results in increased intestinal permeability, which is reflected by low levels of circulating citrulline.[126]

One pilot study in patients hospitalised with septic shock suggested that plasma citrulline correlates with bacterial translocation.[134] Additional studies are needed to clarify the role of citrulline as a potential circulating biomarker of intestinal permeability, as differences in clinical states may have marked effects on its levels. Integration of multiple factors, including details of specific intestinal pathophysiology, citrulline metabolism and clinical status are essential for consideration in analysis.

1.3.5.3 Dietary Macromolecules and Associated Antibodies

As a result of an impaired intestinal barrier, dietary macromolecules themselves may also be present in the circulation. Furthermore, these macromolecules can mount an immune response by means of specific B cell antibody production. The actual presence of dietary macromolecules (e.g. peanut allergen Ara h 6) as well as associated antibodies targeted to dietary antigens (e.g. anti-gliadin antibodies in response to gliadin) may respectively be direct and indirect markers of intestinal barrier dysfunction.

There is currently limited evidence that food-related proteins may be reliable markers of increased intestinal permeability. A trial primarily involving increased gastrointestinal permeability induced by exercise investigated the presence of circulating gliadin peptides as potential biomarkers, though this trial did not assess other intestinal permeability markers for

comparison.[135] The most compelling evidence to date to support circulating dietary protein as an indicator of intestinal permeability has involved the peanut allergen Ara h 6. After ingestion of peanuts, increased circulating levels of Ara h 6 were observed in study participants during an endurance exercise trial, in which the associated mechanisms of exercise-induced gastrointestinal syndrome (as previously discussed) are implicated.[136] The increased levels, measured at 60 minutes cycling at 70% of maximal workload, were significant though variable compared to baseline and correlated with other intestinal permeability markers, including circulating I-FABP levels.[136] These significant correlations may indicate its potential as a marker of intestinal permeability. However, there is the possibility that a low clearance rate of Ara h 6 may explain these results. There is currently limited knowledge on the absorption and clearance mechanisms of the allergen.

Anti-gliadin antibodies, which are produced as part of the adaptive immune response to gliadin peptides, are an indirect measure of intestinal barrier dysfunction. These antibodies are formed when dietary gliadin translocates across an impaired intestinal barrier and initiates an immune response leading to B cell antibody production. Studies beyond primary gastrointestinal conditions (e.g. primary sclerosing cholangitis and cirrhosis) have utilised anti-gliadin antibodies as indicators of an impaired intestinal barrier and resulting immune response, though they remain of particular interest in the study of NCWS due to a suspected immune response to gliadin differential from coeliac disease.[2,137,138] Elevated anti-gliadin antibodies have also been observed in patients with IBD.[139]

1.3.5.4 D-lactate

D-lactate, a small 90 kDa molecule produced from colonic bacterial fermentation, has been a proposed marker of mucosal barrier dysfunction that can be measured in the circulation.[126,140] D-lactate cannot be endogenously produced in large quantities, unlike its stereoisomer L-lactate, though evidence suggests D-lactate is produced in very small concentrations through the glyoxalase pathway, an aldehyde metabolism-related pathway.[141] In this respect, the presence of D-lactate in the body is predominantly attributed to a bacterial source. D-lactate is purported to translocate from luminal bacteria across a compromised colonic barrier and into the circulation. However, as D-lactate levels primarily depend on the quantity and activity of colonic bacteria, circulating levels may not be a direct measure of intestinal barrier dysfunction.

D-lactate has emerged as a potential biomarker, particularly in the study of intestinal ischaemia, which potentiates increased barrier permeability (as previously discussed in the mechanism associated with exercise-induced gastrointestinal syndrome).[142-144] However, the results of studies should be cautiously considered, as levels may instead be indicative of bacterial load and activity. Future research is necessary to clarify the utility of circulating D-lactate concentrations, especially in studies related to colonic permeability.

1.3.5.5 Glutathione S-transferases (GSTs)

Glutathione S-transferases (GSTs) of the α-subclass, in particular, are proposed indicators of intestinal epithelial cell damage. GSTs are cytosolic enzymes that play a primary role in detoxification, as they conjugate foreign electrophilic substrates, including free radicals, to glutathione.[145] GSTs also have peroxidase, isomerase, kinase-inhibition, and ligand-binding activities.[145] Of the four main subgroups (α, μ, π, and θ), αGST is mainly expressed in the

intestines, liver, and kidney.[146] As damaged cells of the intestines, liver, and kidney release cytosolic contents into the extracellular environment, the level of circulating αGST increases. Heightened circulating αGST has been documented in mesenteric ischaemia.[147] However, since αGST is also expressed in the liver and kidneys, it is not a specific indicator of enterocyte dysfunction and intestinal permeability.

1.3.5.6 Tight Junctional Proteins

Proteins that comprise tight junctions have also been measured as circulating markers indicative of intestinal barrier dysfunction. There are more than 40 cortical and transmembrane proteins that have been described as part of the tight junctional complex.[148] Occludin, claudins, marvelD3, and tricellulin belong to the transmembrane protein complex.[149] Cortical proteins, which link membrane proteins to actin cytoskeleton, include those of the zonula occludens (ZO) family; these are ZO-1, ZO-2, and ZO-3.[149] Expression of these proteins reflect epithelial paracellular integrity and their presence in circulation may implicate damage to tight junctional complexes necessary for maintaining epithelial barrier integrity. However, proteins of the tight junctional complex are widely expressed as they are found in both epithelial and endothelial architecture. As such, it can be assumed that the measurement of circulating tight junctional proteins is unlikely to directly express tissue-specific barrier integrity.

1.4 Discussion

Some, though not all, of the featured markers related to intestinal epithelial barrier dysfunction and resulting microbial translocation may provide utility in investigating gastrointestinal disease. There are varying degrees of scientific inquiry into featured markers, and

as such, additional study is warranted to bridge the gaps of knowledge with regards to their utility in specific diseases and conditions. A need for further inquiry is of particular importance in light of increasing popularity and usage of these markers, especially in the investigation of patient cohorts with extra-intestinal dysfunction.

As outlined in previous sections, the featured markers have been investigated in a variety of different disease contexts and studies. Some studies used cross-sectional marker data (i.e. collected at only one point in time) to reflect barrier integrity. Due to inter-individual differences that may occur, this classification of data should be cautiously interpreted. Variables such as fasting and time of day may affect levels of these markers. In this respect, longitudinal data for a particular subject may be able to track inter-individual differences over time and reveal potential sources of variation. In this respect, clinical decisions should not be solely based on a singular cross-sectional marker value.

Several factors should be considered in determining the usage of markers to indicate gastrointestinal disease, particularly with regards to clinical context. In this respect, it should be carefully considered whether the presence of these circulating markers truly reflect an impaired intestinal mucosal barrier. Certain therapeutics (e.g. immunomodulators) are known to influence circulating levels of these markers, particularly endogenous proteins (e.g. LBP) related to an inflammatory response. In addition, some circulating markers (e.g. syndecan-1, αGST, and tight junctional proteins) are not specific to intestinal epithelial cells. In this circumstance, adequate controls are needed to determine whether elevations in nonspecific markers are truly gastrointestinal-tract related. Markers may also be associated with comorbidities. For example, increased circulating levels of syndecan-1 have been associated with the progression of cancers, as it is involved in the differentiation and prognosis of tumours.[150-152]

As previously mentioned, there may be other factors that influence circulating levels of featured markers that are not specifically disease-related (e.g. time of day and fasting). Stress, which may be dietary or psychological, may also play a role in modulating permeability and thus contribute to changes in marker levels. One particular study found that 4-week consumption of low-fat yoghurt reduced levels circulating levels of LBP and sCD14 after study participants consumed high-fat and high-calorie meals, which "stressed" the digestive system.[153] Levels remained high in control groups that did not consume yoghurt. This clearly demonstrates the ability of dietary stress to influence intestinal barrier function, and in turn, featured markers. In respect to psychological stress, corticotropin releasing factor (CRF), a peptide hormone that is released in the stress response, has been shown to trigger increases in paracellular permeability through the release of TNF-α and protease secretion in a mechanism dependent on mast cells.[154]

It is evident that no singular circulating marker featured in this review is adequate to determine mucosal permeability and resulting microbial translocation. A combination of markers, in conjunction with clinical details, may be useful to detect features of gastrointestinal disease attributed to pathogenic processes associated with intestinal permeability and microbial translocation. Further studies to determine useful potential marker combinations and validate their clinical utility in specific gastrointestinal-related conditions are warranted.

CHAPTER 2

AIMS AND SIGNIFICANCE

2.1 Research Aim and Significance

As detailed in Chapter 1, there have been several proposed circulating candidate markers of intestinal barrier dysfunction, which include proteins associated with structural integrity, enterocyte damage, or microbial translocation. Some remain controversial due to methodological shortcomings in detection or investigation with serological samples from patients that have not been well-characterised clinically. As such, questions remain about the efficacy of certain candidate markers to detect features of gastrointestinal disease. Despite the lack of evidence, these markers have been used to study intestinal barrier integrity in numerous extra-intestinal conditions.

The main aim of this research is to evaluate current candidate markers of intestinal barrier dysfunction and characterise them in gastrointestinal disease and damage. The comparative study of featured markers in well-characterised disease states/conditions of damage and associated dietary or drug interventions will provide evidence to support or challenge current pathophysiological mechanisms associated with the intestinal barrier and determine whether the markers are effective in detecting dysfunction.

In the basic research as well as clinical setting, obtaining serum or plasma samples is less invasive, easier to acquire and maintain, and more cost-effective than biopsy samples. The results may potentially assist with diagnostic criteria and clinical evaluation if certain markers are found

to be efficacious in the characterisation of disease. If markers are effective, they can also be applied to the investigation of extra-intestinal disease states with greater confidence.

2.2 Markers and Models of Intestinal Barrier Dysfunction

As described in Figure 4, the featured protein markers under investigation fall into three main categories of intestinal barrier dysfunction: dysfunction of structural barrier integrity (zonulin, syndecan-1), enterocyte damage (I-FABP), and microbial translocation (sCD14 and LBP). All markers were investigated in patients with gastrointestinal diseases (i.e. ASUC, coeliac disease, Crohn's disease, and NCWS) and associated therapeutic conditions, which include dietary and drug interventions. All markers were also investigated in healthy individuals, which include those with exercise-induced gastrointestinal damage. The significance and mechanistic outcomes of investigating the featured markers in each disease state, along with associated dietary or drug interventions, are outlined in Table 4.

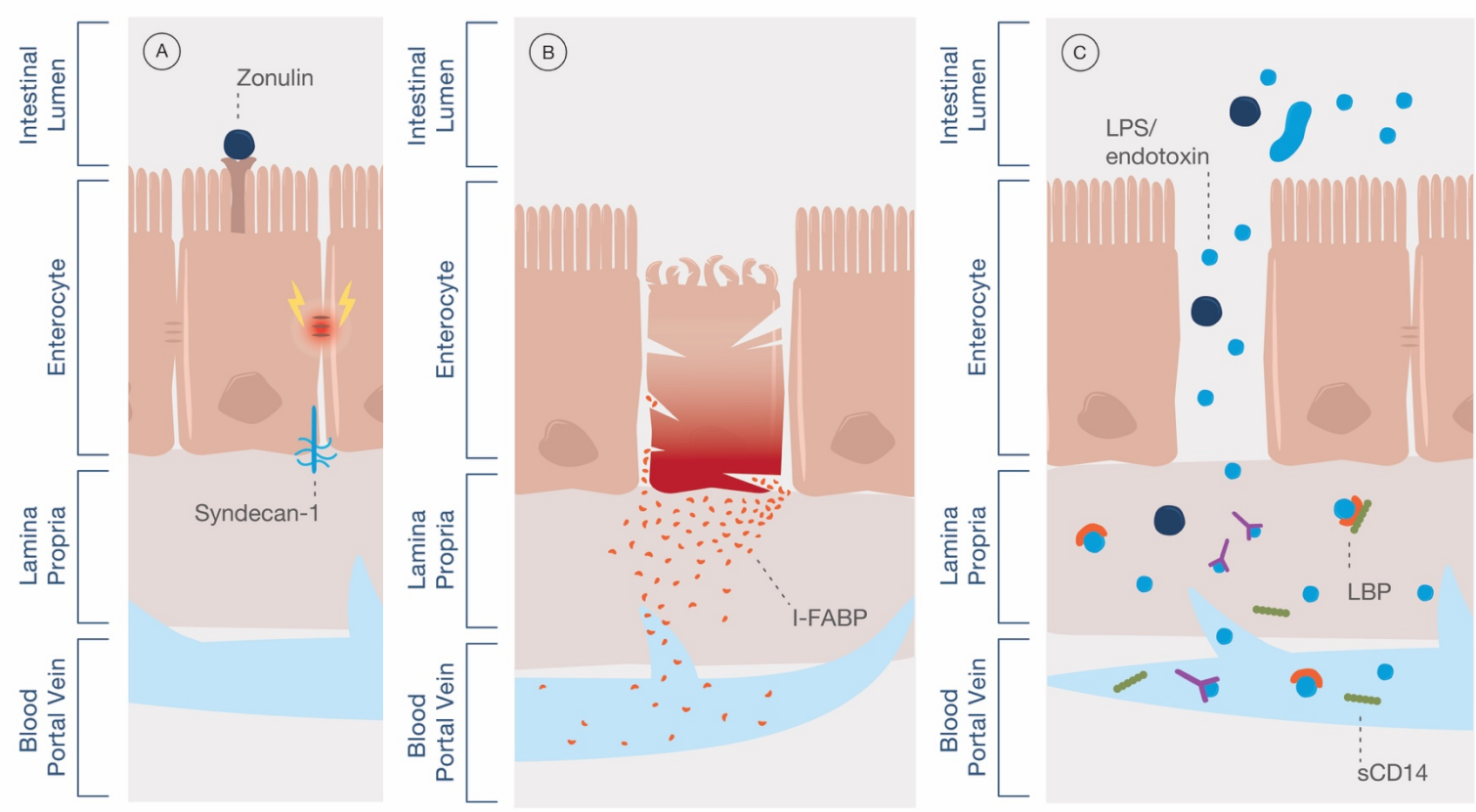

Figure 4. Specific circulating protein markers and associated indications of intestinal barrier dysfunction. Candidate circulating protein markers featured in this research are associated with different indications of intestinal barrier dysfunction. (A) Zonulin and syndecan-1 are associated with the dysfunction of structural barrier integrity. (B) I-FABP is an indicator of enterocyte damage. (C) LBP and sCD14 are markers of microbial translocation.

Table 4. Significance and mechanistic outcomes expected from the investigation of intestinal barrier dysfunction markers in experimental study cohorts.

Cohort	Disease classification	Significance and mechanistic outcome related to barrier integrity
Acute severe ulcerative colitis (ASUC)	Gastrointestinal; inflammatory	Determination of marker efficacy and inferences of associated barrier dysfunction mechanisms in colonic disease
Coeliac disease	Gastrointestinal; autoimmune	Characterisation of the intestinal barrier in stages of mucosal healing on a gluten-free diet with/without steroid intervention
Exercise-induced gut damage model	Unaffected; induced intestinal damage by exercise	Exploration of the effects of ischaemic stress, in conjunction with stress and diet, on the intestinal barrier without prior disease activity
Healthy	Unaffected	Establishment of normal levels for cross-sectional comparison with gastrointestinal disease cohorts
Non-coeliac wheat sensitivity (NCWS)	Gastrointestinal; controversial	Contribution of proposed disease triggers (i.e. gluten, FODMAPs) to barrier dysfunction

2.3 Chapter Aims and Significance

To gain insights into marker efficacy and clinical utility, levels of protein markers were measured in various exercise, dietary, and drug interventions as well as in baseline conditions prior to any intervention. The following section details the experimental aims and significance of each chapter.

2.3.1 Chapter 4: The Utility of Serum Zonulin as a Marker of Gastrointestinal Dysfunction

As previously detailed, controversy lies in zonulin's utility as a serum biomarker of tight junctional integrity and in the widely used commercial enzyme-linked immunosorbent assays (ELISAs) used for its assessment. Zonulin levels were primarily assessed amongst patients with gastrointestinal conditions and healthy individuals. However, several methodological inconsistencies arose as well as the assays' failure to detect zonulin. This chapter features cross-sectional analyses of zonulin levels in gastrointestinal disease cohorts and healthy individuals and specifies the observed methodological inconsistencies.

2.3.2 Chapter 5: Identification of Target Antigens in Zonulin Commercial Assays

This chapter details further inquiry into zonulin detection methodology. The nature of the detected antigens associated with commercial zonulin ELISA assays was investigated using immunoprecipitation followed by mass spectrometric analysis and sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by protein staining. Taken together with the results in Chapter 4, the evidence presented in this chapter resolves whether serum zonulin, as measured by current commercial assays, is a useful marker of gastrointestinal dysfunction.

2.3.3 Chapter 6: Assessment of Intestinal Barrier Integrity in Exercise-Induced Gastrointestinal Damage

Exercise-induced gastrointestinal damage, which occurs due to strenuous exercise in otherwise healthy individuals, is a valuable model to determine the effects of ischaemic stress on the intestinal barrier without prior disease activity. Adjunct factors, including high temperature and diet, may contribute to intestinal barrier modulation in a state of ischaemic stress, though the direct effects of these factors in the absence of disease are currently unknown. This chapter explores whether different ambient temperatures and FODMAPs affect intestinal barrier integrity in a state of ischaemic stress unrelated to disease activity.

2.3.4 Chapter 7: Assessment of Intestinal Barrier Integrity in Non-Coeliac Wheat Sensitivity/Irritable Bowel Syndrome

It has been previously reported that individuals with NCWS have elevated levels of enterocyte damage (i.e. I-FABP) and microbial translocation (i.e. LBP and sCD14) markers compared to coeliac disease patients and healthy individuals.[2] In the current study, the effects of gluten on the intestinal barrier, as indicated by the featured markers, are examined in patients with self-reported NCWS who had IBS symptoms based on Rome III criteria.[36] Further study of an additional NCWS cohort provides a unique opportunity to explore whether intestinal barrier effects are consistent in this controversial diagnosis. In addition, the effects of a background low-FODMAP diet on the intestinal barrier, compared to study participants' normal diets, are investigated.

2.3.5 Chapter 8: Assessment of Intestinal Barrier Integrity in the Treatment of Coeliac Disease

After the commencement of a gluten-free diet in the treatment of coeliac disease, longitudinal mucosal healing is usually assessed by intestinal biopsy. Circulating barrier dysfunction markers, especially I-FABP, may provide less invasive insights into the healing process. The current investigation assesses intestinal barrier integrity, as indicated by the featured markers, in the longitudinal mucosal healing of coeliac disease after patients began a gluten-free diet. Aside from I-FABP, other intestinal barrier dysfunction markers have not been well-characterised in previous studies investigating mucosal healing in the treatment of coeliac disease. The adjunct effects of the steroid budesonide, as indicated by the featured markers and biopsy data indicative of mucosal healing, are also investigated. Budesonide promotes a local anti-inflammatory effect that may support early mucosal healing, particularly in coeliac disease cases with overt malabsorption.[155] The results may provide novel insights into the effect of budesonide on the intestinal mucosal barrier and associated biological mechanisms. The results will also confirm if featured markers are indeed useful to indicate mucosal healing in coeliac disease patients undergoing treatment.

2.3.6 Chapter 9: Cross-sectional Analysis of Gastrointestinal Disease Cohorts and Healthy Controls

This chapter profiles featured markers of intestinal barrier dysfunction markers in different gastrointestinal diseases prior to any associated dietary or drug interventions. The cross-sectional analyses featured in this chapter determine whether singular markers or combinations of markers may be useful indicators of intestinal barrier dysfunction in gastrointestinal disease.

CHAPTER 3

GENERAL MATERIALS AND METHODS

3.1 Materials

3.1.1 ELISA Assays

Human intestinal fatty acid-binding protein/fatty acid-binding protein 2 (I-FABP/FABP2)	R&D Systems, USA
Human lipopolysaccharide binding protein (LBP)	Hycult Biotech, The Netherlands
Human soluble CD14 (sCD14)	R&D Systems, USA
Human syndecan-1 (CD138)	Diaclone, France
Human zonulin	CUSABIO, China
Human zonulin	Immundiagnostik AG, Germany

3.1.2 Protein Standards

Haptoglobin 1-1	Sigma Chemical Company, USA
Haptoglobin 2-2	Sigma Chemical Company, USA
Recombinant zonulin produced by pFastBac1 Bac-to-Bac Baculovirus Expression System	Kindly provided by Dr. Alessio Fasano, manufactured by GenScript, USA
Complement C3c	Athens Research, USA
Bovine serum albumin (BSA)	Sigma Chemical Company, USA

3.1.3 SDS-PAGE and Immunoprecipitation Materials

2-mercaptoethanol	Sigma Chemical Company, USA
Novex 16% Tris-Glycine Mini Gels, WedgeWell format, 15-well, 1.0 mm	Life Technologies, USA
Novex 8-16% Tris-Glycine Mini Gels, WedgeWell format, 15-well, 1.0 mm	Life Technologies, USA
Novex Mini Gel Tank Electrophoresis System	Life Technologies, USA
Novex Tris-Glycine Sodium Dodecyl Sulphate (SDS) Running Buffer (10X)	Life Technologies, USA
Novex Tris-Glycine Sodium SDS Sample Buffer (2X)	Life Technologies, USA
Precision Plus Kaleidoscope Prestained Protein Standards	Bio-Rad Laboratories, USA

3.1.4 Immunoblotting and Gel Staining Materials and Reagents

Acetic acid	Sigma Chemical Company, USA
Ethanol	Sigma Chemical Company, USA
Goat anti-mouse haptoglobin IgG heavy + light chains (H+L) secondary antibody, Alexa Fluor 680 conjugate	Life Technologies, USA
iBlot 2 Dry Blotting System	Life Technologies, USA

iBlot2 Transfer Stacks, polyvinylidene difluoride (PVDF) membranes	Life Technologies, USA
Li-Cor/Odyssey infrared image system	Li-Cor Biosciences, USA
Mouse anti-human haptoglobin IgG monoclonal antibody (clone 26E11)	AbFrontier, South Korea
Pierce Silver Stain Kit	Life Technologies, USA
Pierce Tris-Buffered Saline with Tween 20 (TBST) Buffer (20X)	Life Technologies, USA
SimplyBlue SafeStain	Life Technologies, USA

3.1.5 Buffers and Solutions

All solutions, including pre-manufactured assay, kit, and buffers detailed above, were prepared using Milli Q filtered (Millipore, USA) H_2O according to the manufacturers' protocols unless otherwise stated. Pre-made buffer solutions were diluted to 1X concentration with Milli Q filtered H_2O before use unless otherwise stated.

Blocking Solution

This solution was prepared prior to use in immunoblotting experiments and consisted of a 5% (w/v) solution of BSA in TBST.

Immunoprecipitation Buffer

This buffer was prepared prior to immunoprecipitation experiments. The buffer consisted of 50 µL of undiluted Novex Tris-Glycine SDS Sample Buffer (2X) and 1 µL of 2-mercaptoethanol.

Silver Stain Gel Fixation Solution

This solution was prepared prior to use of the Pierce Silver Stain Kit. The solution consisted of 30% (v/v) ethanol and 10% acetic acid (v/v) in H_2O.

Silver Stain Gel Stopping Solution

This solution was prepared prior to use of the Pierce Silver Stain Kit. The solution consisted of 5% (v/v) acetic acid in H_2O.

Silver Stain Gel Washing Solution

This solution was prepared prior to use of the Pierce Silver Stain Kit. The solution consisted of 10% (v/v) ethanol in H_2O.

3.2 Patients and Controls

Samples of serum or plasma were collected from well-characterised patients and healthy individuals between the ages of 16 and 70 living in Melbourne, Australia. All patient samples were de-identified and featured in previous studies, which had been approved by respective ethics boards, peer-reviewed and published. Serum or plasma from peripheral blood were stored at -80 ºC to maintain stability. All study participants gave written, informed consent. The thesis study

protocols were approved by the Monash University Human Research Ethics Committee in Melbourne, Australia and the use for the purposes of the current evaluation was likewise approved. Featured cohorts are further described in the following chapters.

3.3 Methods

3.3.1 ELISA Assays

Levels of circulating I-FABP, LBP, sCD14, syndecan-1 and purported zonulin were measured by commercial ELISA in duplicate according to the manufacturers' protocols. Details of the assays and suppliers are shown in 2.1.1. The average coefficient of variation (CV) between duplicates was below 10%. Averages of duplicates were determined and absolute values are expressed in the following units: ng/mL for I-FABP, syndecan-1, and zonulin; μg/mL for LBP and sCD14.

3.3.2 Determination of Solubilised Protein Standard Concentrations

Concentrations of solubilised protein standards were determined by NanoDrop Microvolume Spectrophotometer (Life Technologies, USA). A 2 μL sample of protein standard solution was pipetted on the instrument's sample pedestal and protein concentration was measured by the instrument using an absorbance reading at 280 nm (A280) with background solution subtraction. Samples were measured in triplicate for each protein standard and the median sample concentration was used in subsequent experiments.

3.3.3. SDS-PAGE and Haptoglobin Phenotyping Immunoblots

A protocol to determine the haptoglobin phenotypes of study participants by immunoblotting was adapted from information generously provided by Dr Alessio Fasano and Craig Sturgeon of the Harvard Medical School Celiac Research Program (USA). Serum proteins were separated by SDS-PAGE using the Novex Mini Gel Tank Electrophoresis System followed by dry transfer to PVDF membranes for use in immunoblotting. To prepare proteins for separation by SDS-PAGE, 1 μL of neat serum and approximately 5 μg of protein standards for human haptoglobin phenotypes 1-1 and 2-2 were combined with 2-mercaptoethanol and Novex Tris-Glycine SDS Sample Buffer, heated at 100°C for 10 minutes, then added to wells of 16% Tris-Glycine Mini Gels. Proteins were then transferred to PVDF membranes (as part of the iBlot 2 Transfer Stacks) by the iBlot2 Dry Transfer Device. Membranes were then blocked for 1 hour at room temperature or 4°C overnight in blocking solution. After blocking, mouse anti-human haptoglobin primary antibody at a 1:1000 dilution in blocking solution was added to membranes and left to incubate for 2 hours at room temperature or 4°C overnight. Membranes were then washed in TBST then incubated with fluorescent goat anti-mouse IgG (H+L) secondary antibody at a 1:5000 dilution in the dark for 30 minutes at room temperature. Membranes were then washed and imaged at 700 nm by the Li-Cor/Odyssey infrared image system.

3.3.4 Immunoprecipitation of Serum Antigens Bound to Commercial Kit Antibodies

To collect the target antigens of CUSABIO and Immundiagnostik zonulin commercial assays, protocols for the immunoprecipitation of the serum antigen-immobilised antibody complex were developed as previously described with modifications.[156] Undiluted serum samples with high purported zonulin levels, as determined by each respective commercial assay, were selected as

antigen sources. Recombinant zonulin was selected as a positive control. Immunoprecipitation buffer was selected as a negative control. Biotinylated zonulin tracer, which competes with serum antigen in binding to immobilised plate antibodies of the Immundiagnostik assay, was also subject to the immunoprecipitation protocol to determine any potential interactions.

Serum and controls were added to wells of both types of commercial assays in duplicate. Biotinylated zonulin tracer was also added to wells of the Immundiagnostik assay. Wells were then incubated and washed according to the manufacturers' protocols for each assay to form any potential serum antigen-immobilised antibody complexes. To release bound antigens from the immobilised assay antibodies, 50 µL of heated immunoprecipitation buffer was then added to the duplicate wells containing serum, controls, or tracer, and incubated for 5 minutes. The initial 50 µL of immunoprecipitation buffer was then transferred to the next set of duplicate wells containing sample, controls, or tracer and incubated for 5 minutes. The procedure was repeated in further wells to increase the concentration of any captured antigens for mass spectrometry analysis.

3.3.5 Mass Spectrometric Preparation and Acquisition

Mass spectrometric preparation and acquisition was conducted by the Monash Biomedical Proteomics Facility in Clayton, Victoria, Australia. All experimental materials and methods detailed below were reported by study collaborators. Common abbreviations, with the exception of specific spectrometric software parameters and proper names of mass spectrometric equipment, are indicated.

Immunoprecipitation samples were prepared for comparative mass spectrometry analysis. CUSABIO assay standard, Immundiagnostik tracer, and recombinant zonulin were assay standards selected for analysis. Samples and standards were reduced in 2.5mM dithiothreitol (DTT) at 50°C

for 30 minutes followed by alkylation with 10mM iodoacetamide for 30 minutes in the dark at room temperature. Following alkylation, a solution containing 1 μg trypsin (Promega Corp., USA) in 20mM ammonium bicarbonate was added and the samples incubated at 37°C overnight.

Tryptic digests were analysed by liquid chromatography-mass spectrometry (LC-MS/MS) using the QExactive mass spectrometer (Thermo Scientific, Germany) coupled online with a RSLC Nano High Performance Liquid Chromatography (HPLC) Ultimate 3000 (Thermo Scientific, Germany). Samples were concentrated on a 100 μm, 2 cm nanoviper pepmap100 trap column with 98% buffer A (0.1% formic acid) at a flow rate of 15 μL/minute. The peptides then eluted and separated with a 50 cm Thermo RSLC pepmap100, 75 μm id, 100Å pore size, reversed phase nano column starting with 97.5% buffer A (0.1% formic acid) to 40% B (80% acetonitrile, 0.1% formic acid) over a 30 minute gradient, at a flow rate of 250 nL/minute. The eluant was nebulised and ionised using the Thermo nano electrospray source with a distal coated fused silica emitter (New Objective, USA) with a capillary voltage of 1900V. Peptides were selected for tandem mass spectrometry (MS/MS) analysis in Full MS/dd-MS^2 (TopN) mode with the following parameter settings: TopN 10, resolution 17500, MSMS AGC target 1e5, 120ms Max IT, NCE 27 and 2 m/z isolation window.

Data from LC-MS/MS run was exported to Mascot generic file format (*.mgf) using proteowizard 3.0.3631 (open source software, http://proteowizard.sourceforge.net) and searched against Swiss-Prot databases using the MASCOT search engine (version 2.4, Matrix Science Inc., UK) with all taxonomy selected. The following search parameters were used: missed cleavages, 1; peptide mass tolerance, ± 10 ppm Da; peptide fragment tolerance, ± 0.02 Da; peptide charge, 2+, 3+ and 4+; fixed modifications, carbamidomethyl; variable modification, oxidation (Met).

3.3.6 SDS-PAGE of Purported Zonulin Assay Immunoprecipitation Products and Protein Staining

Proteins in immunoprecipitation samples were separated by SDS-PAGE and visualised by gel staining for comparison to protein standards that appeared as top matches in mass spectrometry analysis. Immunoprecipitation samples, 5 μg of haptoglobin standards, and 5 μg of complement C3c standard were combined with 2-mercaptoethanol, heated to 100°C for 10 minutes, then added to wells of 8-16% Tris-Glycine Mini Gels. Proteins were separated by SDS-PAGE using the Novex Mini Gel Tank Electrophoresis System, then stained with Pierce Silver Stain for immunoprecipitation products or Invitrogen SimplyBlue SafeStain, a Coomassie-based stain, for standards. Staining procedures were followed according to the manufacturers' protocols. For silver staining, appropriate solutions for gel fixing, washing, and stopping solutions were prepared with the solutions detailed in section 2.1.5.

3.4 Statistical Analysis

Statistical analyses were performed by IBM SPSS Statistics Version 24 (IBM Corp., USA) and GraphPad Prism 6 (GraphPad Software, USA). Figures were generated with GraphPad Prism 6. Normality of distribution with regards to marker levels and clinical indicators in study cohorts were determined by Shapiro-Wilk tests. For repeated-measures pairwise comparisons, repeated-measures t-tests were used for normally-distributed marker levels or Wilcoxon tests for nonparametric marker distributions. For cross-sectional pairwise comparisons, independent-measures t-tests were used for normally-distributed marker levels and Mann-Whitney U tests were used for nonparametric marker distributions. Friedman's tests were used for repeated-measures

comparisons between marker levels with at least one or more nonparametric distributions. Kruskal-Wallis tests were used for cross-sectional comparisons between marker levels with one or more nonparametric distributions. All multiple pairwise comparisons, including those featured in figures, met criteria for statistical significance after controlling the false discovery rate (FDR) at 5% unless otherwise stated.[157] P-values of false discoveries are not included in figures. Pearson's r correlations between marker data were performed when both variables had normal distributions, whereas Spearman's r correlations were performed when either or both variables had nonparametric distributions. Bland-Altman plots were also used for comparative analyses between zonulin assays. All p-values were two sided and determined to be statistically significant at $p \leq 0.05$.

CHAPTER 4

THE UTILITY OF SERUM ZONULIN AS A MARKER OF INTESTINAL BARRIER DYSFUNCTION

4.1 Introduction

As detailed in Chapter 1, the protein zonulin is capable of tight junction disassembly and is therefore implicated in the regulation of mucosal permeability through the paracellular pathway. It has received considerable attention for its potential involvement in the pathogenesis of gastrointestinal diseases and candidacy as a biomarker of intestinal barrier dysfunction. However, the strength of evidence that it is a specific, reliable serum marker of disease has yet to be examined. In the present study, the primary aim was to measure serum zonulin levels in patients with well-characterised NCWS, coeliac disease, and acute severe ulcerative colitis as well as healthy controls using commercial zonulin assays. However, due to methodological shortcomings in these assays, we sought to determine whether the assays are reliably detecting zonulin as prehaptoglobin-2 and if not, what they may be detecting instead. Along with the results from Chapter 5, the current results resolve whether serum zonulin, as measured by current commercial assays, is a useful marker of gastrointestinal dysfunction and mucosal barrier integrity.

4.2 Materials and Methods

4.2.1 Patients and Controls

General information regarding study cohorts are detailed in Chapter 3.2. Specifics of gastrointestinal disease cohorts are detailed in Chapter 7 for individuals with NCWS and Chapter 8 for newly diagnosed coeliac disease patients.

4.2.1.1 Acute Severe Ulcerative Colitis Cohort

Serum samples were obtained from a previous study of patients with acute severe ulcerative colitis as per modified Truelove-Witts criteria.[158,159] The patients were between the ages of 18 and 70 years and had not adequately responded to 400 mg/day intravenous hydrocortisone for at least 3 days. All had severe inflammation based upon endoscopic criteria and faecal calprotectin as previously published.[158] The study was examining the effect of infliximab infusions on serum infliximab concentrations and how they related to response. Only sera taken during the baseline period (when patients were on hydrocortisone but not infliximab) was evaluated in the current study.

4.2.1.2 Healthy Control Cohort

Healthy individuals (n=49) between the ages of 18 and 65 years were recruited from online and local advertisements. A screening questionnaire was used to evaluate the health of potential study participants. Individuals were excluded if they were on a gluten-free diet or believed they had an allergy or sensitivity to gluten. Other exclusion criteria included evidence of gastrointestinal disease, active infection or inflammation, immune abnormalities, diabetes, liver disease or known abnormal liver test results. Sera was extracted from peripheral blood collected at the Alfred

Hospital Department of Gastroenterology (Melbourne, Australia). Fasting serum samples were collected in the early morning before the study participants' first meal.

4.2.2 ELISA Assays

Information regarding ELISA assays and procedures are provided in Chapter 3.1.1 and 3.3.1, respectively.

4.2.3 Determination of Haptoglobin Phenotype

Procedures to determine haptoglobin phenotype in all study participants are outlined in Chapter 3.3.3.

4.2.4 Recombinant Zonulin Protein Manufacturing and Acquisition

A recombinant zonulin protein sequence from a foundational study confirming the identity of zonulin as prehaptoglobin-2 was generously provided by Dr. Alessio Fasano.[154] Recombinant protein was produced by GenScript (Piscataway, NJ, USA) using the pFastBac1 Bac-to-Bac Baculovirus Expression System. These materials are detailed in Chapter 3.1.2.

4.2.5 Statistical Analysis

Chapter 3.4 details general statistical analysis materials and methods used in this chapter.

4.3 Results

4.3.1 Demographics of Study Participants and Haptoglobin Phenotyping

The details and haptoglobin phenotype of study participants are shown in Table 5. Typical immunoblot analysis of three subjects to determine whether individuals were able to produce zonulin is shown in Figure 5. The majority of study participants were zonulin-producers; 32 of 36 (89%) individuals with NCWS, 34 of 37 (92%) patients with untreated coeliac disease, 19 of 20 (95%) patients with ulcerative colitis, and 46 of 49 (94%) healthy individuals had the Hp2-1 or Hp2-2 phenotype (Table 1). The overall haptoglobin phenotype distribution of our 142 study participants was as follows: 11 (8%) were Hp1-1, 84 (59%) were Hp2-1, and 47 (33%) were Hp2-2. These results are in accordance with previous studies that have determined haptoglobin phenotype distributions within study cohorts as well as in the general population. [46,160,161]

4.3.2 Serum Zonulin Levels Measured by Commercial Assay

Concentrations of purported zonulin for cohorts of gastrointestinal disease and healthy individuals as determined by the CUSABIO assay are shown in Figure 6. Compared with the cohort of healthy individuals with a median (interquartile range [IQR]) of 0.00 (0.00) ng/mL, patient median (IQR) values for purported zonulin were elevated (all $p<0.0001$) at levels of 0.032 (0.90) ng/mL in NCWS, 0.07 (1.27) ng/mL in coeliac disease, and 1.73 (2.17) ng/mL in ulcerative colitis. Levels in ulcerative colitis were higher than those in NCWS ($p=0.004$) and coeliac disease ($p=0.005$) with no significant differences between NCWS and coeliac disease.

A limited number of patients had serum zonulin measured using the Immundiagnostik assay. There was a poor relationship between the results of the two commercially available assays.

Table 5. Demographics and haptoglobin phenotype of study participants.

Subject group	Number of subjects	Mean age, years (SD)	Female sex, n (%)	Haptoglobin phenotype: Hp1-1; Hp2-1; Hp2-2; n (%)
Non-coeliac wheat sensitivity[a]	36	42.6 (12.8)	30 (83)	4(11); 18 (50); 14 (39)
Coeliac disease[b]	37	36.9 (15.7)	27 (73)	3 (8); 24 (65); 10 (27)
Ulcerative colitis[c]	20	36.9 (11.5)	9 (45)	1 (5); 14 (70); 5 (25)
Healthy	49	39.1 (12.9)	32 (65)	3 (6); 28 (57); 18 (37)

[a]Patients had self-reported, non-coeliac wheat sensitivity and irritable bowel syndrome based on Rome III criteria, and did not have other significant gastrointestinal-related diseases. Coeliac disease was ruled out by the absence of HLA-DQ2 or HLA-DQ8 haplotype or by normal duodenal biopsy.[36]

[b]Patients were newly diagnosed and on a gluten-free diet for less than 4 weeks. All had duodenal histology showing a maximum severity of at least Marsh IIIA lesion.[162]

[c]Patients were hospitalised with acute severe disease, refractory to intravenous corticosteroid treatment and receiving medical rescue therapy with infliximab.[158]

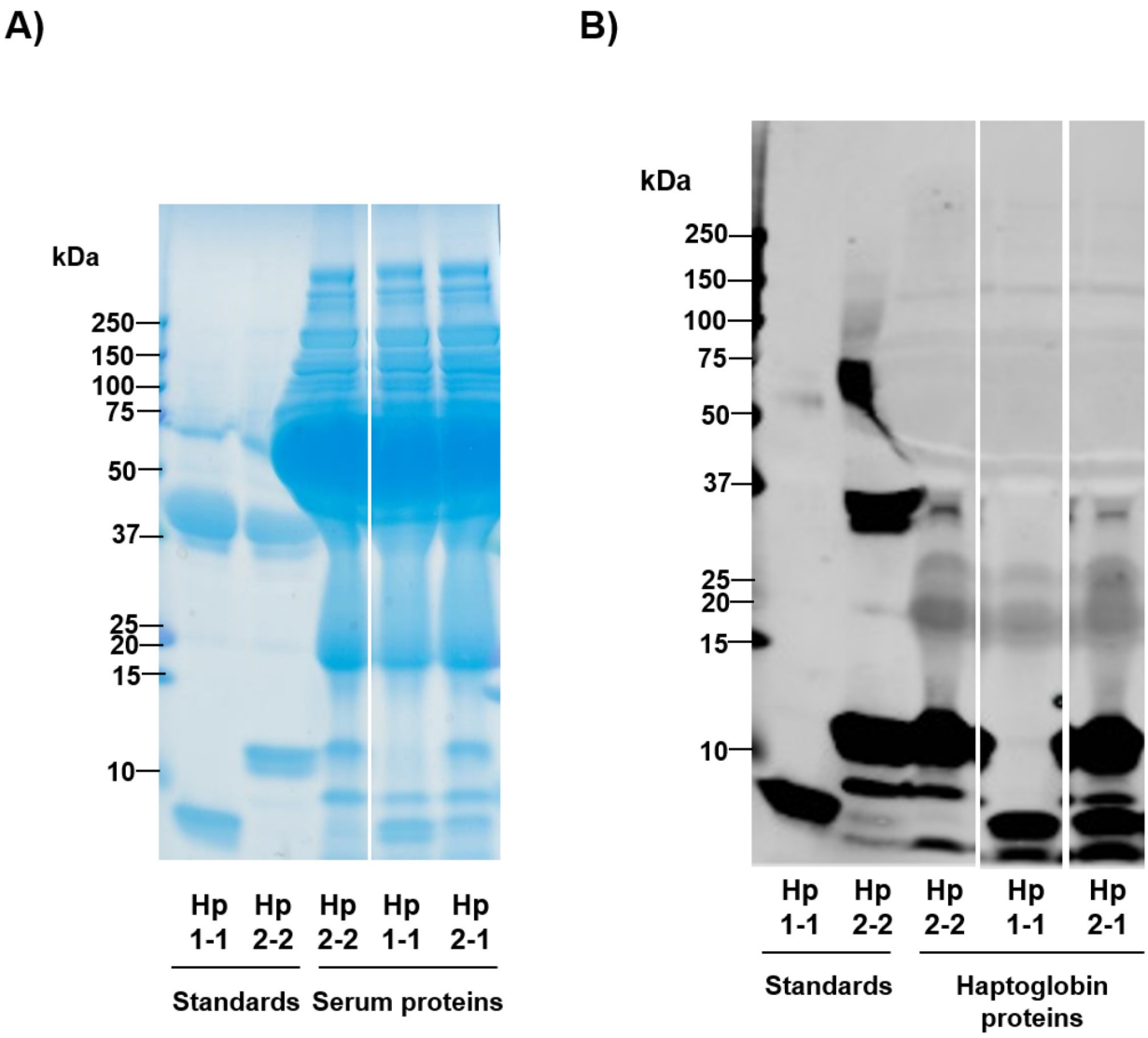

Figure 5. Haptoglobin phenotyping analysis by immunoblot. Haptoglobin proteins in serum samples of patients and controls were detected by anti-haptoglobin polyclonal antibodies and compared to protein standards to determine phenotype. (A) depicts SDS-PAGE separation followed by Coomassie-based staining of haptoglobin standards and proteins in patient sera. The first two lanes from the left contain Hp1-1 and Hp2-2 protein standards, respectively. The third, fourth, and fifth lanes from the left contain study participant sera. As expected in sera, a high abundance of albumin protein was detected in the 66.5 kDa range. (B) depicts an immunoblot of the standards and study participant sera in which haptoglobin proteins are specifically detected by anti-haptoglobin polyclonal antibodies. The first lane from the left contains contains Hp1-1 standard, which has a characteristic band at ~9 kDa representative of the haptoglobin α_1 chain. The second lane from the left contains Hp2-2 standard, with slower migration bands at higher molecular weights representative of the haptoglobin α_2 chain. Hp2-2, Hp1-1, and Hp2-1 phenotypes were detected, in the third, fourth, and fifth lanes from the left, respectively.

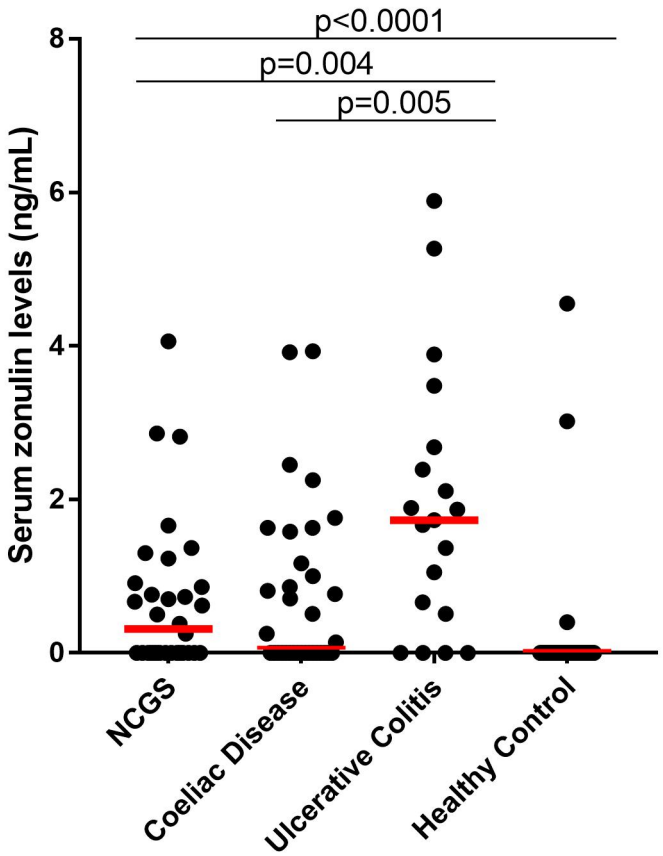

Figure 6. Purported serum zonulin levels in zonulin-producers detected by CUSABIO ELISA assay. Levels of zonulin (ng/mL), as advertised, were determined in NCWS (n=36), coeliac disease, (n=37), and ASUC (n=20) patients as well as healthy individuals (n=49). Compared with the cohort of healthy individuals, patient zonulin levels were elevated (all $p<0.0001$). Levels in ulcerative colitis were higher than those in NCWS ($p=0.004$) and coeliac disease ($p=0.005$) with no significant differences between NCWS and coeliac disease. Red horizontal bars represent median levels for each cohort. Differences in levels between study cohorts were evaluated by Mann-Whitney U tests for nonparametric distributions.

No significant correlation between the same samples tested with both assays was observed (n=28, p=0.14, r=0.29; Figure 7A), nor was there significant agreement between the two methods of measurement (bias/average discrepancy between methods was -31.62 ng/mL, 95% limits of agreement were from -88.51 to 25.28 ng/mL; Figure 7B).

Recombinant zonulin as a positive control was not detected by either CUSABIO or Immundiagnostik assay reliably and no significant dose-responses or signals to saturated concentrations were observed. In addition, 2 of 19 participants who were zonulin non-producers had levels detected by CUSABIO assay. Thus, these results cast doubt on the validity of commercially-available zonulin assays to detect the recombinant protein.

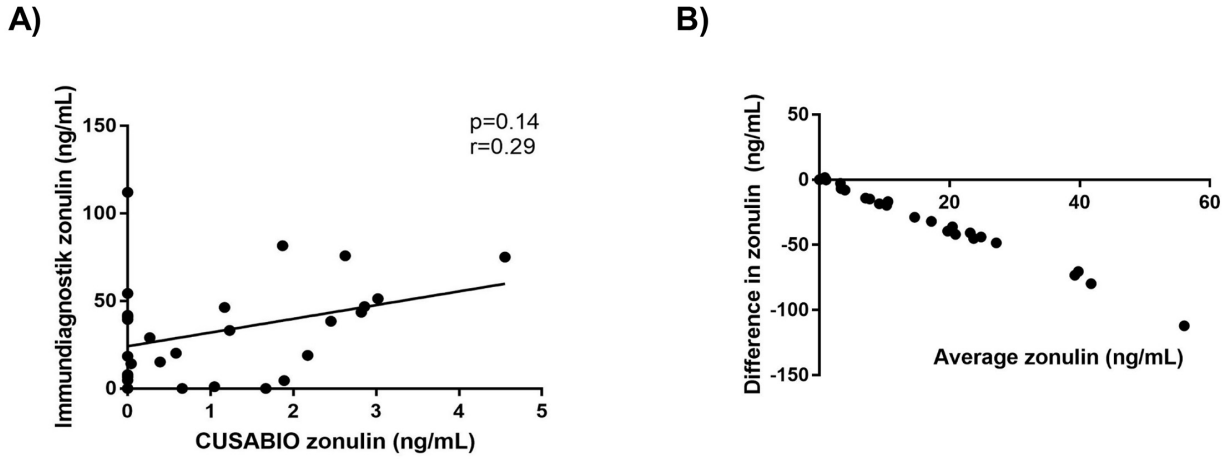

Figure 7. Comparison of purported serum zonulin levels between CUSABIO and Immundiagnostik ELISA assays. Selected study samples (n=28), in which serum zonulin levels (ng/mL) were determined in both CUSABIO and Immundiagnostik ELISA assays were compared. (A) Correlation between the two assays (p=0.14, Spearman's r=0.29). (B) Bland-Altman plot calculating difference in zonulin levels vs average of zonulin levels (bias/average discrepancy between methods was -31.62 ng/mL, 95% limits of agreement were from -88.51 to 25.28 ng/mL).

4.4 Discussion

Due to its putative role in reversible tight junction disassembly, circulating concentration of zonulin has emerged as an increasingly popular biological marker of mucosal barrier integrity. Despite its wide use to assess intestinal mucosal barrier integrity in clinical conditions where a "leaky gut" is suspected, there has been limited information on zonulin levels in patients with gastrointestinal dysfunction. In the current study, sera from well-characterised patient cohorts and controls were used to assess zonulin's utility as a serological marker of gastrointestinal dysfunction and intestinal mucosal barrier integrity. However, the current commercial assays had significant methodological inconsistencies, as detailed in this chapter.

The first indicator of methodological inconsistency involved an inquiry into the assays' capture antibodies. The epitope to which CUSABIO assay capture antibodies were raised remained unknown and enquiries to the manufacturer remained unanswered (communication with customer service, CUSABIO, Wuhan, China). In contrast, the alternative commercial assay by Immundiagnostik clearly indicated an epitope to which capture antibodies are raised (communication with customer service, Immundiagnostik AG, Bensheim, Germany). This epitope is GGVLVQPG, a peptide sequence synthetically manufactured as AT-1001 or larazotide acetate [14,52,53]. Although the purported zonulin receptor has an affinity for this epitope, it remained unclear whether the generation of capture antibodies raised to this sequence would bind to actual circulating zonulin. Another inconsistency was the assays' apparent detection of zonulin in individuals bearing the Hp1-1 phenotype (i.e. in zonulin non-producers). There was also poor strength of relationship between both commercial assays advertised to detect the same protein (Figure 7). Taken together, these inconsistencies casted initial doubt on the utility of the commercial assays to detect circulating zonulin.

In order to confirm whether these assays are actually detecting zonulin, the recombinant zonulin protein as prehaptoglobin-2, which has been used in principal studies that characterise the protein and demonstrate its ability to decrease transepithelial electrical resistance, was tested in both assays.[13] Dose-responses or signals to high concentrations were not observed; as such, the inability of recombinant zonulin to bind to captured antibodies was confirmed. In support of our ongoing observations, a publication was concurrently released which claimed that the Immundiagnostik assay was not detecting zonulin as advertised, but complement C3 instead.[156] Research efforts were then focused on determining the target antigens of these assays, which is detailed and further discussed in Chapter 5.

CHAPTER 5

IDENTIFICATION OF TARGET ANTIGENS IN ZONULIN COMMERCIAL ASSAYS

5.1 Introduction

As detailed in Chapter 4, methodological inconsistencies observed casted doubt on commercial zonulin ELISA assays to detect actual serum zonulin. The aim of the current chapter is to discover the potential identity of serological components captured by commercial zonulin assays. The nature of the assays' detected antigen was investigated using immunoprecipitation followed by mass spectrometric analysis and SDS-PAGE followed by protein staining. The results confirm whether current commercial zonulin assays are detecting the actual protein.

5.2 Methods

Immunoprecipitation experiments, followed by mass spectrometric preparation and acquisition, and protein staining of immunoprecipitation products are detailed in Chapter 3.3.4, 3.3.5, and 3.3.6, respectively.

5.3 Results

5.3.1 Mass Spectrometry Analysis of Immunoprecipitated Proteins and Assay Standards

The potential identity of serological components captured by both commercial assays was determined by mass spectrometry. A direct comparison approach was used instead of separating immunoprecipitated proteins by SDS-PAGE and excising certain bands of interest to conserve

patient serum samples and identify unknown quantities of proteins. The predominant proteins identified for each immunoprecipitation experiment are summarised in Table 6; major contaminants such as keratin, common background proteins, low confidence single peptide protein matches, and isoforms of matched proteins were excluded.

Incubation with serum followed by immunoprecipitation yielded complement C3, haptoglobin, and albumin as top matches using the CUSABIO assay and complement C3 along with albumin as top matches using the Immundiagnostik assay. Incubation with recombinant zonulin followed by immunoprecipitation yielded haptoglobin and albumin as top matches using the CUSABIO assay and albumin using the Immundiagnostik assay. The negative control for both CUSABIO and Immundiagnostik assays yielded albumin as a top match along with nonspecific proteins.

CUSABIO assay standard, Immundiagnostik tracer, and recombinant zonulin were also analysed by mass spectrometry. The most significant and abundant match for CUSABIO assay standard was bovine serum albumin. However, the composition of this product has not been published and, if a recombinant protein was present, the sequence will not have been in the database against which a search can be made. Aside from a collagen alpha protein, there was no strong match in the Immundiagnostik tracer. Similarly, the composition of this product is also unknown and may be a recombinant protein of unknown sequence or a non-protein formulation. The top match for recombinant zonulin was haptoglobin in addition to serum albumin and viral proteins associated with baculovirus expression systems.

Table 6. Identification of proteins immunoprecipitated from commercial zonulin ELISA assays by LC-MS/MS.

Commercial Assay	Incubation before immunoprecipitation	Complement C3	Haptoglobin	Albumin
CUSABIO	Serum	+	+	+
	Zonulin	-	+	+
	Negative control	-	-	+
Immundiagnostik	Serum	+	-	+
	Zonulin	-	-	+
	Negative control	-	-	+

+, protein present; -, protein absent.

5.3.2 Immunoprecipitation Product Staining and Confirmation of Commercial Assays' Inability to Bind Recombinant Zonulin

Immunoprecipitated protein products and standards were separated by SDS-PAGE followed by gel staining for visualisation and are depicted in Figure 8. Protein standards of complement C3c, haptoglobin, and recombinant zonulin each show characteristic bands that were compared visually to immunoprecipitation products. The conserved complement C3 β-chain at approximately 70 kDa, the haptoglobin β-chain at 40 kDa, and the 47 kDa band indicative of recombinant zonulin are clearly indicated by Coomassie stain (Figure 8A). Since the concentration of immunoprecipitation protein products detected by LC-MS/MS was unknown, silver stain was chosen as a more sensitive technique for protein visualisation. The results of staining the immunoprecipitated protein products corresponded to those of the candidate proteins indicated by mass spectrometry. Staining of immunoprecipitated protein products from incubation of the CUSABIO assay with serum revealed distinct bands at 70 kDa, 65 kDa, and 40 kDa, suggestive of the complement C3 β-chain, albumin, and the haptoglobin β-chain, respectively (Figure 8B, Lane 1). The complement C3 β-chain at 70 kDa, albumin at 65 kDa, and faint staining below 50 kDa appeared in the immunoprecipitation sample from incubation of the Immundiagnostik assay with serum (Figure 8B, Lane 2). Addition of recombinant zonulin to assays prior to immunoprecipitation did not yield significant protein products, which confirms the assays' inability to capture recombinant zonulin (Figure 8B, Lanes 3 and 4). Mass spectrometry analysis for the immunoprecipitated product of CUSABIO capture antibodies incubated with zonulin indicated haptoglobin as a top match, but in trace amounts. Staining of negative control

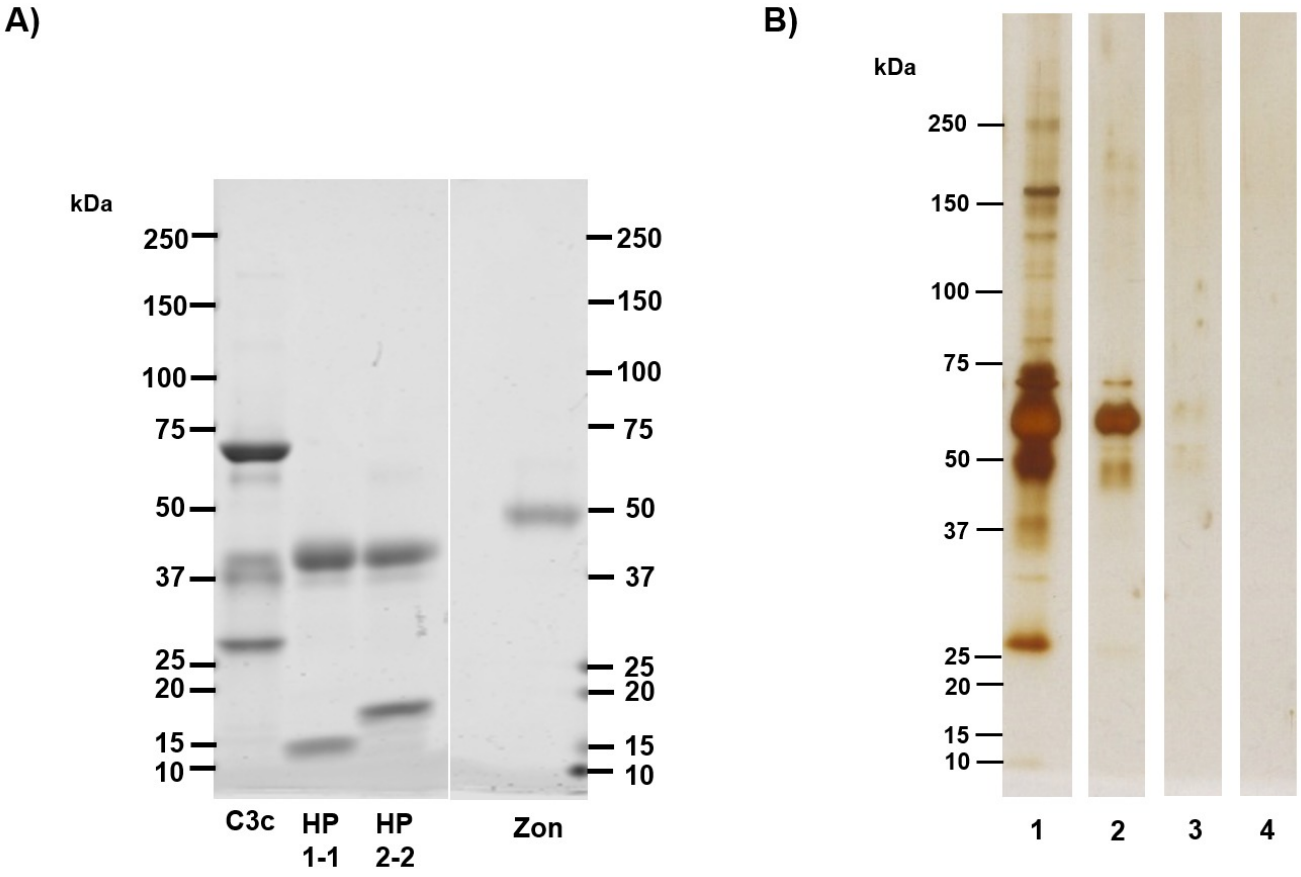

Figure 8. Visualisation of immunoprecipitated protein products and standards. (A) depicts 5 µg of human complement C3c, HP1-1, HP2-2 and recombinant zonulin (Zon) standards separated by SDS-PAGE and stained with Coomassie gel stain. Characteristic bands of standards include the conserved C3c β-chain at 70 kDa, the haptoglobin β-chain at 40 kDa, and the 47 kDa band indicative of recombinant zonulin. (B) Silver staining of immunoprecipitated protein samples detected by LC-MS/MS. Lanes (1) and (2) contain immunoprecipitated proteins from incubation with serum in commercial assays. Lane (1) contains serum proteins captured by CUSABIO assay, which includes complement C3 and haptoglobin as identified by mass spectrometry. Bands at 70 kDa, suggestive of complement C3, and haptoglobin at 40 kDa are present. Lane (2) contains serum proteins captured by Immundiagnostik assay, which includes complement C3 as identified by mass spectrometry. Lanes (3) and (4) contain immunoprecipitated product from incubation of commercial kits with recombinant zonulin. Lane (3), which includes immunoprecipitated product captured by CUSABIO assay, contains trace amounts of protein around the 45-65 kDa range. Mass spectrometry results also indicated a small amount of haptoglobin, which remained undetected by silver stain. Lane (4) contained immunoprecipitated product captured by Immundiagnostik assay; no significant detectable proteins aside from albumin were detected by mass spectrometry or indicated by silver stain.

immunoprecipitated products from both commercial assays indicated a wide array of nonspecific proteins, which corresponded to results from mass spectrometry analysis.

5.3.3 Determination of Whether Candidate Proteins Detected by Mass Spectrometry are Also Detected by CUSABIO Assay

We sought to determine whether candidate proteins detected by mass spectrometry (i.e. complement C3 and haptoglobin) are also detected by the CUSABIO assay. We spiked complement C3c, haptoglobin 1-1, and haptoglobin 2-2 standards into the CUSABIO assay and did not observe any dose-responses or reactivity to saturated concentrations.

5.4 Discussion

As examined in Chapter 5, methodological inconsistencies indicated that actual zonulin protein is not detected by the commercial assays applied. Research efforts, as detailed in the current chapter, focused on determining the identity of serological proteins that the commercial kits were detecting instead. Using an adapted protocol, the captured serum antigens as well as other components from both assays were immunoprecipitated then analysed by mass spectrometry. Our results paralleled those of the concurrent publication by Scheffler et al. (previously discussed in Chapter 4), as the top match for the Immundiagnostik assay immunoprecipitation product when incubated with serum was complement C3.[156] The serum protein product immunoprecipitated from the CUSABIO assay was best matched with haptoglobin and complement C3. However, addition of recombinant zonulin to both assays did not yield any significant immunoprecipitated protein product as indicated by silver staining (Figure 8B, Lanes 3 and 4). Trace amounts of haptoglobin were detected by mass spectrometry and very slight silver staining was observed for the CUSABIO

assay immunoprecipitated product as a result of incubation with recombinant zonulin (Figure 8B, Lane 3). Albumin was the only significant mass spectrometry match and no visual staining was observed for the Immundiagnostik assay immunoprecipitated product as a result of incubation with recombinant zonulin (Figure 8B, Lane 4). These results confirmed that the current commercial assays are not detecting the protein as advertised and were sufficient evidence to discontinue any further testing of our cohorts and use of our resources.

A follow-up publication by Scheffler et al. took further steps in the characterisation of captured antigens.[160] However, they found that the principal mass spectrometry candidate protein, complement C3, was not detected by the immunoassay. Instead, properdin, a complement-associated and "zonulin-like" protein emerged as the likely candidate of assay detection. Properdin was not detected in our mass spectrometry results for either assay and was a low abundance match in the group's mass spectrometry results. The group found that Immundiagnostik assay antibodies as well as antibodies raised to recombinant zonulin cross-reacted to this protein, which is of a similar molecular weight (~50 kDa) compared to zonulin. As such, results remain inconclusive, indicating the necessity of additional studies to establish the true identity of target proteins associated with the Immundiagnostik assay, which they assume to be part of the zonulin family.

Our results also hold the same conclusion for the inefficacy of the CUSABIO assay, which does not detect recombinant zonulin. In addition, neither complement C3 nor haptoglobin, despite both being candidate target proteins as determined by mass spectrometry, was detected by the CUSABIO assay. Consequently, we are unable to make valid interpretations of our results with respect to our study cohorts (Figure 6). However, we found this assay to have a different capture antibody than the Immundiagnostik assay, which differs from conclusions made by Scheffler et al. In both primary and follow-up publications, the authors list a table with studies that use the

Immundiagnostik assay with a note that "the kit sold by other companies (e.g., ALPCO) is the same as the Immundiagnostik kit."[156,160] Our results do not agree with this claim, as there was poor strength of relationship between both commercial assays (Figure 7) and the immunoprecipitation protein products of both assays differed (Figure 8). This indicates distinctive capture antibodies for both commercial assays.

Complement-associated and haptoglobin proteins share similar homology, and both complement C3 and haptoglobin have also been detected together as candidate serological biomarkers by mass spectrometry in an extra-intestinal study.[163-165] The α-chain of haptoglobin contains a complement control protein domain, which is a characteristic component of proteins involved in the regulation of complement (e.g., complement factors H and C1r, mannose-binding lectin-associated serine proteinases and C1 receptor).[164,166] Prohaptoglobin is cleaved by complement C1r-like protein, though it does not cleave to the preform of C1s, which is a similar protein to prehaptoglobin-2.[10,47] It has been, therefore, hypothesised that the activity of Cr-like protein modulates zonulin production.[10] As such, the close association and shared homology between haptoglobin and complement-associated proteins introduces difficulty in the identification of specific markers by current methods and their roles in the mechanisms of disease. Recent studies show a potential role for complement C3, which is synthesised by murine intestinal epithelial cells, in modulating intestinal barrier integrity.[167,168] Whether complement C3 acts in synergy with or is independent of the proposed zonulin pathway in the modulation of the intestinal epithelial barrier is an avenue for further study.

In conclusion, the current commercial zonulin ELISA assays investigated in this study detect different proteins, neither of which was zonulin. Therefore, there can be no value of circulating concentrations in assessing intestinal mucosal barrier dysfunction and permeability

until the target proteins are indeed identified. The identification of such anomalies should be highlighted to researchers to avoid wasting expenditure of time, money, and precious clinical samples. A monoclonal antibody directed towards human zonulin has been developed for use in western blot analysis, as indicated in the methods of Scheffler et al., though screening zonulin levels by this method of analysis is less quantitative and not preferable for use in the clinical setting compared to an ELISA assay.[160] Commercial ELISA detection methodology may be improved with the development of specific and reliable monoclonal capture and detection antibodies to recombinant zonulin/prehaptoglobin-2 protein. Until assay methodology is improved, we urge the greater scientific and medical community to exercise caution in considering the measurement of serum zonulin as a marker of intestinal barrier dysfunction and permeability.

CHAPTER 6

ASSESSMENT OF INTESTINAL BARRIER INTEGRITY IN EXERCISE-INDUCED GASTROINTESTINAL DAMAGE

6.1 Introduction

Prolonged physical exertion has been demonstrated to induce damage in the gastrointestinal tract. As previously detailed in Section 1.3.2.4 and Figure 3, mechanisms of damage include those in circulatory-gastrointestinal and neuroendocrine-gastrointestinal pathways. The primary cause of intestinal barrier damage is a prolonged redistribution of blood flow from the gut, which leads to an ischaemic state. As a result, individuals may experience mucosal damage, epithelial injury, and microbial translocation, which may contribute to the onset of acute (e.g. septic shock, ischaemic colitis) and chronic (e.g. IBD, chronic fatigue) health complications.

Heat is a primary factor that contributes to the effects of ischaemic stress in exercise-induced gastrointestinal damage. There is current evidence that exercise in hot ambient temperatures exacerbates stress damage through both pathways, as outlined in Figure 3.[40] The neuroendocrine-gastrointestinal pathway may also be affected by heat, as stress hormone (i.e., cortisol) and catecholamines are released due to heightened ambient temperatures, which increase sympathetic nervous system drive.[169] This process directly affects gut motility, transit, and nutrient transporter activity, leading to malabsorption. An increased sympathetic drive may also contribute to damage associated with the circulatory-gastrointestinal pathway, as it exerts an inhibitory effect

over gastrointestinal muscle and mucosal secretion, and also decreases local splanchnic blood flow by vasoconstriction.[170] The central nervous system (CNS), which plays a direct role in core body temperature control, also modulates motor output and central drive in high heat environments, which may influence splanchnic blood flow.[171] Taken together, these processes promote an ischaemic state associated with circulatory-gastrointestinal pathway damage.

Diet is an additional factor that may modulate intestinal barrier damage due to gastrointestinal ischaemic stress. In murine models, dietary supplementation with the amino acids L-arginine as well as glutamine prevented increases in intestinal permeability during exercise under heat stress, which appeared independent of core body temperature regulation by the CNS.[172,173] However, there is currently insufficient evidence in human trials to suggest any lasting, substantial benefits of amino acid supplementation. The most promising evidence of a dietary intervention to prevent symptoms of exercise-induced gastrointestinal damage involves the reduction of FODMAPs. A recent case study of an ultramarathon athlete, who was susceptible to experience post-exercise gastrointestinal symptoms, found that following a short-term (i.e., 6 day) low-FODMAP diet prior to physical training improved symptoms in comparison with the athlete's habitual diet.[174] Whether symptom improvement relates to physiological processes of intestinal barrier damage remains unknown; there have been no comprehensive trials of exercise-induced gastrointestinal damage to date that investigate the effects of FODMAPs on gastrointestinal symptoms and markers of intestinal barrier dysfunction and microbial translocation.

The aims of the research in this chapter are to determine the effects of a) heat stress and b) FODMAPs on the intestinal barrier, as indicated by featured circulating markers of dysfunction and microbial translocation, in a model of exercise-induced gastrointestinal damage. In this model, otherwise healthy athletes were subject to 2 hours of running at 60% VO_{2max}, which was

determined as a cut-off level for gastrointestinal damage regardless of fitness by a recent review.[40] The inquiry into heat stress seeks to characterise marker levels that have not yet been measured (i.e. purported zonulin, syndecan-1, LBP and sCD14) in experimental trials of exercise-induced gastrointestinal damage. Investigating the effects of a low-FODMAP diet in this model of damage may provide insights into the diet's utility in preserving intestinal barrier integrity. The evidence of both trials will provide insights into the utility of markers in longitudinal studies and characterise mechanisms of ischaemic stress related to the intestinal mucosal barrier. Since individuals subject to experimental trials were otherwise healthy, this model of induced gastrointestinal damage provides a unique opportunity to study the effects of ischaemic stress in the absence of prior inflammation and gastrointestinal disease.

6.2 Materials and Methods

6.2.1 Study Participants and Protocols

Plasma samples were available from two trials investigating exercise-induced gastrointestinal syndrome at the Monash University Be Active Sleep Eat (BASE) Facility in Notting Hill, Victoria, Australia. The first study investigated the effects of exertional stress in different ambient temperatures on gastrointestinal integrity, gastrointestinal symptoms, systemic endotoxin, and cytokine profile.[102] Healthy trained athletes (n=6) were subject to exercise interventions of 2 hours of running at 60% maximal oxygen update (VO_{2max}), which was previously determined as a threshold for gut damage.[40] All participants exercised at approximately 20 ºC, 30 ºC, and 35 ºC ambient temperature conditions with 4-6% relative humidity in a cross-over study in which the order of temperatures was randomised. There was a 1-week washout period between exercise periods. Study participants were not heat-acclimatised prior to the trials and interventions were

conducted over cooler seasonal periods (with outdoor temperatures consistently below 20 ºC) to avoid seasonal heat acclimatisation. Exclusion criteria for study participants was as previously described.[175] Before each experimental trial, participants were provided a low-FODMAP diet for 24 hours and refrained from strenuous exercise for 48 hours; compliance was determined by a dietary and exercise log. Water intake was provided ad libitum to minimise the occurrence of gastrointestinal symptoms, as participants did not undergo prior gut-training with set fluid volumes.[175,176]

Plasma was obtained from peripheral blood of the participants just prior to and at the end of the exercise intervention. Thus, paired plasma samples were evaluated in the present study under the following conditions:

- Pre- and post-exercise at 20 ºC
- Pre- and post-exercise at 30 ºC
- Pre- and post-exercise at 35 ºC

The second study investigated the effects of FODMAPs on exercise-induced gastrointestinal syndrome. Healthy trained athletes (n=15) were subject to two experimental trials in which they consumed low- or high-FODMAP diets the day before running on a treadmill at 60% VO_{2max} for 2 hours in 35 ºC heat. The experimental trials were assigned to study participants in a random order usually conducted one week apart. Plasma was obtained from peripheral blood of the participants just prior to and at the end of each exercise intervention. Participants completed an initial assessment prior to commencing experimental trials that included anthropometrical measures and a maximal aerobic exercise test on a treadmill. The specific dietary interventions for the two experimental trials were as follows:

- Low-FODMAP experimental trial

    - Low-FODMAP (<5 g/day) foods consumed the day before experimental trial, which were inclusive of breakfast, lunch, dinner, and snacks.
    - A low-FODMAP breakfast consumed 2 hours before the experimental trial.
    - A low-FODMAP recovery beverage consumed within 30 minutes post-exercise.
- High-FODMAP experimental trial
    - High-FODMAP (>50 g/day) foods consumed the day before experimental trial, which were inclusive of breakfast, lunch, dinner and snacks.
    - A high-FODMAP breakfast consumed 2 hours before the experimental trial.
    - A high-FODMAP recovery beverage consumed within 30 minutes post exercise.

Water intake was set at 12 mL/kg per day for each intervention. Participants refrained from strenuous exercise, alcohol, and non-steroidal anti-inflammatory medications for 48 hours prior to each experimental trial. Participants also refrained from consuming any dietary supplements, including probiotics, one month prior to and during the study. Compliance was determined by a dietary and exercise log.

6.2.2 Commercial Assays

Levels of featured plasma protein markers were determined by commercially-available ELISA assays. Details of assays are indicated in Section 3.1.1 and assay methodology is indicated in Section 3.3.1.

6.2.3 Statistical Analysis

Chapter 3.4 details general statistical analysis materials and methods used in this chapter. The following results in Section 6.3 report specific methods used for analysis.

6.3 Results

6.3.1 Heat Stress Trials

Absolute levels of featured protein markers pre- and post-exercise were determined for each participant in ambient temperature trials at 20 °C, 30 °C, and 35 °C. The results are reported in the figures of this section, wherein each individual colour represents a particular study participant. Pre- and post-exercise percent changes were also determined for each protein marker and temperature intervention. Correlations between markers were also reported.

6.3.1.1 Purported Zonulin

Purported zonulin levels were investigated in this cohort prior to commercial assay verification, as detailed in Chapter 5. Figure 9A-C depicts pre- and post-exercise absolute levels of purported zonulin (ng/mL) of study participants in each temperature intervention. Shapiro-Wilk tests indicated a normal distribution of absolute purported zonulin levels in all temperature interventions. As indicated by paired t-tests, participants had significant increases in purported zonulin levels pre- to post-exercise in the 20 °C ($p=0.021$) and 35 °C ($p=0.030$) conditions. A significant difference in purported zonulin levels pre- and post-exercise intervention was observed ($F(1,5)=20.42$, $p=0.0063$), as indicated by two-way repeated measures analysis of variance (ANOVA). However, temperature intervention yielded no significant difference in purported zonulin levels. No interaction was observed between exercise and temperature interventions.

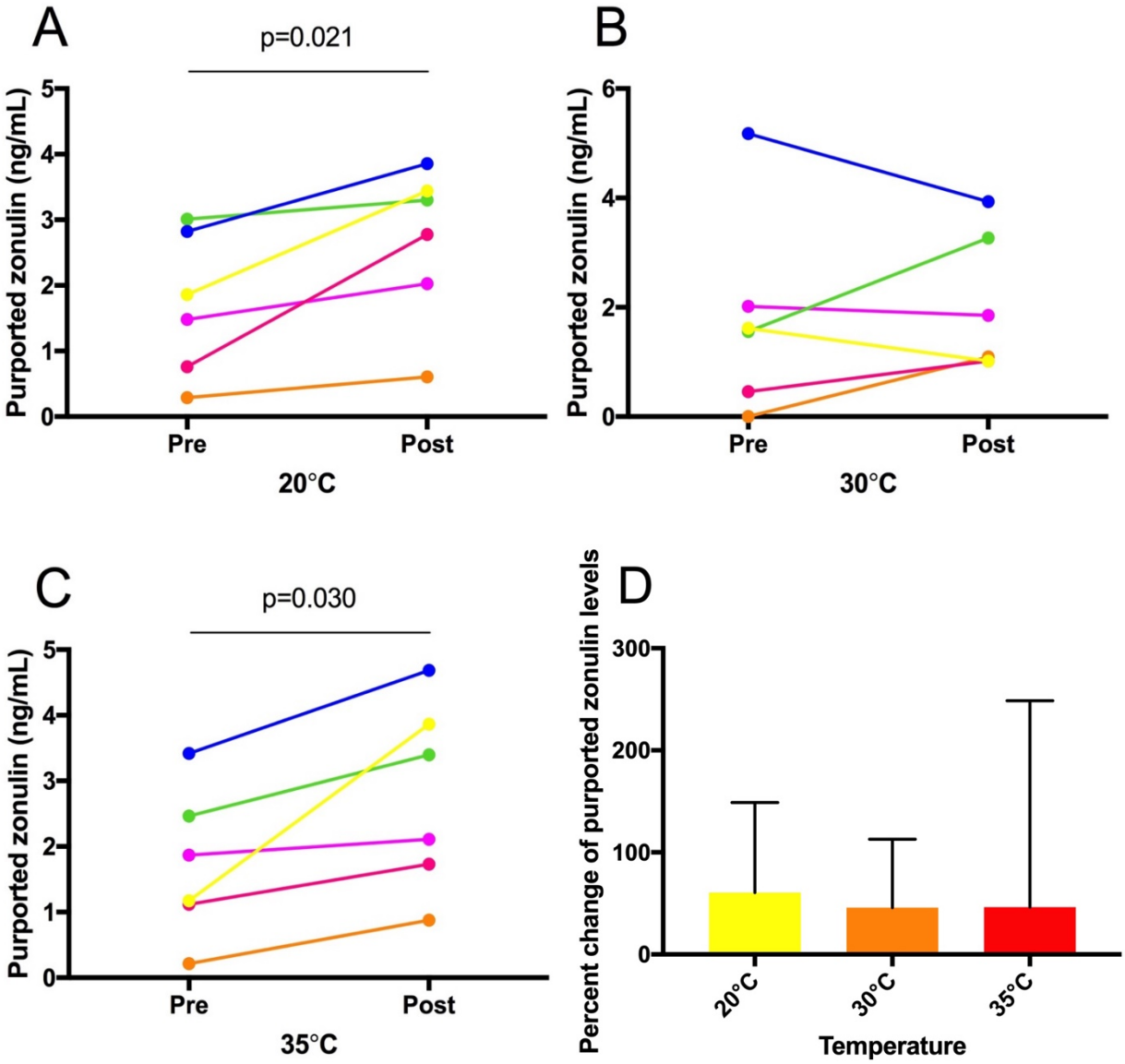

Figure 9. Absolute levels and percent change of purported plasma zonulin in a model of exercise-induced gastrointestinal damage subject to varying ambient temperatures. Figures represent pre- and post-exercise purported plasma zonulin absolute levels (ng/mL), measured by CUSABIO assay, of healthy athletes (n=6) who ran for 2 hours at 60% VO_{2max} in different ambient temperature interventions. (A) indicates absolute zonulin levels at 20°C (B) at 30 °C, and (C) at 35 °C. Significant differences between pre- and post-exercise purported plasma zonulin levels were detected in 20 °C (p=0.021) and 35 °C (p=0.030) interventions. Each colour represents the results of an individual study participant. (D) indicates pre- and post-exercise percent change of plasma zonulin levels in each temperature intervention. No significant differences in percent changes of purported zonulin levels were observed between temperature interventions. Bars represent means with standard deviations.

Percent changes pre- and post-exercise for each temperature intervention are shown in Figure 9D. Shapiro-Wilk tests indicated normal percent change distributions of purported zonulin levels in 20 °C and 30 °C conditions and a nonparametric percent change distribution of levels in the 35 °C condition. Positive median percent changes were observed in each temperature intervention; participants expressed the highest median percent change (IQR) of purported zonulin in the 20 °C intervention at 61% (119%), followed by the 35 °C intervention at 46% (218%), and the 30 °C intervention at 46% (140%). Percent changes between temperature interventions were not significantly different, as indicated by Friedman's test as well as paired t-tests and Wilcoxon tests.

6.3.1.2 Syndecan-1

Figure 10A-C depicts pre- and post-exercise absolute levels of syndecan-1 (ng/mL) of study participants in each temperature intervention. Shapiro-Wilk tests indicated nonparametric distributions of absolute syndecan-1 levels in all temperature interventions. Participants had significant increases in syndecan-1 levels pre- to post-exercise in all temperature interventions ($p=0.031$ for all interventions), as indicated by Wilcoxon tests. A significant difference in syndecan-1 levels pre- and post-exercise intervention was observed ($F(1,5)=8.61$, $p=0.033$), as indicated by two-way repeated measures ANOVA. However, temperature intervention yielded no significant difference in syndecan-1 levels. No interaction was observed between exercise and temperature interventions.

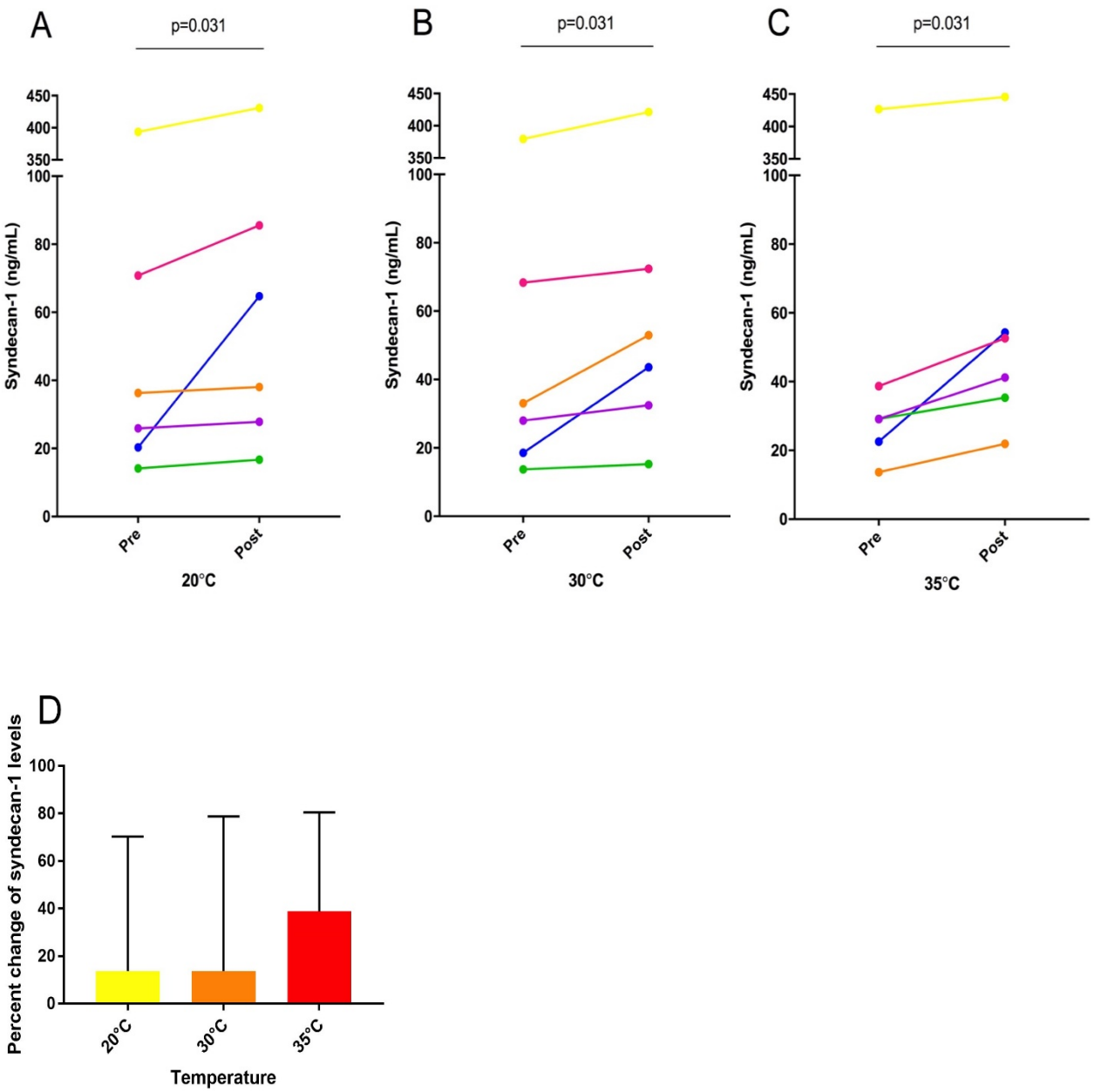

Figure 10. Absolute levels and percent change of plasma syndecan-1 in a model of exercise-induced gastrointestinal damage subject to varying ambient temperatures. Pre- and post-exercise plasma syndecan-1 absolute levels (ng/mL) of healthy athletes (n=6) who ran for 2 hours at 60% VO_{2max} in different ambient temperature interventions. (A) Indicates absolute syndecan-1 levels at 20 °C (B) at 30 °C, and (C) at 35 °C. Participants had significant increases pre- to post-exercise in all temperature interventions (p=0.031). Each colour represents the results of an individual study participant. (D) indicates pre- and post-exercise percent changes of plasma syndecan-1 levels in each temperature intervention. No significant differences in percent changes of syndecan-1 levels were observed between temperature interventions. Bars represent medians with IQRs.

Percent changes pre- and post-exercise for each temperature intervention are shown in Figure 10D. Shapiro-Wilk tests indicated nonparametric percent change distribution of syndecan-1 levels in 20 °C and 30 °C conditions and a normal percent change distribution of levels in the 35 °C condition. Positive median percent changes were observed in each temperature intervention. Participants expressed the highest median percent change (IQR) of syndecan-1 in the 35 °C intervention at 39% (64%), followed by the 20 °C intervention at 14% (64%), and the 30 °C intervention at 14% (64%). Percent changes between interventions were not significantly different, as indicated by Friedman's test as well as Wilcoxon tests.

6.3.1.3 I-FABP

Pre- and post-exercise absolute I-FABP levels (ng/mL) of study participants in each temperature intervention were previously reported by collaborators and adapted into Figure 11A-C.[102] Shapiro-Wilk tests indicated normal distributions in both 20 °C and 35 °C temperature interventions and a nonparametric distribution in the 30 °C intervention. Participants had significant increases in plasma I-FABP levels pre- to post-exercise in all temperature interventions, as indicated by paired samples t-tests in the 20 °C ($p=0.0133$) and 35 °C ($p=0.0121$) interventions and by Wilcoxon test in the 30 °C ($p=0.0312$) intervention. As indicated by two-way repeated measures ANOVA, a significant difference in I-FABP levels pre- and post-exercise intervention was observed ($F(1,5)=17.13$, $p=0.0090$). A significant difference in I-FABP levels was also observed in temperature intervention ($F(2,10)=7.89$, $p=0.0088$). There was a significant interaction observed between exercise and temperature interventions ($F(2,10)=8.154$, $p=0.0079$). As predicted, heat exacerbated increases in I-FABP levels during exercise interventions.

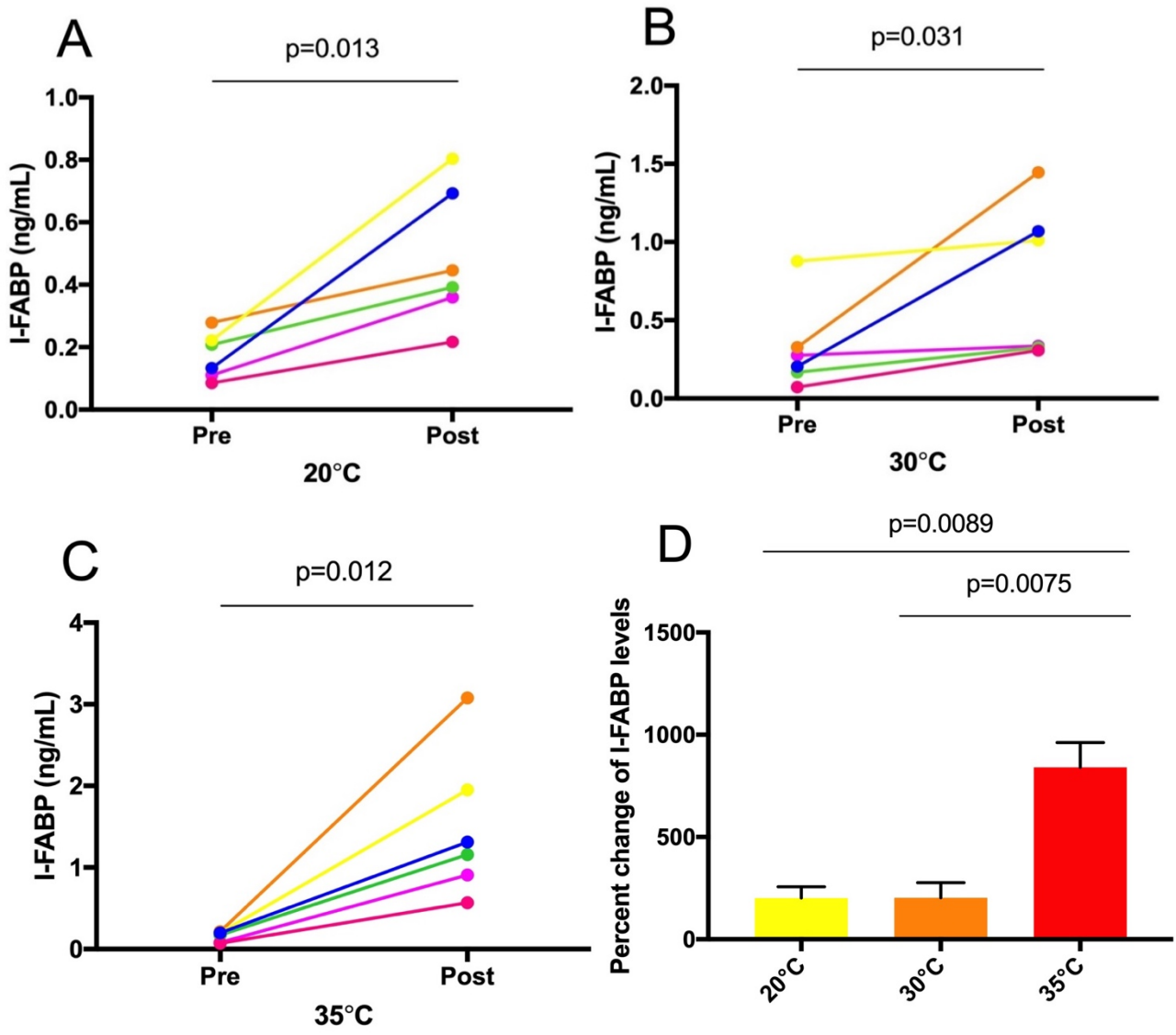

Figure 11. Absolute levels and percent change of plasma I-FABP in a model of exercise-induced gastrointestinal damage subject to varying ambient temperatures. Pre- and post-exercise plasma I-FABP absolute levels (ng/mL) of healthy athletes (n=6) who ran for 2 hours at 60% VO_{2max} in different ambient temperature conditions are represented. (A) indicates I-FABP levels at 20 °C (B) at 30 °C, and (C) at 35 °C. Study participants showed significant increases pre- to post-exercise in all temperature interventions. Each colour represents the results of an individual study participant. Graphs were adapted from previously published data.[102] (D) indicates pre- and post-exercise percent changes of I-FABP levels in each temperature intervention. Significant percent changes were found between the 20 °C and 35 °C interventions (p=0.0089) and between the 30 °C and 35 °C interventions (p=0.0075) as indicated by paired t-tests. Bars represent means with SEM.

Percent changes pre- and post-exercise for each temperature intervention are shown in Figure 11D. Positive percent changes of I-FABP levels were observed in each temperature intervention, as shown in Figure 3D. Shapiro-Wilk tests indicated normal distributions of percent change in I-FABP levels across all temperature interventions. Participants expressed the highest mean percent change ± standard error of the mean (SEM) of I-FABP in the 35 °C intervention at 841% ± 122%, followed by the 30 °C intervention at 203% ± 74%, and the 20 °C intervention at 202% ± 54%. A one-way repeated measures ANOVA showed that the effect of temperature on the percent change of I-FABP levels was significant, $F(2,5)=15.56$, $p=0.0037$. Significant percent changes were found between the 20 °C and 35 °C interventions ($p=0.0089$) and between the 30 °C and 35 °C interventions ($p=0.0075$) as indicated by paired t-tests.

6.3.1.4 LBP

Figure 12A-C depicts pre- and post-exercise absolute levels of plasma LBP (μg/mL) of study participants in each temperature intervention. Shapiro-Wilk tests indicated normal distributions in both 30 °C and 35 °C temperature interventions conditions and a nonparametric distribution in the 20 °C intervention. No significant differences in plasma LBP levels pre- to post-exercise were observed as indicated by paired t-tests and Wilcoxon tests. No significant differences in LBP levels were observed in exercise or temperature interventions as indicated by a two-way repeated measures ANOVA. However, a significant interaction was observed between exercise and temperature interventions ($F(2,10)=4.787$, $p=0.035$). The effect of heat on exercise intervention increased LBP levels.

Percent changes pre- and post-exercise for each temperature intervention are shown in Figure 12D. Shapiro-Wilk tests indicated normal distributions in all temperature interventions.

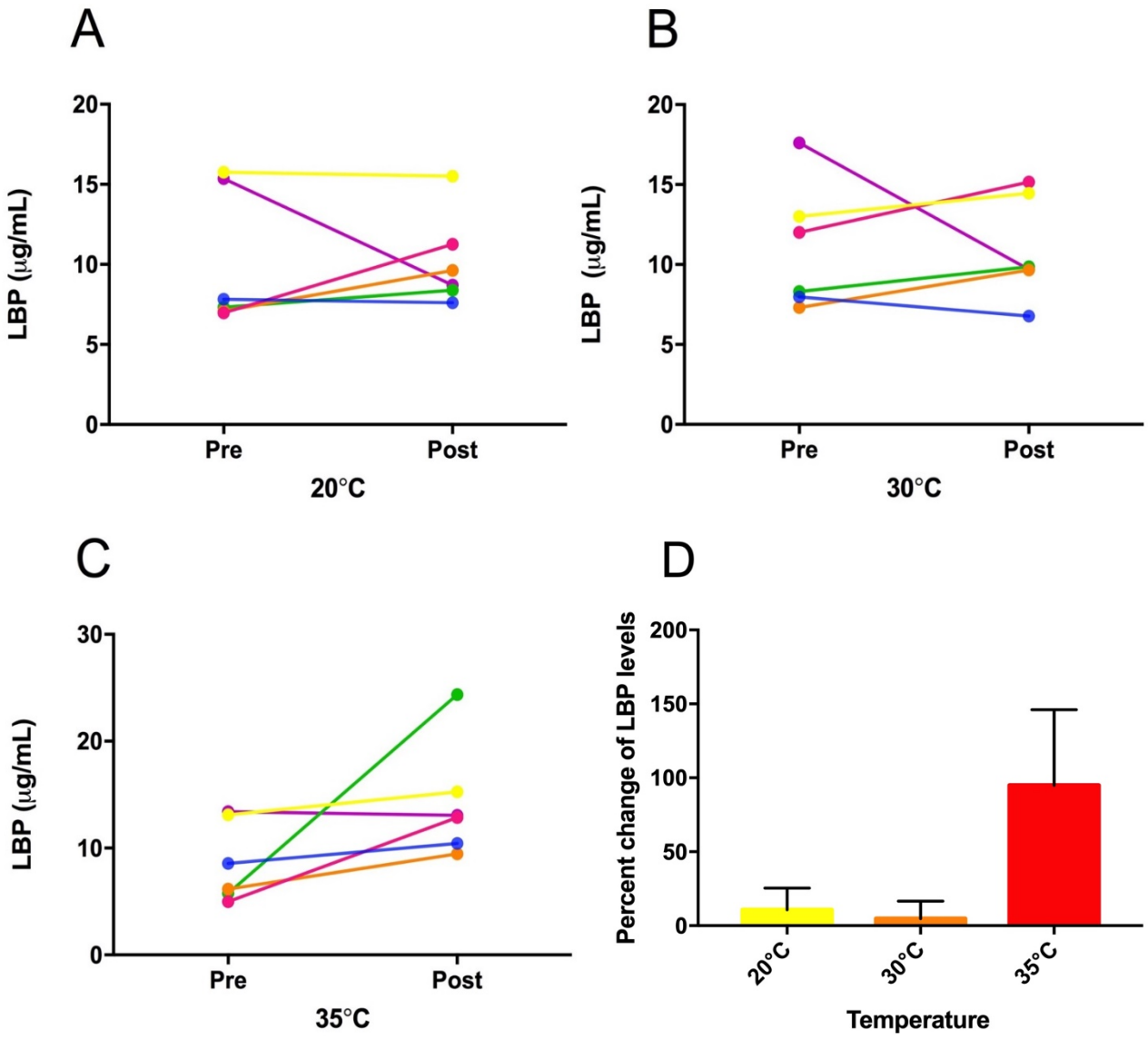

Figure 12. Absolute levels and percent change of plasma LBP in a model of exercise-induced gastrointestinal damage subject to varying ambient temperatures. Pre- and post-exercise plasma LBP absolute levels (μg/mL) of healthy athletes (n=6) who ran for 2 hours at 60% VO_{2max} in different ambient temperature conditions are represented. (A) indicates LBP levels at 20 °C (B) at 30 °C, and (C) at 35 °C. Study participants did not show significant increases pre- to post-exercise in any temperature intervention. Each colour represents the results of an individual study participant. (D) indicates pre- and post-exercise percent changes of LBP levels in each temperature intervention. No significant differences in percent changes of LBP levels were observed between temperature interventions. Bars represent means with SEM.

Participants expressed the highest mean percent change ± SEM of LBP in the 35 °C intervention at 95% ± 51%, followed by the 20 °C intervention at 11% ± 15%, and the 30 °C intervention at 5% ± 12%. Percent changes between temperature interventions were not significantly different, as indicated by repeated-measures ANOVA and paired t-tests.

6.3.1.5 sCD14

Figure 13A-C depicts pre- and post-exercise absolute levels of sCD14 (μg/mL) for each study participant in the ambient temperature interventions. Shapiro-Wilk tests indicated normal distributions in the 30 °C intervention and a nonparametric distribution in both 20 °C and 30 °C interventions. No significant differences in plasma sCD14 levels pre- to post exercise were observed in any temperature intervention as indicated by paired t-tests and Wilcoxon test. No significant differences in sCD14 levels were observed in exercise or temperature interventions as indicated by a two-way repeated measures ANOVA. No significant interaction was observed between exercise and temperature interventions.

Percent changes pre- and post-exercise for each temperature intervention are shown in Figure 13D. Shapiro-Wilk tests indicated normal distributions in 20 °C and 30 °C interventions and a nonparametric distribution in the 35 °C intervention. Participants expressed the highest median percent change (IQR) in the 35 °C intervention at 27% (51%), followed by the 30 °C intervention at -0.92% (49%), and the 20 °C intervention at -9% (56%). Percent changes between temperature interventions were not significantly different, as indicated by repeated-measures analysis of variance (ANOVA) and paired t-tests.

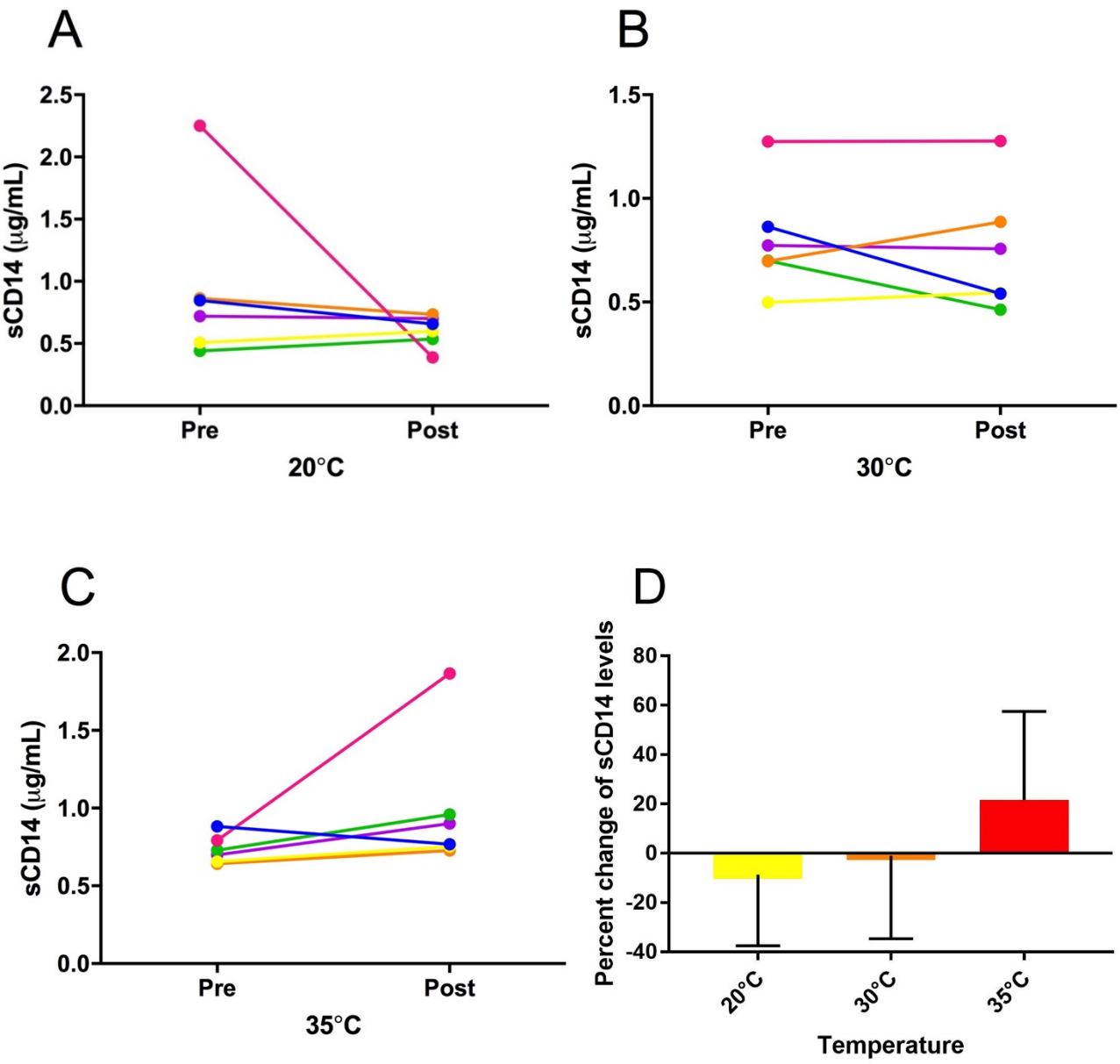

Figure 13. Absolute levels and percent change of plasma sCD14 in a model of exercise-induced gastrointestinal damage subject to varying ambient temperatures. Pre- and post-exercise plasma sCD14 levels (μg/mL) of healthy athletes (n=6) who ran for 2 hours at 60% VO_{2max} in different ambient temperature conditions are represented. (A) indicates sCD14 levels at 20 °C (B) at 30 °C, and (C) at 35 °C. Study participants did not show significant increases pre- to post-exercise in temperature intervention. Each colour represents the results of an individual study participant. (D) indicates pre- and post-exercise percent changes of sCD14 levels in each temperature intervention. No significant differences in percent changes of sCD14 levels were observed between temperature interventions. Bars represent medians with IQRs.

6.3.1.6 Marker Correlations

Change in pre- to post-exercise levels, post-exercise, and percent change of marker levels were correlated in each intervention for study participants. No significant marker correlations were found for change in pre- and post-exercise levels or percent change. A significant correlation of post-exercise levels between I-FABP and sCD14 was observed ($r=-0.943$, $p=0.017$, $n=6$).

6.3.2 FODMAP Dietary Trials

Absolute levels of featured protein markers pre- and post-exercise were determined for every participant in each FODMAP experimental trial. Pre- and post-exercise percent changes were also determined for each protein marker and FODMAP experimental trial. Correlations between markers were also reported. Purported zonulin results are not available, as experiments for this cohort commenced after zonulin commercial assay verification, as detailed in Chapter 5. Differences in sample size amongst markers (i.e., difference in syndecan-1 sample size compared with other markers) was due to lack of plasma available for testing; these differences are indicated in each individual section.

6.3.2.1 Syndecan-1

Figure 14A and B depicts pre- and post-exercise absolute values of syndecan-1 (ng/mL) for each study participant in low- ($n=12$) and high- ($n=13$) FODMAP dietary interventions. Shapiro-Wilk tests indicated normal distributions in the low-FODMAP intervention and a nonparametric distribution in the high-FODMAP distribution. Significant differences of levels

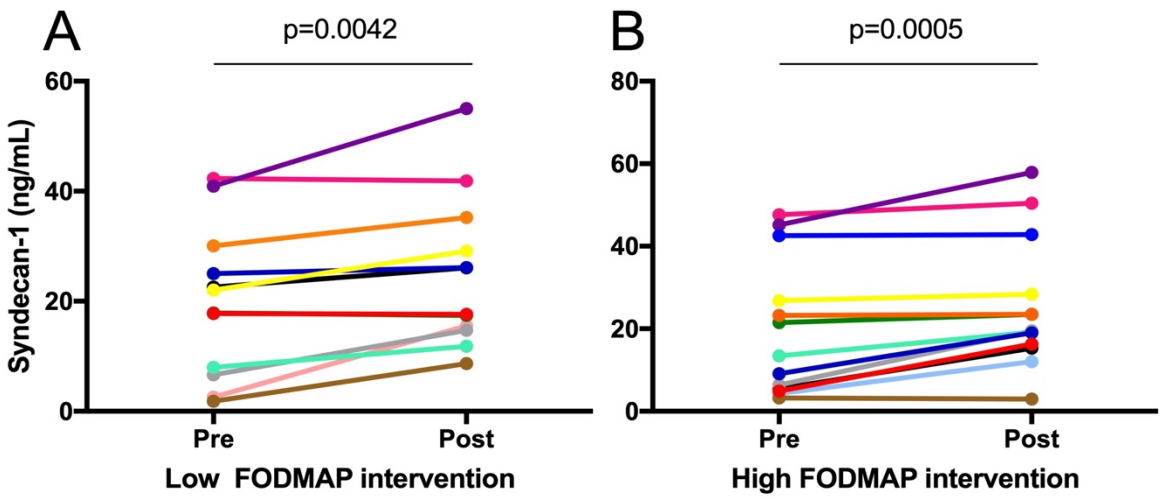

Figure 14. Absolute levels and percent change of plasma syndecan-1 in a model of exercise-induced gastrointestinal damage subject to low- and high-FODMAP dietary interventions. Pre- and post-exercise plasma syndecan-1 levels (ng/mL) of healthy athletes who ran for 2 hours at 60% VO_{2max} in 35 °C heat and underwent low- (n=12) and high- (n=13) FODMAP dietary interventions are represented. (A) indicates pre- and post-exercise plasma syndecan-1 levels during the low-FODMAP intervention and (B) represents pre- and post-exercise plasma syndecan-1 levels during the high-FODMAP intervention. Significant differences pre- to post-exercise were detected in both low-FODMAP (p=0.0042) and high-FODMAP (p=0.0005) dietary interventions, as indicated by paired t-test and Wilcoxon test, respectively. Each colour represents the results of an individual study participant.

were observed pre- to post-exercise in both low-FODMAP (p=0.0042) and high-FODMAP (p=0.0005) interventions, as indicated by paired t-test and Wilcoxon test, respectively. A significant difference pre- and post-exercise intervention was observed (p=0.011), but not for dietary intervention, as indicated by two-way repeated measures ANOVA (n=11). No interaction was found between exercise and FODMAP interventions.

Percent changes pre- and post-exercise for each dietary intervention (n=11) were determined. Shapiro-Wilk tests indicated nonparametric distributions in the dietary interventions. Participants expressed the highest median percent change (IQR) of syndecan-1 levels in the low-FODMAP intervention at 33% (120%) followed by the high-FODMAP intervention at 29% (174%). Percent changes between dietary interventions were not significantly different, as indicated by Wilcoxon test.

6.3.2.2 I-FABP

Figure 15A and B depicts pre- and post-exercise absolute levels of I-FABP (ng/mL) for each study participant in low- and high-FODMAP dietary interventions. Shapiro-Wilk tests indicated nonparametric distributions in both low- and high-FODMAP interventions. Significant differences of levels were observed pre- to post-exercise in both low-FODMAP (p=0.0085) and high-FODMAP (p=0.0031) interventions, as indicated by Wilcoxon tests. A significant difference pre- and post-exercise intervention was observed (p=0.0031), but not for dietary intervention, as indicated by two-way repeated measures ANOVA. No interaction was found between exercise and FODMAP interventions.

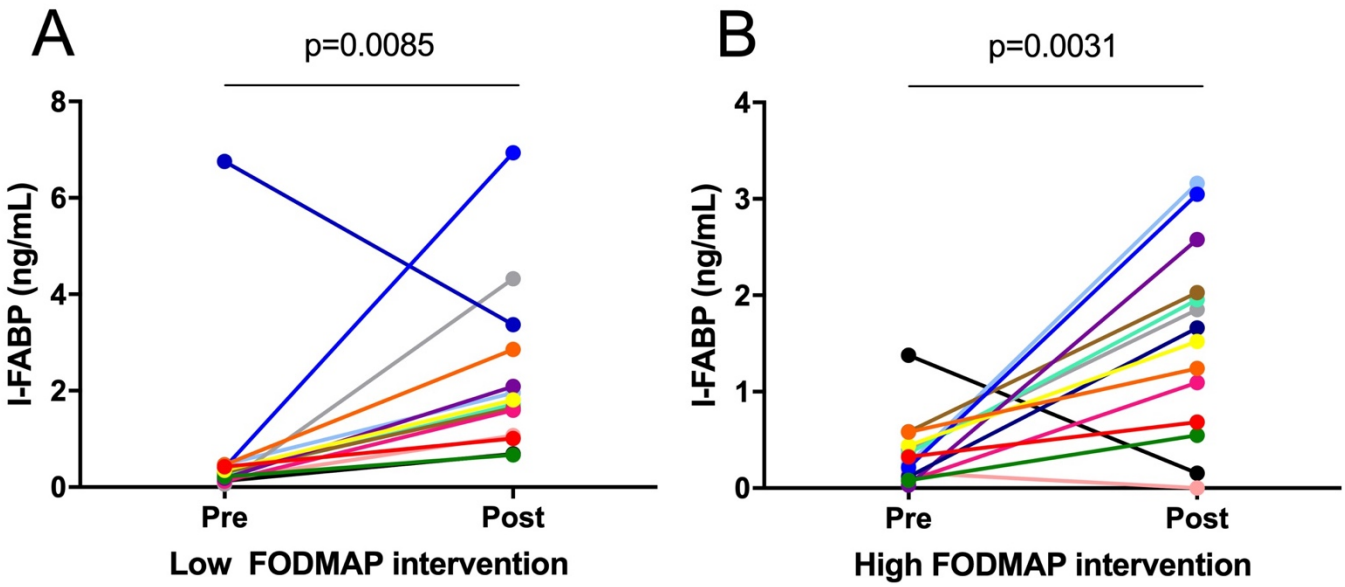

Figure 15. Absolute levels and percent change of plasma I-FABP in a model of exercise-induced gastrointestinal damage subject to low- and high-FODMAP dietary interventions. Pre- and post-exercise plasma I-FABP levels (ng/mL) of healthy athletes (n=14) who ran for 2 hours at 60% VO_{2max} in 35 °C heat and underwent low- and high-FODMAP dietary interventions are represented. (A) indicates pre- and post-exercise plasma I-FABP levels during the low-FODMAP intervention and (B) represents pre- and post-exercise plasma I-FABP levels during the high-FODMAP intervention. Significant differences pre- to post-exercise were detected in both low-FODMAP (p=0.0085) and high-FODMAP (p=0.0031) dietary interventions. Each colour represents the results of an individual study participant.

Percent changes pre-and post-exercise for each dietary intervention were determined. Shapiro-Wilk tests indicated nonparametric distributions in the dietary interventions. Participants expressed the highest median percent change (IQR) of I-FABP levels in the low-FODMAP intervention at 477% (777%) followed by the high-FODMAP intervention at 404% (1181%). Percent changes between dietary interventions were not significantly different, as indicated by Wilcoxon test.

6.3.2.3 LBP

Figure 16A and B depicts pre- and post-exercise absolute levels of plasma LBP (μg/mL) for each study participant in low- and high-FODMAP dietary interventions. Shapiro-Wilk tests indicated normal distributions in the low-FODMAP intervention and nonparametric distributions in the high-FODMAP intervention. There were no significant differences of levels observed pre- to post-exercise in either dietary intervention. As indicated by two-way repeated measures ANOVA, no significant differences in LBP levels pre- and post-exercise or in FODMAP interventions were observed.

Percent changes pre-and post-exercise for each dietary intervention were determined. Shapiro-Wilk tests indicated a nonparametric distribution for the low-FODMAP dietary intervention. Participants expressed the highest median percent change (IQR) of LBP levels in the low-FODMAP intervention at 4% (60%) followed by the high-FODMAP intervention at -6% (45%). Percent changes between dietary interventions were not significantly different, as indicated by Wilcoxon test.

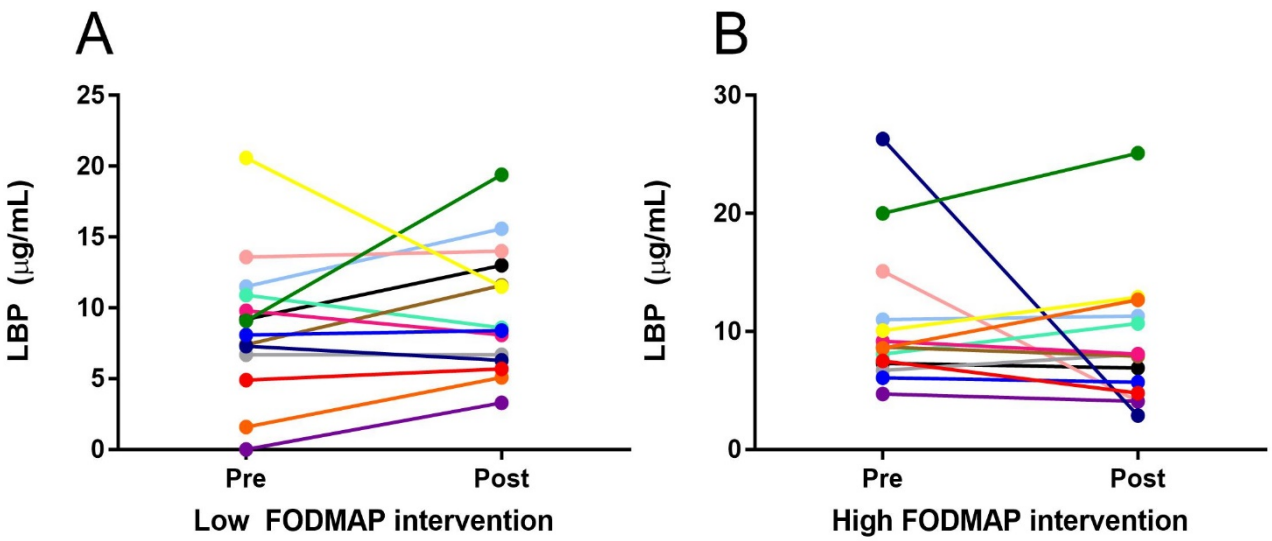

Figure 16. Absolute levels and percent change of plasma LBP in a model of exercise-induced gastrointestinal damage subject to low- and high-FODMAP dietary interventions. Pre- and post-exercise plasma LBP levels (μg/mL) of healthy athletes (n=14) who ran for 2 hours at 60% 60% VO_{2max} in 35 °C heat and underwent low- and high-FODMAP dietary interventions are represented. (A) indicates pre- and post-exercise plasma LBP levels during the low-FODMAP intervention and (B) represents pre- and post-exercise plasma LBP levels during the high-FODMAP intervention. No significant differences in plasma LBP levels pre- to post-exercise in any dietary intervention were detected. Each colour represents the results of an individual study participant.

6.3.2.4 sCD14

Figure 17A and B depicts pre- and post-exercise absolute levels of plasma sCD14 (μg/mL) for each study participant in low- and high-FODMAP dietary interventions. Shapiro-Wilk tests indicated a nonparametric distribution in the low-FODMAP dietary intervention and normal distributions in the high-FODMAP intervention. There were no significant differences of levels observed pre- to post-exercise in either dietary intervention. As indicated by two-way repeated measures ANOVA, no significant differences in sCD14 levels pre- and post-exercise or in FODMAP interventions were observed. No interaction was found between exercise and FODMAP interventions.

Percent changes pre- and post-exercise for each dietary intervention were determined. Shapiro-Wilk tests indicated normal distributions for both dietary interventions. Participants on a low-FODMAP diet experienced higher percent changes of sCD14 levels at a median (IQR) of 20% (72%) compared to -6 (45%) while on the high-FODMAP diet. No significant differences in percent change between the dietary interventions were observed.

6.3.2.5 Marker Correlations

Change in pre- to post-exercise levels, post-exercise, and percent change of marker levels were correlated in each intervention for study participants. No significant marker correlations were found for change in pre- and post-exercise levels or percent change. A significant correlation of post-exercise levels between I-FABP and sCD14 was observed ($r=0.59$, $p=0.029$, $n=14$).

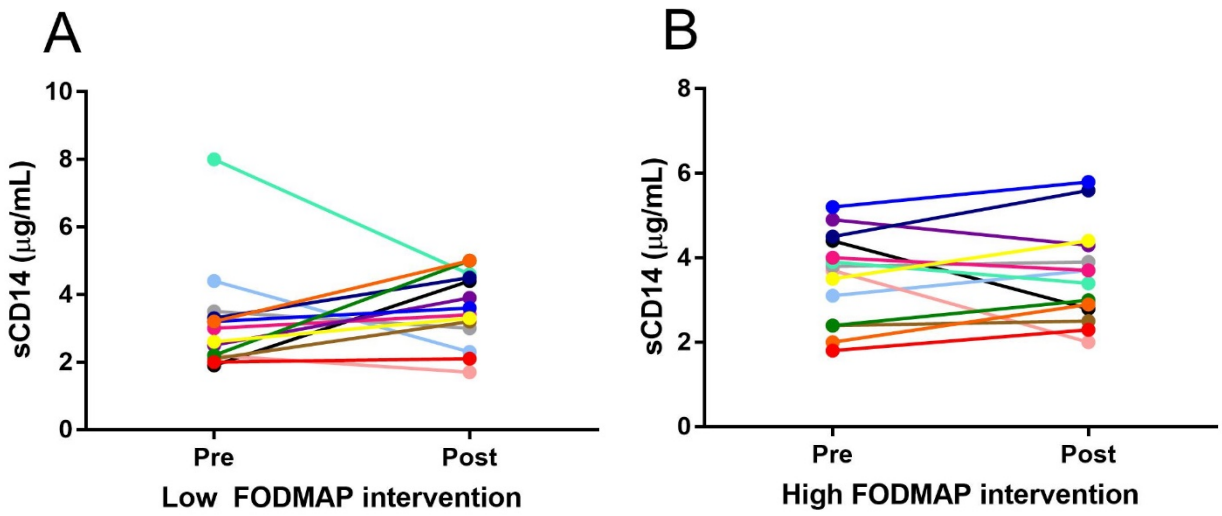

Figure 17. Absolute levels and percent change of plasma sCD14 in a model of exercise-induced gastrointestinal damage subject to low- and high-FODMAP dietary interventions. Pre- and post-exercise plasma sCD14 levels (μg/mL) of healthy athletes (n=14) who ran for 2 hours at 60% VO_{2max} in 35 °C heat and underwent low- and high-FODMAP dietary interventions are represented. (A) indicates pre- and post-exercise plasma sCD14 levels during the low-FODMAP intervention and (B) represents pre- and post-exercise plasma sCD14 levels during the high-FODMAP intervention. Study participants did not show any significant differences pre- to post-exercise in any dietary intervention. Each colour represents the results of an individual study participant.

6.4 Discussion

Exercise-induced gastrointestinal damage in otherwise healthy athletes is an important phenomenon, as upper gastrointestinal symptoms are a major problem in limiting further exertion and achieving better performance. It is also a subject of interest since injury and symptoms are exaggerated at hot ambient temperatures, as recently shown by the elevation of circulating I-FABP levels in several studies, which are featured in Table 3, and described in the present study. In addition, recent published and unpublished work has indicated that manipulation of the FODMAP intake in the diet prior to the exercise can also reduce symptoms without theoretical expectation of reducing intestinal injury. Indeed, I-FABP levels also increased in the current experiments when pre-exercise diet was varied in its FODMAP content, but no differences were observed between the diets, despite the improved symptoms reported in the low FODMAP arm (Costa RJ, personal communication). A low- or high-FODMAP diet did not affect any marker levels and no interactions between exercise and dietary interventions were observed. As such, the results indicate that a low- or high-FODMAP diet does not influence damage of the mucosal barrier during the induction of intestinal ischaemia. In this respect, a low-FODMAP is not necessary to recommend to the general population of athletes for therapeutic benefit. However, the same result may not be the case for individuals with pre-existing inflammation or disease. The therapeutic value of a low-FODMAP diet in athletes who consistently experience gastrointestinal symptoms upon exercise or have a pre-existing gastrointestinal condition may be assessed in future trials.

For the current study, exercise-induced gastrointestinal damage also presents as an excellent model in which effects on other markers and physiological processes, such as epithelial injury not specific to the small intestine, may influence circulating levels of syndecan-1 and markers of bacterial translocation. The additional strength of the model is that it studies subjects

without disease and who are in a state of excellent health and nutrition, avoiding the problems in studying those with diseases that may introduce unwanted confounders. In this respect, the current study has enabled insight into physiological effects beyond small intestinal injury. No previous literature exists on characterising the circulating markers syndecan-1, LBP, and sCD14 in exercise-induced gastrointestinal damage, moreover investigating the adjunct effects of heat and FODMAP dietary interventions. The results in this chapter provide novel findings for longitudinal marker utility and insights into biological mechanisms associated with intestinal barrier damage primarily due to ischaemic stress and the adjunct effects of heat and diet.

In the heat stress trial, significant increases in markers of mucosal barrier integrity, i.e. purported zonulin (Figure 9), syndecan-1 (Figure 10), and I-FABP (Figure 11), were detected in absolute levels pre- to post-exercise intervention. Significant pre- to post-exercise syndecan-1 and I-FABP levels were found in all temperature trials, whereas only the 20 °C and 30 °C interventions yielded significant changes in pre- to post-exercise levels of purported zonulin. These results are novel for syndecan-1 as a marker of epithelial barrier function in exercise-induced gastrointestinal damage. However, the results for purported zonulin should be considered cautiously. As previously mentioned, study participants from this cohort were tested for purported zonulin before experiments from Chapter 5 verified that current commercial assays are not measuring recombinant zonulin.

In contrast to mucosal barrier integrity markers, no significant increases in absolute levels pre- to post-exercise intervention were found in the microbial translocation markers, i.e. LBP (Figure 12) or sCD14 (Figure 13). The same results were confirmed in the dietary FODMAP trial; significant differences in the absolute levels of mucosal barrier integrity markers were detected pre- to post-exercise (Figures 14 and 15), yet no significant differences were detected for the

absolute levels of microbial translocation markers (Figures 16 and 17). This might suggest that, although epithelial injury occurs (as demonstrated by heightened syndecan-1 and I-FABP levels), physical barrier function remains largely intact, preventing microbial translocation. Heat appears to have a slight effect on physical barrier function, as it was observed that the percent change in levels of microbial translocation markers were consistently, though not significantly, elevated in the 35 °C interventions compared to other ambient temperatures. The observed elevation may indicate the beginnings of an immune response to translocated bacterial products due to impaired barrier integrity in high heat, though it is likely that the physical barrier remains largely intact.

It should be noted that there was no significant correlation between I-FABP and syndecan-1. This confirms different mechanisms of damage associated with both markers, which are associated with mucosal barrier integrity. It can therefore be concluded that syndecan-1 does not detect effects specific to small intestinal epithelial damage. This information may also reveal details about location of damage in exercise-induced gastrointestinal damage. As mentioned in Chapter 1, previous studies resolved that I-FABP is mainly expressed in the small intestines though can be found in smaller amounts in the colon. There have been no current studies to date that quantify differences in syndecan-1 expression in epithelial tissue, particularly in the gastrointestinal tract. However, as syndecan-1 is a ubiquitous epithelial and endothelial protein, its expression is most likely not specific to a particular region of the gastrointestinal tract. It is therefore likely that syndecan-1 may be a useful marker to detect an impaired barrier in other locations, such as the colon. Future studies may investigate syndecan-1 expression in the intestinal tract as well as investigate levels in colonic versus small intestinal disease for further understanding.

CHAPTER 7

ASSESSMENT OF INTESTINAL BARRIER INTEGRITY IN NON-COELIAC WHEAT SENSITIVITY/IBS

7.1 Introduction

As discussed in Chapter 1, non-coeliac wheat sensitivity is a controversial diagnosis in which individuals lack the genetic, immune and serological biomarkers of coeliac disease or wheat protein allergy, yet present with similar symptoms that appear to resolve when following a gluten-free diet. There are currently no established biomarkers of disease, yet a recent study by Uhde, Ajamian et al. of an Italian cohort observed levels of associated intestinal barrier dysfunction and microbial translocation markers, i.e. I-FABP, LBP, and sCD14, that were distinctive from coeliac disease and healthy controls in individuals with NCWS.[2] Study participants with NCWS also experienced a significant decline in levels in these markers of immune activation and gut epithelial cell damage, in conjunction with symptom improvement when gluten-containing foods were omitted from their diet. The results implicate dysregulation at the intestinal barrier that may provide clues into NCWS disease mechanism and potential biomarker utility.

Dietary triggers of NCWS are a current topic of debate, as recent studies have sought to determine whether gluten or other factors may be causative agents of gastrointestinal symptoms in individuals with self-reported NCWS. The aforementioned study by Uhde, Ajamian et al. recruited patients who believed they were sensitive to gluten, as they reported experiencing intestinal and/or

extra-intestinal symptoms after ingestion of gluten-containing foods. However, it has been demonstrated in an Australian cohort by Biesiekierski et al. that FODMAPs were the more likely causative factors for gastrointestinal symptoms experienced in individuals with self-reported NCWS and IBS symptoms based on Rome III criteria.[36] In this particular cohort, the specific effects of gluten were examined after dietary reduction of FODMAPs in a placebo-controlled, randomised, double-blind cross-over trial. In all participants, gastrointestinal symptoms consistently and significantly improved during a reduced FODMAP intake. The results provided no evidence of specific or dose-dependent effects of gluten in their study cohort. Only 8% of study participants experienced gluten-specific gastrointestinal effects and, when these subjects were blindly rechallenged many months later, gluten specificity to induction of symptoms was not observed. The evidence suggests that the current description of NCWS as a disease entity is poorly defined, as gluten intake was not a specific trigger of gastrointestinal symptoms in this cohort of patients. This conclusion was reinforced by the results of a Norwegian triple cross-over re-challenge study, in which fructans (i.e., a FODMAP), not gluten, were more likely to be the inciting culprit.[37]

One problem in studies of NCWS has been patient selection.[37] In the Australian and Norwegian studies, only subjects who were HLA -DQ2 and -DQ8 negative or with normal duodenal biopsies were studied. In contrast, Italian studies have in general not excluded patients with mild histological abnormalities such as an increased density of intraepithelial lymphocytes; such patients would have been excluded by the other investigating groups. While the Italian study did not report duodenal histology, it is likely that at least a proportion of the patients had duodenal abnormalities, which another group claim are due to immune reactions to wheat, milk and other proteins.[177]

One way of addressing the potential heterogeneity of patient selection between studies is to determine whether patients from the Australian study also had the abnormalities in marker levels of intestinal barrier integrity and microbial translocation observed in the Italian cohort. Thus, in the current study, serological samples of NCWS study participants from the Biesiekierski et al. study were analysed, using the tests and methods previously used by Uhde, Ajamian et al. in addition to syndecan-1, to examine gluten's effects on the intestinal barrier. The results pertaining to syndecan-1 will be a novel investigation of this marker in NCWS/IBS. Further characterisation of an additional, well-characterised NCWS cohort provides a unique opportunity to explore whether these individuals share similar biomarker profiles despite potential differences in study recruitment. This may provide insights into pathophysiological mechanisms of disease in a seemingly disparate and controversial disease entity.

7.2 Materials and Methods

7.2.1 Study Participants and Protocols

Serum samples for the NCWS cohort (n=36) were obtained from a previous study in which individuals above 16 years of age had self-reported NCWS and irritable bowel syndrome (IBS) symptoms based on Rome III criteria, but not coeliac disease or other significant gastrointestinal-related diseases.[36] Subjects had coeliac disease excluded by HLA studies or had normal duodenal histology. In addition to gastrointestinal disease, study participants who had immune-related or poorly-controlled psychiatric diseases were excluded. Additional exclusion criteria were intake of excessive alcohol, nonsteroidal anti-inflammatory agents, and immunosuppressants.

Study participants underwent a randomised, placebo-controlled, double-blinded cross-over trial to determine whether gluten is a causative agent of gastrointestinal symptoms in NCWS.[36]

Serum samples for each individual study participant were available from baseline when participants consumed their habitual gluten-free diets (in which FODMAPs were not purposely reduced), and while consuming diets low in FODMAPs and containing low amounts of gluten, high amounts of gluten, or whey protein (as placebo). The detailed protocol has been previously outlined and published. Briefly, during the 1-week baseline period, individuals recorded their usual diet and symptoms, then underwent a 2-week run-in period in which they were educated on a low-FODMAP diet. At the end of the run-in period, participants were provided their entire diet, which had a background of being low FODMAP and gluten-free, and was spiked with one of three combinations: (a) 2 g per day of whole-wheat gluten and 14 g per day of whey isolate during low-gluten intervention; (b) 16 g per day of whole-wheat gluten during the high-gluten intervention; and (c) 16 g per day of whey protein isolate for the placebo intervention. Participants received 1 of the 3 dietary interventions for one week followed by a washout period of at least 2 weeks and until symptoms induced during the dietary trial resolved. Participants then crossed over to the next randomly-assigned dietary condition. Participants then resumed the next assigned dietary intervention after the washout period. Patient blood samples were collected and processed after 6 days of each dietary intervention. Thus, paired serum samples for each study participant were associated with the following dietary regimens:

- Gluten-free, normal-FODMAP
- Low gluten, low-FODMAP
- High gluten, low-FODMAP
- Gluten-free, low-FODMAP

The nutritional composition was evaluated by food diary input and analysis using FoodWorks (Xyris Software, Australia) and, for the provided diets, food content and laboratory food analysis for FODMAPs as previously detailed.[36]

7.2.2 Commercial Assays

Levels of featured plasma protein markers were determined by commercially-available ELISA assays. Details of assays are indicated in Section 3.1.1 and assay methodology is indicated in Section 3.3.1.

7.2.3 Statistical Analysis

Chapter 3.4 details general statistical analysis materials and methods used in this chapter. The following results in Section 7.3 report specific methods used for analysis.

7.3 Results

Absolute levels and percentage changes of featured protein markers were determined for each study participant in baseline (normal-FODMAP) and study intervention arms of high-gluten, low-gluten, and placebo, all with a background low-FODMAP diet. Differences in sample size amongst markers due to lack of sera available or otherwise are indicated in each individual section.

7.3.1 Syndecan-1

Shapiro-Wilk tests indicated nonparametric distributions of serum syndecan-1 levels in all dietary interventions. Figure 18A depicts absolute median syndecan-1 levels (ng/mL) in each study participant (n=34) for baseline and dietary intervention arms and significant differences for

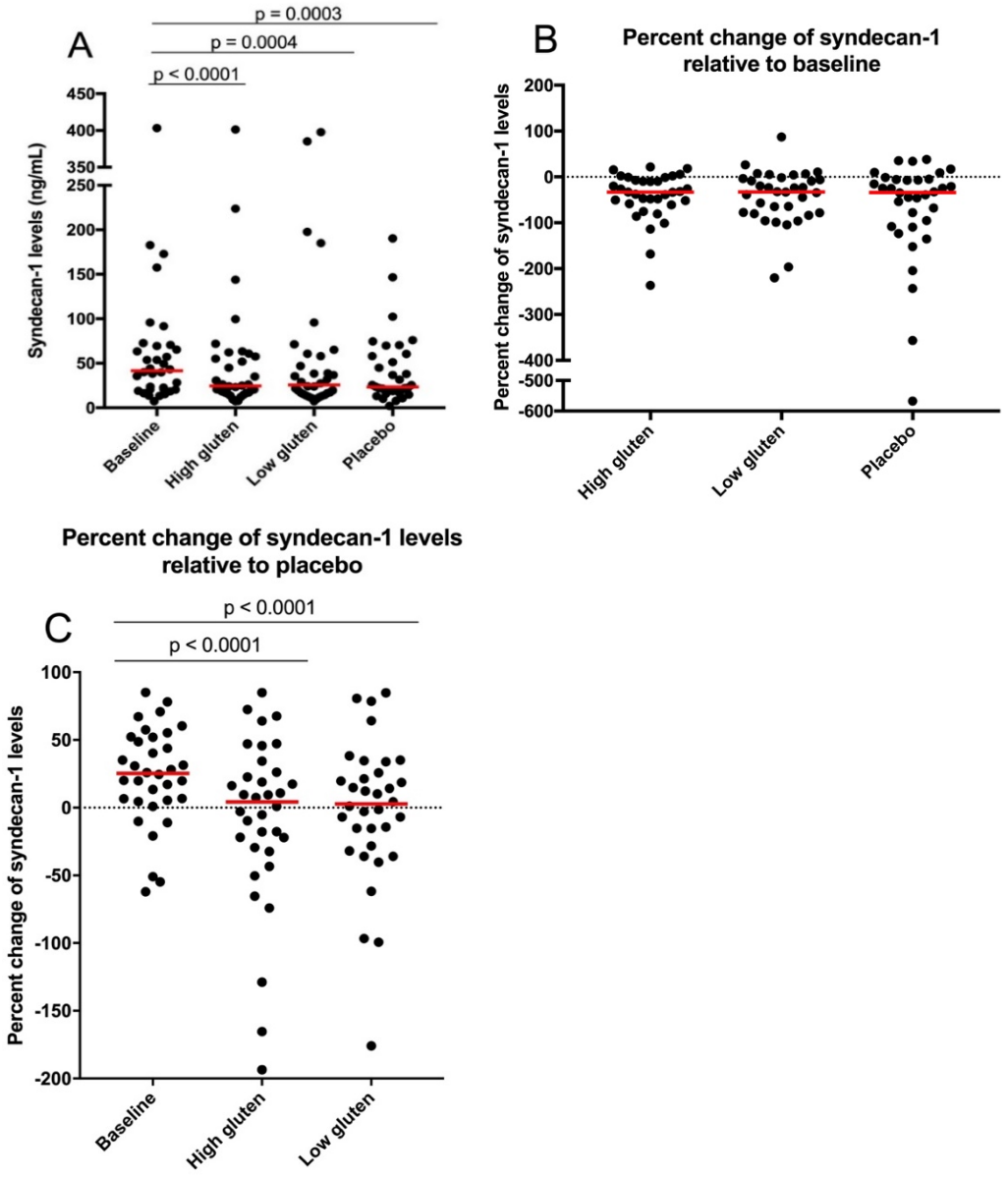

Figure 18. Absolute and percent changes of serum syndecan-1 levels in participants with NCWS undergoing featured high-gluten, low-gluten, and placebo dietary interventions on a background low-FODMAP diet and at baseline condition. Absolute serum syndecan-1 levels and percent changes are represented for individuals with NCWS (n=34) in each dietary intervention and at baseline condition. (A) indicates absolute serum syndecan-1 levels (ng/mL) during dietary interventions arms and at baseline condition. Significant pairwise comparisons were found between baseline and high-gluten ($p<0.0001$), baseline and low-gluten ($p=0.0004$), and baseline and placebo ($p=0.0003$) interventions. (B) represents percent change in syndecan-1 levels relative to baseline. No significant differences were detected in pairwise comparisons. (C) represents percent change in syndecan-1 levels relative to placebo. Significant pairwise comparisons were observed between baseline and high-gluten ($p<0.0001$) and baseline and low-gluten ($p<0.0001$) interventions. Red bars represent medians.

pairwise comparisons between the dietary interventions. Study participants had the highest median (IQR) of syndecan-1 levels in the baseline condition at 42 (49), followed by the low-gluten intervention at 26 (44), the high-gluten intervention at 24 (42), and the placebo intervention at 41 (23). A significant difference between repeated-measures group medians was observed by Friedman's test ($\chi^2_F(3)=24.67$, $p<0.0001$). Significant pairwise comparisons, as indicated by Wilcoxon tests, were observed between baseline and high-gluten ($p<0.0001$), baseline and low-gluten ($p=0.0004$), and baseline and placebo ($p=0.0003$) interventions.

The percent changes relative to baseline and placebo interventions are indicated in Figure 18B and C, respectively. Shapiro-Wilk tests indicated nonparametric distributions in percent changes relative to baseline. Negative percentage changes were observed across all dietary interventions by nearly the same amount; the placebo intervention had the lowest median (IQR) syndecan-1 levels at -34% (103%), followed by the high-gluten -33% (53%), and the low-gluten intervention at -33% (76%) (Figure 18B). No significant differences were detected by Friedman's test or in pairwise comparisons. Shapiro-Wilk tests indicated nonparametric distributions in percent changes relative to placebo. Increased syndecan-1 levels were observed for the baseline condition at 25% (47%) compared to the high-gluten intervention at 4% (58%) as well as the low-gluten intervention at 3% (57%) (Figure 18C). A significant difference in medians was detected by Friedman's test ($\chi^2_F(3)=20.18$, $p<0.0001$) with significant pairwise comparisons between baseline and high-gluten ($p<0.0001$) and baseline and low-gluten ($p<0.0001$) conditions, as indicated by Wilcoxon tests.

7.3.2 I-FABP

Shapiro-Wilk tests indicated nonparametric distributions of serum I-FABP levels in dietary interventions. Figure 19A depicts absolute median I-FABP levels (ng/mL) in each study participant (n=34) for baseline and dietary intervention arms. Study participants had the highest median (IQR) I-FABP levels in the baseline condition at 0.9 (0.8), followed by the high-gluten intervention at 0.8 (0.7), the placebo intervention at 0.8 (0.5), and the low-gluten intervention at 0.7 (0.4). A significant difference in medians was detected by Friedman's test ($\chi^2_F(3)=8.82$, p=0.032). As indicated by Wilcoxon tests, significant pairwise comparisons were found between baseline and low-gluten intervention (p=0.0131) and baseline and placebo intervention (p=0.031), though these were not significant discoveries when the FDR was controlled (q=0.083 and q=0.098, respectively).

The percent changes relative to baseline and placebo interventions are indicated in Figure 19B and C, respectively. Shapiro-Wilk tests indicated nonparametric distributions in percent changes relative to baseline. Decreased I-FABP levels were observed across all dietary interventions; the low-gluten intervention had the lowest median (IQR) levels at -41% (102%), followed by the placebo intervention at -34% (118%), and the high-gluten intervention at -22% (99%) (Figure 19B). No significant differences between medians were detected by Friedman's test or in pairwise comparisons by Wilcoxon tests. Shapiro-Wilk tests indicated nonparametric distributions in percent changes relative to placebo. The highest percent change in I-FABP levels were observed for the high-gluten intervention at 13% (44%), followed by the low-gluten intervention at 2% (61%), and the baseline condition at 0.3% (0.7%) (Figure 19C). No significant difference between medians were detected by Friedman's test or in pairwise comparisons by Wilcoxon tests.

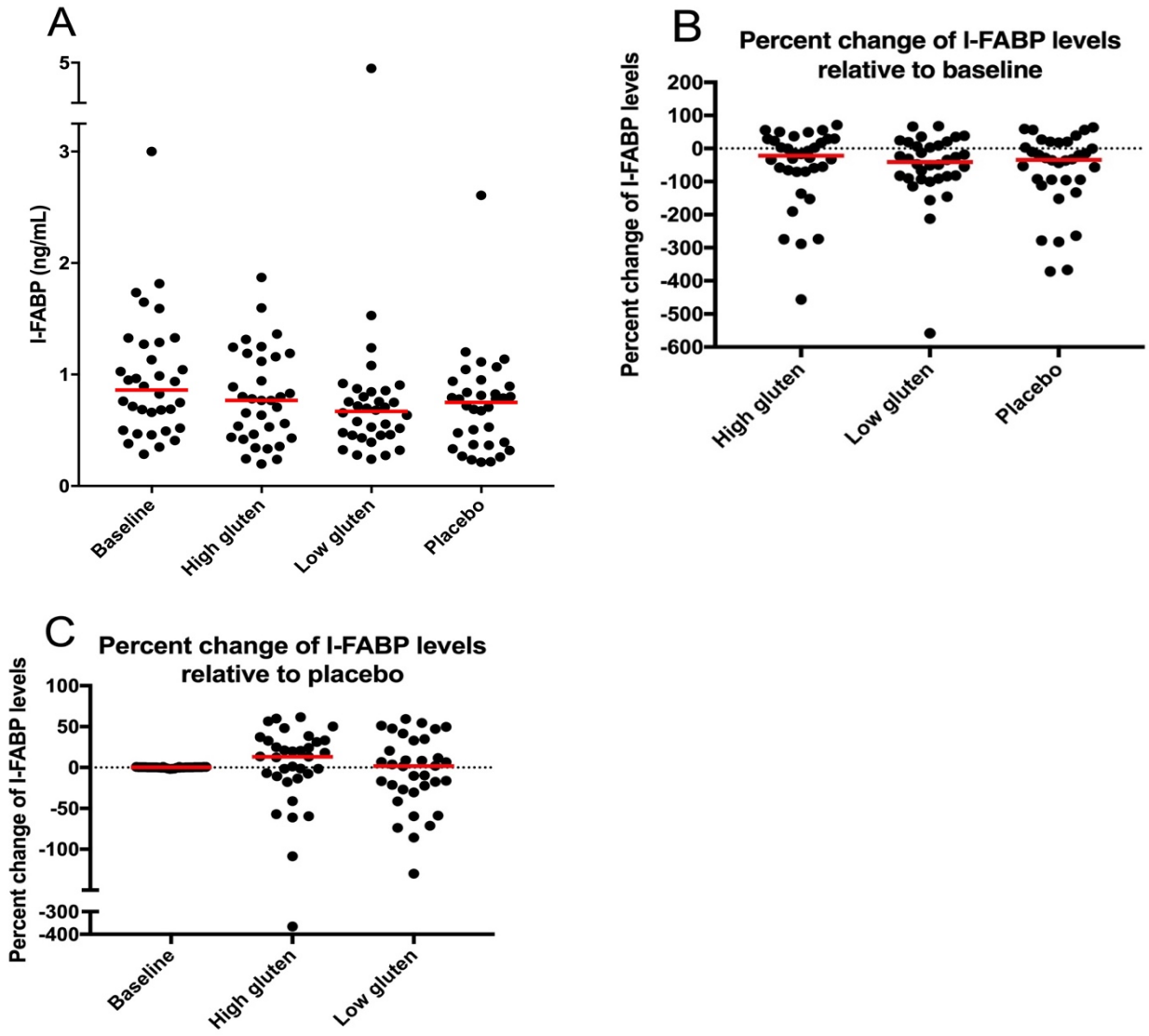

Figure 19. Absolute and percent changes of serum I-FABP levels in participants with NCWS undergoing featured high-gluten, low-gluten, and placebo dietary interventions on a background low-FODMAP diet and at baseline condition. Absolute serum I-FABP levels and percent changes are represented for individuals with NCWS (n=34) in each dietary intervention and at baseline condition. (A) indicates absolute serum I-FABP levels (ng/mL) during dietary interventions arms and at baseline condition. (B) represents percent change in I-FABP levels relative to baseline. (C) represents percent change in I-FABP levels relative to placebo. Red bars represent medians. No significant pairwise comparisons between means or medians were observed in absolute or percent change levels.

7.3.3 LBP

Shapiro-Wilk tests indicated nonparametric distributions of serum LBP levels in dietary interventions. Figure 20A depicts absolute median LBP levels (μg/mL) for each study participant (n=34) in baseline and dietary intervention arms. Study participants had the highest median (IQR) LBP levels in the baseline condition at 12 (6), followed by the placebo intervention at 9 (5), the high-gluten intervention at 9 (6), and the low-gluten intervention at 9 (6). Significant differences between medians were detected by Friedman's test ($\chi^2_F(3)=9.37$, $p=0.023$). A significant difference between high-gluten and baseline interventions was detected ($p=0.0038$) by a paired t-test, which was a significant discovery when the FDR was controlled ($q=0.0228$). Significant pairwise comparisons were also found between low-gluten and baseline interventions ($p=0.024$) as well as placebo and baseline interventions ($p=0.048$) as indicated by Wilcoxon tests, though these were not significant discoveries when the FDR was controlled ($q=0.071$ and $q=0.097$, respectively).

The percent changes relative to baseline and placebo are indicated in Figure 20B and C, respectively. Shapiro-Wilk tests indicated normal distributions in all percent changes relative to baseline. Negative percent changes in LBP levels were observed across all dietary interventions by similar amounts; the lowest mean ± SEM percent change relative to baseline was found in the placebo intervention at -28% ± 10%, followed by the low-gluten intervention at -28% ± 10% and the high-gluten intervention at -27% ± 9%. No significant percent changes between groups were found in a one-way repeated measures ANOVA or in paired t-tests. Shapiro-Wilk tests indicated nonparametric distributions in all percent changes relative to placebo. The highest median (IQR) percent change was observed in the baseline condition 18% (44%), followed by the low-gluten

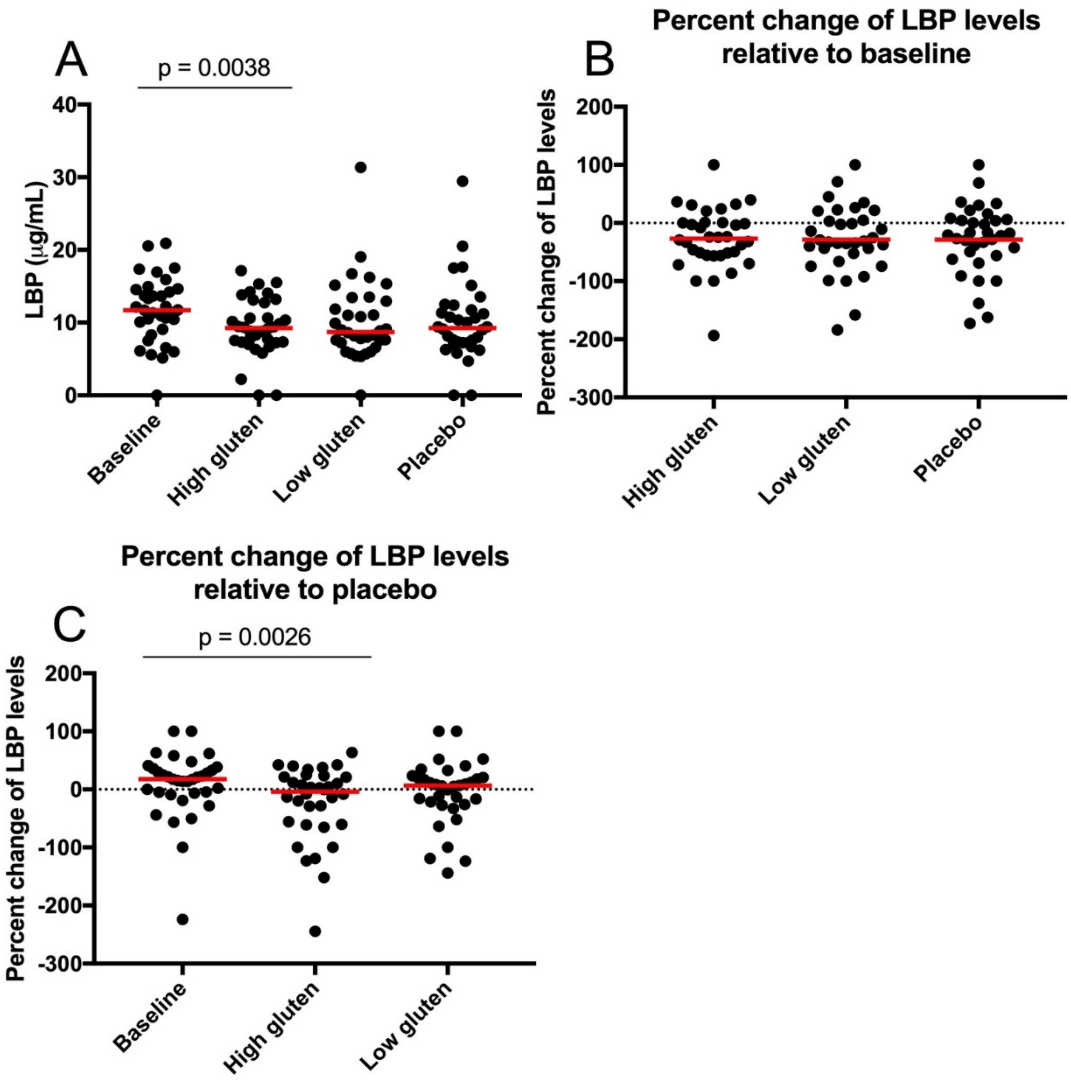

Figure 20. Absolute and percent changes of serum LBP levels in participants with NCWS undergoing featured high-gluten, low-gluten, and placebo dietary interventions on a background low-FODMAP diet and at baseline condition. Absolute serum LBP levels and percent changes are represented for individuals with NCWS (n=34) in each dietary intervention and at baseline condition. (A) indicates absolute median serum LBP levels (ng/mL) during dietary interventions arms and at baseline condition. Significant pairwise comparisons were found between baseline and high-gluten interventions (p=0.0038). (B) represents mean percent change in LBP levels relative to baseline. No significant differences were observed in pairwise comparisons. (C) represents median percent change in LBP levels relative to placebo. A significant pairwise comparison was observed between baseline and high gluten interventions (p=0.0026).

intervention at 6% (48%) and the high-gluten intervention at -4% (82%). Significant differences between medians were detected by Friedman's test ($\chi^2_F(3)=10.45$, $p=0.0054$). A significant pairwise comparison was also found between the high-gluten intervention and baseline by a Wilcoxon test ($p=0.0026$), which was a significant discovery when the FDR was controlled ($q=0.0078$). Another significant pairwise comparison was found between the low-gluten and baseline intervention by a Wilcoxon test ($p=0.0433$), though it was not a significant discovery when the FDR was controlled ($q=0.065$).

7.3.4 sCD14

Shapiro-Wilk tests indicated normal distributions of serum sCD14 levels in all dietary interventions. Figure 21A depicts absolute mean sCD14 levels (μg/mL) for each study participant (n=34) in baseline and dietary intervention arms. Study participants had the highest mean ± SEM sCD14 levels in the baseline condition at 1.4 ± 0.1, followed by the low-gluten condition at 1.4 ± 0.1. The high-gluten and placebo interventions shared the same mean values at 1.3 ± 0.1. No significant differences between intervention means were observed by a repeated measures one-way ANOVA or in paired t-tests.

The percent changes relative to baseline and placebo are indicated in Figures 21B and C, respectively. Shapiro-Wilk tests indicated a nonparametric distribution in percent changes relative to baseline. Negative percent changes in sCD14 levels were observed across all dietary interventions; the lowest median (IQR) percent change was observed in the placebo intervention at -21% (52%), followed by the high-gluten intervention at -16% (65%) and the low-gluten

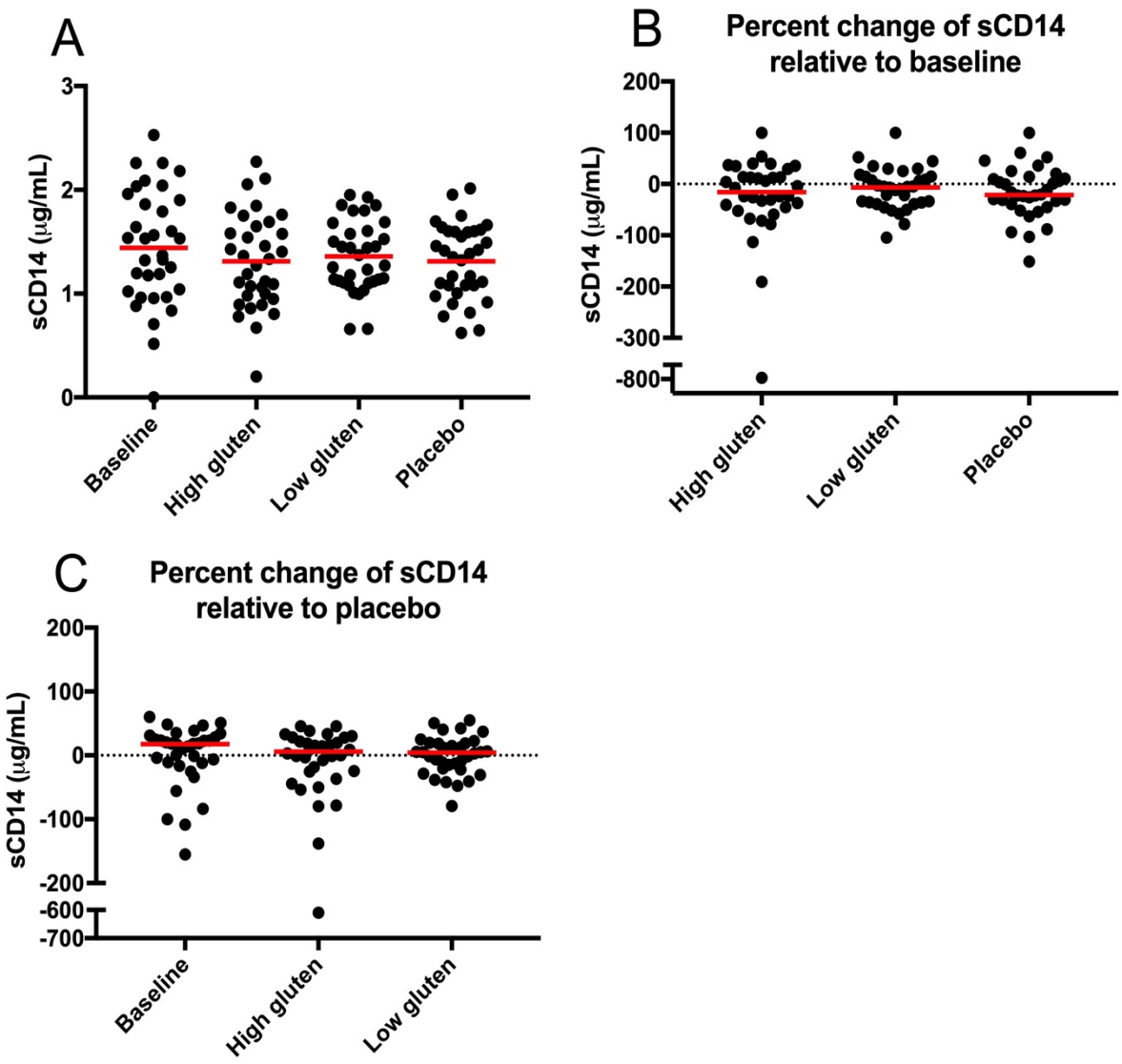

Figure 21. Absolute and percent changes of serum sCD14 levels in participants with NCWS undergoing featured high-gluten, low-gluten, and placebo dietary interventions on a background low-FODMAP diet and at baseline condition. Absolute serum sCD14 levels and percent changes are represented for individuals with NCWS (n=34) in each dietary intervention and at baseline condition. (A) indicates absolute mean serum sCD14 levels (μg/mL) during dietary interventions arms and at baseline condition. (B) represents median percent change in sCD14 levels relative to baseline. (C) represents percent change in sCD14 levels relative to placebo. No significant pairwise comparisons between means or medians were observed in absolute or percent change levels.

condition at -9% (53%). No significant differences between medians were observed by Friedman's test or in pairwise comparisons. Shapiro-Wilk tests indicated nonparametric distributions in percent changes relative to placebo. Positive percent changes in sCD14 levels were observed across all dietary interventions; the highest median (IQR) percent change was observed in the baseline condition at 18% (42%), followed by the high-gluten intervention at 6% (51%) and the low-gluten condition at 4% (40%). No significant difference between medians were observed by Friedman's test or in pairwise comparisons.

7.3.5 Marker Correlations

Marker correlations between absolute levels as well as percent changes to baseline and placebo were analysed within each intervention for study participants (n=33). As previously mentioned, individuals were omitted from analysis if there was incomplete testing of markers due to lack of available sera. A positive, significant correlation was found between absolute LBP and sCD14 levels ($p=0.020$, $r=0.403$). With regards to percent change from baseline, positive, significant correlations were observed between baseline and the low-gluten intervention for syndecan-1 and sCD14 ($p=0.017$, $r=0.412$) as well as LBP and sCD14 ($p=0.029$, $r=0.381$). Similarly, with regards to percent change from placebo, the only significant correlations were observed between placebo and the low-gluten intervention for syndecan-1 and I-FABP ($p=0.006$, $r=-0.467$), syndecan-1 and sCD14 ($p=0.029$, $r=0.381$), and LBP and sCD14 ($p=0.047$, $r=0.348$).

7.4 Discussion

7.4.1 Comparison of Italian and Australian NCWS Cohorts

NCWS is a controversial diagnosis with uncertain pathophysiology. The findings of a unique combination of systemic immune activation and compromised intestinal barrier integrity in an Italian cohort with NCWS defined by the so-called Salerno criteria (based upon consensus opinion of an international group who believe that gluten is the cause of NCWS) has suggested a potential pattern of biomarkers that identify the condition.[178] However, other studies such as the Australian blinded randomised re-feeding controlled trial in patients with self-reported NCWS used different inclusion criteria (as outlined in Chapter 1). This raises the very real concern that differences in responses to gluten might relate to patient selection. The application of the biomarkers from the previous study by Uhde, Ajamian et al. (i.e. the study that tested the Italian NCWS cohort) to the Australian cohort was an opportunity to test this hypothesis.

Compared to the current study, heightened median absolute levels of I-FABP, LBP, and sCD14 were observed in the Italian cohort; the median I-FABP level in the current study was 0.9 ng/mL compared to 1.6 ng/mL in the previous study, the median LBP level in the current study was 12 μg/mL compared to 19 μg/mL, and median sCD14 level in the current study was 1.4 μg/mL compared to 2.5 μg/mL (all previous study values have been extrapolated from figures).[2] The results confirm a difference in study participant recruitment between both NCWS cohorts. Furthermore, marker levels in the current study match those found in IBS cohorts. A mean ± SEM level of 0.7 ± 0.4 ng/mL was observed for I-FABP in a previous study, which matches the current study's observed median (IQR) level of 0.9 (0.8) ng/mL.[179] In the same study, a mean ± SEM level of 2 ± 0.6 μg/mL was observed for sCD14; this level was at the higher end, though within range of the current study's median (IQR) level of 1.4 (0.6).[179]

Despite the difference in study participant recruitment, it remains uncertain why heightened levels were observed in the Italian cohort. One possible explanation might be due to the inclusion and emphasis of extra-intestinal symptoms as outlined in the Salerno criteria and consequently featured in the Italian NCWS cohort. Only gastrointestinal symptoms and fatigue were considered in the current study's participants, whereas gastrointestinal and/or extra-intestinal symptoms were featured in the Italian NCWS study participants. These manifestations consist of fatigue, headache, anxiety, memory and cognitive disturbances, and numbness in the arms and legs.[2] Hence, although the current study ruled out gluten as a primary causative agent of gastrointestinal symptoms in self-reported NCWS, there remains the possibility that gluten may be involved in the pathogenesis of extra-intestinal manifestations, which is somehow linked to the integrity of intestinal mucosal barrier. In neurobehavioural disorders, there is some evidence of gluten-specific immune activation, which implicates the translocation of pathogenic gluten fragments across a compromised intestinal mucosal barrier. In subsets of children with autism and cerebral palsy, increased levels of gluten-specific antibodies have been observed compared to healthy controls.[180,181] Anecdotal reports indicate improvement in symptom severity upon adherence to a gluten-free diet, much like individuals with extra-intestinal manifestations of NCWS. However, there have been no comprehensive placebo-controlled, randomised double-blinded crossover trials designed to investigate whether gluten influences extra-intestinal symptoms alone and whether it has a potential effect on the intestinal barrier in NCWS or in neurobehavioural disorders. Further inquiry into the subset of NCWS patients who experience extra-intestinal manifestations may provide the key to understanding the divergent results observed in the current study's NCWS cohort and that of the Italian cohort.

7.4.2 Insights into the Effect of Reducing Dietary FODMAPs

The present study confirms that dietary gluten is unlikely to modulate intestinal barrier function and microbial translocation in patients who have wheat-induced symptoms that fit criteria for IBS, but no evidence of coeliac disease or intestinal histopathological abnormalities. However, the consumption of the provided diets somewhat unexpectedly led to consistent changes in the concentrations of three of the four biomarkers relative to their concentrations when the subjects were consuming their habitual gluten-free diet. The dominating change was in the syndecan-1 levels, which fell by about 30% irrespective of the gluten or whey protein content of the diet ($p<0.001$). Levels of the small intestinal epithelial marker, I-FABP, and the marker of bacterial translocation, LBP, also fell, but these changes just failed to reach statistical significance when correction for multiple comparisons was applied. Levels of sCD14 were not different. In other words, the provided diets, irrespective of gluten content, led to reduced epithelial injury and possibly less bacterial translocation. The predominance of changes in syndecan-1 over I-FABP might indicate the main effect was in the colon rather than proximal small intestine, particular from what has been found in coeliac disease, a model of small intestine injury, as further discussed in Chapters 1 and 6.

The reason for this likely change in the epithelial barrier is not simple to define since altering diets changes components that might potentially affect the epithelium, such as emulsifier and thickener content.[182] Analysis of the dietary intake during these phases was performed.[36] There were no differences in macronutrients. However, the two components where differences were noted were in the alcohol intake (reducing from a mean of 12 g/d to about 3 g/d) and in the FODMAP content (reducing from 19 g/d to about 5 g/d). Alcohol can injure epithelium, but it is unlikely that the intake reported, which were less than recommended at-risk levels in nearly all

patients, would be responsible, particularly in the colon was the main target.[183] However, the effect of alcohol on these markers is not known and may be worthy of further investigation.

The major mechanism of action of FODMAPs is likely to be reduction of luminal distension in the intestines, which in turn reduces mechanostimulation and subsequent sensation of pain and bloating. However, it is suggested that intestinal wall stretching may potentially reduce barrier function by stretching the intercellular epithelial junctions. Alterations of FODMAP intake changes the structure and density of the colonic microbiota, which might affect barrier function, as observed by Halmos et al.[184] Indeed, in adult male Wistar rats, a high FODMAP diet reduces colonic epithelial barrier function and induces mild colonic inflammation, an effect that was reversed by a low FODMAP diet or rifaximin therapy. Both approaches reduced bacterial abundance and it was subsequently shown that the abnormalities were induced by LPS.[185] Thus, the concept was introduced that the reduction of the absolute abundance of colonic bacteria and LPS (consistent with findings from Halmos et al.) by the low FODMAP diet improves colonic barrier function, and reduces visceral nociception and colonic inflammation as an additional mechanism for the longer-term efficacy of the low FODMAP diet. The findings of the current study support this concept. Thus, epithelial injury is reduced and LPS exposure possibly reduced by lowering FODMAP intake in patients with self-reported NCWS. This speculative interpretation of the biomarker findings warrants further investigation.

7.4.3 Potential Marker Utility

Taken together, the results provide insights into the utility of featured markers in the investigation of NCWS/IBS. A very minimal median increase for percent change of I-FABP levels relative to placebo in the baseline condition was observed at 0.3%. As I-FABP is primarily

expressed in the small intestines, these results point to a minimal effect of the low-FODMAP diet on small bowel enteropathy and implies that effects are localised to the colon.

Syndecan-1 absolute levels were significantly heightened in the baseline normal-FODMAP condition compared to all background low-FODMAP intervention arms. These levels did not correlate with I-FABP absolute levels. This supports previous observations, as detailed in Chapter 6, that syndecan-1 may instead be indicating colonic barrier integrity effects. In support of this observation, a negative correlation was found between syndecan-1 and I-FABP levels in the percent change from placebo to low-gluten/low-FODMAP condition. Significant correlations between syndecan-1 and microbial translocation markers were also observed in this particular condition as well as in the percent change from baseline to low-gluten condition. These results suggest that syndecan-1 may reflect the potential effects of a low-FODMAP diet in reducing epithelial injury and thus limiting LPS exposure.

Taken together, the outcomes provide new evidence that syndecan-1 may be a useful marker for determining colonic epithelial barrier integrity, which may be helpful for the many emerging trials that investigate the effects of colonic bacteria on epithelial integrity. Comparison of syndecan-1 levels to biopsy data in which it is clear that colonic epithelial integrity has been compromised may further support the use of syndecan-1 levels as a reliable circulating marker indicative of colonic epithelial integrity.

7.4.4 Summary

In summary, the results of this study:

1) Demonstrate the heterogeneity of the NCWS population by the very different patterns of circulating levels of biomarkers of intestinal barrier dysfunction and microbial translocation in two populations recruited by different inclusion criteria;
2) Show that gluten had no effects on biomarkers of the intestinal epithelial barrier and microbial translocation in a cohort that did not have specific symptomatic responses to gluten;
3) Provide evidence to support the notion that reducing dietary FODMAP intake has a protective effect on the colonic epithelium; and
4) Provide insights into the utility of featured circulating markers, particularly with the use of syndecan-1 to indicate colonic epithelial barrier integrity.

CHAPTER 8

ASSESSMENT OF INTESTINAL BARRIER INTEGRITY IN THE TREATMENT OF COELIAC DISEASE

8.1 Introduction

As detailed in Chapter 1, strict adherence to a gluten-free diet is necessary for mucosal healing and the amelioration of symptoms in coeliac disease. However, there are some patients who do not respond well to a gluten-free diet alone. These poorly responsive patients are classified as having refractory coeliac disease if they have persistent or recurrent small intestinal villous atrophy with symptoms of malabsorption despite ≥ 12 months of a strict gluten-free diet in the absence of a condition that causes villous atrophy, such as lymphoma.[186] In addition to keeping a strict gluten-free diet, patients with refractory coeliac disease may be prescribed the synthetic glucocorticoid budesonide as treatment. Budesonide has low systemic bioavailability and high active topical delivery in the formulation commonly used. Delivery is particularly to the distal small intestines and colon, though about 30% is released and absorbed in the upper small intestines.[187]

To date, there has been one primary study (n=34) that has reported a 76% response to the medication, and a complete response in 55% when budesonide was used alone or with oral corticosteroids in patients with refractory coeliac disease.[188] Aside from this evidence, there exist only expert opinions and observations on the efficacy of budesonide in the treatment of any type of coeliac disease. In the treatment of other intestinal inflammatory conditions, including

microscopic colitis, eosinophilic colitis, and collagenous sprue, which can resemble coeliac disease, patients also appear to respond to budesonide.[189,190] Due to the drug's target specificity in the intestinal tract, limited systemic effects, and positive outcomes in response, it has been hypothesised that it may accelerate healing for newly-diagnosed coeliac disease patients in conjunction with a gluten-free diet.

The current study examines circulating mucosal barrier integrity and microbial translocation markers in serological samples from the first double-blinded, randomised placebo controlled study designed to determine the effects of budesonide in addition to gluten-free diet in the mucosal healing of newly-diagnosed coeliac disease patients. The investigation of the featured mucosal barrier integrity and microbial translocation markers in the current study will: 1) provide evidence for the efficacy of budesonide in the treatment of newly-diagnosed coeliac disease, 2) provide insights into mucosal barrier healing and 3) provide evidence for the longitudinal utility of featured markers in coeliac disease.

8.2 Materials and Methods

8.2.1 Study Participants and Protocols

Serum samples for the coeliac disease cohort (n=37) were obtained from newly diagnosed patients between the ages of 16 and 60 years. All patients had villous atrophy (Marsh III lesions) in endoscopic duodenal biopsies. Patients were excluded if duodenal lesions were of Marsh I-II grade as well as those with significant co-morbidity, including gastrointestinal and immune-related diseases or active infection. Additional exclusion criteria were abnormal liver test results, evidence of liver cirrhosis, use of inhaled steroids, treatment with anti-fungals, existing or intended pregnancy, known intolerance or hypersensitivity to the study drug or those similar in

pharmacological profile, participation in another coeliac disease clinical trial within the last 30 days prior to enrolment or simultaneously, and well-founded doubt about patient compliance due to drug or alcohol addiction. In addition, patients who were on a gluten-free diet for more than 4 weeks prior to commencement of the study were excluded from participation.

Patients were participating in a phase IIb, randomised, double-blinded placebo-controlled trial to evaluate the efficacy and safety of oral budesonide in the induction of mucosal healing.[162] Participants were randomised to receive 9 mg once per day of an effervescent budesonide preparation or placebo for 8 weeks. All study participants were counselled to undertake a gluten-free diet by an accredited practising dietitian to which adherence was assessed by direct questioning to be excellent. The patients had duodenal biopsies repeated after 3 months and 12 months, in which the degree of mucosal healing was assessed by a pathologist blinded to the treatment. Sera evaluated in the current study was obtained from peripheral blood during baseline (i.e., 'untreated', at the time of study enrolment after the diagnosis of coeliac disease), at 4 weeks of the intervention, and at 8 weeks of the intervention. Thus, paired sera were available for the following scenarios:

- Untreated coeliac disease
- Gluten-free diet for 4 weeks
    - With or without budesonide treatment
    - With or without mucosal healing at 3 months
- Gluten-free diet for 8 weeks
    - With or without budesonide treatment
    - With or without mucosal healing at 3 months

8.2.2 Commercial Assays

Levels of featured plasma and serum protein markers were determined by commercially-available ELISA assays. Details of assays are indicated in Section 3.1.1 and assay methodology is indicated in Section 3.3.1.

8.2.3 Statistical Analysis

Chapter 3.4 details general statistical analysis materials and methods used in this chapter. The following results in Section 8.3 report specific methods used for analysis.

8.3 Results

Complete mucosal healing, as indicated by duodenal biopsy Marsh score of 0, was achieved in 9 patients at the 3-month assessment; of these patients, 6 received budesonide treatment and 3 received placebo. Where noted, nonparametric tests were utilised for analysis due to the small number of patients who achieved mucosal healing; these tests were particularly utilised to analyse levels or percent changes of markers in the small placebo group.

Absolute levels of featured protein markers at baseline/untreated, 4-week, and 8-week time points after following a gluten-free diet were determined for each study participant. These results are reported in the figures of this section. Percent changes were also determined for each protein marker. In the following figures, blue coloured points and/or lines denote patients who received budesonide (n=16), whereas patients who received placebo (n=14) are indicated in green. Individuals who achieved mucosal healing by the 3-month assessment are indicated in purple.

8.3.1 Syndecan-1

Shapiro-Wilk tests indicated nonparametric distributions of serum syndecan-1 levels for study participants. Figures 22A and B depicts absolute median syndecan-1 levels (ng/mL) in patients who received budesonide and placebo, respectively. Study participants who took budesonide had the highest median (IQR) of syndecan-1 levels in the baseline condition at 27 (77); they had similar median levels at the 4-week and 8-week time points at 23 (49) and 23 (55), respectively. No significant differences were detected by Friedman's test, though a significant difference in levels was detected at the 4-week time point compared to baseline ($p=0.008$). Study participants who took placebo had the highest median syndecan-1 levels in the 8-week time point at 42 (143), followed by the 4-week time point at 35 (155), and the baseline condition at 19 (233). No significant differences were detected by Friedman's test or in pairwise comparisons for the placebo group. A two-way repeated measures ANOVA did not indicate significant differences in syndecan-1 levels in relation to intervention or time. No interaction between intervention and time was observed.

Among the study participants who achieved mucosal healing at the 3-month assessment, those who took budesonide ($n=6$) had the highest median levels in the baseline condition at 61 (126), closely followed by levels in the 8-week time point at 61 (122), then followed by levels at the 4-week intervention at 41 (103). Those who took placebo ($n=3$) and achieved complete mucosal healing had lower overall median levels; the highest levels were observed in the 4-week time point at 30 (25), followed by the baseline condition at 21 (35), and the 8-week time point at 15 (47). No significant differences were detected by Friedman's test or in pairwise comparisons in both budesonide and placebo groups. A two-way repeated measures ANOVA did not indicate

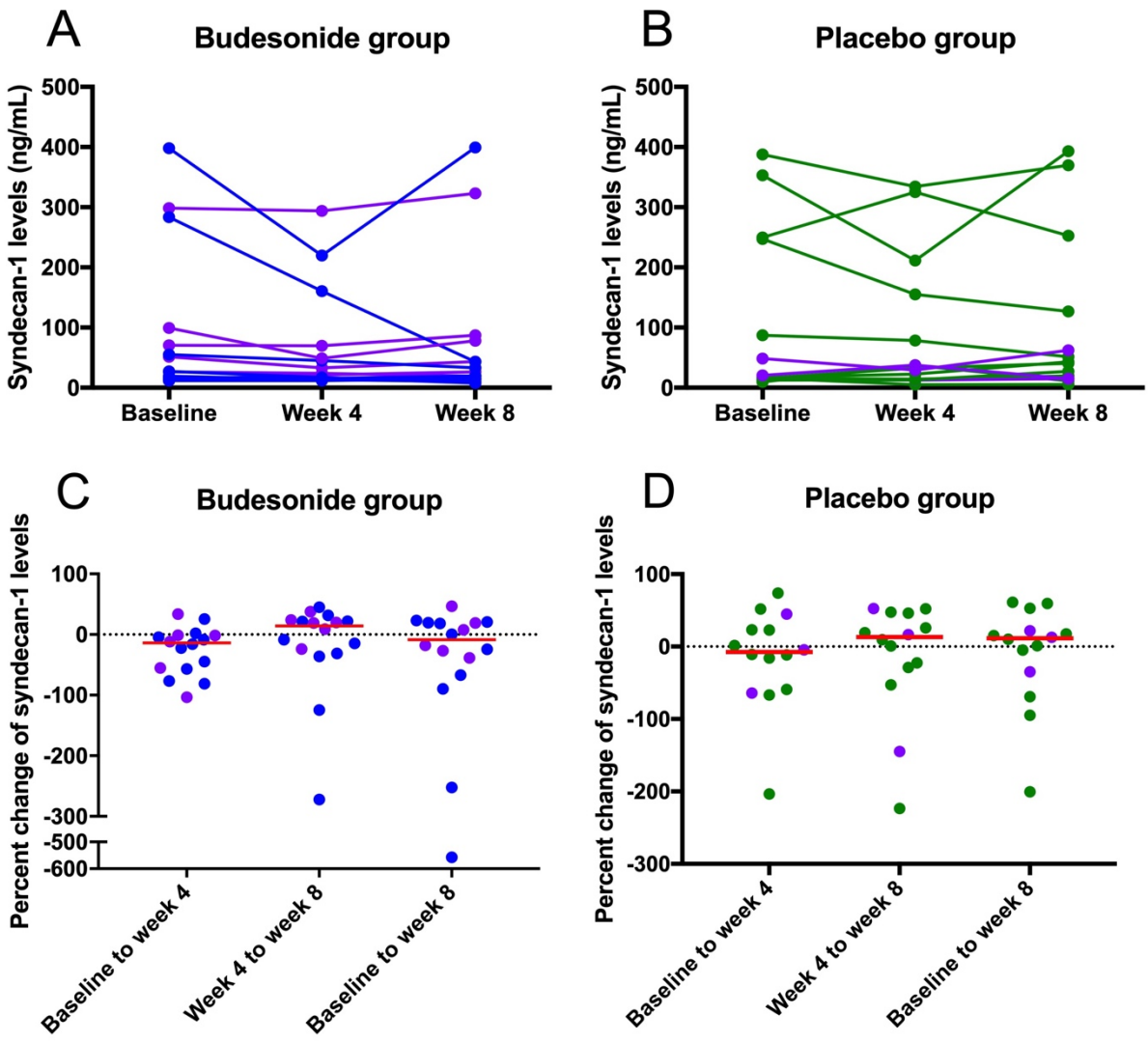

Figure 22. Absolute and percent changes of serum syndecan-1 levels in newly-diagnosed coeliac disease patients undergoing treatment with budesonide or placebo and a gluten-free diet. Absolute serum syndecan-1 levels and percent changes are represented for newly-diagnosed coeliac disease patients. (A) indicates absolute median syndecan-1 levels (ng/mL) from baseline condition to week 8 in study participants who received budesonide (n=16) and (B) indicates levels in those who received placebo (n=14). Median percent changes are indicated for patients who received budesonide (C) and for those who received placebo (D). Absolute levels or percent changes of syndecan-1 in patients who achieved mucosal healing are indicated in purple; these include 6 patients in the budesonide group and 3 patients in the placebo group. Red bars represent medians for all study participants in the specified treatment group in (C) and (D).

significant differences in syndecan-1 levels with relation to intervention or time. No interaction between intervention and time was observed.

Absolute syndecan-1 levels were compared in those with and without achievement of mucosal healing at the 3-month assessment in both budesonide and placebo groups. Two-way repeated measures ANOVAs did not indicate significant difference in syndecan-1 levels in relation to mucosal healing status or time in both groups. No interaction between mucosal healing status and time was observed.

The percent changes of serum syndecan-1 levels between different study time points are represented in Figures 22C and D. Shapiro-Wilk tests indicated nonparametric distributions of percent changes in both groups of study participants. Study participants who took budesonide had the highest median percent change of syndecan-1 levels from the 4-week to 8-week time point at 14% (53%). The overall percent change (i.e. from baseline to the 8-week time point) followed at -9% (80%), then the baseline to 4-week time point at -14% (55%). No significant differences were detected by Friedman's test, though a significant pairwise comparison was found between the overall percent change and the percent change between the 4-week to 8-week time point ($p=0.011$). Study participants who took placebo had the highest percent change of syndecan-1 levels from the 4-week to 8-week time point at 13% (81%), followed by the overall percent change at 12% (73%), and the baseline to 4-week time point at -8% (89). No significant differences were detected by Friedman's test or in pairwise comparisons. A two-way repeated measures ANOVA did not indicate significant differences in syndecan-1 levels in relation to intervention or time. No interaction between intervention and time was observed.

Among patients who achieved mucosal healing at the 3-month assessment, those who took budesonide had the highest median syndecan-1 percent change occur in the baseline condition to

4-week time point at 61% (126%), closely followed by the overall percent change at 60% (122%), and the 4-week to 8-week time point at 41% (103%). Among mucosal healing achievers who took placebo, the highest median syndecan-1 percent change occurred in the 4-week to 8-week time point at 30% (25%), followed by the baseline condition to 4-week time point at 21% (35%), and the overall percent change at 15% (47%). No significant differences were detected by Friedman's test or in pairwise comparisons for both budesonide and placebo groups. A two-way repeated measures ANOVA did not indicate significant differences in syndecan-1 levels in relation to intervention or time. No interaction between intervention and time was observed.

Percent changes of syndecan-1 levels were compared in those with and without achievement of mucosal healing at the 3-month assessment in both budesonide and placebo groups. A two-way repeated measures ANOVA did not indicate significant difference in percent changes of syndecan-1 in relation to mucosal healing status or time in both groups. No interaction between mucosal healing status and time was observed.

8.3.2 I-FABP

Shapiro-Wilk tests indicated nonparametric distributions of serum I-FABP levels for study participants. Figures 23A and B depicts absolute median syndecan-1 levels (ng/mL) in patients who received budesonide (n=12) and placebo (n=13), respectively. Study participants who took budesonide had the highest median I-FABP levels in the baseline condition at 2.2 (1.6), followed by the 4-week time point at 1.9 (1.5), and the 8-week time point at 1.3 (1.7). Study participants who took placebo had the highest median I-FABP levels in the baseline condition at 2.7 (1.6),

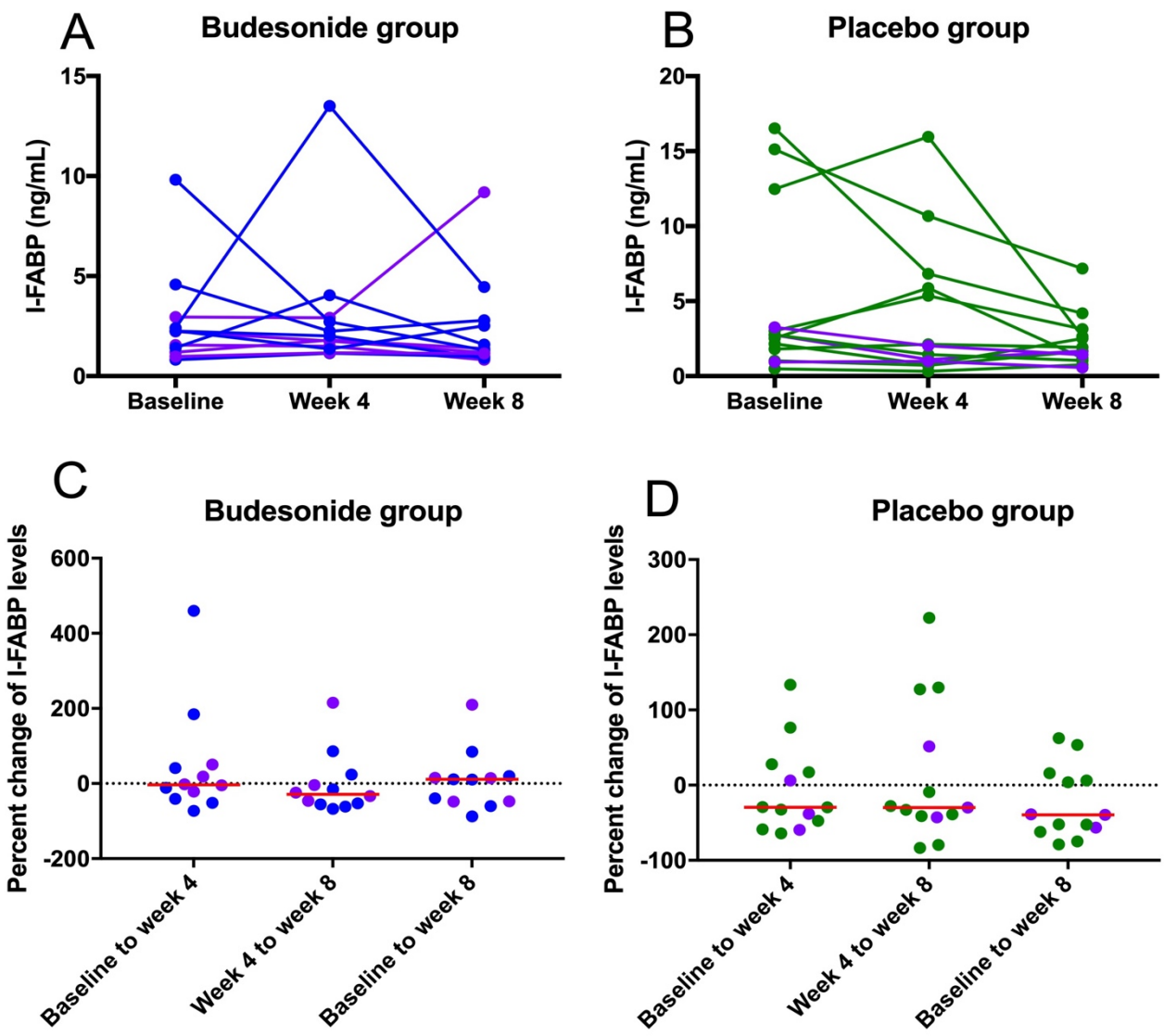

Figure 23. Absolute and percent changes of serum I-FABP levels in newly-diagnosed coeliac disease patients undergoing treatment with budesonide or placebo and a gluten-free diet. Absolute serum I-FABP levels and percent changes are represented for newly-diagnosed coeliac disease patients. (A) indicates absolute median I-FABP levels (ng/mL) from baseline condition to week 8 in study participants who received budesonide (n=12) and (B) indicates levels in those who received placebo (n=13). Median percent changes are indicated for patients who received budesonide (C) and for those who received placebo (D). Absolute levels or percent changes of I-FABP in patients who achieved mucosal healing are indicated in purple; these include 5 patients in the budesonide group and 3 patients in the placebo group. Red bars represent medians for all study participants in the specified treatment group in (C) and (D).

followed by the 4-week time point at 2.0 (5.5), and the 8-week time point at 1.7 (1.8). No significant differences were detected by Friedman's test or in pairwise comparisons in both budesonide and placebo groups. A two-way repeated measures ANOVA did not indicate significant differences in I-FABP levels with relation to intervention or time. No interaction between intervention and time was observed.

Shapiro-Wilk tests indicated a nonparametric distribution in serum I-FABP levels for who achieved mucosal healing at the three-month assessment and took budesonide (n=5). These participants had the highest median levels observed at the 4-week time point at 1.8 (1.0), followed by the baseline condition at 1.6 (1.5), and the 8-week time point at 1.2 (4.3). Participants who achieved mucosal healing and took placebo (n=3) had the highest median levels were observed in the baseline condition at 2.7 (2.3), followed by the 8-week time point at 1.4 (1.1), and the 4-week time point at 1.1 (1.0). No significant differences were detected by Friedman's test or in pairwise comparisons in both budesonide and placebo groups. A two-way repeated measures ANOVA did not indicate significant differences in I-FABP levels in relation to intervention or time. No interaction between intervention and time was observed.

Absolute I-FABP levels were compared in those with and without achievement of mucosal healing at the 3-month assessment in both budesonide and placebo groups. Two-way repeated measures ANOVAs did not indicate significant differences in I-FABP levels in relation to mucosal healing status or time in both groups. No interaction between mucosal healing status and time was observed.

The percent changes of serum I-FABP between study time points are represented in Figures 23C and D. The highest median percent change was observed overall at 11% (67%), followed by the baseline to 4-week time point percent change at -3% (84), and the 4-week to 8-week percent

change at -28% (72%) in study participants who took budesonide. In those who took placebo, negative median percent changes were observed across all time point comparisons; the baseline condition to 4-week time point had the highest percent change at -29% (76), closely followed by percent change in the 4-week to 8-week time point at -29% (132%), and the overall percent change at -39% (70%). No significant differences were detected by Friedman's test or in pairwise comparisons in both budesonide and placebo groups.

Percent changes of I-FABP levels were compared in those with and without achievement of mucosal healing at the 3-month assessment in both budesonide and placebo groups. Two-way repeated measures ANOVAs did not indicate significant differences in percent changes of I-FABP in relation to mucosal healing status or time in both groups. No interaction between mucosal healing status and time was observed.

8.3.3 LBP

Shapiro-Wilk tests indicated a nonparametric distribution of absolute LBP levels for study participants who took budesonide (n=14) and normal distributions for participants who took placebo (n=14). Figure 24A depicts median absolute LBP levels (μg/mL) in the budesonide group and Figure 24B depicts mean absolute LBP levels in the placebo group. Study participants who took budesonide had the highest median absolute LBP levels in the 8-week time point at 13 (7), followed by the 4-week time point at 11 (8), and the baseline condition at 10 (9). No significant differences were detected by Friedman's test or in pairwise comparisons. Study participants who took placebo had the highest mean ± SEM in the baseline condition at 11 ± 2, followed by the 8-week time point at 10 ± 1, and the 4-week time point at 9 ± 2. No significant differences were

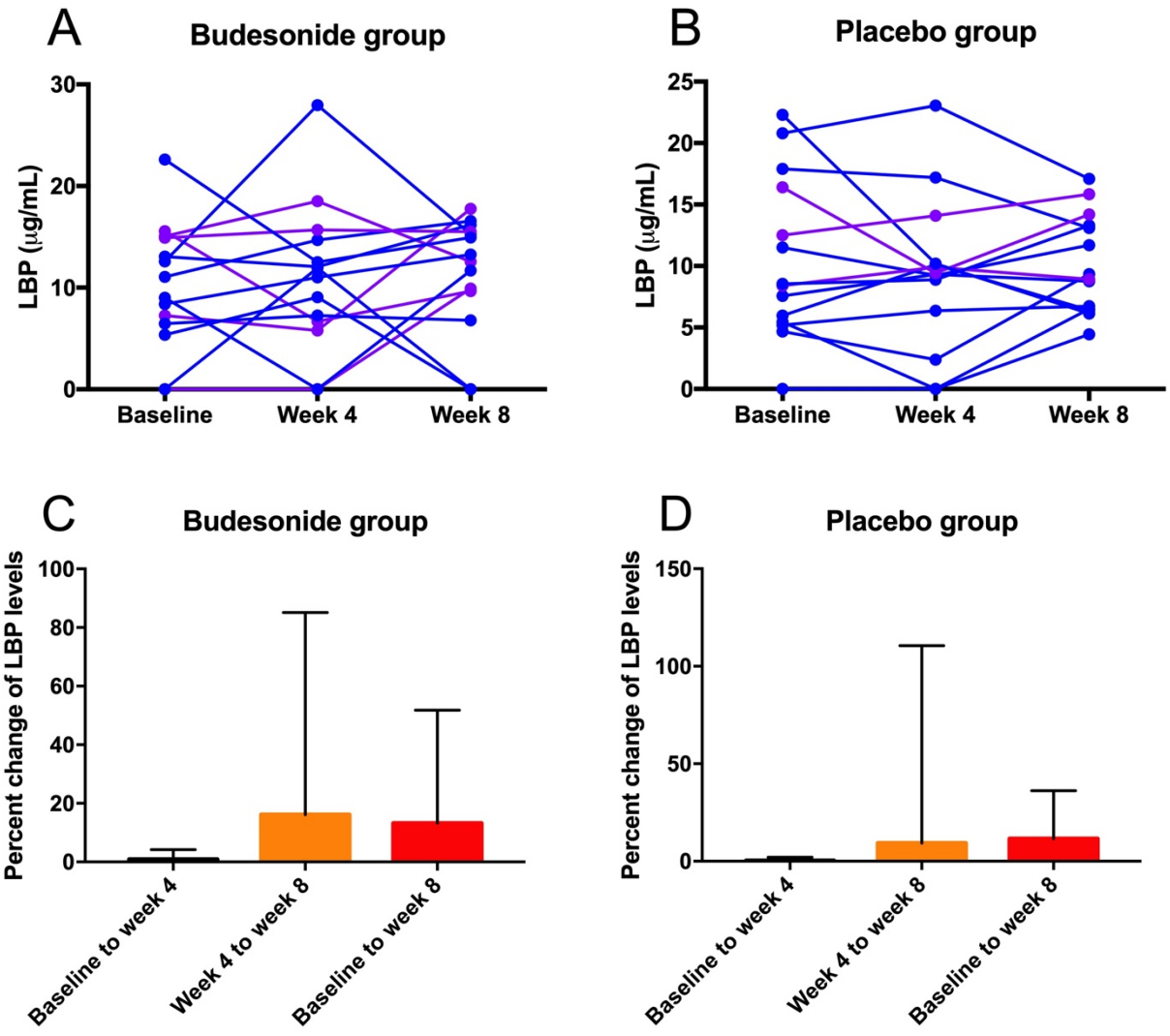

Figure 24. Absolute and percent changes of serum LBP levels in newly-diagnosed coeliac disease patients undergoing treatment with budesonide or placebo and a gluten-free diet. Absolute serum LBP levels and percent changes are represented for newly-diagnosed coeliac disease patients. (A) indicates absolute median LBP levels (μg/mL) from baseline condition to week 8 in study participants who received budesonide (n=14) and (B) indicates levels in those who received placebo (n=14). Median percent changes and IQR are indicated for patients who received budesonide (C) and for those who received placebo (D). Absolute levels or percent changes of LBP in patients who achieved mucosal healing are indicated in purple; these include 5 patients in the budesonide group and 3 patients in the placebo group. Bars represent medians and IQRs in (C) and (D).

detected by a repeated measures one-way ANOVA or in pairwise comparisons. A two-way repeated measures ANOVA did not indicate significant differences in LBP levels with relation to intervention or time. No interaction between intervention and time was observed.

Absolute LBP levels were compared in those with and without achievement of mucosal healing at the 3-month assessment in both budesonide and placebo groups. Two-way repeated measures ANOVAs did not indicate significant differences in LBP levels in relation to mucosal healing status or time in both groups. No interaction between mucosal healing status and time was observed.

Among study participants who achieved mucosal healing at the three-month assessment, those who took budesonide (n=5) had normal distributions of absolute serum LBP levels according to Shapiro-Wilk tests. The highest mean absolute LBP levels were observed at the 8-week time point at 13 ± 2, followed by the baseline condition at 11 ± 3, and the 4-week time point at 9 ± 3. No significant differences were detected by a repeated measures one-way ANOVA or in pairwise comparisons. Study participants who achieved mucosal healing at the three-month assessment and took placebo (n=3) had the highest median absolute LBP levels observed at the 8-week time point at 14 (7), followed by the baseline condition at 13 (8) and the 4-week time point at 10 (5). No significant differences were detected by a repeated measures one-way ANOVA or in pairwise comparisons. A two-way repeated measures ANOVA did not indicate significant differences in LBP levels with relation to intervention or time. No interaction between intervention and time was observed.

Shapiro-Wilk tests indicated nonparametric distributions in the percent changes of serum LBP levels for both budesonide and placebo groups. The percent changes of serum LBP between study time points are represented in Figures 24C and D. Study participants who took budesonide

experienced the highest percent changes in levels from the 4-week to 8-week time point at 16 (121), followed by the overall percent change at 13 (87), and the baseline to 4-week time point at 0.9 (7). Participants in the placebo group experienced the highest percent change overall at 12 (51), followed by the 4-week to 8-week percent change at 9 (135), and the baseline to 4-week percent change at 0.2 (6.3). No significant differences were detected by Friedman's test or in pairwise comparisons for both budesonide and placebo groups. A two-way repeated measures ANOVA did not indicate significant differences in LBP levels with relation to intervention or time. No interaction between intervention and time was observed.

Percent changes of LBP levels were compared in those with and without achievement of mucosal healing at the 3-month assessment in both budesonide and placebo groups. Two-way repeated measures ANOVAs did not indicate significant differences in percent changes of LBP in relation to mucosal healing status or time in both groups. No interaction between mucosal healing status and time was observed.

8.3.4 sCD14

Shapiro-Wilk tests indicated normal distributions of absolute sCD14 levels for study participants who took budesonide (n=12) and placebo (n=14). Figure 25A depicts median absolute sCD14 levels (μg/mL) in the budesonide group and Figure 25B depicts mean absolute sCD14 levels in the placebo group. The highest mean levels in the budesonide group were detected at the 4-week time point at 1.4 ± 0.1, followed by the 8-week time point at 1.3 ± 0.2, and the baseline condition at 1.3 ± 0.1. The highest mean levels for the placebo group were detected in the baseline condition at 1.4 ± 0.1, followed by the 8-week condition at 1.2 ± 0.1, and the 4-week condition at 1.1 ± 0.1. No significant differences were detected by Friedman's test or in pairwise comparisons

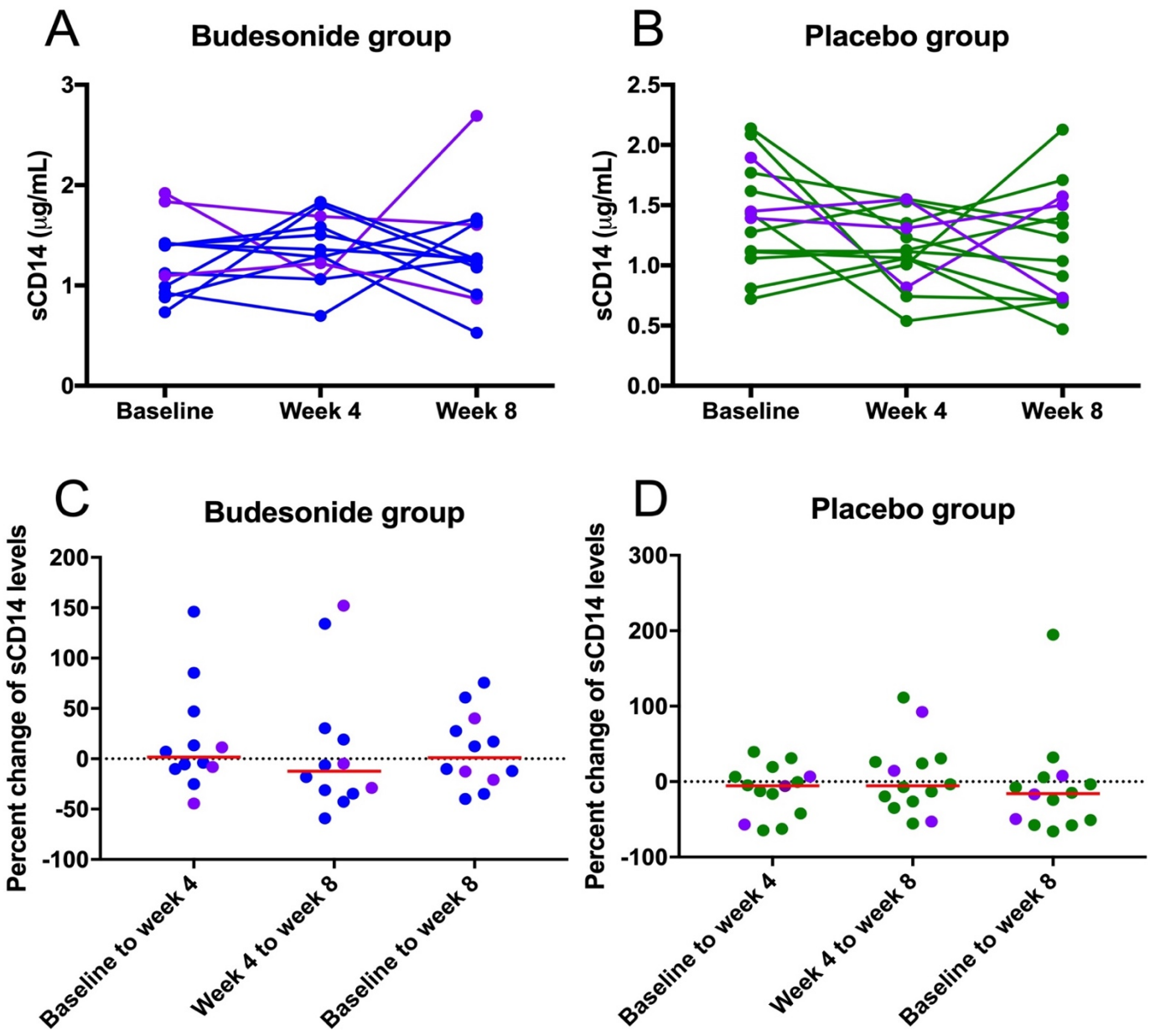

Figure 25. Absolute and percent changes of serum sCD14 levels in newly-diagnosed coeliac disease patients undergoing treatment with budesonide or placebo and a gluten-free diet. Absolute serum sCD14 levels and percent changes are represented for newly-diagnosed coeliac disease patients. (A) indicates absolute median sCD14 levels (μg/mL) from baseline condition to week 8 in study participants who received budesonide (n=12) and (B) indicates levels in those who received placebo (n=14). Median percent changes and IQR are indicated for patients who received budesonide (C) and for those who received placebo (D). Absolute levels or percent changes of sCD14 in patients who achieved mucosal healing are indicated in purple; these include 3 patients in the budesonide group and 3 patients in the placebo group. Red bars represent medians for all study participants in the specified treatment group in (C) and (D).

for both budesonide and placebo groups. A two-way repeated measures ANOVA did not indicate significant differences in sCD14 levels with relation to intervention or time. No interaction between intervention and time was observed.

As Shapiro-Wilk tests indicated normal distributions in both mucosal healers and non-healers at the 3-month assessment for absolute sCD14 levels in both study cohorts, tests associated with normal distributions were utilised for analysis regarding mucosal healers. The highest mean levels in the budesonide group (n=3) were observed in the 8-week time point at 1.7 ± 0.5, followed by the baseline condition at 1.6 ± 0.3, and the 4-week time point at 1.3 ± 0.2. The highest mean levels in the placebo group (n=3) were observed at the baseline condition at 1.5 ± 0.2, followed by the 8-week time point at 1.3 ± 0.3, and the 4-week time point at 1.2 ± 0.2. No significant differences were detected by Friedman's test or in pairwise comparisons for both budesonide and placebo groups. A two-way repeated measures ANOVA did not indicate significant differences in sCD14 levels with relation to interaction or time. No interaction between intervention and time was observed.

Absolute sCD14 levels were compared in those with and without achievement of mucosal healing at the 3-month assessment in both budesonide and placebo groups. Two-way repeated measures ANOVAs did not indicate significant differences in sCD14 levels in relation to mucosal healing status or time in both groups. No interaction between mucosal healing status and time was observed.

Shapiro-Wilk tests indicated nonparametric distributions in the percent changes of serum sCD14 levels for both budesonide and placebo groups. The percent changes of serum sCD14 between study time points are represented in Figures 25C and D. In the budesonide group, the highest median percent change occurred in the baseline to 4-week time point at 1.6% (48%),

followed by the overall percent change at 1.2% (56%), and the percent change in the 4-week to 8-week time point at -12% (61%). Median percent changes in the placebo group were negative; the highest percent change occurred overall at -16% (59%), followed by both the baseline to 4-week time point and the 4-week to 8-week time point at -5% (56%). No significant differences were detected by Friedman's test or in pairwise comparisons for both budesonide and placebo groups. A two-way repeated measures ANOVA did not indicate significant differences in sCD14 levels with relation to interaction or time. No interaction between intervention and time was observed.

Percent changes of sCD14 levels were compared in those with and without achievement of mucosal healing at the 3-month assessment in both budesonide and placebo groups. Two-way repeated measures ANOVAs did not indicate significant differences in percent changes of LBP in relation to mucosal healing status or time in both groups. No interaction between mucosal healing status and time was observed.

8.3.5 Marker Correlations

All absolute levels as well as percent changes of each marker were correlated within each respective intervention for study participants. As previously mentioned, individuals were omitted from analysis if there was incomplete testing of markers due to lack of available sera. No correlation analysis was conducted in the mucosal healing cohorts due small sample sizes.

A positive, significant correlation was found between absolute LBP and sCD14 levels in the baseline condition ($p=0.0008$, Pearson's $r = 0.53$, $n=34$). Aside from this correlation, there were no other significant correlations between absolute markers levels. Significant correlations of percent changes were observed for markers within the same period of time and treatment; a positive, significant correlation was observed between syndecan-1 and LBP percent changes in

individuals who took budesonide from the baseline condition to 4-week time point (p=0.012, r=0.662, n=14) and a negative, significant correlation was observed between syndecan-1 and sCD14 (p=0.002, Spearman's r=-0.811, n=12) in these same conditions. A positive, significant correlation was observed between I-FABP and sCD14 percent changes in individuals who took placebo from the baseline condition to 4-week time point (p=0.019, r=0.648, n=10). Another positive, significant correlation was also observed between LBP and sCD14 percent changes in participants who took placebo from the 4-week to 8-week time point (p=0.040, r=0.56, n=12).

8.4 Discussion

The number of these patients who achieved complete mucosal healing at the 3-month assessment and took budesonide along with a gluten-free diet was double the amount of those who took placebo, which was essentially a gluten-free diet alone. This evidence suggests that budesonide may enhance mucosal healing in some newly-diagnosed coeliac disease patients. However, as shown by the results, it is unclear whether levels of circulating protein markers are reliable indicators of mucosal healing when patients are undergoing treatment with budesonide, particularly with regards to syndecan-1, I-FABP, and sCD14, as these markers, which have been suggested for use in monitoring mucosal healing coeliac disease, followed a similar pattern in these patients. Longitudinal differences in signals of these proteins, particularly with regard to percent change of protein levels, were not as robust in budesonide-treated patients compared to those who took placebo.

As previously mentioned, depletion of syndecan-1 alters cell morphology and organization at the epithelial barrier. As individuals who achieved mucosal healing at the 3-month assessment had lower median syndecan-1 levels than their counterparts who did not heal, the observed

decrease may be associated with increased healing; this result is consistent with previous research in paediatric coeliac patients.[78] However, levels of syndecan-1 remained heightened in study participants who took budesonide in patients who achieved mucosal healing than those who took placebo. This may suggest that budesonide may interact with syndecan-1 levels and not be indicative of mucosal healing. Curiously, the percent change in levels from baseline to the 4-week time point were positively and significantly correlated with LBP levels, yet the inverse was found with sCD14 in the budesonide group in the same conditions; however, this trend was not sustained from the 4-week to 8-week period nor was it sustained overall (i.e. from baseline to the 8-week time point, which was closer to the 3-month biopsy assessment). As the current evidence stands, the data support the evidence that syndecan-1 may be a promising longitudinal marker to indicate mucosal healing in coeliac patients undergoing a traditional, gluten-free diet treatment regimen alone. However, no statistically significant associations between healing status and intervention time point nor treatment and intervention time point were observed.

The current I-FABP results support, though not strongly, the evidence for the marker's use in investigating coeliac disease. The median I-FABP levels of mucosal healers were lower than those who did not heal, though these results were not statistically significant. A decrease in the percent changes of I-FABP levels were observed for individuals who underwent treatment with placebo; as such, the placebo results, which show the effects of a gluten-free diet alone on mucosal healing in coeliac disease supports previous evidence. However, the same trend was not observed in those who were treated with budesonide; the percent changes of median I-FABP levels over time were not as consistent and fluctuated from positive to negative. Similar to the results of syndecan-1 levels, the results suggest an interaction of budesonide with I-FABP levels. However,

there were no overall statistically significant associations observed between healing status and intervention time point nor treatment and intervention time point.

No significant differences in absolute or percentage changes in LBP levels were observed for either budesonide and placebo groups as well as patients who achieved mucosal healing. Large variations of LBP levels were observed within the percent changes of protein levels over time in both budesonide and coeliac disease groups. As this is the first evidence of the measurement of LBP in the treatment of coeliac disease, this marker alone does not appear to be of value for the indication of mucosal healing. Results for sCD14 had less variation and decreases in levels as well as percent changes over time were observed, which may be associated with less microbial translocation during the progression of the healing process. Although these results were not statistically significant, they are consistent with previous evidence that suggests lower sCD14 levels are associated with a healthy mucosal barrier.[89] A similar pattern to I-FABP percent changes was observed; the placebo group had consistently lower, negative sCD14 percent changes over time whereas higher percent changes were observed for the budesonide group. Taken together, usage these markers alone do not provide a comprehensive representation of mucosal healing and associated decrease in microbial translocation, though their use together may provide a more accurate indication.

Similar to the results found in the NCWS cohort, a significant, positive correlation was observed for LBP and sCD14 in absolute values at the baseline condition and in percent changes in participants who took placebo from the 4-week to 8-week time point. These results provide evidence for microbial translocation associated with coeliac disease pathology at the baseline condition. Evidence of positive, significant correlations between LBP and sCD14 in the latter 4-week to 8-week time point on a gluten-free diet alone suggests that these proteins may remain in

concordance with one another in a lack of mucosal healing and subsequent state of increased microbial translocation; the same results during this time period were not observed for the budesonide group, in which more participants achieved mucosal healing. However, as previously mentioned, small sample sizes of mucosal healers were limitations to knowing the longitudinal value of LBP and sCD14 level concordance.

In conclusion, although few significant associations or relationships were observed in absolute levels or percent changes of markers, possibly reflecting small numbers, trends in levels, particularly with regard to syndecan-1, I-FABP, and sCD14 were observed. There were similar findings with budesonide therapy, such that no specific budesonide effects were noted. Levels of syndecan-1 and I-FABP have been proposed as useful longitudinal non-invasive markers of mucosal healing, or at least improvement, as outlined in Chapter 1. However, despite trends, the current evidence cannot support the clinical value of any of the markers evaluated in following the individual's progress. In the current study, they are altered to a point that does not indicate their longitudinal utility in signalling mucosal barrier integrity and microbial translocation. A potential variable that might be responsible for the discrepancy in results compared to past research includes time point of disease assessment. In this respect, serological samples utilised in the current study are from patients early in their disease course and are carefully assessed in histology. Future studies may extend the assessment of marker levels and their association with mucosal healing to later time points associated with biopsy-proven mucosal healing and determine whether the effects of budesonide are observed on long-term levels.

CHAPTER 9

CROSS-SECTIONAL ANALYSIS OF GASTROINTESTINAL DISEASE COHORTS AND HEALTHY INDIVIDUALS

9.1 Introduction

Aside from the investigation of zonulin in Chapters 4 and 5, each previous chapter investigates levels of featured circulating markers in interventions associated with different gastrointestinal diseases or models of disease. The results of these chapters provide information on the longitudinal value of each marker in specific disease states and associated interventions. The current chapter brings together the collective results in a cross-sectional analysis of featured markers in each gastrointestinal disease investigated and in healthy controls. Marker levels of serological samples from baseline conditions of serological samples are investigated. The information in this chapter characterises marker levels in gastrointestinal diseases and in healthy controls. The analysis will help determine whether certain markers or combinations thereof may be indicative of gastrointestinal disease.

9.2 Materials and Methods

9.2.1 Study Participants and Protocols

Baseline levels of featured markers in serological samples from gastrointestinal disease cohorts (NCWS, untreated coeliac disease, and ASUC) and a cohort of healthy individuals are included in this chapter. Correlations between absolute marker levels are also included in the analysis.

9.2.2 Commercial Assays

Levels of featured plasma protein markers were determined by commercially-available ELISA assays. Details of assays are indicated in Section 3.1.1 and assay methodology is indicated in Section 3.3.1.

9.2.3 Statistical Analysis

Chapter 3.4 details general statistical analysis materials and methods used in this chapter. The following results in Section 9.3 report specific methods used for analysis.

9.3 Results

Absolute levels of featured protein markers were determined for each gastrointestinal disease patient at baseline condition (prior to any dietary or drug intervention) and in healthy individuals. The results are indicated in the following figures for each section pertaining to a featured marker. Differences in sample size amongst markers due to lack of sera available or otherwise are indicated in each section.

9.3.1 Syndecan-1

Shapiro-Wilk tests indicated nonparametric distributions of absolute serum syndecan-1 levels at baseline conditions (i.e. before any dietary or drug interventions) in all gastrointestinal disease cohorts and in healthy individuals. Figure 26 depicts absolute syndecan-1 levels (ng/mL) in these patients and controls at baseline conditions. The highest median (IQR) of absolute syndecan-1 levels were found in the ASUC cohort (n=12) at 97 (111), followed by the NCWS cohort (n=34) at 42 (50), then the untreated coeliac disease cohort (n=36) at 24 (76), and healthy individuals (n=49) at 23 (45). The overall difference in distribution amongst cohorts, as indicated by Kruskal-Wallis test, was significant ($H=14.55$, $p=0.0022$). Significant pairwise comparisons, as observed by Mann-Whitney U tests, were found between NCWS and ASUC ($p=0.025$) cohorts, untreated coeliac cohort disease and ASUC cohorts ($p=0.021$), NCWS cohort and healthy controls ($p=0.018$), and the ASUC cohort and healthy controls ($p<0.001$).

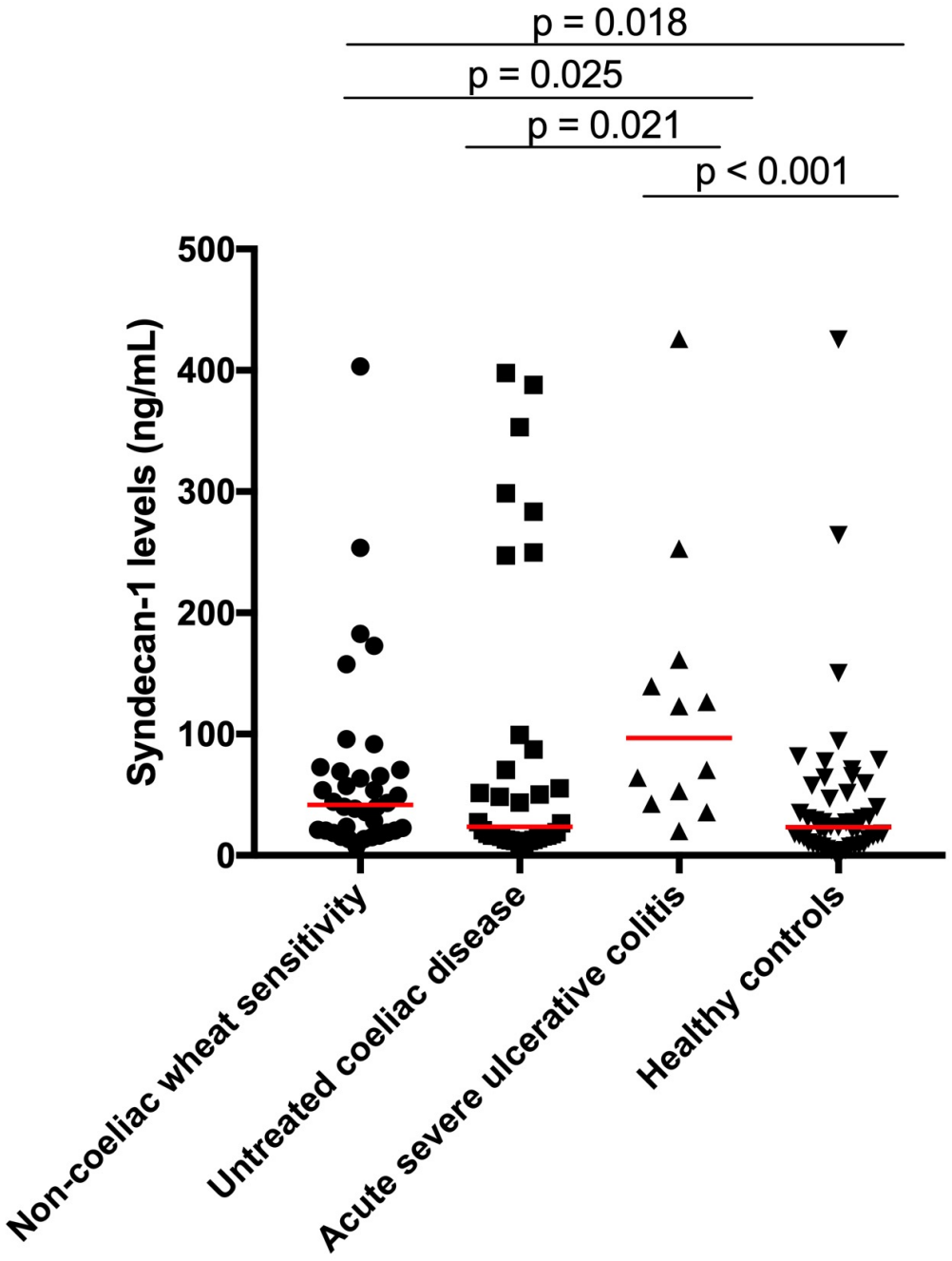

Figure 26. Absolute syndecan-1 levels in gastrointestinal disease and healthy individuals at baseline conditions. Absolute serum syndecan-1 levels are represented for individuals with NCWS (n=34), untreated coeliac disease (n=36), ASUC (n=12), and healthy controls (n=49). Significant pairwise comparisons were found between NCWS and ASUC (p=0.025), untreated coeliac disease and ASUC (p=0.021), NCWS and healthy controls (p=0.018) and ASUC and healthy controls (p<0.001). Red bars represent medians of syndecan-1 levels per group.

9.3.2 I-FABP

Shapiro-Wilk tests indicated nonparametric distributions of absolute serum I-FABP levels in gastrointestinal disease cohorts and in healthy individuals. Figure 27 depicts absolute I-FABP levels (ng/mL) in these patients and controls at baseline conditions. The highest median (IQR) of absolute I-FABP levels were found in the untreated coeliac disease cohort (n=32) at 2.2 (1.8), followed by healthy individuals (n=49) at 2.0 (3.1), then the NCWS cohort (n=34) at 0.86 (0.76), and the ASUC cohort (n=20) at 0.6 (0.5). As indicated by Kruskal-Wallis test, the overall difference in distribution amongst cohorts was significant ($H=62.24$, $p<0.0001$). Significant pairwise comparisons, as observed by Mann-Whitney U tests, were found between the NCWS and untreated coeliac disease cohorts ($p<0.0001$), NCWS and ASUC ($p=0.013$) cohorts, NCWS cohort and healthy individuals ($p<0.0001$), untreated coeliac disease cohort and ASUC cohort ($p<0.0001$), and the ASUC cohort and healthy individuals ($p<0.0001$).

9.3.3 LBP

Shapiro-Wilk tests indicated nonparametric distributions of absolute serum LBP levels in gastrointestinal disease cohorts and in healthy individuals. Figure 28 depicts absolute LBP levels (μg/mL) in these patients and controls at baseline conditions. The highest median (IQR) of absolute serum LBP levels was observed in the ASUC cohort (n=20) at 18 (12), followed by the healthy controls (n=49) at 16 (19), then the NCWS cohort (n=35) at 12 (6), and the untreated coeliac disease cohort at 8 (10). A Kruskal-Wallis test indicated the overall difference in distribution amongst cohorts was significant ($H=16.13$, $p=0.0011$). Significant pairwise comparisons, as determined by Mann-Whitney U tests, were found between NCWS and ASUC cohorts ($p=0.0006$),

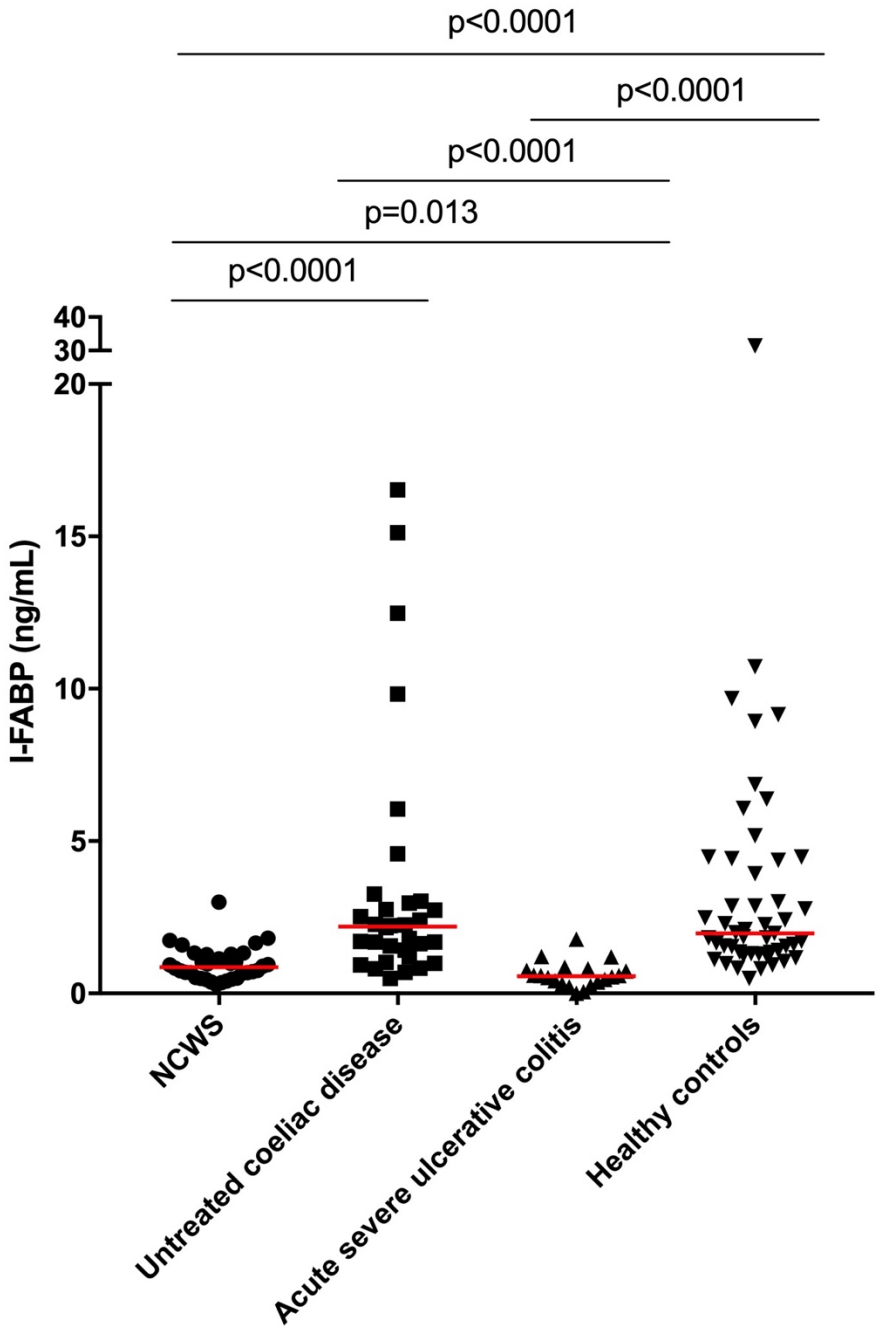

Figure 27. Absolute I-FABP levels in gastrointestinal disease and healthy individuals at baseline conditions. Absolute serum I-FABP levels are represented for individuals with NCWS (n=34), untreated coeliac disease (n=32), ASUC (n=20), and healthy controls (n=49). Significant pairwise comparisons were found between NCWS and untreated coeliac disease ($p<0.0001$), NCWS and ASUC ($p=0.0128$), NCWS and healthy controls ($p<0.0001$), untreated coeliac disease and ASUC ($p<0.0001$), and ASUC and healthy controls ($p<0.0001$). Red bars represent medians of I-FABP levels per group.

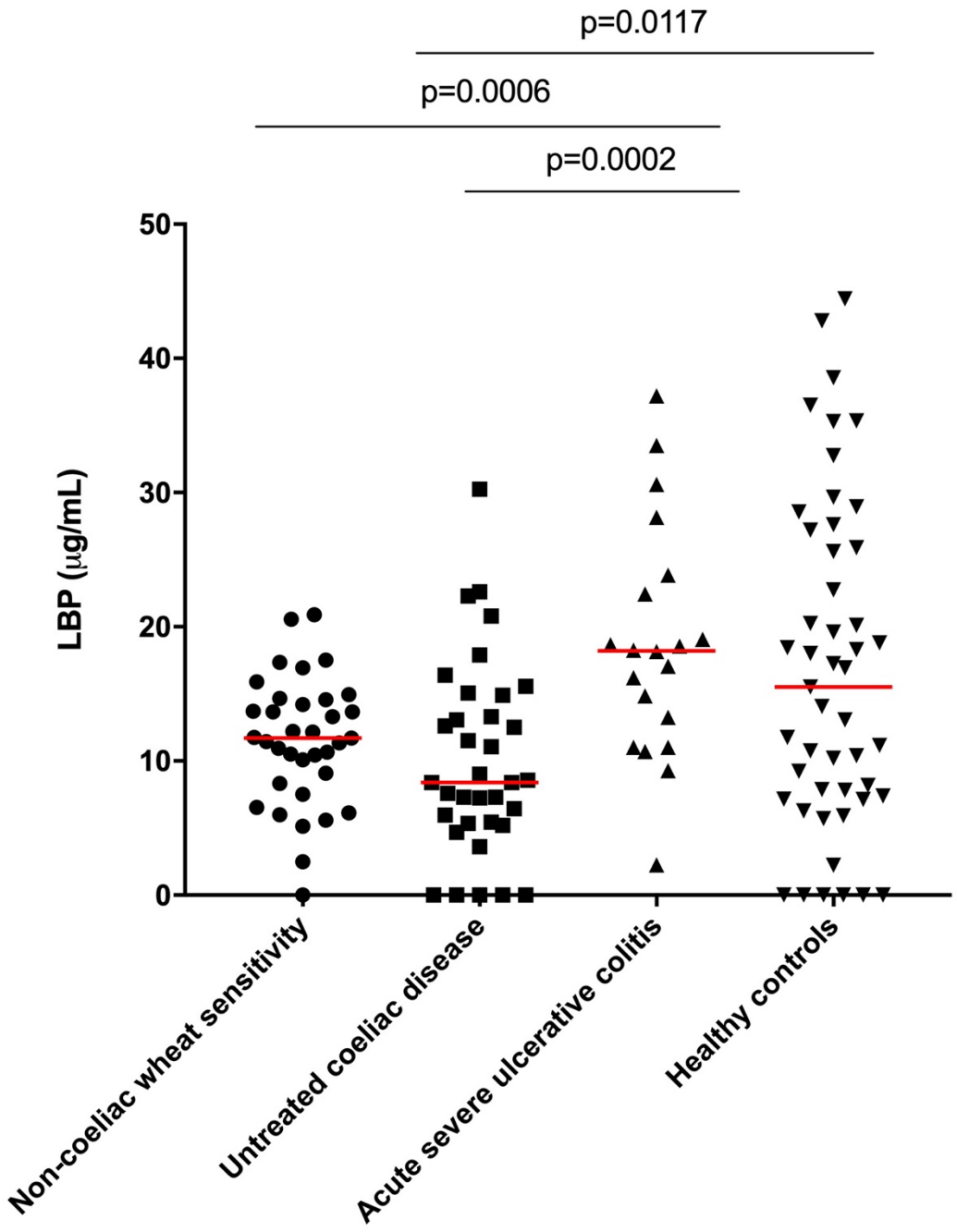

Figure 28. Absolute LBP levels in gastrointestinal disease and healthy individuals at baseline conditions. Absolute serum LBP levels are represented for individuals with NCWS (n=34), untreated coeliac disease (n=34), ASUC (n=20), and healthy controls (n=49). Significant pairwise comparisons were found between NCWS and ASUC cohorts (p=0.0006), untreated coeliac disease and ASUC cohorts (p=0.0002), and the untreated coeliac disease cohort and healthy controls (p=0.0117). Red bars represent medians of LBP levels per group.

untreated coeliac disease and ASUC cohorts (p=0.0002), and the untreated coeliac disease cohort and healthy controls (p=0.0117).

9.3.4 sCD14

Shapiro-Wilk tests indicated nonparametric distributions of absolute serum sCD14 levels in gastrointestinal disease cohorts and in healthy individuals. Figure 29 depicts absolute sCD14 levels (μg/mL) in these patients and controls at baseline conditions. The highest median (IQR) of absolute serum sCD14 levels were observed in the untreated coeliac disease cohort (n=34) at 1.4 (0.7), followed by the NCWS cohort (n=35) at 1.4 (1.0), the ASUC cohort (n=20) at 1.1 (0.9), and the healthy controls (n=49) at 1.2 (1.5). A Kruskal-Wallis test indicated the overall difference in distribution amongst cohorts was not significant and no significant pairwise comparisons were detected.

9.3.5 Marker Correlations

Baseline levels of markers within cohorts of gastrointestinal disease and healthy individuals were correlated with one another using Spearman's r. Figure 30 depicts the significant correlations found within cohorts. A positive, significant correlation was observed between LBP and sCD14 for all patient cohorts, though no significant correlation was found for healthy individuals. A significant correlation was also observed between syndecan-1 and sCD14 in healthy controls (p=0.010, r=0.356).

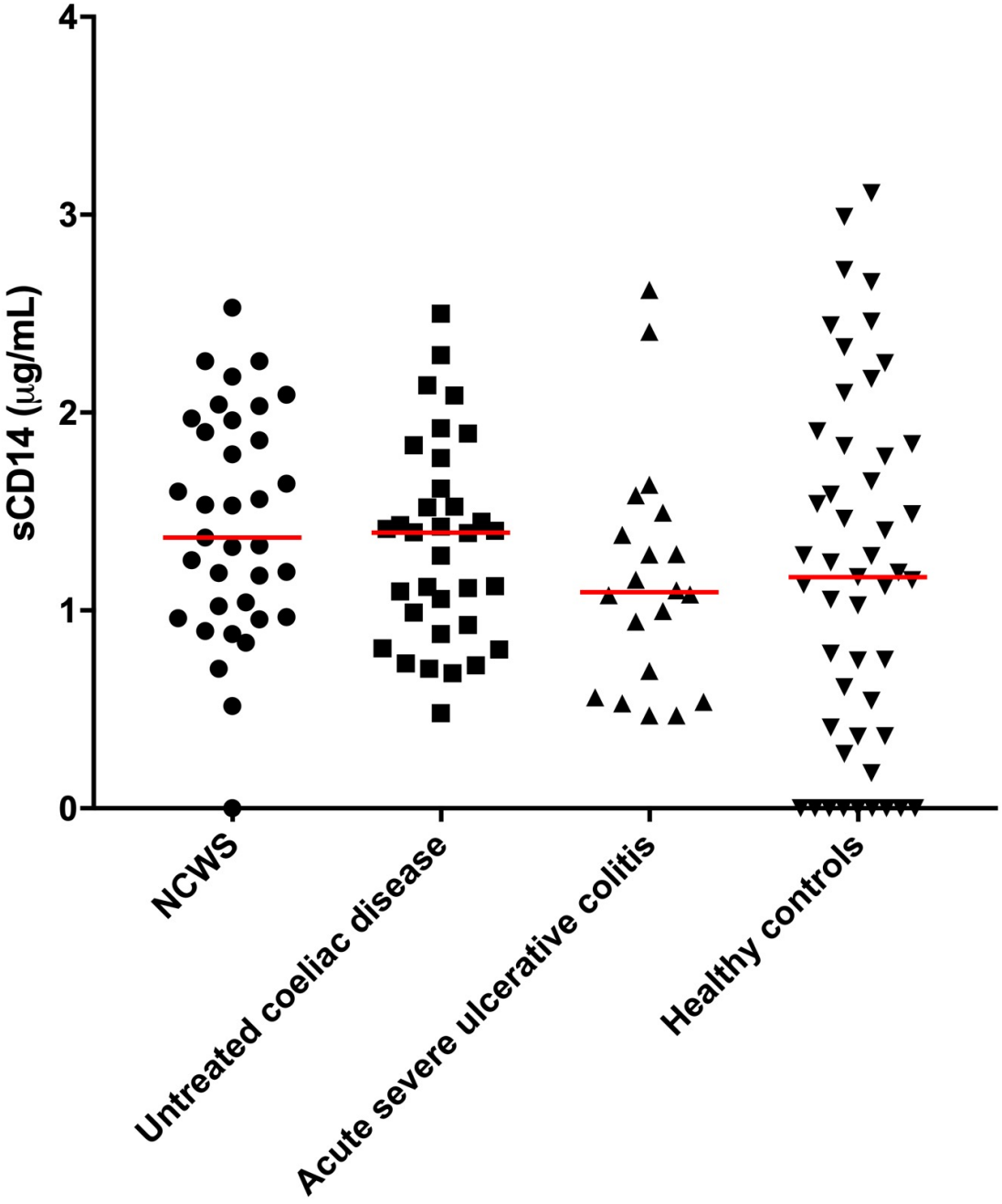

Figure 29. Absolute sCD14 levels in gastrointestinal disease and healthy individuals at baseline conditions. Absolute serum sCD14 levels are represented for individuals with NCWS (n=35), untreated coeliac disease (n=34), ASUC (n=20), and healthy controls (n=49). No significant pairwise comparisons were found between groups. Red bars represent medians of sCD14 levels per group.

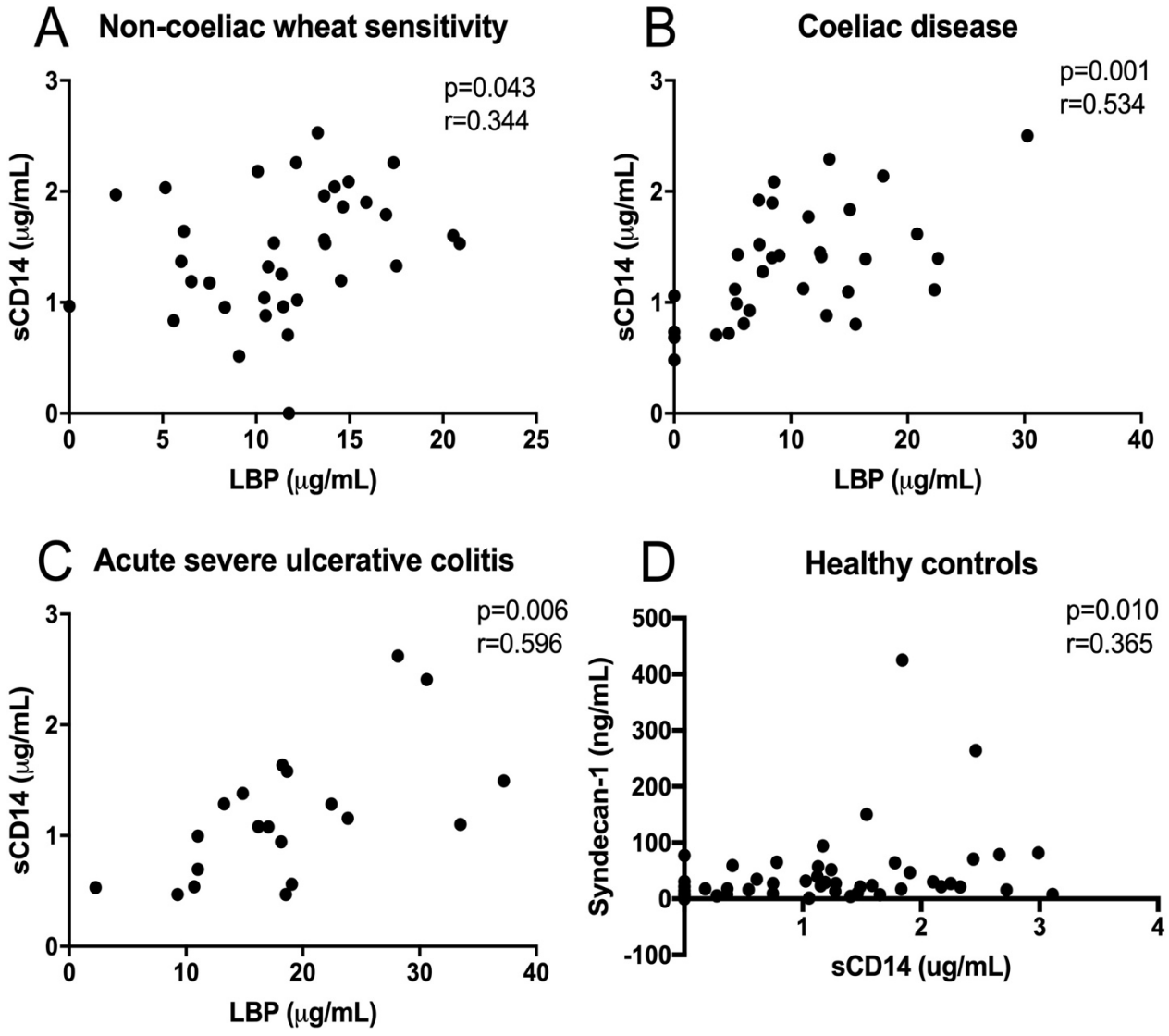

Figure 30. Significant correlations of absolute marker levels in patients with gastrointestinal disorders and in healthy controls. A positive, significant correlation of absolute marker levels were observed between LBP and sCD14 in all patient cohorts and between syndecan-1 and sCD14 in healthy controls. (A), (B), and (C) describes the correlation between LBP and sCD14 levels in individuals with non-coeliac wheat sensitivity (p=0.043, r=0.344, n=35), coeliac disease (p=0.001, r=0.0534, n=34), and acute severe ulcerative colitis (p=0.006, r=0.596, n=20), respectively. (D) describes the correlation between syndecan-1 and sCD14 levels in healthy controls (p=0.010, r=0.365).

9.4 Discussion

The results of this chapter provide a characterisation of featured marker levels in patients with different gastrointestinal diseases, previously featured in specific trials, and in healthy individuals, at baseline conditions before any dietary or drug interventions. The cross-sectional analysis conducted allows for the comparison of marker levels to the literature, establishment of marker levels that have not previously been characterised, and determination of the utility of markers in the indication of disease/site-specificity of abnormal levels.

9.4.1 Syndecan-1

The median absolute syndecan-1 levels observed matched previous levels reported in the literature for healthy controls. A study that profiled syndecan-1 levels in IBD reported levels of 21 ng/mL in healthy individuals with a median age of 38, which closely paralleled the observed results of 23 ng/mL in healthy individuals with a mean age of 39.[68] However, it is not reported whether a normal range was claimed and/or whether outliers were observed in the previous study. The most significant elevation in syndecan-1 levels was found in the ASUC cohort. Our results are consistent with the current literature; as previously mentioned in Chapter 1, the evidence of syndecan-1 as a reliable marker of disease remains strongest for IBD, presumably due to its close association with inflammatory cascades. Heightened syndecan-1 levels were also found in the NCWS/IBS cohort, as outlined in Chapter 7. There is currently no previous literature that details levels; the current research is the first to report syndecan-1 levels in a cohort of individuals with NCWS/IBS. As ulcerative colitis and IBS are colonic-associated conditions, the current results suggest that heightened syndecan-1 levels may be indicative of localised colonic epithelial injury.

Three outliers with heightened levels were observed in the healthy control cohort. The particular control subject with the highest syndecan-1 levels had a history of anxiety, though it was unknown whether the subject had a relapse of psychiatric illness and/or high stress levels at the time of study enrolment. The effects of psychiatric illness/stress on syndecan-1 levels is currently unknown; there has been no previous research examining potential links.

Syndecan-1 has also been recommended as a marker for coeliac disease in a previous study that observed heightened levels in paediatric patients compared to controls (with abdominal pain), which also correlated to mucosal damage.[78] However, syndecan-1 levels in the current study's untreated coeliac disease cohort did not significantly differ from that of healthy controls, though the proportion with marked elevation in levels was much higher, similar to the previous study. However, the most significant commonality between the previous study and current research is syndecan-1's ability to indicate mucosal healing longitudinally. Therefore, as a cross sectional marker, syndecan-1 may not be a robust indicator of disease, particularly in the case of coeliac disease.

9.4.2 I-FABP

The current study's results indicate heightened median I-FABP levels in untreated coeliac disease and healthy controls compared to colonic conditions (i.e. ASUC and NCWS/IBS). Similar median levels and distributions were found in a study by Uhde, Ajamian et al. for both coeliac disease and healthy control cohorts.[2] As I-FABP is a marker of enterocyte damage and is primarily found in the jejunum, heightened levels in untreated coeliac disease would be expected. However, levels between the untreated coeliac disease and healthy controls group remained similar and not

statistically different from one another. This result signals the inability of the marker to differentiate healthy controls from diseased cohorts.

Unforeseen or temporary intestinal damage, perhaps due to diet, alcohol, exercise, or medications that individuals in the healthy control cohort consumed may certainly be a contributor to heightened levels. When clinical notes were consulted, one particular healthy individual with heightened levels consumed 5 alcoholic drinks the night before blood collection. In this case, levels may have been heightened in response to alcohol-induced damage. Among the individuals with the top 5 highest levels, 4 were males between the ages of 25-32. Unfortunately, no further information on drinks recently consumed or drinking habits for other subjects was available. A recent study in the United Kingdom has determined that alcohol consumption peaks at age 25 around 13 drinks per week, which fits the demographic of high I-FABP producers in the current study's healthy cohort.[191] Further studies are warranted to investigate alcohol-induced intestinal damage and its consequences on circulating markers. Another individual with heightened levels recently passed a biliary stone and was on the medications Valdoxan and statins. No further information on current psychiatric state or triglyceride levels were available for this particular subject. However, as suggested in previous chapters, the strength of evidence remains that I-FABP's utility as a marker of mucosal barrier dysfunction remains in longitudinal, rather than cross-sectional studies.

9.4.3 LBP and sCD14

The highest median LBP levels were found in ASUC patients; these levels were statistically significant from those observed in non-coeliac wheat sensitivity and untreated coeliac disease. However, heightened levels were also found in the healthy controls cohort, which surpassed

NCWS and untreated coeliac disease levels. A wide distribution of LBP levels in healthy controls has been previously reported in Uhde, Ajamian et al. These results indicate that cross-sectional LBP levels are unable to distinguish between gastrointestinal disease cohorts and healthy controls. No significant medians amongst groups or in pairwise comparisons were observed for sCD14. The results also confirm sCD14's inability to distinguish between gastrointestinal disease cohorts and healthy controls in cross-sectional analysis.

9.4.4 Marker Correlations

As indicated in Figure 5, each gastrointestinal disease cohort had a significant, positive correlation between LBP and sCD14 levels, which confirms concordance between the two endotoxin-related markers and immune activation characteristic of microbial translocation. A negative, non-significant correlation was observed for healthy controls, which provides evidence that the endotoxin-associated response associated with microbial translation is unlikely to be observed in healthy individuals. The significant correlation between syndecan-1 and sCD14 in healthy individuals has also been paralleled in cohorts with gastrointestinal disease within the previous chapters that feature longitudinal studies; these collective results are discussed in Chapter 10. The results may suggest concurrent inflammatory cascade and macrophage-associated activation.

9.4.5 Summary

The results from cross-sectional comparison of featured markers confirm that they have the best utility in longitudinal studies. Aside from syndecan-1, absolute levels of I-FABP, LBP, and sCD14 in patients with gastrointestinal disease (with the exception of LBP levels in ASUC)

were not significantly different than levels in healthy individuals. In this respect, the individual markers were unable to detect disease. The results also contribute to evidence that syndecan-1 may have utility in the indication of colonic epithelial injury. A positive, significant correlation of LBP and sCD14 levels appeared to discriminate between individuals with gastrointestinal disease and healthy individuals. This result demonstrates that marker combinations may be more effective in the indication of disease than individual markers.

CHAPTER 10

GENERAL DISCUSSION

10.1 Introduction

Impaired intestinal barrier integrity has received increasing attention as a proposed mechanism of gastrointestinal disease pathogenesis. Some circulating plasma or serological protein candidates have arisen as markers to assess the extent of intestinal barrier damage and resulting microbial translocation. Previous studies have noted elevations in these markers linked to gastrointestinal disease, though it is unclear whether these markers may be of clinical use due to methodological shortcomings in detection or investigation with samples that have not been well-characterised.

The results found in this thesis help resolve whether featured markers of impaired intestinal barrier integrity and microbial translocation are indeed helpful to indicate gastrointestinal disease. Featured markers of intestinal barrier damage (i.e., zonulin and syndecan-1) and markers of resulting microbial translocation (i.e., LBP and sCD14) were investigated in well-characterised cohorts of gastrointestinal disease, an exercise-induced gastrointestinal damage model in otherwise healthy subjects, and healthy individuals. The investigation yielded several important facts, specific indications, and efficacy of use for the markers.

A summary of results for each featured marker is presented in the following sections. Common themes with regards to marker levels between diseases, interventions and models are explored. In addition, efficacy of use pertaining to each marker is indicated. Future directions for further study are also recommended.

10.2 Zonulin

Chapters 4 and 5 explore the role of zonulin in indicating gastrointestinal damage. As previously detailed, zonulin is a putative reversible modulator of intestinal epithelial tight junctions. Heightened zonulin levels are linked to "gut leakiness," which is implicated in a variety of gastrointestinal disorders and suggested in various other extra-intestinal conditions. Despite its growing popularity as a biomarker, limited evidence supported its efficacy as a marker of gastrointestinal dysfunction. Commercially-available assays for measuring zonulin concentrations in plasma or serum were chosen for use, as most of the concepts and current understanding of the response of zonulin in health and disease states was built around results from these assays. As reported in the Supplementary Table, over 70 independent original studies used these assays. However, inconsistencies in the results obtained were noted and it was determined that current commercial assays are not detecting actual zonulin protein. In this respect, there can be no confidence in the current detection methods; therefore, as it currently stands, circulating zonulin concentration is not a useful marker. Research efforts in Chapter 5 aimed to determine the identity of the proteins detected by the commercial assays. Complement C3 and haptoglobin, of which zonulin is a pre-form protein, were top matches. However, neither protein bound to the commercial assay capture antibodies. A concurrent publication observed similar results.[156]

As detailed in Chapter 5, complement-associated proteins and haptoglobin share similar homology and closely interact with one another. These associations warrant further investigation in the context of intestinal epithelial barrier function. It has been shown that complement C3 may also modulate the intestinal epithelial barrier, though whether this regulation is in a zonulin-dependent manner is to be determined.

Taking a purely pragmatic approach, there are assays currently used in the clinical setting of which the actual proteins or protein behaviours being measured are not known. The erythrocyte sedimentation rate is one example.[192] In this respect, perhaps levels of purported zonulin, as advertised by commercial assay, may still have utility as a marker. However, there was no consistency of the findings in the human models of intestinal injury and barrier dysfunction that could be translated into clinical value.

Our findings regarding the inability of commercial assays to detect serum zonulin have been recently published in PLOS ONE, which is an initial step in transmitting this necessary information to the scientific community. Despite this, continuing use of the assays may occur. Next steps include informing companies that have manufactured the assays (i.e. CUSABIO and Immundiagnostik) of the results from both studies. It is imperative that these companies take responsibility to remove the product and notify those who have bought it.

In conclusion, the protein zonulin, as it currently stands, is not recommended for use as a circulating marker of gastrointestinal dysfunction. Until methodology of detection is improved, we urge the scientific and clinical communities to exercise caution in considering the measurement of serum zonulin as a biomarker of intestinal epithelial barrier integrity and permeability.

10.3 Syndecan-1

Syndecan-1, a proteoglycan involved in adhesion between cells and extracellular matrices, has also emerged as a candidate biomarker of intestinal epithelial barrier integrity. Like zonulin, syndecan-1 is linked to maintenance of the paracellular pathway. As such, it is implicated in the pathogenesis of gastrointestinal disease. However, there is limited previous research into its role as a specific serological marker of gastrointestinal disease.

The current studies provide evidence that the utility of syndecan-1 lies in longitudinal analysis and detecting intestinal injury. Specifically, in concert with I-FABP, it may indicate colonic epithelial barrier injury. In all studies of gastrointestinal cohorts included in this thesis, syndecan-1 levels did not significantly correlate with I-FABP levels, which is a specific marker for small intestinal injury. The strongest observation for this case was demonstrated in the NCWS cohort featured in Chapter 7. The data suggest that serum syndecan-1 levels may reflect the potential effects of a low-FODMAP diet in reducing colonic epithelial injury and thus limiting LPS exposure in individuals with NCWS. Trends were observed in the ability of syndecan-1 to track longitudinal mucosal healing in coeliac disease (Chapter 8), though no significant results were detected.

As indicated in Chapter 9, extremely high syndecan-1 levels were detected in a few healthy individuals. Recruitment for healthy controls was primarily based on the absence of gastrointestinal or immune abnormalities. Unless the information was voluntarily stated or inferred, no information about lifestyle (e.g. physical fitness and smoking status), diet, or psychiatric illness was collected. Indeed, variables such as dietary or psychological stress, as previously discussed, may affect the intestinal epithelial barrier. Unforeseen comorbidities, which may or may not be linked to the intestinal barrier, may also be causative factors linked to high levels. Prolonged, intense physical exertion may contribute to heightened levels as well, and heat exacerbates the effect, as demonstrated in Chapter 5.

In addition to confounders related to intestinal effects, there exist extra-intestinal confounders that influence circulating syndecan-1 levels. As syndecan-1 is ubiquitously expressed throughout epithelia and endothelia, it can be difficult to determine whether heightened levels are actually due to gastrointestinal effects in the case of comorbidities (e.g. cancer). As such,

localisation of syndecan-1 levels to the gastrointestinal tract may be very difficult to pinpoint and a degree of interpretation is required. In this respect, patients must be rigorously screened for possible comorbidities. Shed syndecan-1, as evidenced by heightened circulating levels, correlates with growth- and angiogenic-related factors linked to tumour development in cancers.[152]

The best evidence to localise elevated syndecan-1 levels to the intestinal tract may be its correlation with other related markers as well as investigation of protein expression in longitudinal biopsies. As detailed in Chapter 1, the loss of syndecan-1 core protein and ectodomain components has been shown to increase protein efflux into the gut lumen, indicating dysregulation of the intestinal epithelial barrier through intercellular spaces.[55] An interpretation that compares levels of syndecan-1 expression and circulating protein may provide evidence for localisation. For example, an individual with gastrointestinal disease may have low syndecan-1 expression in a particular intestinal biopsy sample, though high circulating syndecan-1 levels at that particular time point. If syndecan-1 expression in biopsy samples increases in the healing process, along with decreases in circulating levels, disease location (as evidenced by biopsy sample) and mechanism relating to syndecan-1, may be inferred. Similar analyses have been made in neoplastic disease in regards to cellular localisation of syndecan-1.[152]

In summary, it is evident that one cross-sectional value of syndecan-1 levels is not enough to glean a comprehensive clinical picture due to inter-individual factors. Longitudinal levels, in combination with vigilance of aforementioned variables and comprehensive clinical information, are required for its utility in tracking intestinal epithelial injury. Avenues for future research regarding the potential of syndecan-1 as a biomarker involve affirming its ability to detect colonic epithelial injury. This can be achieved by investigating longitudinal mucosal healing, as evidenced

by biopsy samples, in patients with colonic-specific disturbances. In addition, an inquiry into how stress, particularly psychological, may affect syndecan-1 levels is also an area of interest.

10.4 I-FABP

I-FABP is a protein released by intestinal epithelial cells in response to damage. I-FABP is primarily expressed in the small intestine and thus is a specific marker for epithelial damage in this location, though it is also expressed in the large intestine in small quantities. In this respect, I-FABP was utilised as a localising marker for small intestinal epithelial damage in the featured investigations.

Elevated levels were found in coeliac disease (Chapters 8 and 9) compared to colonic disease (i.e. ASUC) and healthy controls (although it was a slight elevation, most likely due to confounding variables discussed), which reflects its role in detecting small-bowel enteropathy, and in the model of exercise-induced gastrointestinal damage (Chapter 6). The results in Chapter 7 indicated that I-FABP levels were not as strongly affected as those of syndecan-1 in the NCWS cohort, which agrees with the assumption it has specificity in detecting small intestinal injury. Taken together, the experiments support the use of I-FABP as a marker of small intestinal epithelial damage. As with the other circulating markers, the utility of I-FABP lies in longitudinal, rather than cross-sectional analysis.

The results in Chapter 8 reveal that I-FABP may not be a strong indicator in terms of mucosal healing in coeliac disease. Trends in a decrease of I-FABP levels were observed longitudinally during the course of a gluten-free diet administered to newly-diagnosed coeliac patients, similar to the findings in previous studies (Table 2), though the current results were not statistically significant. A limitation is the low number of participants who reached complete

mucosal healing by a 3-month biopsy assessment. Future studies that examine a larger group of patients who reached mucosal healing, in conjunction with I-FABP levels, may be able to add greater strength to the marker's utility in indicating mucosal healing longitudinally.

10.5 LBP and sCD14

LBP and sCD14 are endogenous proteins that are produced in response to the presence of LPS/endotoxin from gram-negative bacteria. Mechanistically, both proteins act in synergy to bind LPS and activate innate immune processes. Both have been used as surrogate circulating markers in recent studies to reflect microbial translocation across the intestinal barrier. In cross-sectional analysis, the correlation of LBP and sCD14 seemed to be indicative of disease. The results in Chapter 9 indicate positive, significant correlations of both markers at baseline conditions (i.e. before any dietary or drug intervention) for all cohorts of gastrointestinal disease, but not for healthy controls. This was the strongest evidence in their indication of disease from a cross-sectional standpoint; their levels alone were not able to distinguish disease. In addition, the results from Chapter 8 also indicate that their individual values do not provide a comprehensive representation of mucosal healing, though their use together may deliver a more accurate indication. As with other markers, additional strength of utility may be found in longitudinal, rather than cross-sectional analysis. This is supported by the correlation analysis in Chapters 7 and 8.

In addition, LBP and sCD14 did not necessarily correlate with heightened markers of intestinal epithelial damage (i.e. syndecan-1 and I-FABP), indicating that despite the occurrence of epithelial injury, physical barrier defences may remain largely intact, thus preventing the translocation of LPS and endotoxin-related products into the lamina propria. This observation was demonstrated by the results in Chapter 8, though as previously discussed, the evidence could be

strengthened by increasing the sample size of coeliac disease patients who have achieved biopsy-proven mucosal healing.

10.6 Overview of Potential Marker Use

In the clinical or experimental scenarios studied in this thesis, mucosal barrier injury is likely due to the results of several previous investigations using different modes of assessment, such as histology for coeliac disease and ulcerative colitis, other biomarkers as in the exercise model, or a similar spectrum of biomarkers as for patients with NCWS. Furthermore, the interventions that improve or worsen the severity of barrier injury were studied. It is clear from this approach that injury to the mucosal barrier may manifest in many structurally and functionally different ways. For example, there may be evidence of epithelial injury, though no evidence of microbial translocation, as observed in Chapters 6 and 8. Therefore, the use of a panel of markers is important in any study in which an intervention's effect on barrier function is being investigated. Table 7 provides an overview of featured marker utility.

The usage of a panel of markers may be an efficacious lens in further understanding mechanisms of disease. As evidenced in the current studies, the panel provides insight on the nature of how certain factors (e.g. drugs, diets, or diseases) are affecting intestinal barrier function. As in the previous example, increased I-FABP levels indicative of epithelial injury were observed in coeliac disease patients (Chapter 8) without correlating elevations in LBP or sCD14. This may indicate that, although epithelial injury occurs, physical barrier defences that prevent microbial translocation remain largely intact. In accordance with previous evidence, this result indicates that microbial translocation is not a significant factor of coeliac disease pathogenesis.[2]

Combinations of markers as described in a panel may also help determine the location of injury. For example, I-FABP, which indicates enterocyte damage in the small intestine, may be used in conjunction with syndecan-1, which is presumably expressed throughout the intestinal epithelial tract, to determine whether injury is occurring specifically in the small or large intestinal epithelium. If correlations occur between the two markers, intestinal injury is likely localised in the small intestine. If no correlations occur and/or a predominance in syndecan-1 is detected, the colon instead may be the area of disease localisation, as suggested in Chapter 7. In this respect, exploration of marker ratios along with composite scores may have clinical benefit and should be investigated in future studies. Statistical models may then be applied to determine appropriate end-point values. However, the difficulty lies in the fact that no gold standards relating to the mechanisms of markers (e.g. paracellular permeability) currently exist.

As discussed above, two of the potential applications for these markers are in the detection of injury/barrier dysfunction and in the measurement of changes in injury/barrier dysfunction. The importance of the data derived from the healthy controls was highlighted by the wide range of values that were observed for most of the markers. This indicates that the use of a single measurement in a cross-sectional study is unlikely to be able to identify, with confidence, injury in the individual, unless there was gross elevation. However, the markers may still be useful in comparing populations as demonstrated in the thesis. Reasons for the wide variation in healthy subjects was not explored, but studies could be mounted to examine many of the potential confounders (e.g., effects of food, exercise, time of day) for those markers. The true value in the clinical or experimental setting of these markers may well lie in their ability of detect change following an intervention. Controlling such potential confounders might assist in the precision by which changes can be detected. Clearly, more work is needed in this area.

Table 7. Overview of marker characteristics and their potential clinical or experimental utility

Circulating Marker	Relationship/mechanism specific to intestinal barrier	Tissue localisation	Indication	Utility
Zonulin	Protein capable of reversible tight junction disassembly	Epithelial and endothelial cell tight junctions; presumably ubiquitous	Increased intestinal permeability and leakiness between tight junctions/through paracellular route	Not useful; inconsistencies in current detection methodology
Syndecan-1	Proteoglycan involved in the maintenance of tight junctions and cell adhesion	Epithelial and endothelial cell tight junctions; presumably ubiquitous	Non-specific epithelial and endothelial injury	Potential marker of changes to epithelial injury, but should be interpreted in light of the clinical situation and better used in conjunction with I-FABP to assist with localisation
I-FABP	Protein released as a result of enterocyte damage	Highest expression in jejunum; minimal expression in colon	Small intestinal epithelial injury	Useful as a localising marker; potential longitudinal indicator of mucosal healing in coeliac disease
LBP	Endogenous protein produced by hepatocytes that binds to bacterial LPS/endotoxin that has translocated across epithelial barrier	Appendix, liver, pancreas, stomach, duodenum, small intestine, colon, rectum, circulation[193]	Microbial translocation; innate immune activation	Useful in the indication of endotoxaemia and microbial translocation, in conjunction with sCD14
sCD14	Endogenous glycoprotein secreted by monocytes/ macrophages in response to LPS/endotoxin that has translated across epithelial barrier	Circulation and ubiquitous as it is a component of monocytes/macrophages	Microbial translocation; innate immune activation	Useful in the indication of endotoxaemia and microbial translocation, in conjunction with LBP

10.7 Conclusion

The results from the previous chapters show that the featured circulating markers, aside from zonulin (as it currently stands), may have some clinical and experimental utility in detecting and defining the nature of intestinal barrier injury/dysfunction, but intra-individual differences may limit cross-sectional usage of marker data and may not be indicative of a comprehensive clinical picture. However, there appears clear utility of the markers in defining changes of barrier function/integrity over time, which is particularly valuable in characterising the effects of interventions. Care must also be considered in the selection of study cohorts and with regards to lifestyle, diet, stress and potential comorbidity, as these variables may have an influence on marker levels. For example, a diet low in FODMAPs had considerable influence over the levels of syndecan-1 (Chapter 7). Studies that examine extra-intestinal conditions must consider additional gastrointestinal-related clinical measures before claiming that marker levels truly reflect intestinal barrier dysfunction. To confirm that marker levels (i.e. syndecan-1 and I-FABP) may be predictive of mucosal healing in coeliac disease, further studies should be conducted due to small sample sizes of study participants who achieved mucosal healing (Chapter 8). In addition, a longitudinal study that assesses colonic healing in, for example, patients with ulcerative colitis as evidenced by endoscopic and histological criteria in conjunction with syndecan-1 levels, may confirm the utility of syndecan-1 in indicating the status of colonic epithelial injury in the individual. As it currently stands, markers alone are unable to accurately determine disease and caution should be exercised in solely using these markers for clinical decisions. However, when used in conjunction together with one another and with clinical data, they may provide a lens that helps us further understand mechanisms of disease.

REFERENCES

1. Ahmad R, Sorrell MF, Batra SK, Dhawan P, Singh AB. Gut permeability and mucosal inflammation: bad, good or context dependent. *Mucosal immunology*. 2017;10(2):307-317.

2. Uhde M, Ajamian M, Caio G, et al. Intestinal cell damage and systemic immune activation in individuals reporting sensitivity to wheat in the absence of coeliac disease. *Gut.* 2016;65(12):1930-1937.

3. Mu Q, Kirby J, Reilly CM, Luo XM. Leaky Gut As a Danger Signal for Autoimmune Diseases. *Frontiers in immunology.* 2017;8:598.

4. Sandler NG, Douek DC. Microbial translocation in HIV infection: causes, consequences and treatment opportunities. *Nature reviews Microbiology.* 2012;10(9):655-666.

5. Morris G, Berk M, Carvalho A, et al. The Role of the Microbial Metabolites Including Tryptophan Catabolites and Short Chain Fatty Acids in the Pathophysiology of Immune-Inflammatory and Neuroimmune Disease. *Molecular neurobiology.* 2017;54(6):4432-4451.

6. Yang T, Richards EM, Pepine CJ, Raizada MK. The gut microbiota and the brain–gut–kidney axis in hypertension and chronic kidney disease. *Nature Reviews Nephrology.* 2018.

7. Thaiss CA, Levy M, Grosheva I, et al. Hyperglycemia drives intestinal barrier dysfunction and risk for enteric infection. *Science.* 2018;359(6382):1376-1383.

8. Denno DM, VanBuskirk K, Nelson ZC, Musser CA, Hay Burgess DC, Tarr PI. Use of the lactulose to mannitol ratio to evaluate childhood environmental enteric dysfunction: a systematic review. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America.* 2014;59 Suppl 4:S213-219.

9. Odenwald MA, Turner JR. Intestinal permeability defects: is it time to treat? *Clinical gastroenterology and hepatology : the official clinical practice journal of the American Gastroenterological Association.* 2013;11(9):1075-1083.

10. Fasano A. Zonulin and its regulation of intestinal barrier function: the biological door to inflammation, autoimmunity, and cancer. *Physiological reviews.* 2011;91(1):151-175.

11. Turner JR. Intestinal mucosal barrier function in health and disease. *Nature reviews Immunology.* 2009;9(11):799-809.

12. Fasano A, Shea-Donohue T. Mechanisms of disease: the role of intestinal barrier function in the pathogenesis of gastrointestinal autoimmune diseases. *Nature clinical practice Gastroenterology & hepatology.* 2005;2(9):416-422.

13. Tripathi A, Lammers KM, Goldblum S, et al. Identification of human zonulin, a physiological modulator of tight junctions, as prehaptoglobin-2. *Proc Natl Acad Sci U S A.* 2009;106(39):16799-16804.

14. Wang W, Uzzau S, Goldblum SE, Fasano A. Human zonulin, a potential modulator of intestinal tight junctions. *Journal of cell science.* 2000;113 Pt 24:4435-4440.

15. Ramani VC, Pruett PS, Thompson CA, DeLucas LD, Sanderson RD. Heparan sulfate chains of syndecan-1 regulate ectodomain shedding. *J Biol Chem.* 2012;287(13):9952-9961.

16. Wang Z, Li R, Tan J, et al. Syndecan-1 Acts in Synergy with Tight Junction Through Stat3 Signaling to Maintain Intestinal Mucosal Barrier and Prevent Bacterial Translocation. *Inflamm Bowel Dis.* 2015;21(8):1894-1907.

17. Pelsers MM, Hermens WT, Glatz JF. Fatty acid-binding proteins as plasma markers of tissue injury. *Clin Chim Acta.* 2005;352(1-2):15-35.

18. Ludvigsson JF, Leffler DA, Bai JC, et al. The Oslo definitions for coeliac disease and related terms. *Gut.* 2013;62(1):43-52.

19. Fasano A, Berti I, Gerarduzzi T, et al. Prevalence of celiac disease in at-risk and not-at-risk groups in the United States: a large multicenter study. *Archives of internal medicine.* 2003;163(3):286-292.

20. Husby S, Murray JA. Diagnosing coeliac disease and the potential for serological markers. *Nature Reviews Gastroenterology &Amp; Hepatology.* 2014;11:655.

21. Alaedini A, Green PH. Narrative review: celiac disease: understanding a complex autoimmune disorder. *Ann Intern Med.* 2005;142(4):289-298.

22. M'Koma AE. Inflammatory bowel disease: an expanding global health problem. *Clin Med Insights Gastroenterol.* 2013;6:33-47.

23. Laass MW, Roggenbuck D, Conrad K. Diagnosis and classification of Crohn's disease. *Autoimmun Rev.* 2014;13(4-5):467-471.

24. Torres J, Mehandru S, Colombel JF, Peyrin-Biroulet L. Crohn's disease. *Lancet.* 2017;389(10080):1741-1755.

25. Kalla R, Ventham NT, Satsangi J, Arnott ID. Crohn's disease. *BMJ.* 2014;349:g6670.

26. Hindryckx P, Jairath V, D'Haens G. Acute severe ulcerative colitis: from pathophysiology to clinical management. *Nat Rev Gastroenterol Hepatol.* 2016;13(11):654-664.

27. Pimentel M, Chang M, Chow EJ, et al. Identification of a prodromal period in Crohn's disease but not ulcerative colitis. *Am J Gastroenterol.* 2000;95(12):3458-3462.

28. Gajendran M, Loganathan P, Catinella AP, Hashash JG. A comprehensive review and update on Crohn's disease. *Dis Mon.* 2018;64(2):20-57.

29. Sehgal R, Koltun WA. Scoring systems in inflammatory bowel disease. *Expert Rev Gastroenterol Hepatol.* 2010;4(4):513-521.

30. Walsh AJ, Bryant RV, Travis SP. Current best practice for disease activity assessment in IBD. *Nat Rev Gastroenterol Hepatol.* 2016;13(10):567-579.

31. Pastor Rojo O, Lopez San Roman A, Albeniz Arbizu E, de la Hera Martinez A, Ripoll Sevillano E, Albillos Martinez A. Serum lipopolysaccharide-binding protein in endotoxemic patients with inflammatory bowel disease. *Inflamm Bowel Dis.* 2007;13(3):269-277.

32. Vermeire S, Van Assche G, Rutgeerts P. Laboratory markers in IBD: useful, magic, or unnecessary toys? *Gut.* 2006;55(3):426-431.

33. de Souza HS, Fiocchi C. Immunopathogenesis of IBD: current state of the art. *Nat Rev Gastroenterol Hepatol.* 2016;13(1):13-27.

34. Hollander D, Vadheim CM, Brettholz E, Petersen GM, Delahunty T, Rotter JI. Increased intestinal permeability in patients with Crohn's disease and their relatives. A possible etiologic factor. *Ann Intern Med.* 1986;105(6):883-885.

35. Lundin KE, Alaedini A. Non-celiac gluten sensitivity. *Gastrointest Endosc Clin N Am.* 2012;22(4):723-734.

36. Biesiekierski JR, Peters SL, Newnham ED, Rosella O, Muir JG, Gibson PR. No effects of gluten in patients with self-reported non-celiac gluten sensitivity after dietary reduction of fermentable, poorly absorbed, short-chain carbohydrates. *Gastroenterology.* 2013;145(2):320-328 e321-323.

37. Skodje GI, Sarna VK, Minelle IH, et al. Fructan, Rather Than Gluten, Induces Symptoms in Patients With Self-Reported Non-Celiac Gluten Sensitivity. *Gastroenterology.* 2018;154(3):529-539 e522.

38. Reig-Otero Y, Manes J, Manyes L. Amylase-Trypsin Inhibitors in Wheat and Other Cereals as Potential Activators of the Effects of Nonceliac Gluten Sensitivity. *J Med Food.* 2018;21(3):207-214.

39. Dlugosz A, Nowak P, D'Amato M, et al. Increased serum levels of lipopolysaccharide and antiflagellin antibodies in patients with diarrhea-predominant irritable bowel syndrome. *Neurogastroenterol Motil.* 2015;27(12):1747-1754.

40. Costa RJS, Snipe RMJ, Kitic CM, Gibson PR. Systematic review: exercise-induced gastrointestinal syndrome-implications for health and intestinal disease. *Aliment Pharmacol Ther.* 2017;46(3):246-265.

41. Costa RJ, Snipe R, Camoes-Costa V, Scheer V, Murray A. The Impact of Gastrointestinal Symptoms and Dermatological Injuries on Nutritional Intake and Hydration Status During Ultramarathon Events. *Sports Med Open.* 2016;2:16.

42. Pfeiffer B, Stellingwerff T, Hodgson AB, et al. Nutritional intake and gastrointestinal problems during competitive endurance events. *Med Sci Sports Exerc.* 2012;44(2):344-351.

43. Fasano A, Baudry B, Pumplin DW, et al. Vibrio cholerae produces a second enterotoxin, which affects intestinal tight junctions. *Proc Natl Acad Sci U S A.* 1991;88(12):5242-5246.

44. Cenac N, Chin AC, Garcia-Villar R, et al. PAR2 activation alters colonic paracellular permeability in mice via IFN-gamma-dependent and -independent pathways. *The Journal of physiology.* 2004;558(Pt 3):913-925.

45. Fasano A, Fiorentini C, Donelli G, et al. Zonula occludens toxin modulates tight junctions through protein kinase C-dependent actin reorganization, in vitro. *The Journal of clinical investigation.* 1995;96(2):710-720.

46. Langlois MR, Delanghe JR. Biological and clinical significance of haptoglobin polymorphism in humans. *Clin Chem.* 1996;42(10):1589-1600.

47. Wicher KB, Fries E. Prohaptoglobin is proteolytically cleaved in the endoplasmic reticulum by the complement C1r-like protein. *Proceedings of the National Academy of Sciences of the United States of America.* 2004;101(40):14390-14395.

48. Fasano A. Leaky gut and autoimmune diseases. *Clin Rev Allergy Immunol.* 2012;42(1):71-78.

49. Fasano A. Zonulin, regulation of tight junctions, and autoimmune diseases. *Ann N Y Acad Sci.* 2012;1258:25-33.

50. Hollon J, Puppa EL, Greenwald B, Goldberg E, Guerrerio A, Fasano A. Effect of gliadin on permeability of intestinal biopsy explants from celiac disease patients and patients with non-celiac gluten sensitivity. *Nutrients.* 2015;7(3):1565-1576.

51. Barbaro MR CC, Caio G, Bellacosa L, De Giorgio R, Volta U, Stanghellini V, Barbara G. Increased Zonulin Serum Levels and Correlation with Symptoms in Non-Celiac Gluten Sensitivity and Irritable Bowel Syndrome with Diarrhea. *United European Gastroenterology Journal.* 2014;2:Supplement 1.

52. Di Pierro M, Lu R, Uzzau S, et al. Zonula occludens toxin structure-function analysis. Identification of the fragment biologically active on tight junctions and of the zonulin receptor binding domain. *J Biol Chem.* 2001;276(22):19160-19165.

53. Khaleghi S, Ju JM, Lamba A, Murray JA. The potential utility of tight junction regulation in celiac disease: focus on larazotide acetate. *Therapeutic advances in gastroenterology.* 2016;9(1):37-49.

54. Alexopoulou AN, Multhaupt HA, Couchman JR. Syndecans in wound healing, inflammation and vascular biology. *The international journal of biochemistry & cell biology.* 2007;39(3):505-528.

55. Bode L, Salvestrini C, Park PW, et al. Heparan sulfate and syndecan-1 are essential in maintaining murine and human intestinal epithelial barrier function. *The Journal of clinical investigation.* 2008;118(1):229-238.

56. Elenius V, Gotte M, Reizes O, Elenius K, Bernfield M. Inhibition by the soluble syndecan-1 ectodomains delays wound repair in mice overexpressing syndecan-1. *J Biol Chem.* 2004;279(40):41928-41935.

57. Couchman JR. Syndecans: proteoglycan regulators of cell-surface microdomains? *Nature reviews Molecular cell biology.* 2003;4(12):926-937.

58. Qing Q, Zhang S, Chen Y, Li R, Mao H, Chen Q. High glucose-induced intestinal epithelial barrier damage is aggravated by syndecan-1 destruction and heparanase overexpression. *J Cell Mol Med.* 2015;19(6):1366-1374.

59. Yan Z, Zhongqiu W, Jun L, et al. Cell surface-anchored syndecan-1 ameliorates intestinal inflammation and neutrophil transmigration in ulcerative colitis. *Journal of Cellular and Molecular Medicine.* 2017;21(1):13-25.

60. Henry-Stanley MJ, Hess DJ, Erlandsen SL, Wells CL. Ability of the heparan sulfate proteoglycan syndecan-1 to participate in bacterial translocation across the intestinal epithelial barrier. *Shock.* 2005;24(6):571-576.

61. Hess DJ, Henry-Stanley MJ, Erlandsen SL, Wells CL. Heparan sulfate proteoglycans mediate Staphylococcus aureus interactions with intestinal epithelium. *Med Microbiol Immunol.* 2006;195(3):133-141.

62. Smith AJ, Schacker TW, Reilly CS, Haase AT. A role for syndecan-1 and claudin-2 in microbial translocation during HIV-1 infection. *J Acquir Immune Defic Syndr.* 2010;55(3):306-315.

63. Ierardi E, Giorgio F, Zotti M, et al. Infliximab therapy downregulation of basic fibroblast growth factor/syndecan 1 link: a possible molecular pathway of mucosal healing in ulcerative colitis. *J Clin Pathol.* 2011;64(11):968-972.

64. Tursi A, Elisei W, Principi M, et al. Mucosal expression of basic fibroblastic growth factor, syndecan 1 and tumour necrosis factor-alpha in Crohn's disease in deep remission under treatment with anti-TNFalpha antibodies. *J Gastrointestin Liver Dis.* 2014;23(3):261-265.

65. Brule S, Charnaux N, Sutton A, et al. The shedding of syndecan-4 and syndecan-1 from HeLa cells and human primary macrophages is accelerated by SDF-1/CXCL12 and mediated by the matrix metalloproteinase-9. *Glycobiology.* 2006;16(6):488-501.

66. Gan X, Wong B, Wright SD, Cai TQ. Production of matrix metalloproteinase-9 in CaCO-2 cells in response to inflammatory stimuli. *J Interferon Cytokine Res.* 2001;21(2):93-98.

67. Klein RD, Borchers AH, Sundareshan P, et al. Interleukin-1beta secreted from monocytic cells induces the expression of matrilysin in the prostatic cell line LNCaP. *J Biol Chem.* 1997;272(22):14188-14192.

68. Yablecovitch D, Stein A, Shabat-Simon M, et al. Soluble Syndecan-1 Levels Are Elevated in Patients with Inflammatory Bowel Disease. *Dig Dis Sci.* 2015;60(8):2419-2426.

69. Murch SH, MacDonald TT, Walker-Smith JA, Lionetti P, Levin M, Klein NJ. Disruption of sulphated glycosaminoglycans in intestinal inflammation. *The Lancet.* 1993;341(8847):711-714.

70. Kainulainen V, Nelimarkka L, Jarvelainen H, Laato M, Jalkanen M, Elenius K. Suppression of syndecan-1 expression in endothelial cells by tumor necrosis factor-alpha. *J Biol Chem.* 1996;271(31):18759-18766.

71. Fernig DG, Gallagher JT. Fibroblast growth factors and their receptors: an information network controlling tissue growth, morphogenesis and repair. *Prog Growth Factor Res.* 1994;5(4):353-377.

72. Day R, Forbes A. Heparin, cell adhesion, and pathogenesis of inflammatory bowel disease. *Lancet.* 1999;354(9172):62-65.

73. Beck PL, Podolsky DK. Growth factors in inflammatory bowel disease. *Inflamm Bowel Dis.* 1999;5(1):44-60.

74. Kato M, Saunders S, Nguyen H, Bernfield M. Loss of cell surface syndecan-1 causes epithelia to transform into anchorage-independent mesenchyme-like cells. *Molecular Biology of the Cell.* 1995;6(5):559-576.

75. Kamboj AK, Oxentenko AS. Clinical and Histologic Mimickers of Celiac Disease. *Clinical and translational gastroenterology.* 2017;8(8):e114.

76. Salvestrini C, Lucas M, Lionetti P, et al. Matrix expansion and syncytial aggregation of syndecan-1+ cells underpin villous atrophy in coeliac disease. *PLoS One.* 2014;9(9):e106005.

77. Shiner M. Ultrastructural changes suggestive of immune reactions in the jejunal mucosa of coeliac children following gluten challenge. *Gut.* 1973;14(1):1-12.

78. Yablecovitch D, Oren A, Ben-Horin S, et al. Soluble Syndecan-1: A Novel Biomarker of Small Bowel Mucosal Damage in Children with Celiac Disease. *Dig Dis Sci.* 2017;62(3):755-760.

79. Cekic C, Kirci A, Vatansever S, et al. Serum Syndecan-1 Levels and Its Relationship to Disease Activity in Patients with Crohn's Disease. *Gastroenterology research and practice.* 2015;2015:850351.

80. Zhang S, Qing Q, Wang Q, et al. Syndecan-1 and heparanase: potential markers for activity evaluation and differential diagnosis of Crohn's disease. *Inflamm Bowel Dis.* 2013;19(5):1025-1033.

81. Johansson PI, Bergstrom A, Aachmann-Andersen NJ, et al. Effect of acute hypobaric hypoxia on the endothelial glycocalyx and digital reactive hyperemia in humans. *Front Physiol.* 2014;5:459.

82. Furuhashi M, Hotamisligil GS. Fatty acid-binding proteins: role in metabolic diseases and potential as drug targets. *Nature reviews Drug discovery.* 2008;7(6):489-503.

83. Derikx JP, Schellekens DH, Acosta S. Serological markers for human intestinal ischemia: A systematic review. *Best practice & research Clinical gastroenterology.* 2017;31(1):69-74.

84. Haunerland NH, Spener F. Fatty acid-binding proteins--insights from genetic manipulations. *Prog Lipid Res.* 2004;43(4):328-349.

85. Chmurzynska A. The multigene family of fatty acid-binding proteins (FABPs): function, structure and polymorphism. *J Appl Genet.* 2006;47(1):39-48.

86. Pelsers MM, Namiot Z, Kisielewski W, et al. Intestinal-type and liver-type fatty acid-binding protein in the intestine. Tissue distribution and clinical utility. *Clin Biochem.* 2003;36(7):529-535.

87. Derikx JP, Vreugdenhil AC, Van den Neucker AM, et al. A pilot study on the noninvasive evaluation of intestinal damage in celiac disease using I-FABP and L-FABP. *J Clin Gastroenterol.* 2009;43(8):727-733.

88. Adriaanse MP, Tack GJ, Passos VL, et al. Serum I-FABP as marker for enterocyte damage in coeliac disease and its relation to villous atrophy and circulating autoantibodies. *Aliment Pharmacol Ther.* 2013;37(4):482-490.

89. Hoffmanova I, Sanchez D, Habova V, Andel M, Tuckova L, Tlaskalova-Hogenova H. Serological markers of enterocyte damage and apoptosis in patients with celiac disease, autoimmune diabetes mellitus and diabetes mellitus type 2. *Physiol Res.* 2015;64(4):537-546.

90. Bottasso Arias NM, Garcia M, Bondar C, et al. Expression Pattern of Fatty Acid Binding Proteins in Celiac Disease Enteropathy. *Mediators Inflamm.* 2015;2015:738563.

91. Vreugdenhil AC, Wolters VM, Adriaanse MP, et al. Additional value of serum I-FABP levels for evaluating celiac disease activity in children. *Scand J Gastroenterol.* 2011;46(12):1435-1441.

92. Adriaanse MPM, Mubarak A, Riedl RG, et al. Progress towards non-invasive diagnosis and follow-up of celiac disease in children; a prospective multicentre study to the usefulness of plasma I-FABP. *Sci Rep.* 2017;7(1):8671.

93. Oldenburger IB, Wolters VM, Kardol-Hoefnagel T, Houwen RHJ, Otten HG. Serum intestinal fatty acid-binding protein in the noninvasive diagnosis of celiac disease. *APMIS.* 2018;126(3):186-190.

94. Adriaanse MP, Leffler DA, Kelly CP, et al. Serum I-FABP Detects Gluten Responsiveness in Adult Celiac Disease Patients on a Short-Term Gluten Challenge. *Am J Gastroenterol.* 2016;111(7):1014-1022.

95. Not T, Ziberna F, Vatta S, et al. Cryptic genetic gluten intolerance revealed by intestinal antitransglutaminase antibodies and response to gluten-free diet. *Gut.* 2011;60(11):1487-1493.

96. Rodriguez-Martin L, Vaquero L, Vivas S. Letter: serum I-FABP as marker for enterocyte damage in first-degree relatives of patients with coeliac disease. *Aliment Pharmacol Ther.* 2015;42(1):121-122.

97. Sarikaya M, Ergul B, Dogan Z, Filik L, Can M, Arslan L. Intestinal fatty acid binding protein (I-FABP) as a promising test for Crohn's disease: a preliminary study. *Clin Lab.* 2015;61(1-2):87-91.

98. Bodelier AG, Pierik MJ, Lenaerts K, et al. Plasma intestinal fatty acid-binding protein fails to predict endoscopic disease activity in inflammatory bowel disease patients. *Eur J Gastroenterol Hepatol.* 2016;28(7):807-813.

99. Michelini Z, Baroncelli S, Fantauzzi A, et al. Reduced Plasma Levels of sCD14 and I-FABP in HIV-infected Patients with Mesalazine-treated Ulcerative Colitis. *HIV Clin Trials.* 2016;17(2):49-54.

100. Wiercinska-Drapalo A, Jaroszewicz J, Siwak E, Pogorzelska J, Prokopowicz D. Intestinal fatty acid binding protein (I-FABP) as a possible biomarker of ileitis in patients with ulcerative colitis. *Regul Pept.* 2008;147(1-3):25-28.

101. Haskell H, Andrews CW, Jr., Reddy SI, et al. Pathologic features and clinical significance of "backwash" ileitis in ulcerative colitis. *Am J Surg Pathol.* 2005;29(11):1472-1481.

102. Snipe RMJ, Khoo A, Kitic CM, Gibson PR, Costa RJS. The impact of exertional-heat stress on gastrointestinal integrity, gastrointestinal symptoms, systemic endotoxin and cytokine profile. *Eur J Appl Physiol.* 2018;118(2):389-400.

103. Sessions J, Bourbeau K, Rosinski M, et al. Carbohydrate gel ingestion during running in the heat on markers of gastrointestinal distress. *Eur J Sport Sci.* 2016;16(8):1064-1072.

104. van Wijck K, Pennings B, van Bijnen AA, et al. Dietary protein digestion and absorption are impaired during acute postexercise recovery in young men. *Am J Physiol Regul Integr Comp Physiol.* 2013;304(5):R356-361.

105. Van Wijck K, Lenaerts K, Van Bijnen AA, et al. Aggravation of exercise-induced intestinal injury by Ibuprofen in athletes. *Med Sci Sports Exerc.* 2012;44(12):2257-2262.

106. Lis D, Stellingwerff T, Kitic CM, Ahuja KD, Fell J. No Effects of a Short-Term Gluten-free Diet on Performance in Nonceliac Athletes. *Med Sci Sports Exerc.* 2015;47(12):2563-2570.

107. Morrison SA, Cheung SS, Cotter JD. Bovine colostrum, training status, and gastrointestinal permeability during exercise in the heat: a placebo-controlled double-blind study. *Appl Physiol Nutr Metab.* 2014;39(9):1070-1082.

108. Barberio MD, Elmer DJ, Laird RH, Lee KA, Gladden B, Pascoe DD. Systemic LPS and inflammatory response during consecutive days of exercise in heat. *Int J Sports Med.* 2015;36(3):262-270.

109. van Wijck K, Lenaerts K, van Loon LJ, Peters WH, Buurman WA, Dejong CH. Exercise-induced splanchnic hypoperfusion results in gut dysfunction in healthy men. *PLoS One.* 2011;6(7):e22366.

110. Grube BJ, Cochane CG, Ye RD, et al. Lipopolysaccharide binding protein expression in primary human hepatocytes and HepG2 hepatoma cells. *J Biol Chem.* 1994;269(11):8477-8482.

111. Klein RD, Su GL, Aminlari A, Alarcon WH, Wang SC. Pulmonary LPS-binding protein (LBP) upregulation following LPS-mediated injury. *J Surg Res.* 1998;78(1):42-47.

112. Wright SD, Ramos RA, Tobias PS, Ulevitch RJ, Mathison JC. CD14, a receptor for complexes of lipopolysaccharide (LPS) and LPS binding protein. *Science.* 1990;249(4975):1431-1433.

113. Tapping RI, Tobias PS. Cellular binding of soluble CD14 requires lipopolysaccharide (LPS) and LPS-binding protein. *J Biol Chem.* 1997;272(37):23157-23164.

114. Landmann R, Knopf HP, Link S, Sansano S, Schumann R, Zimmerli W. Human monocyte CD14 is upregulated by lipopolysaccharide. *Infection and immunity.* 1996;64(5):1762-1769.

115. da Silva Correia J, Soldau K, Christen U, Tobias PS, Ulevitch RJ. Lipopolysaccharide is in close proximity to each of the proteins in its membrane receptor complex. transfer from CD14 to TLR4 and MD-2. *J Biol Chem.* 2001;276(24):21129-21135.

116. Akira S, Takeda K. Toll-like receptor signalling. *Nature reviews Immunology.* 2004;4(7):499-511.

117. Brenner SA, Zacheja S, Schaffer M, Feilhauer K, Bischoff SC, Lorentz A. Soluble CD14 is essential for lipopolysaccharide-dependent activation of human intestinal mast cells from macroscopically normal as well as Crohn's disease tissue. *Immunology.* 2014;143(2):174-183.

118. Shive CL, Jiang W, Anthony DD, Lederman MM. Soluble CD14 is a nonspecific marker of monocyte activation. *AIDS.* 2015;29(10):1263-1265.

119. Thompson PA, Tobias PS, Viriyakosol S, Kirkland TN, Kitchens RL. Lipopolysaccharide (LPS)-binding protein inhibits responses to cell-bound LPS. *J Biol Chem.* 2003;278(31):28367-28371.

120. Knapp S, Florquin S, Golenbock DT, van der Poll T. Pulmonary lipopolysaccharide (LPS)-binding protein inhibits the LPS-induced lung inflammation in vivo. *J Immunol.* 2006;176(5):3189-3195.

121. Lequier LL, Nikaidoh H, Leonard SR, et al. Preoperative and postoperative endotoxemia in children with congenital heart disease. *Chest.* 2000;117(6):1706-1712.

122. Opal SM, Scannon PJ, Vincent JL, et al. Relationship between plasma levels of lipopolysaccharide (LPS) and LPS-binding protein in patients with severe sepsis and septic shock. *J Infect Dis.* 1999;180(5):1584-1589.

123. Lakatos PL, Kiss LS, Palatka K, et al. Serum lipopolysaccharide-binding protein and soluble CD14 are markers of disease activity in patients with Crohn's disease. *Inflamm Bowel Dis.* 2011;17(3):767-777.

124. Pasternak BA, D'Mello S, Jurickova, II, et al. Lipopolysaccharide exposure is linked to activation of the acute phase response and growth failure in pediatric Crohn's disease and murine colitis. *Inflamm Bowel Dis.* 2010;16(5):856-869.

125. Griffiths AM, Nguyen P, Smith C, MacMillan JH, Sherman PM. Growth and clinical course of children with Crohn's disease. *Gut.* 1993;34(7):939-943.

126. Bischoff SC, Barbara G, Buurman W, et al. Intestinal permeability--a new target for disease prevention and therapy. *BMC gastroenterology.* 2014;14:189.

127. Currie CG, McCallum K, Poxton IR. Mucosal and systemic antibody responses to the lipopolysaccharide of Escherichia coli O157 in health and disease. *J Med Microbiol.* 2001;50(4):345-354.

128. Funderburg NT, Stubblefield Park SR, Sung HC, et al. Circulating CD4(+) and CD8(+) T cells are activated in inflammatory bowel disease and are associated with plasma markers of inflammation. *Immunology.* 2013;140(1):87-97.

129. Kronborg G, Fomsgaard A, Galanos C, Freudenberg MA, Hoiby N. Antibody responses to lipid A, core, and O sugars of the Pseudomonas aeruginosa lipopolysaccharide in chronically infected cystic fibrosis patients. *J Clin Microbiol.* 1992;30(7):1848-1855.

130. Barahona-Garrido J, Sarti HM, Barahona-Garrido MK, et al. Serological markers in inflammatory bowel disease: a review of their clinical utility. *Rev Gastroenterol Mex.* 2009;74(3):230-237.

131. Choung RS, Princen F, Stockfisch TP, et al. Serologic microbial associated markers can predict Crohn's disease behaviour years before disease diagnosis. *Aliment Pharmacol Ther.* 2016;43(12):1300-1310.

132. Curis E, Crenn P, Cynober L. Citrulline and the gut. *Current opinion in clinical nutrition and metabolic care.* 2007;10(5):620-626.

133. Piton G, Capellier G. Biomarkers of gut barrier failure in the ICU. *Current opinion in critical care.* 2016;22(2):152-160.

134. Crenn P, Neveux N, Chevret S, et al. Plasma L-citrulline concentrations and its relationship with inflammation at the onset of septic shock: a pilot study. *J Crit Care.* 2014;29(2):315 e311-316.

135. Matsuo H, Morimoto K, Akaki T, et al. Exercise and aspirin increase levels of circulating gliadin peptides in patients with wheat-dependent exercise-induced anaphylaxis. *Clin Exp Allergy.* 2005;35(4):461-466.

136. JanssenDuijghuijsen LM, van Norren K, Grefte S, et al. Endurance Exercise Increases Intestinal Uptake of the Peanut Allergen Ara h 6 after Peanut Consumption in Humans. *Nutrients.* 2017;9(1).

137. Tornai T, Palyu E, Vitalis Z, et al. Gut barrier failure biomarkers are associated with poor disease outcome in patients with primary sclerosing cholangitis. *World J Gastroenterol.* 2017;23(29):5412-5421.

138. Reiberger T, Ferlitsch A, Payer BA, et al. Non-selective betablocker therapy decreases intestinal permeability and serum levels of LBP and IL-6 in patients with cirrhosis. *J Hepatol.* 2013;58(5):911-921.

139. Koninckx CR, Giliams JP, Polanco I, Pena AS. IgA antigliadin antibodies in celiac and inflammatory bowel disease. *J Pediatr Gastroenterol Nutr.* 1984;3(5):676-682.

140. Smith SM, Eng RH, Campos JM, Chmel H. D-lactic acid measurements in the diagnosis of bacterial infections. *J Clin Microbiol.* 1989;27(3):385-388.

141. Talasniemi JP, Pennanen S, Savolainen H, Niskanen L, Liesivuori J. Analytical investigation: assay of D-lactate in diabetic plasma and urine. *Clin Biochem.* 2008;41(13):1099-1103.

142. Shi H, Wu B, Wan J, Liu W, Su B. The role of serum intestinal fatty acid binding protein levels and D-lactate levels in the diagnosis of acute intestinal ischemia. *Clin Res Hepatol Gastroenterol.* 2015;39(3):373-378.

143. Nielsen C, Kirkegard J, Erlandsen EJ, Lindholt JS, Mortensen FV. D-lactate is a valid biomarker of intestinal ischemia induced by abdominal compartment syndrome. *J Surg Res.* 2015;194(2):400-404.

144. van der Voort PH, Westra B, Wester JP, et al. Can serum L-lactate, D-lactate, creatine kinase and I-FABP be used as diagnostic markers in critically ill patients suspected for bowel ischemia. *BMC Anesthesiol.* 2014;14:111.

145. Sheehan D, Meade G, Foley VM, Dowd CA. Structure, function and evolution of glutathione transferases: implications for classification of non-mammalian members of an ancient enzyme superfamily. *The Biochemical journal.* 2001;360(Pt 1):1-16.

146. Sundberg AG, Nilsson R, Appelkvist EL, Dallner G. Immunohistochemical localization of alpha and pi class glutathione transferases in normal human tissues. *Pharmacology & toxicology.* 1993;72(4-5):321-331.

147. Treskes N, Persoon AM, van Zanten ARH. Diagnostic accuracy of novel serological biomarkers to detect acute mesenteric ischemia: a systematic review and meta-analysis. *Intern Emerg Med.* 2017;12(6):821-836.

148. Gonzalez-Mariscal L, Betanzos A, Nava P, Jaramillo BE. Tight junction proteins. *Prog Biophys Mol Biol.* 2003;81(1):1-44.

149. Runkle EA, Mu D. Tight junction proteins: from barrier to tumorigenesis. *Cancer Lett.* 2013;337(1):41-48.

150. Seidel C, Sundan A, Hjorth M, et al. Serum syndecan-1: a new independent prognostic marker in multiple myeloma. *Blood.* 2000;95(2):388-392.

151. Ibrahim SA, Gadalla R, El-Ghonaimy EA, et al. Syndecan-1 is a novel molecular marker for triple negative inflammatory breast cancer and modulates the cancer stem cell phenotype via the IL-6/STAT3, Notch and EGFR signaling pathways. *Mol Cancer.* 2017;16(1):57.

152. Szatmari T, Otvos R, Hjerpe A, Dobra K. Syndecan-1 in Cancer: Implications for Cell Signaling, Differentiation, and Prognostication. *Dis Markers.* 2015;2015:796052.

153. Pei R, DiMarco DM, Putt KK, et al. Low-fat yogurt consumption reduces biomarkers of chronic inflammation and inhibits markers of endotoxin exposure in healthy premenopausal women: a randomised controlled trial. *Br J Nutr.* 2017;118(12):1043-1051.

154. Overman EL, Rivier JE, Moeser AJ. CRF induces intestinal epithelial barrier injury via the release of mast cell proteases and TNF-alpha. *PLoS One.* 2012;7(6):e39935.

155. Ciacci C, Maiuri L, Russo I, et al. Efficacy of budesonide therapy in the early phase of treatment of adult coeliac disease patients with malabsorption: an in vivo/in vitro pilot study. *Clin Exp Pharmacol Physiol.* 2009;36(12):1170-1176.

156. Scheffler L, Crane A, Heyne HO, et al. Widely used commercial ELISA for human Zonulin reacts with Complement C3 rather than pre-Haptoglobin 2. *bioRxiv.* 2017.

157. Benjamini Y, Hochberg Y. Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing. *Journal of the Royal Statistical Society Series B (Methodological).* 1995;57(1):289-300.

158. Beswick L, Rosella O, Rosella G, et al. Exploration of predictive biomarkers of early infliximab response in acute severe colitis: A prospective pilot study. *J Crohns Colitis.* 2017.

159. Truelove SC, Witts LJ. Cortisone in ulcerative colitis; final report on a therapeutic trial. *Br Med J.* 1955;2(4947):1041-1048.

160. Scheffler L, Crane A, Heyne H, et al. Widely Used Commercial ELISA Does Not Detect Precursor of Haptoglobin2, but Recognizes Properdin as a Potential Second Member of the Zonulin Family. *Frontiers in endocrinology.* 2018;9:22.

161. Koch W, Latz W, Eichinger M, et al. Genotyping of the common haptoglobin Hp 1/2 polymorphism based on PCR. *Clin Chem.* 2002;48(9):1377-1382.

162. Newnham E, Srikanth J, Hosking P, Shepherd S, Gibson P. Adjunctive induction therapy with oral effervescent budesonide in newly diagnosed celiac disease: Results of a pilot, randomized, double-blind, placebo-controlled trial. Paper presented at: JOURNAL OF GASTROENTEROLOGY AND HEPATOLOGY2017.

163. Tosi M, Duponchel C, Meo T, Couture-Tosi E. Complement genes C1r and C1s feature an intronless serine protease domain closely related to haptoglobin. *J Mol Biol.* 1989;208(4):709-714.

164. Wicher KB, Fries E. Haptoglobin, a hemoglobin-binding plasma protein, is present in bony fish and mammals but not in frog and chicken. *Proceedings of the National Academy of Sciences of the United States of America.* 2006;103(11):4168-4173.

165. Kuo S-J, Wang F-S, Sheen J-M, Yu H-R, Wu S-L, Ko J-Y. Complement component C3: Serologic signature for osteogenesis imperfecta. Analysis of a comparative proteomic study. *Journal of the Formosan Medical Association.* 2015;114(10):943-949.

166. Lehtinen MJ, Meri S, Jokiranta TS. Interdomain contact regions and angles between adjacent short consensus repeat domains. *J Mol Biol.* 2004;344(5):1385-1396.

167. Sunderhauf A, Skibbe K, Preisker S, et al. Regulation of epithelial cell expressed C3 in the intestine - Relevance for the pathophysiology of inflammatory bowel disease? *Mol Immunol.* 2017;90:227-238.

168. Sina C, Kemper C, Derer S. The intestinal complement system in inflammatory bowel disease: Shaping intestinal barrier function. *Semin Immunol.* 2018.

169. Brenner I, Shek PN, Zamecnik J, Shephard RJ. Stress hormones and the immunological responses to heat and exercise. *Int J Sports Med.* 1998;19(2):130-143.

170. Browning KN, Travagli RA. Central nervous system control of gastrointestinal motility and secretion and modulation of gastrointestinal functions. *Compr Physiol.* 2014;4(4):1339-1368.

171. Vargas N, Marino F. Heat stress, gastrointestinal permeability and interleukin-6 signaling - Implications for exercise performance and fatigue. *Temperature (Austin).* 2016;3(2):240-251.

172. Costa KA, Soares AD, Wanner SP, et al. L-arginine supplementation prevents increases in intestinal permeability and bacterial translocation in male Swiss mice subjected to physical exercise under environmental heat stress. *J Nutr.* 2014;144(2):218-223.

173. Soares AD, Costa KA, Wanner SP, et al. Dietary glutamine prevents the loss of intestinal barrier function and attenuates the increase in core body temperature induced by acute heat exposure. *Br J Nutr.* 2014;112(10):1601-1610.

174. Lis D, Ahuja KD, Stellingwerff T, Kitic CM, Fell J. Case Study: Utilizing a Low FODMAP Diet to Combat Exercise-Induced Gastrointestinal Symptoms. *Int J Sport Nutr Exerc Metab.* 2016;26(5):481-487.

175. Costa RJS, Miall A, Khoo A, et al. Gut-training: the impact of two weeks repetitive gut-challenge during exercise on gastrointestinal status, glucose availability, fuel kinetics, and running performance. *Appl Physiol Nutr Metab.* 2017;42(5):547-557.

176. Lambert GP, Lang J, Bull A, Eckerson J, Lanspa S, O'Brien J. Fluid tolerance while running: effect of repeated trials. *Int J Sports Med.* 2008;29(11):878-882.

177. Carroccio A, Mansueto P, Iacono G, et al. Non-celiac wheat sensitivity diagnosed by double-blind placebo-controlled challenge: exploring a new clinical entity. *Am J Gastroenterol.* 2012;107(12):1898-1906; quiz 1907.

178. Catassi C, Elli L, Bonaz B, et al. Diagnosis of Non-Celiac Gluten Sensitivity (NCGS): The Salerno Experts' Criteria. *Nutrients.* 2015;7(6):4966-4977.

179. Undseth R, Berstad A, Valeur J. Systemic symptoms in irritable bowel syndrome: An investigative study on the role of enterocyte disintegrity, endotoxemia and inflammation. *Mol Med Rep.* 2016;14(6):5072-5076.

180. Lau NM, Green PH, Taylor AK, et al. Markers of Celiac Disease and Gluten Sensitivity in Children with Autism. *PLoS One.* 2013;8(6):e66155.

181. Stenberg R, Schollin J. Is there a connection between severe cerebral palsy and increased gluten sensitivity? *Acta Paediatr.* 2007;96(1):132-134.

182. Halmos EP, Mack A, Gibson PR. Review article: emulsifiers in the food supply and implications for gastrointestinal disease. *Aliment Pharmacol Ther.* 2019;49(1):41-50.

183. Patel S, Behara R, Swanson GR, Forsyth CB, Voigt RM, Keshavarzian A. Alcohol and the Intestine. *Biomolecules.* 2015;5(4):2573-2588.

184. Halmos EP, Christophersen CT, Bird AR, Shepherd SJ, Gibson PR, Muir JG. Diets that differ in their FODMAP content alter the colonic luminal microenvironment. *Gut.* 2015;64(1):93-100.

185. Zhou SY, Gillilland M, 3rd, Wu X, et al. FODMAP diet modulates visceral nociception by lipopolysaccharide-mediated intestinal inflammation and barrier dysfunction. *The Journal of clinical investigation.* 2018;128(1):267-280.

186. Kelly CP, Bai JC, Liu E, Leffler DA. Advances in diagnosis and management of celiac disease. *Gastroenterology.* 2015;148(6):1175-1186.

187. Edsbacker S, Larsson P, Wollmer P. Gut delivery of budesonide, a locally active corticosteroid, from plain and controlled-release capsules. *Eur J Gastroenterol Hepatol.* 2002;14(12):1357-1362.

188. Brar P, Lee S, Lewis S, Egbuna I, Bhagat G, Green PH. Budesonide in the treatment of refractory celiac disease. *Am J Gastroenterol.* 2007;102(10):2265-2269.

189. Villanueva MS, Alimi Y. Microscopic colitis (lymphocytic and collagenous), eosinophilic colitis, and celiac disease. *Clin Colon Rectal Surg.* 2015;28(2):118-126.

190. Jain A, Sebastian K, Quigley B. Collagenous sprue, an enigma in the spectrum of celiac disease. *Clinical gastroenterology and hepatology : the official clinical practice journal of the American Gastroenterological Association.* 2014;12(1):e2-3; quiz e4-6.

191. Britton A, Ben-Shlomo Y, Benzeval M, Kuh D, Bell S. Life course trajectories of alcohol consumption in the United Kingdom using longitudinal data from nine cohort studies. *BMC Med.* 2015;13:47.

192. Brigden ML. Clinical utility of the erythrocyte sedimentation rate. *Am Fam Physician.* 1999;60(5):1443-1450.

193. Ponten F, Jirstrom K, Uhlen M. The Human Protein Atlas--a tool for pathology. *J Pathol.* 2008;216(4):387-393.

SUPPLEMENTARY TABLE

Supplementary Table 1. Plasma or serum CUSABIO and Immundiagnostik commercial assay use in publications. Google Scholar and PubMed search term: "zonulin." Content was last updated August 7, 2018.

Assay	Author (Year)	Title	Journal
CUSABIO	Çakir M et al. (2017)	Effects of long-term symbiotic supplementation in addition to lifestyle changes in children with obesity-related non-alcoholic fatty liver disease.	The Turkish Journal of Gastroenterology
CUSABIO	Dong S et al. (2018)	Protective effect of 1, 25-dihydroxy vitamin D3 on pepsin-trypsin-resistant gliadin-induced tight junction injuries.	Digestive Diseases and Sciences
CUSABIO	Feuerecker M et al. (2016)	Headache under simulated microgravity is related to endocrine, fluid distribution, and tight junction changes.	Pain
CUSABIO	Greis C et al. (2017)	Intestinal T lymphocyte homing is associated with gastric emptying and epithelial barrier function in critically ill: a prospective observational study.	Critical Care

CUSABIO	Jayashree B et al. (2013)	Increased circulatory levels of lipopolysaccharide (LPS) and zonulin signify novel biomarkers of proinflammation in patients with type 2 diabetes.	Molecular and Cellular Biochemistry
CUSABIO	Küme T et al. (2017)	The relationship between serum zonulin level and clinical and laboratory parameters of childhood obesity.	Journal of Clinical Research in Pediatric Endocrinology
CUSABIO	Küme T et al. (2017)	The relationship between serum zonulin level and clinical and laboratory parameters of childhood obesity.	Journal of Clinical Research in Pediatric Endocrinology
CUSABIO	Mall JPG et al. (2018)	Are self-reported gastrointestinal symptoms among older adults associated with increased intestinal permeability and psychological distress?	BMC Geriatrics
CUSABIO	Ramos CI et al. (2018)	Effect of prebiotic (fructooligosaccharide) on uremic toxins of chronic kidney disease patients: a randomized controlled trial.	Nephrology Dialysis Transplantation
CUSABIO	Zhang D et al. (2014)	Circulating zonulin levels in newly diagnosed Chinese type 2 diabetes patients.	Diabetes Research and Clinical Practice
Immundiagnostik	Barceló A et al. (2016)	Gut epithelial barrier markers in patients with obstructive sleep apnea.	Sleep Medicine

Immundiagnostik	Carrera-Bastos P et al. (2018)	Serum zonulin and endotoxin levels in exceptional longevity versus precocious myocardial infarction.	Aging and Disease
Immundiagnostik	Chuanwei L et al. (2016)	Zonulin regulates intestinal permeability and facilitates enteric bacteria permeation in coronary artery disease.	Scientific Reports
Immundiagnostik	Chwist A et al. (2014)	A composite model including visfatin, tissue polypeptide-specific antigen, hyalruronic acid, and hematological variables for the diagnosis of moderate-to-severe fibrosis in non-alcoholic fatty liver disease: a preliminary study.	Polish Archives of Internal Medicine
Immundiagnostik	Damms-Machado A et al. (2017)	Gut permeability is related to body weight, fatty liver disease, and insulin resistance in obese individuals undergoing weight reduction.	The American Journal of Clinical Nutrition
Immundiagnostik	de Roos et al. (2017)	The effects of a multispecies probiotic on migraine and markers of intestinal permeability – results of a randomized placebo-controlled study.	European Journal of Clinical Nutrition
Immundiagnostik	Ficek J et al. (2017)	Relationship between plasma levels of zonulin, bacterial lipopolysaccharides, D-lactate and markers of inflammation in haemodialysis patients.	International Urology and Nephrology

Immundiagnostik	Gentser L et al. (2018)	Increased jejunal permeability in human obesity is revealed by a lipid challenge and is linked to inflammation and type 2 diabetes.	The Journal of Pathology
Immundiagnostik	Gerdes S et al. (2011)	Zonulin may not be a marker of autoimmunity in patients with psoriasis.	Acta Dermato Venereologica
Immundiagnostik	Houttu N et al. (2017)	Overweight and obesity status in pregnant women are related to intestinal microbiota and serum metabolic and inflammatory profiles	Clinical Nutrition
Immundiagnostik	Hunt P et al. (2014)	Gut epithelial barrier dysfunction and innate immune activation predict mortality in treated HIV infection.	The Journal of Infectious Diseases
Immundiagnostik	Józefczuk J et al. (2018)	The occurrence of antibodies against gluten in children with autism spectrum disorders does not correlate with serological markers of impaired intestinal permeability.	Journal of Medicinal Food
Immundiagnostik	Karhu E et al. (2017)	Exercise and gastrointestinal symptoms: running-induced changes in intestinal permeability markers of gastrointestinal function in asymptomatic and symptomatic runners.	European Journal of Applied Physiology

Immundiagnostik	Kim AS et al. (2018)	Plasma concentrations of zonulin are elevated in obese men with fatty liver disease.	Diabetes, Metabolic Syndrome and Obesity: Targets and Therapy
Immundiagnostik	Kim JH et al. (2018)	Zonulin level, a marker of intestinal permeability, is increased in association with liver enzymes in young adolescents.	Clinica Chimica Acta
Immundiagnostik	Kim S et al. (2018)	Imbalance of gut microbiome and intestinal epithelial barrier dysfunction in patients with high blood pressure.	Clinical Science
Immundiagnostik	Klaus DA et al. (2013)	Increased plasma zonulin levels in patients with sepsis.	Biochemica Medica (Zagreb)
Immundiagnostik	Koay WLA et al. (2018)	Intestinal integrity biomarkers in early antiretroviral-treated perinatally HIV-1-intected infants.	The Journal of Infectious Diseases
Immundiagnostik	Kvehaugen AS et al. (2018)	Is perceived intolerance to milk and wheat associated with the corresponding IgG and IgA food antibodies? A cross sectional study in subjects with morbid obesity and gastrointestinal symptoms.	BMC Gastroenterology

Immundiagnostik	Lamprecht M et al. (2015)	Effects of zeolite supplementation on parameters of intestinal barrier integrity, inflammation, redoxbiology and performance in aerobically trained subjects.	Journal of the International Society of Sports Nutrition
Immundiagnostik	Li C et al. (2016)	Zonulin regulates intestinal permeability and facilitates enteric bacteria permeation in coronary artery disease.	Scientific Reports
Immundiagnostik	Łukaszyk E et al. (2018)	Zonulin, inflammation and iron status in patients with early stages of chronic kidney disease.	International Urology and Nephrology
Immundiagnostik	Łukaszyk M et al. (2015)	Iron status and inflammation in early stages of chronic kidney disease.	Kidney and Blood Pressure Research
Immundiagnostik	Malyszko et al. (2014)	Zonulin, iron status, and anemia in kidney transplant recipients: are they related?	Transplantation Proceedings
Immundiagnostik	Mokkala K et al. (2016)	Gut microbiota richness and composition and dietary intake of overweight pregnant women are related to serum zonulin concentration, a marker for intestinal permeability.	The Journal of Nutrition
Immundiagnostik	Mokkala K et al. (2017)	Evaluation of serum zonulin for use as an early predictor for gestational diabetes.	Nutrition and Diabetes
Immundiagnostik	Mokkala K et al. (2017)	Increased intestinal permeability, measured by serum zonulin, is associated with metabolic risk markers in overweight pregnant women.	Metabolism

Immundiagnostik	Mokkala K et al. (2018)	The impact of probiotics and n-3 long-chain polyunsaturated fatty acids on intestinal permeability in pregnancy: a randomised clinical trial.	Beneficial Microbes
Immundiagnostik	Mondal D et al. (2012)	Contribution of enteric infection, altered intestinal barrier function, and maternal malnutrition to infant malnutrition in Bangladesh.	Clinical Infectious Diseases
Immundiagnostik	Moreno-Navarrete JM et al. (2012)	Circulating zonulin, a marker of intestinal permeability, is increased in association with obesity-associated insulin resistance.	PLoS One
Immundiagnostik	Mörkl S et al. (2018)	Gut microbiota, dietary intakes and intestinal permeability reflected by serum zonulin in women.	European Journal of Nutrition
Immundiagnostik	Muñoz-Cano R et al. (2017)	Distinct transcriptome profiles differentiate NSAID-dependent from NSAID-independent food anaphylaxis.	The Journal of Allergy and Clinical Immunology
Immundiagnostik	Mwape I et al. (2017)	Immunogenicity of rotavirus vaccine (Rotarix^{TM}) in infants with environmental enteric dysfunction.	PLoS One

Immundiagnostik	Ohlsson B et al. (2016)	Two meals with different carbohydrate, fat and protein contents render equivalent postprandial plasma levels of calprotectin, cortisol, triglycerides and zonulin.	International Journal of Food Sciences and Nutrition
Immundiagnostik	Ohlsson B et al. (2017)	Calprotectin in serum and zonulin in serum and faces are elevated after introduction of a diet with lower carbohydrate content and higher fiber, fat and protein contents.	Biomedical Reports
Immundiagnostik	Ohlsson B et al. (2017)	Higher levels of zonulin may rather be associated with increased risk of obesity and hyperlipidemia, than with gastrointestinal symptoms or disease manifestations.	International Journal of Molecular Sciences
Immundiagnostik	Orlando A et al. (2014)	Lactobacillus GG restoration of the gliadin induced epithelial barrier disruption: the role of cellular polyamines.	BMC Microbiology
Immundiagnostik	Ott B et al. (2017)	Effect of caloric restriction on gut permeability, inflammation markers, and fecal microbiota in obese women.	Scientific Reports
Immundiagnostik	Ott B et al. (2018)	Short-term overfeeding with dairy cream does not modify gut permeability, the fecal microbiota, or glucose metabolism in young healthy men.	The Journal of Nutrition

Immundiagnostik	Pacifico L et al. (2014)	Increased circulating zonulin in children with biopsy-proven non-alcoholic fatty liver disease.	World Journal of Gastroenterology
Immundiagnostik	Pacifico L et al. (2014)	Increased circulating zonulin in children with biopsy-proven non-alcoholic fatty liver disease.	World Journal of Gastroenterology
Immundiagnostik	Pärtty A et al. (2017)	Infantile colic is associated with low-grade systemic inflammation.	Journal of Pediatric Gastroenterology and Nutrition
Immundiagnostik	Pei R et al. (2017)	Low-fat yogurt consumption reduces biomarkers of chronic inflammation and inhibits markers of endotoxin exposure in healthy premenopausal women: a randomised controlled trial.	British Journal of Nutrition
Immundiagnostik	Qi Y et al. (2017)	Intestinal permeability biomarker zonulin is elevated in healthy aging.	Journal of the American Medical Directors Association
Immundiagnostik	Rees D et al. (2018)	A randomised, double-blind, cross-over trial to evaluate bread, in which gluten has been pre-digested by prolyl endoprotease treatment, in subjects self-reporting benefits of adopting a gluten-free or low-gluten diet.	British Journal of Nutrition

Immundiagnostik	Ruiz-Núñez B et al. (2018)	Higher prevalence of “low T3 syndrome” in patients with chronic fatigue syndrome: a case-control study.	Frontiers in Endocrinology (Lausanne)
Immundiagnostik	Russo F et al. (2012)	Inulin-enriched pasta improves intestinal permeability and modifies the circulating levels of zonulin and glucagon-like peptide 2 in healthy young volunteers.	Nutrition Research
Immundiagnostik	Russo F et al. (2013)	The effects of fluorouracil, epirubicin, and cyclophosphamide (FEC60) on the intestinal barrier function and gut peptides in breast cancer patients: an observational study.	BMC Cancer
Immundiagnostik	Sanches-Alcoholado L et al. (2017)	Role of gut microbiota on cardio-metabolic parameters and immunity in coronary artery disease patients with and without type-2 diabetes mellitus.	Frontiers in Microbiology
Immundiagnostik	Scorletti E et al. (2018)	Design and rationale of the INSYTE study: A randomised, placebo controlled study to test the efficacy of a symbiotic on liver fat, disease biomarkers and intestinal microbiota in non-alcoholic fatty liver disease.	Contemporary Clinical Trials
Immundiagnostik	Sheen YH et al. (2018)	Serum zonulin is associated with presence and severity of atopic dermatitis in children, independent of total IgE and eosinophil.	Clinical and Experimental Allergy

Immundiagnostik	Šket R et al. (2017)	Hypoxia and inactivity related physiological changes (constipation, inflammation) are not reflected at the level of gut metabolites and butyrate producing microbial community; the PlanHab Study.	Frontiers in Physiology
Immundiagnostik	Stadlbauer V et al. (2015)	Lactobacillus casei Shirota supplementation does not restore gut microbiota composition and gut barrier in metabolic syndrome: a randomized pilot study.	PLoS One
Immundiagnostik	Stenman LK et al. (2016)	Probiotic with or without fiber controls body fat mass, associated with serum zonulin, in overweight and obese adults – randomized controlled trial.	EBioMedicine
Immundiagnostik	Stevens BR et al. (2018)	Increased human intestinal barrier permeability plasma and FABP2 correlated with plasma LPS and altered gut microbiome in anxiety and depression.	Gut
Immundiagnostik	Strewe C et al. (2018)	PlanHab Study: Consequences of combined normobaric hypoxia and bed rest on adenosine kinetics.	Scientific Reports
Immundiagnostik	Tarko A et al. (2017)	Zonulin: a potential marker of intestinal injury in newborns.	Disease Markers

Immundiagnostik	Wilms E et al. (2016)	Effects of supplementation of the Synbiotic Ecologic® 825/FOS P6 on intestinal barrier function in healthy humans: a randomized control trial.	PLoS One
Immundiagnostik	Wosiewicz P et al. (2016)	Portal vein thrombosis in cirrhosis is not associated with intestinal barrier disruption or increased platelet aggregability.	Clinics and Research in Hepatology and Gastroenterology
Immundiagnostik	Żak-Gołab A et al. (2013)	Gut microbiota, microinflammation, metabolic profile and zonulin concentration in obese and normal weight subjects.	International Journal of Endocrinology
Immundiagnostik	Zhang D et al. (2015)	Serum zonulin is elevated in women with polycystic ovary syndrome and correlates with insulin resistance and severity of anovulation.	European Journal of Endocrinology