Diploma thesis

Zonulin as biomarker of gut barrier dysfunction in different diseases: A systematic review

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Graz, 23 June 2026

Declaration of Academic Integrity

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Graz, 23 June 2026 Priska Hopfgartner m.p.

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Zusammenfassung

Hintergrund:

Eine erhöhte Darmpermeabilität, oft als „Leaky-Gut-Syndrom“ bezeichnet, spielt vermutlich bei verschiedenen Erkrankungen eine Rolle, insbesondere bei Magen-Darm- Erkrankungen wie Zöliakie. Zonulin wird häufig als Biomarker für die Darmpermeabilität herangezogen. Seine Zuverlässigkeit ist jedoch umstritten, da verschiedene Antikörper unterschiedliche Proteine nachweisen. Ein häufig verwendeter Assay der Immundiagnostik AG (Deutschland) nutzt einen polyklonalen, affinitätsgereinigten IgG-Antikörper, der gegen das Oktapeptid GGVLVQPG gerichtet ist. Diese systematische Übersicht hatte zum Ziel, die klinische Relevanz von Zonulin-Messungen, die mit diesem spezifischen Antikörper bei verschiedenen Erkrankungen gewonnen wurden, zu bewerten.

Methoden:

Zwischen dem 2. November 2021 und dem 31. Januar 2022 wurde eine systematische Literaturrecherche unter Verwendung von PubMed, der Cochrane Library und Google Scholar mit den Suchbegriffen „zonulin“ UND „human“ durchgeführt. Für Fall-Kontroll-Studien mit gesunden Kontrollpersonen wurden Metaanalysen durchgeführt. Interventionsstudien wurden zusammengefasst, um signifikante Veränderungen zu identifizieren. Es wurden nur Krankheitsgruppen mit mindestens fünf Studien analysiert. Standardisierte Mittelwertdifferenzen wurden unter Verwendung eines Random-Effects-Modells berechnet. Die Heterogenität wurde anhand des I^2-Wertes bewertet. Zudem wurden Metaregressions-und Sensitivitätsanalysen durchgeführt.

Ergebnisse:

Insgesamt wurden 62 Studien in die Arbeit einbezogen, von denen 22 für die Metaanalyse geeignet waren. Fall-Kontroll-Studien zeigten bei Patienten mit metabolischen (SMD = 0.63 [0.13; 1.13]) und gastrointestinalen (SMD = 1.00 [0.40; 1.59]) Erkrankungen im Vergleich zu gesunden Kontrollpersonen signifikant höhere Zonulinwerte. Im Gegensatz dazu waren die Ergebnisse für Leber- und andere Erkrankungen uneinheitlich. Insgesamt war die Heterogenität zwischen den

Studien hoch (I^2 = 90%). Es wurden zehn Interventionsstudien einbezogen, von denen drei über eine signifikante Senkung der Zonulinwerte nach der Intervention berichteten.

Schlussfolgerung:

Insgesamt zeigte Zonulin, gemessen mit dem Antikörper gegen das Oktapeptid GGVLVQPG, klinisch relevante Effekte. Dies unterstreicht seinen Wert als Marker für Darmpermeabilität. Aufgrund der hohen Heterogenität und des unbekannten Zielproteins sollten die Ergebnisse jedoch mit Vorsicht interpretiert werden, und weitere Untersuchungen sind erforderlich.

Abstract

Background:

Increased intestinal permeability, often called as “leaky gut”, is thought to play a role in several diseases, especially in gastrointestinal diseases such as celiac disease. Zonulin is commonly used as a biomarker for intestinal permeability. However, its reliability is debated because different antibodies detect different proteins. One commonly used assay from Immundiagnostik AG (Germany) uses a polyclonal, affinity-purified IgG antibody directed against the octapeptide GGVLVQPG. This systematic review aimed to evaluate the clinical relevance of zonulin measurements obtained with this specific antibody across different diseases.

Methods:

A systematic literature search was conducted between November 2, 2021, and January 31, 2022, using PubMed, the Cochrane Library, and Google Scholar with the terms “zonulin” AND “human”. Meta-analyses were performed for case-control studies with healthy controls. Intervention studies were summarized to identify significant changes. Only disease groups with at least five studies were analysed. Standardized mean differences were calculated using a random-effects model. Heterogeneity was assessed using I^2. Meta-regression and sensitivity analyses were also performed.

Results:

A total of 62 studies were included in the review, of which 22 were eligible for meta-analysis. Case-control studies demonstrated significantly higher zonulin levels in patients with metabolic (SMD = 0.63 [0.13; 1.13]) and gastrointestinal (SMD = 1.00 [0.40; 1.59]) diseases compared to healthy controls. In contrast, results for liver and other diseases were inconsistent. Overall, heterogeneity between studies was high (I^2 = 90%). Ten intervention studies were included, of which three reported significant reductions in zonulin levels following intervention.

Conclusion:

Overall, zonulin measured with the antibody against the octapeptide GGVLVQPG showed clinically relevant effects, supporting its value as a marker of intestinal permeability. However, due to high heterogeneity and the unknown target protein, results should be interpreted with caution and further research is needed.

Information on previous publications

Parts of this research were previously presented as a poster titled “Zonulin als Biomarker von Darmbarrierestörungen bei verschiedenen Krankheiten: Ein systematisches Review“ at the 57th Annual Meeting of the Austrian Society of Gastroenterology and Hepatology (ÖGGH), Salzburg, 2024 (1).

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Abbreviations

ADHD	attention-deficit/hyperactivity disorder
AJs	adherens junctions
AMPs	antimicrobial proteins
ASD	autism spectrum disorder
BAs	bile acids
ELISA	enzyme-linked immunosorbent assay
ENS	enteric nervous system
FOS	fructo-oligosaccharides
GALT	gut-associated lymphoid tissue
GLP	glucagon-like petpid
GOS	galacto-oligosaccharides
Hb	haemoglobin
I-FABP	intestinal fatty acid-binding protein
IBD	inflammatory bowel diseases
IBS	irritable bowel syndrome
ICD	implantable cardioverter-defibrillator
IECs	intestinal epithelial cells
IELs	intraepithelial lymphocytes
IGT	impaired glucose tolerance
JAMs	junctional adhesion molecules
L/M	lactulose/mannitol
LBP	LPS-binding protein
LPS	Lipopolysaccharide
MUC2	mucin 2
MASLD	metabolic dysfunction-associated steatotic liver disease
MASH	metabolic dysfunction-associated steatohepatitis
PCOS	polycystic ovarian syndrome
PPI	proton pump inhibitor
pre-HP2	pre-haptoglobin-2
SCFAs	short-chain fatty acids

sIgA	secretory immunoglobulin A
SMD	standardized mean difference
T1DM	type 1 diabetes mellitus
T2DM	type 2 diabetes mellitus
TJs	tight junctions
TLR2	toll-like receptor 2
WHO	World Health Organization
ZO	zonula occludens
Zot	zonula occludens toxin

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1 Introduction

1.1 The intestinal barrier

The intestinal barrier consists of several layers that work together to provide physical and immune protection. The outer layer includes the mucus layer with commensal gut microbiota. Beneath this is a monolayer formed by specialized intestinal epithelial cells (IECs). The inner layer, known as the lamina propria, contains immune cells. Each layer plays an important role in maintaining the integrity and function of the intestinal barrier, which is essential for overall health (2, 3).

1.1.1 Microbiological barrier

The outermost layer is the microbiological barrier. It is located in the intestinal lumen, on top of and within the mucus. This barrier consists of a diverse community of microorganisms including bacteria, fungi, yeasts, archaea, and viruses that colonize the gastrointestinal (GI) tract. In a healthy adult, the gut hosts over 100 trillion microorganisms with bacteria from the phyla *Bacteroidota* and *Bacillota* being the most dominant. They assist in digestion, produce vital metabolites like short-chain fatty acids (SCFAs) and vitamins. Additionally, they support immune system regulation and defend against pathogens. An imbalance in this microbial ecosystem, known as dysbiosis, can weaken the barrier and contribute to various diseases (4).

1.1.2 Chemical barrier

Located beneath the microbiological barrier is the chemical barrier which is made up of various secreted molecules that help neutralize potential threats. These include bile acids (BAs), antimicrobial peptides (AMPs), and secretory immunoglobulin A (sIgA). The direct antimicrobial effect from BAs helps fighting bacteria by damaging their membranes through their detergent-like characteristics (4).

AMPs, mainly produced by enterocytes and Paneth cells, are especially important in the small intestine, where the mucus layer is thinner and more permeable to bacteria and potential toxins (2, 4, 5).

sIgA is the main immunoglobulin secreted at the intestinal mucosal surface within the mucus layer and produced by plasma cells in the mucosa. It prevents colonization and damage to the epithelial barrier through immune exclusion. Reduced or absent sIgA is associated with increased mucosal immune activation and a higher susceptibility to infections and gastrointestinal disorders, suggesting that sIgA plays a broader role in mucosal immunity (4).

1.1.3 Physical barrier

The physical barrier consists of the mucus layers, the epithelial cell layer and endothelial cells (2).

1.1.3.1 Mucus Layer

The mucus layer is covered by a hydrated gel composed primarily of water and heavily glycosylated mucin proteins (6). Mucin 2 (MUC2) is the most secreted mucin by goblet cells and essential for disease protection, as MUC2 knockout mice spontaneously develop colitis (7). Also, the importance of mucus gel hydration is evident in cystic fibrosis, where the production of hyper viscous mucus contributes to diseases of the lungs, pancreas, and intestines. The mucus layer serves as the first physical barrier, preventing external substances from entering the intestinal lumen and stopping bacteria from reaching the epithelial cells. In the small intestine, there is only one mucus layer, whereas the large intestine has an outer loose layer inhabited by bacteria and an inner dense layer that is bacteria-free (8).

1.1.3.2 Intestinal epithelial cells

The epithelial cells beneath the mucus layer represent the most important component of the physical intestinal barrier. This layer prevents harmful substances such as foreign antigens, microorganisms, and their toxins from passing through. Pluripotent stem cells located in the intestinal crypts develop to five specialized cell types. Goblet cells produce mucus, enterocytes absorb nutrients, enteroendocrine cells release hormones, Paneth cells contribute to antimicrobial defence, and microfold cells transport antigens to immune cells. Together, these cells create a single layer that forms a barrier between the intestinal lumen and the lamina propria (2, 8).

The epithelial layer also serves as a selective filter, enabling the absorption of essential nutrients, electrolytes, and water from the gut lumen into the bloodstream. Transport between the cells is regulated by three specialized cell junctions, including tight junctions (TJs), adherens junctions (AJs), and desmosomes, which regulate paracellular permeability and provide structural stability (2, 8).

Transport across the intestinal epithelium happens either via the transcellular route (through the cells) or the paracellular route (between the cells) (8).

1.1.3.3 Intestinal endothelial cells

A third physical barrier is formed by endothelial cells in the lamina propria. Together with pericytes and enteric glial cells, they form the gut-vascular barrier. This barrier controls the passage of substances into the bloodstream (9).

1.1.4 Immune barrier

The immunological component of the intestinal barrier consists of several elements that work together to ensure effective defence against harmful microorganisms, toxins, and other environmental factors. The immune defense of the intestine is provided by several types of cells, including intraepithelial lymphocytes (IELs), IECs and Paneth cells. The lamina propria also contains immune cells from both the innate and adaptive immune systems. In the small intestine, gut-associated lymphoid tissue (GALT) appears as large clusters, known as Peyer's patches. It is also found as single lymphoid follicles in the upper small intestine and the colon (5).

Intestinal defence is also supported by the muscular tissue of the gut as well as the enteric nervous system (ENS) (10).

1.2 Tight Junctions

Tight junctions (TJs) are special complexes connecting neighbouring epithelial and endothelial cells. The complexes are made of multiple transmembrane proteins

and scaffold proteins including occludins, claudins, junctional adhesion molecules (JAMs), zonula occludens (ZOs) and cingulin (11, 12).
Occludin was the first identified TJ protein. Its two functions are maintaining structural integrity and barrier function of TJs (11).
Claudins are a large family of transmembrane proteins that regulate paracellular transport. They can function as either barrier-forming or pore-forming proteins (12). Pore-forming claudins, for example claudin-2, increase permeability and are upregulated in diseases like IBD. In contrast, if claudin-5 and claudin-8 are downregulated, the barrier integrity is reduced (11).
JAMs are also transmembrane proteins that belong to the immunoglobulin superfamily. These proteins regulate the formation of TJs and immune cell migration (11, 12).
Zonula occludens proteins (ZOs) and cingulin are intracellular scaffolding proteins that connect transmembrane TJ components to the cytoskeleton (11).

The coordinated action of all these proteins is fundamental to the structural and functional integrity of the intestinal epithelial barrier. Disruptions in these proteins can increase intestinal permeability and contribute to the pathogenesis of several diseases (11, 12).

1.3 Intestinal Permeability

Intestinal permeability is described as the rate of flux of defined molecules across the intestinal epithelium that can be measured (10). Normal function of intestinal permeability is crucial for human health. It helps to maintain a selective boundary between the intestinal lumen and the internal environment. This allows the absorption of essential nutrients while restricting the passage of harmful substances such as pathogens, toxins, and antigens. Disruption of this regulated barrier, often called as "leaky gut", is associated with the development and progression of several gastrointestinal and systemic diseases (5).

1.3.1 Factors that can influence intestinal permeability

Multiple factors influence intestinal permeability. These include diet, dysbiosis, infections, stress and genetic susceptibility. Moreover, alcohol and several drugs can cause increased permeability (13).

1.3.1.1 Diet

Western-style diets, which are typically rich in saturated fatty acids, sugar, salt and low in fiber are connected to increased permeability. The consumption of high-fat diets can develop intestinal dysbiosis. High-fat diets have been shown to reduce beneficial intestinal bacteria such as *Lactobacillus* and *Bifidobacterium* (14).

In contrast, dietary fibers and fermentable oligosaccharides (FODMAPs) may support barrier integrity. SCFAs produced by fiber fermentation, especially butyrate, enhance epithelial integrity by upregulating TJ proteins (15).

Adequate intake of micronutrients also plays a role. Vitamin A is essential for intestinal barrier integrity, as it regulates epithelial cell differentiation, mucin production, and antimicrobial defences, while deficiency is linked to a higher risk of infection and impaired barrier function. Vitamin D and zinc deficiencies also contribute to increased intestinal permeability (2, 10).

Beyond vitamins and fatty acids, other dietary components such as plant-derived flavonoids have been studied. Quercetin, found in foods like grapes and onions, has been shown to increase epithelial resistance and upregulate claudin-4 expression in intestinal epithelial cells (10).

1.3.1.2 Alcohol

Alcohol consumption is a well-established factor that impairs intestinal barrier integrity. Both ethanol and its metabolite acetaldehyde directly disrupt tight junctions and damage epithelial cells, leading to increased intestinal permeability. Alcohol and its metabolite acetaldehyde damage the intestinal barrier by harming epithelial cells, weakening TJs and AJs, and increasing oxidative stress. Gut bacteria also produce acetaldehyde, which strengthens these effects. Chronic alcohol consumption changes the gut microbiota, increasing

gram-negative bacteria and worsening barrier damage, which develops endotoxin leakage and inflammation (2).

1.3.1.3 Smoking

Cigarette smoking also influences intestinal permeability. Tobacco has been widely studied for its effects on gut health, particularly in inflammatory bowel disease, where it shows opposite effects. While it increases the risk of Crohn's disease, it reduces the development of ulcerative colitis. Although smoking is a major environmental factor in IBD, studies examining its impact on intestinal permeability have shown inconsistent results (2, 16).

1.3.1.4 Medication

Several commonly used medications affect intestinal permeability. Drugs such as nonsteroidal anti-inflammatory agents and paracetamol can injure the gastric and intestinal mucosa and increase intestinal permeability. Antibiotics can weaken the barrier by altering the balance of the gut microbiota. The effects depend on the type, dose, and duration of the treatment (17).

1.3.1.5 Stress

Psychological and physiological stress are important regulators of intestinal permeability. Stress activates neuroendocrine pathways, including the release of corticotropin-releasing factor and cortisol, which can increase paracellular permeability and promote low-grade intestinal inflammation. Both acute and chronic stress have been shown to increase intestinal permeability (2, 17).

1.3.1.6 Infections

Specific pathogens like Helicobacter pylori can also harm TJ structures. On the other hand, certain probiotic strains, including *Escherichia coli* and *Lactobacillus plantarum*, have been shown to improve intestinal barrier function by upregulating TJ proteins and reducing inflammation in both in vitro and in vivo studies (17).

1.4 Diseases associated with gut barrier dysfunction

1.4.1 Gastrointestinal diseases

Intestinal barrier dysfunction plays a central role in several gastrointestinal diseases, most prominently inflammatory bowel diseases (IBD) and celiac disease. In IBD, including Crohn's disease and ulcerative colitis, increased intestinal permeability is linked to microbial imbalance, inflammation, and alterations of TJ structure (2, 18).

The gut microbiota in patients with IBD shows an increased abundance of inflammation – promoting bacteria and changes in cytokine levels, which further contributes to epithelial damage and barrier dysfunction (5, 18).
Importantly, increased intestinal permeability has been detected in asymptomatic individuals before the onset of clinical IBD, suggesting that barrier dysfunction may precede and contribute to disease development rather than being only a consequence of inflammation (2, 18).
In celiac disease, gluten-derived peptides can cross the epithelium and activate immune responses in genetically predisposed individuals. Exposure to gliadin triggers the release of zonulin, a protein which can modulate TJs and leads to increased intestinal permeability (2, 17).

1.4.2 Metabolic diseases

Metabolic disorders such as obesity, type 1 diabetes (T1DM), type 2 diabetes (T2DM) and metabolic syndrome are associated with intestinal barrier impairment. In T1DM, increased intestinal permeability and TJ alterations have been observed before the clinical onset of disease. Changes in the composition of the gut microbiota have been reported in both T1DM and T2DM , suggesting a possible involvement in disease pathogenesis. Moreover, persistent hyperglycaemia in patients with T2DM contributes to the persistence of systemic inflammation (18, 19).
Obesity is similarly linked to dysbiosis, increased permeability, and ongoing mild inflammation, partly mediated by immune processes in adipose tissue (17, 18).

1.4.3 Liver diseases

Liver diseases, particularly metabolic dysfunction-associated steatotic liver disease (MASLD) and metabolic dysfunction-associated steatohepatitis (MASH), are strongly influenced by intestinal barrier dysfunction through the gut–liver axis. Increased intestinal permeability and dysbiosis allow bacterial products to reach the liver via the portal circulation, triggering hepatic inflammation and fibrosis. Inflammation increases liver damage and promotes the progression to liver cirrhosis (17, 18).

Experimental and clinical data show that the combined disruption of the intestinal epithelial barrier and the intestinal vascular barrier contributes to the progression from simple steatosis to MASH (18).

In alcoholic liver disease, intestinal barrier dysfunction is observed, as alcohol and its metabolites directly damage intestinal epithelial cells, disrupt TJ integrity, and promote bacterial translocation. This further enhances liver inflammation and disease progression (18, 19).

1.4.4 Neurological and psychiatric diseases

Gut barrier dysfunction has been linked to neurological and psychiatric disorders through the microbiota–gut–brain axis. In neurodegenerative diseases like Alzheimer's and Parkinson's disease, increased gut permeability may allow pro-inflammatory substances to enter the bloodstream, amplifying neuroinflammation (20).

High serum zonulin levels and increased permeability have been observed in children with autism spectrum disorder (ASD) and attention-deficit/hyperactivity disorder (ADHD) (21).

1.4.5 Others

Many other conditions are linked to a "leaky gut". Increased serum zonulin levels, dysbiosis, and increased permeability have been observed in autoimmune disorders like rheumatoid arthritis and ankylosing spondylitis, supporting the concept of an intestinal–articular axis (18).

Barrier dysfunction has also been associated with neoplastic diseases, infections, and allergic diseases (21, 22).

1.5 Therapeutic potential of barrier modulation

Apart from diet, probiotics and prebiotics have attracted increasing attention for their potential effects on intestinal health.

1.5.1 Probiotics

According to the definiton, “oral probiotics are living microorganisms which upon ingestion in certain numbers, exert health benefits beyond inherent basic nutrition” (23). Common probiotic strains include *Lactobacillus*, *Bifidobacterium*, and *Saccharomyces*, which are found in fermented foods or supplements. Experimental studies indicate that probiotics can improve intestinal barrier function by increasing the expression of TJ proteins, protecting epithelial cells from inflammation-induced damage, and reducing pathogen adhesion (5, 10).

Several probiotic strains have been shown to stabilize TJ models. For example, *Lactobacillus plantarum*, enhance TJ proteins and help maintain barrier integrity during inflammatory stress (10).

In addition to classical probiotics, next-generation probiotics derived from commensal gut bacteria, have gained attention due to their anti-inflammatory and barrier-supporting properties. While their clinical use is still limited, these microbes represent promising future therapeutic options (5).

1.5.2 Prebiotics

Prebiotics are dietary components, mainly fibers, that remain undigested and support beneficial gut bacteria (24). Common prebiotics include fructo-oligosaccharides (FOS), galacto-oligosaccharides (GOS), and inulin. These compounds are fermented by gut microbiota into SCFAs, such as butyrate, which provide energy to intestinal cells and support barrier function (10).

SCFAs contribute to mucus production, tight junction stability, and immune regulation. Prebiotic supplementation has been shown to improve gut barrier function, reduce endotoxin levels, and lower inflammation (19).

1.5.3 Snybiotics

Synbiotics combine probiotics and prebiotics to enhance their effects. Emerging evidence suggests that synbiotics may improve intestinal barrier function and reduce inflammation more effectively than probiotics or prebiotics alone (19).

1.6 Measurement of intestinal permeability

1.6.1 Lactulose/mannitol ratio test

Intestinal permeability can be measured in several methods. The most common method is the lactulose/mannitol (L/M) ratio test. The L/M test is based on the differential absorption of two non-metabolized sugar probes. Mannitol, a small monosaccharide, is absorbed transcellularly, and lactulose, a larger disaccharide, can only cross via paracellular routes. Its uptake increases when TJs are widened. After drinking an oral solution containing both sugars, urine is collected over a defined period, typically over four to five hours, and the ratio of recovered lactulose to mannitol is calculated. An elevated L/M ratio indicates increased permeability and impaired barrier function (25). Although this test is regarded as the gold standard, it is impractical in clinical practice. Moreover, it has been suggested that lactulose and mannitol have very similar three-dimensional sizes. Therefore, it is unlikely that both sugars pass through the epithelium via different pathways (26). It requires patient fasting, long urine collection periods and specialized laboratory analysis. In addition, factors such as urine collection and medication use can affect the results (27, 28).

1.6.2 Biomarkers

Due to this practical limitations, non-invasive biomarkers have gained increasing relevance for the assessment of intestinal permeability. Intestinal fatty acid-binding protein (I-FABP) can indicate enterocyte damage. Lipopolysaccharide (LPS) and LPS-binding protein (LBP) indicate bacterial translocation and endotoxemia. Other markers, such as citrulline and glucagon-like peptide (GLP) - 2, are also being studied. Among these, zonulin has been proposed as a promising biomarker of intestinal permeability, as it directly modulates TJs. Overall, non-

invasive biomarkers are easier to use and more suitable for routine clinical settings (10, 13, 29).

1.7 Zonulin

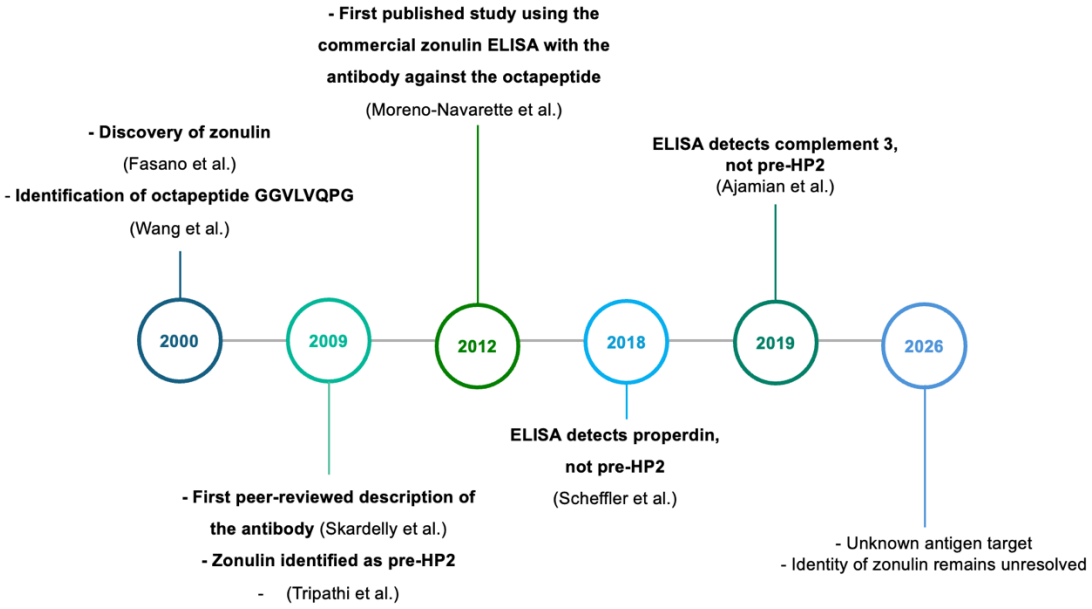

Figure 1: Timeline of key discoveries and analytical controversies in zonulin research

1.7.1 Discovery of zonulin

Zonulin was first discovered in 2000 by Fasano et al. (30). In the process of developing a vaccine for *Vibrio cholerae*, zonula occludens toxin (Zot), a protein produced by *V. cholerae*, was discovered (31, 32). In following studies, it has been shown that Zot can reversibly open TJs between intestinal cells. It increases the permeability of TJs by reorganizing the actin cytoskeleton mediated by Proteinkinase C (32). Based on the hypothesis that an endogenous analogue of Zot might also exist in the human body, a human protein with similar effects on intestinal permeability, named zonulin, was identified (30). It was found in both foetal and adult human intestinal tissue which differ in the amino acid sequence. Furthermore, they discovered zonulin not only in the intestine but also in the heart and brain (33).

During this work, Wang et al. identified an eight-amino-acid sequence (GGVLVQPG) in the N-terminal region of zonulin, which inhibited zonulin's interaction with its epithelial receptor (33).
In 2009, Tripathi et al. discovered that zonulin is the same molecule as pre-haptoglobin-2 (pre-HP2), which is an inactive precursor of haptoglobin 2. Haptoglobins are acute-phase plasma proteins. They bind free haemoglobin (Hb) released from lysed red blood cells and form a stable complex with it to prevent oxidative tissue damage (34).

1.7.2 Development of commercial zonulin ELISA kits

Due to the association of zonulin with various immune-mediated and inflammatory diseases, a quantitative sandwich enzyme-linked immunosorbent assay (ELISA) was developed. Based on the eight-amino-acid sequence identified by Wang, Immundiagnostik (Bensheim, Germany) generated a polyclonal antibody directed against the octapeptide and designed commercial ELISA kits, intended for measuring zonulin in serum and stool (35).

1.7.3 Analytical problems with current ELISA assays

However, Scheffler et al. (2018) demonstrated that the widely used commercial ELISA, raised against the octapeptide GGVLVQPG, does not actually detect pre-HP2. In a study of 376 subjects, measured zonulin levels did not correlate with HP genotype. Mass spectrometry and western blot analyses failed to detect pre-HP2 in ELISA-positive samples. They suggested that the antibody binds properdin, which was however underestimated by the ELISA kit. (36).

Another study by Ajamian et al. (2019) performed immunoprecipitation of human sera, using the same Immundiagnostik ELISA kits, followed by mass spectrometry. As potential target protein they identified complement C3, which however was not detected by the antibody. (37).

Despite extensive research, the exact structure and regulation of zonulin remain unclear. At the same time, widely used commercial ELISA kits, developed using the octapeptide GGVLVQPG, do not reliably detect pre-HP2, the protein later

proposed as zonulin. Instead, these assays may detect properdin, complement C3 or other serum proteins. Therefore, zonulin data in clinical studies must be interpreted with caution (36, 37).

1.7.4 Larazotide acetate as a therapeutic target

Targeting the zonulin pathway presents a potential therapeutic option to treat diseases associated with elevated zonulin levels. The most studied zonulin inhibitor is Larazotide acetate (AT-1001). It is a synthetic octapeptide (GGVLVQPG) that acts as a competitive zonulin receptor antagonist. Several human and animal studies have shown promising results, especially in celiac disease. Moreover, the zonulin inhibitor is being considered as a possible treatment for metabolic diseases (21). Larazotide acetate is now undergoing phase III clinical trials. It is administered orally to adults with celiac disease as an add-on therapy to help restore intestinal barrier function impaired by gliadin-induced immune responses (38).

In addition to larazotide acetate, another synthetic zonulin-related peptide, AT-1002, has been identified. Unlike larazotide acetate, AT-1002 acts as an agonist of zonulin receptors and is used to increase permeability for drug delivery (21).

2 Materials and Methods

2.1 Registration

This systematic review was registered at the International prospective register of systematic reviews (PROSPERO; No. CRD42021288925).

2.2 Search strategy

A comprehensive literature search was performed between November 2, 2021, and January 31, 2022, using the databases PubMed, the Cochrane Library, and Google Scholar, with the search terms: "*zonulin*" AND "*human*". The search was limited to studies published in English or German language.
In addition to database searches, the vendor (Immundiagnostik AG, Bensheim, Germany) provided a list of publications that used their polyclonal antibody directed against the octapeptide GGVLVQPG. Duplicate removal was conducted using Python (Anaconda 3).

2.3 Eligibility criteria

2.3.1 Inclusion criteria

- studies using the polyclonal antibody against the octapeptide GGVLVQPG (Immundiagnostik AG, Bensheim, Germany)
- studies measuring zonulin in human blood and/or stool samples
- studies with two comparable groups (e.g., patient vs. control or pre- vs. post-intervention)
- studies published in English or German language

2.3.2 Exclusion criteria

- studies using a different antibody or targeting a different epitope
- studies not conducted in human subjects
- studies that misidentified other proteins (e.g. ZO-1) as zonulin
- studies lacking a control or comparison group

2.3.3 Population and conditions studied

The included studies measured zonulin levels in human participants across a wide range of diseases or disease clusters. Participants could be patients with any disease condition in which zonulin was evaluated, compared to healthy controls when available.

2.4 Quality assessment

Each study underwent a structured methodological review using the JBI Appraisal Checklists (39) considering:

- use of correct antibody and sample type
- study design (case control vs. intervention)
- comparability of groups (e.g., sex, age, body mass index (BMI))
- confounding variables
- statistical methodology (e.g., use of Mann–Whitney U test)
- intervention-specific criteria (e.g., blinding, flow diagrams)

Based on these criteria, a study was rated as:

- good: two-thirds of criteria fulfilled
- medium: one-half of criteria fulfilled
- poor: one-third of criteria fulfilled

2.5 Data extraction

Following data was extracted from each publication:

- first author, publication year, country of origin
- participant age, BMI, sex distribution
- disease type and intervention type (if available)
- zonulin levels (mean and standard deviation) for each group
- sample type (blood/stool)
- group sizes and statistical significance between groups

BMI was classified according to the World Health Organization (WHO) guidelines: underweight (<18,5 kg/m2), normal weight (18,5-24,9 kg/m2), overweight (25,0-29,9 kg/m2) and obese (≥30,0 kg/m2).
If zonulin values were missing or published in a different format, corresponding authors were contacted. Studies were excluded if the data could not be retrieved.

2.6 Data synthesis and analysis

Meta-analyses were conducted only on case-control studies, including healthy control groups, and on intervention studies, including baseline and end of treatment data. First, disease clusters were defined, then studies were grouped into these clusters. Disease groups with at least five eligible groups were analysed. For the meta-analysis, the standardized mean difference (SMD) was calculated to compare zonulin levels between patients and healthy controls, as well as between patients before and after intervention. A random-effects model was used to pool the data. Heterogeneity among studies was assessed using τ^2 and the I^2 statistic. I^2 values of 50% or below considered low, values between 50% and 75% classified as moderate, and values above 75% indicating high heterogeneity. To further explore sources of heterogeneity, meta-regressions were conducted using a mixed-effects model. As contributing factors BMI [kg/m^2], sex [percent of female study participants], age [years], appraisal and disease type.
Sensitivity analyses were performed by excluding studies with a high risk of bias, small sample sizes, or potential confounding variables. All statistical analyses were carried out using R version 4.3.3 with the metafor and metasense packages (40, 41).

2.7 Subgroup analysis

When data allowed, subgroup analyses were performed for case-control studies. The examined subgroups included age, sex, BMI category and disease type (e.g., obesity, T2DM). Comparisons were made using SMD of zonulin levels.

3 Results

3.1 Study selection

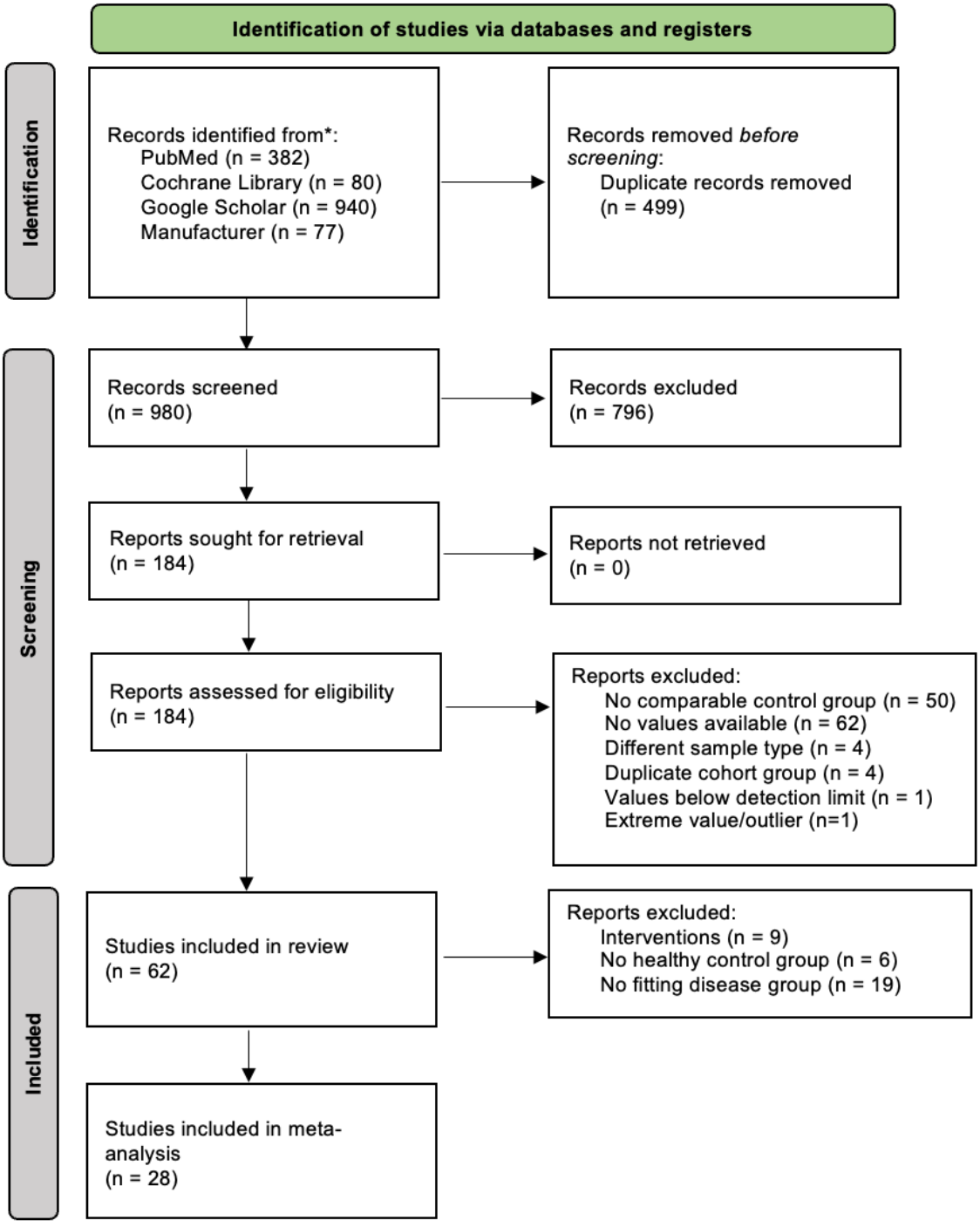

Figure 2: The flow diagram shows the study selection

Source: Page MJ, et al. BMJ 2021;372:n71. doi: 10.1136/bmj.n71. This work is licensed under CC BY 4.0. To view a copy of this license, visit https://creativecommons.org/licenses/by/4.0/

In total, 1479 studies were identified through the systematic search. These originated from the databases PubMed (n = 382), Cochrane Library (n = 80), Google Scholar (n = 940) and from manufacturer information (n = 77). After removing duplicates, 980 publications remained for screening. From these, 184 studies were sought for retrieval, which all of these were successfully obtained. Following eligibility and data extraction, 122 publications were excluded for the following reasons: no comparable control group (n = 50), no values available (n = 62), different sample type (n = 4), duplicate cohort groups (n = 4), values below the detection limit (n = 1), and extreme values/outliers (n = 1). The remaining 62 studies were included in the review. Additional exclusions were made due to intervention studies (n = 9), lack of a healthy control group (n = 6), and non-fitting disease groups (n = 19). Finally, 28 studies were included in the meta-analysis.

3.2 Case control studies

The 47 publications retrieved for the meta-analysis were categorized into four groups:

- Metabolic Disorders (n = 16)
- Gastrointestinal Disorders (n = 5)
- Liver Diseases (n = 7)
- Remaining studies as “others” (n = 19)

Each group was analysed separately using a random-effects model. Heterogeneity was evaluated through Cochran’s Q, I^2, τ^2, and the prediction interval. Because the biomarker was studied across different disease types, high heterogeneity was expected ($I^2 > 75\%$). When data allowed, subgroup analyses were conducted. The examined subgroups included sample type, disease type, BMI category, and age group. To explore sources of heterogeneity, meta-regression analyses were performed, considering factors such as study appraisal, sample type, disease type, BMI, gender, and age.

Table 1: Case control studies retrieved for the meta-analysis

all samples are serum unless otherwise indicated

[a]) stool sample

*) significant differences in zonulin values in the publication

#) values given in pg/ml

Study Year Country	**Disease**	**Control group**				**Patient group**			
		Zonulin [ng/ml]	Age [years]	BMI [kg/m^2]	Sex (m/f)	Zonulin [ng/ml]	Age [years]	BMI [kg/m^2]	Sex (m/f)
Moreno-Navarette 2012 Spain	Type 2 Diabetes Mellitus *	4.5 ± 9.10	48.29 ± 11.7	26.6 ± 3.2	82/ 0	10.9 ± 4.8	55.9 ± 10.3	28.6 ± 3.8	41/0
Pabijasz 2013 Poland	Crohn's Disease	7.84 ± 5.78	12.09	*NA*	18/ 14	18.96 ±14.2	13.4	*NA*	19/17
	Ulcerative Colitis					17.4 ± 16.05	12.4	*NA*	18/17
Zak-Golab 2013 Poland	Obesity *	5.4 ± 0.65	42.5 (32 – 52)	23.7 (21.8 – 24.8)	6/ 24	8.2 ± 1.4	53.5 (42 – 63)	35.4 (30.6 – 38.)	11/39
Malyszko 2014 Poland	Kidney transplant *	48.53 ± 6.44	*NA*	*NA*	*NA* n = 22	11.76 ± 3.25	45.5 ± 12.2	*NA*	48/24
Zhang 2014 China	Impaired Glucose Tolerance	5.54 ± 3.68	34 ± 11	26.4 ± 3.9	48/ 47	6.61 ± 3.9	39 ± 13	27.1 ± 3.1	50/42
	Type 2 Diabetes Mellitus *					7.99 ± 4.24	47 ± 12	26.3 ± 3.1	54/48
Zhang # 2015 China	Polycystic ovary syndrome *	4.57 ± 4.05	30 ± 5	25.3 ± 3	0/ 100	7.78 ± 5.26	29 ± 5	27.6 ± 4	0/100
Stadlbauer 2015 Austria	Metabolic Syndrome*	52.67 ± 7.17	*NA*	*NA*	NA n = 21	44.5 ± 8.26	55 ± 11	33.2 ± 4.5	18/10
		23.73 ± 10.35[a]			NA n = 25[a]	85.93 ± 45.62[a]			17/10[a]
Lukaszyk 2015 Poland	Absolute iron deficiency	34.42 ± 8.2	54.75 ± 16.9	27.19 ± 5.72	*NA* n = 49	34.85 ± 5.6	51.58 ± 19.4	27.7 ± 5	*NA* n = 12
	Functional iron deficiency					37.1 ± 6.7	72.5 ± 13	27.19 ± 5.72	*NA* n = 8
Vors 2015 France	Obesity	18.3 ± 0.45	29 ± 1	22.4 ± 0.5	8/ 0	14.8 ± 4.53	31 ± 2	31.8 ± 0.3	8 / 0
Barcelo 2016 Spain	Obstructive Sleep Apnea	4.5 ± 0.8	49 ± 12	29 ± 6	19/ 19	4.2 ± 0.8	50 ± 12	28 ± 6	21/17
Li 2016 China	Coronary Artery Disease*	4 ± 1.6	62.1 ± 13.2	23.5 ± 3.6	36/ 44	7.3 ± 1.8	63.5 ± 11.1	23.7 ± 3.5	65/61

Mishra 2016 India	Celiac Disease (first-degree relatives)	51.2 ± 26.12	26.8 ± 4.7	*NA*	164/ 34	47.5 ± 23	31.2 ± 12.1	*NA*	105 / 67
Jozefczuk 2017 Poland	Autism Spectrum Disorder	15.3 ± 5.9	8.5	*NA*	*NA* n = 46	17.2 ± 15.7	7.3	*NA*	*NA* n = 75
Mokkala 2017 Finland	Gestational diabetes	45.2 ± 9.7	30.1 ± 4.8	30.4 ± 4.4	0/ 64	53.4 ± 14.3	29.5 ±3.7	30.9 ± 3.2	0 / 16
Al-Obaide 2017 USA	Type 2 Diabetes Mellitus with Chronic Kidney Disorder*	14.2 ± 1.46	54.3 ± 3.2	28.17 ± 1.1	*NA* n = 20	22.59 ± 2.04	64.4 ± 2.3	33.2 ± 2.9	*NA* n = 20
Hasslacher 2018 Germany	Type 2 Diabetes Mellitus*	42.2 ± 9.5	64.6 ± 9.4	*NA*	9/ 10	58.8 ± 15.4	67.3 ± 6.1	*NA*	36 / 27
Kim 2018 USA	High Blood Pressure*	28.40 ± 8.75	*NA*	*NA*	*NA* n = 18	42.64 ± 12.52	*NA*	*NA*	*NA* n = 22
Schwiertz 2018 Germany	Parkinson's disease*[a]	50.7 ± 36.5	62.25 (54 – 75)	*NA*	11/ 17	108.1 ± 76.4	65.53 (44 – 78)	*NA*	22 / 14
Kim 2018 South Korea	Mild fatty liver*	0.618 ± 1.15	44.5 ± 9.5	27.8 ± 2.5	17/ 0	2.143 ± 4.27	44.8 ± 8.9	28.8 ± 2.8	89 / 0
	Moderate to severe fatty liver*					5.815 ± 3.11	44.7 ± 9.2	29.3 ± 2.6	34 / 0
Akao 2018 Japan	Liver cirrhosis*	40.26 ± 10.23	*NA*	*NA*	*NA* n = 27	11.99 ± 4.92	*NA*	*NA*	NA n = 9
	Chronic Hepatitis*					20.1 ± 7.09	*NA*	*NA*	NA n = 17
Carrera-Bastos 2018 Sweden	Centenarians*	5.2 ± 2.7	<40	*NA*	102/ 76	7.6 ± 3.1	≥100	*NA*	39 / 40
	Acute Myocardial Infarction*					4 ± 2.1	<40	*NA*	102 / 76
Leiva-Gea 2018 Spain	Type 1 Diabetes Mellitus*	3.21 ± 1.24	12.25 ± 2.92	17.35 ± 1.82	7/ 6	3.94 ± 1.44	12.56 ± 3.59	17.89 ± 2.01	7 / 8
	Maturity-onset diabetes of the young*					4.80 ± 1.41	13.06 ± 3.20	18.23 ± 1.90	7 / 8
Scheffler 2018 Germany	Impaired Glucose Tolerance	67.25 ± 25.45	45.4 ± 15.6	25.8 ± 5	67/ 124	71.88 ± 29.36	59.7 ± 29.4	29.7 ± 4.6	30 / 49
	Type 2 Diabetes Mellitus*					81.78 ± 25.31	62.9 ± 11.2	30.97 ± 5.53	46 / 60
Stevens 2018 USA	Anxiety and depression*	21.29 ± 4.33	*NA*	*NA*	6/ 17	36.26 ± 8.46	*NA*	*NA*	12 / 13
Genser 2018 France	Obesity*	21.18 ± 4.21	43 ± 2.2	22.5 ± 0.3	2/ 28	24.16 ± 5.39	*NA*	*NA*	*NA* n = 26
Dhillon 2019 Norway	Primary Sclerosing Cholangitis	35.1 ± 8.02	*NA*	*NA*	59/ 41	31.5 ± 11.11	41 (16.3	*NA*	132 / 34

							- 72.4)		
Mörkl 2019 Austria	Anorexia nervosa	57.95 ± 29.67	22.15 ± 3.86	22.15 ± 3.86	0 / 20	120.44 ± 306.24	22.44 ± 3.52	15.30 ± 3.78	0 / 18
Cinkajzlova 2019 Czech Republic	Short Bowel Syndrome	39.48 ± 3.79	53.3 ± 1.5	22.6 ± 0.7	6/ 4	47.24 ± 7.09	64.2 ± 4.3	20.0 ± 3.7	6 / 5
Caviglia 2019 Italy	Crohn's Disease	9 ± 3.2	50 (26 – 63)	*NA*	10/ 13	34.8 ± 15.6	49 (18 – 77)	*NA*	*NA* n = 86
	Ulcerative colitis					41.3 ± 16.1			*NA* n = 31
Kellerer 2019 Germany	Obesity	6.67 ± 0.99	*NA*	21.5 (19.5; 23.3)	*NA* n = 17	5.93 ± 1.12	*NA*	52.5 (47; 56.8)	*NA* n = 17
Aydin 2020 Turkey	Bipolar disorder	35.70 ± 9.69	35.56 ± 12.57	23.1 ± 2.49	14/ 15	38.09 ± 11.33	34.26 ± 8.84	25.77 ± 5.79	14 /16
Bawah 2020 Ghana	Pregnancy induced hypertension*	40.4 ± 8.6	27.98 ± 6.43	27.86 ± 4.19	0/ 60	56.81 ± 7.72	29.10 ± 5.32	28.96 ± 4.121	0 / 88
Blesl 2020 Austria	Secondary Sclerosing Cholangitis[a]	71.7 ± 20.6	58 ± 6.9	25.3 ± 3.1	13/ 5	209.1 ± 137.5	59 ± 12.7	27.2 ± 5.3	9 / 12
Olsson 2020 Denmark	Multiple Sclerosis	41.52 ± 1.45	37 (29 – 48)	22.8 (21.1 – 25.9)	30/ 28	40.26 ± 2.41	35 (30-43)	22.7 (20.9 – 25.5)	17 / 32
Sanchez-Alcoholado 2020 Spain	Colorectal cancer with obesity*	14.72 ± 9.57	61.42 ± 7.40	25.45 ± 3.23	10/ 10	26.57 ± 14.95	64.43 ± 7.31	35.82 ± 3.83	11 / 11
	Colorectal cancer without obesity					20.07 ± 15.23	62.52 ± 7.99	25.32 ± 3.67	12 / 11
Meyer-Mykelstad 2020 Norway	Human immuno-deficiency virus-infected immunological non-responders	33.2 ± 3.62	54.58 (50.7 - 59.2)	25.7 ± (24.5 – 27.4)	20/ 0	32.4 ± 5.68	49.6 (43.9 - 58.9)	25.5 (23.5 – 27.1)	19 / 0
	Human immuno-deficiency virus-infected immunological responders					31.7 ± 4.63	52.5 (48.2 - 59.3)	25.6 (23.2 – 27.2)	20 / 0
Hoshiko 2021 Netherlands	Metabolic Health	33.49 ± 5.27	*NA*	21.7 (0.8)	7/ 7	37.3 ± 7.62	*NA*	27.9 (1.7)	7 / 7
Hoshiko 2021 Netherlands	Metabolic Health*	37.8 ± 6.96	*NA*	22.1 ± 0.3	*NA* n = 40	48.8 ± 10.75	*NA*	29.7 ± 0.6	*NA* n = 40
Jung 2021 Austria	Alcohol related liver disease	2.25 ± 0.37	45 ± 2.3	22.7 ± 0.7	10/ 7	1.83 ± 0.65	49 ± 1.5	27.2 ± 1.09	26 /11

Karim 2021 Pakistan	Chronic obstructive pulmonary disease*	2.19 ± 0.31	67.5 ± 5.2	25.23 ± 4.2	76 / 0	2.691 ± 0.55	64.2 ± 7.3	23.18 ± 3.3	70 / 0
Hsu 2021 Taiwan	Liver cirrhosis*	1.8 ± 0.22	55.9 ± 8	NA	13 / 7	7.9 ± 0.76	53.2 ± 11	*NA*	11 / 4
Lingaiah 2021 Finland	Polycystic ovary syndrome	130.9 ± 14	46	27.9 ± 5.5	0 / 203	128 ± 17	46	27.9 ± 5.5	0 / 104
Seethaler 2021 Germany	Overweight	42.1 ± 10.4	31 (28 – 36)	21.7 (20 – 24)	9 / 22	38.2 ± 9.9	41.5 (29 – 54.8)	28.9 (25 – 32)	6 / 14
						202.4 ± 226.1[a]			
	Obesity	124.9 ± 63				53.9 ± 23.5	45 (38 – 49)	42.2 (41 – 47)	13 / 14
						231.2 ± 190.5[a]			
Baumann 2021 Austria	Metabolic dysfunction-associated steatotic liver disease (previously NAFLD)*	10.99 ± 4.92	46.2 ± 1.6	23.6 ± 0.7	5/10	13.78 ± 4.46	47.7 ± 2.2	31.6 ± 1	24 / 13
Aho 2021 Finland	Parkinson's disease[a]	52.86 ± 30.28	66.38 ± 6.73	26.7 ± 3.56	27 / 29	58.53 ± 42.73	67.63 ± 5.21	27.63 ± 4.74	29 / 26
Kleppe 2022 Norway	Anorexia nervosa*	49.2 ± 5.6	20.8 ± 5.1	23.2 ± 2.4	0/28	44.2 ± 7.4	21 ± 5.5	16.3 ± 1.9	0 / 25
Wang 2022 Sweden	Incident IBD	59.7 ± 10.7	55.9 ± 2.8	24.7 ± 3.3	0/47	61.2 ± 9.9	55.7 ± 2.9	24.7 ± 3.3	0/18
	Prevalent IBD	57.0 ± 9.7	54.6 ± 2.2	24.3 ± 3.2	0/18	63.2 ± 8.5	54.2 ± 2.3	24.3 ± 4.3	0/18

3.2.1 Metabolic disorders

For metabolic diseases (Fig.3), the meta-analysis included 22 effect sizes from 16 different studies published between 2012 and 2021. This group covered a wide range of metabolic diseases, including overweight (42), obesity (42-46), metabolic health (47, 48), metabolic syndrome (49), polycystic ovarian syndrome (PCOS) (50, 51), gestational diabetes (52), impaired glucose tolerance (IGT) (36, 53) and T2DM (36, 51, 54, 55) with chronic kidney disorder (56). Two studies provided both serum and stool data (42, 49), whereas all other studies only used serum. In terms

of study quality, 13 publications were rated as “good” and three as “medium” (43, 48, 56).

Body mass index (BMI) categories were within the overweight and obese range. In two analyses BMI was not reported (45, 55). Age groups also varied but were mainly adults.

The proportion of female participants ranged from 0% to 100%. Three publications included only women (50-52), whereas two included only men (43, 54). In 4 analyses, sex distribution was not reported (44, 45, 48, 56). The remaining studies included mixed sex populations with female proportions ranging from 36% to 80%.

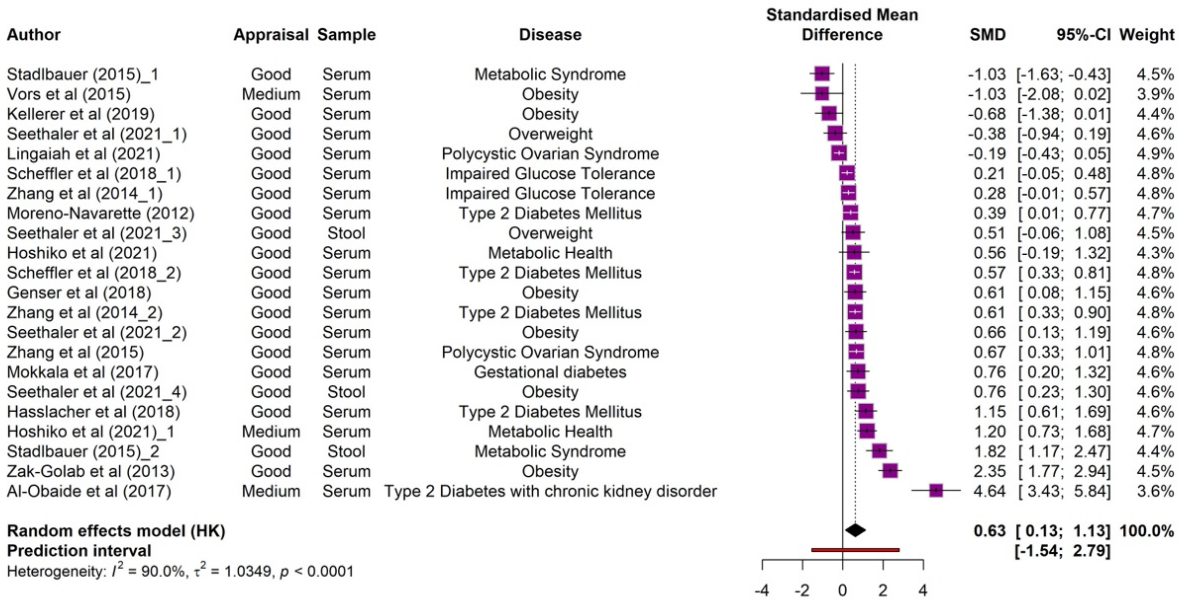

Figure 3: Forest plot of SMDs comparing patients with metabolic diseases and healthy controls. The pooled effect was calculated using a random effects model.

The overall SMD was 0.63, indicating significantly higher zonulin levels in the patient group. It showed a wide range from -1.03 to 4.64. Negative effects were observed in five analyses, of which only one reached statistical significance (49). The remaining four negative effects (42-44, 50) were not statistically significant. In contrast, 17 analyses demonstrated positive effect sizes, with 13 showing statistically significant increases. The strongest positive effect was seen in Al-Obaide et al., 2017 (56).

However, the heterogeneity across the studies is very high (τ^2 = 1.0349) with an I^2 value of 90%. Each study contributed approximately 3.6% to 4.9% of the total weight, indicating an even distribution of influence. The prediction interval ranged from -1.5393 to 2.7947.

For a more detailed interpretation, disease-specific analyses among the patient groups were performed. Obesity was the biggest subgroup (n = 6). However, no significant difference in zonulin levels was observed with high heterogeneity (SMD = 0.5013; 95% CI: -0.7403 - 1.7429, I^2 = 91.2%). In contrast, patients with type 2 diabetes mellitus (n = 4) showed significantly elevated zonulin levels with moderate heterogeneity (SMD = 0.1612; 1.1889, I^2 = 42.4%). Other disease groups included too few studies (n ≤ 2) allowing meaningful analysis. Categorizing studies by BMI, the overweight group (n=11) was the largest and showed a significant increase in zonulin levels. However, the heterogeneity was still high (SMD = 0.4199; 95% CI: 0.1244 - 0.7132; I^2 = 79.6%).
Dividing the studies by BMI groups, overweight patients formed the largest subgroup (n = 11). In this group, zonulin levels were still significantly higher compared to controls (SMD = 0.4199; 95% CI: 0.1244 - 0.7132), although heterogeneity remained high (I^2 = 79.6%). The other BMI groups were smaller and not statistically significant.
When the data were split by age or by sample type, no significant differences were found.
In the regional analysis, only studies from Europe (n = 18) remained significant (SMD = 0.4663; 95% CI: 0.0422 - 0.9105; I^2 = 89.2%). Studies from other continents were not significant.
Age, sex or BMI could not explain the high heterogeneity (R^2 = 0%). Most of the heterogeneity was explained by differences in disease type (R^2 = 22.69%), followed by the quality of the study (R^2 = 4.89%).

3.2.2 Gastrointestinal diseases

The analysis of gastrointestinal diseases included nine study arms with data from five publications between 2013 and 2022 (Fig. 4). Included diseases were IBD (57),

colorectal cancer with and without obesity (58), ulcerative colitis (59, 60), Crohn's disease (59, 60), and short bowel syndrome (61). All studies used serum samples. Patient populations differed in terms of BMI and age, ranging from normal weight to obese class II and from 12 to 64 years. The appraisal of the included publications was rated as "good" in four publications (33, 58, 60, 61), while one study received a medium rating (59).

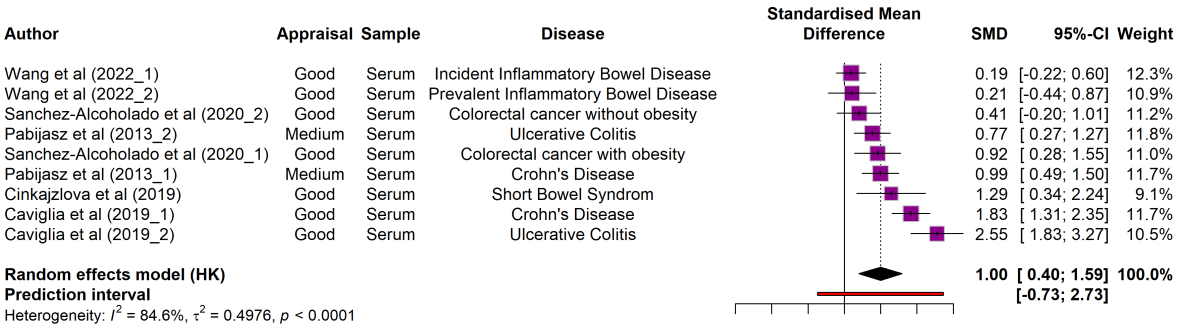

Figure 4: Forest plot of SMDs comparing patients with gastrointestinal diseases and healthy controls. The pooled effect was calculated using a random effects model.

BMI was only reported in three studies, ranging from normal weight to obese. Participants were classified as normal weight in 2 studies (57, 61), overweight and obese in one study (58). Only one study consisted out of a juvenile cohort (59), all others investigated adults. Sex distribution varied across studies. One study only included women (57). In another study, sex distribution was not reported (60). Across the remaining studies, the proportion of female participants was balanced, ranging from 47% to 50%.

The analysis showed a significant effect with an overall SMD of 1.00 (0.40; 1.59). The SMDs were all positive, ranging from 0.19 to 2.55. The largest effects were found in the studies by Caviglia et al (60). The smallest effect was observed in Wang et al., which was not statistically significant (57). At the same time the analysis showed very high heterogeneity between the studies (I^2 = 90% (84.6%; 91.4%), τ^2 = 0.50 (0.17; 2.12)) and a wide prediction interval, with a range from -0.73 to 2.73. The weights of the individual studies were evenly distributed (9.1-12.3%), so no single study dominated the results.

No subgroup analysis was performed due to the small group sizes. Meta-regression showed sex to be the factor that contributed to the high heterogeneity (R^2 = 100%). However, one study did not report the sex of the participants, and another study only included women, which may explain the differences.

3.2.3 Liver diseases

The meta-analysis on zonulin levels in liver diseases included nine effect sizes from seven publications published between 2018 and 2021 (Fig.5). The analysis covered a wide range of liver diseases, including liver cirrhosis (62, 63), chronic hepatitis (62) , alcohol-related liver disease (64), primary sclerosing cholangitis (65), moderate to severe fatty liver (66), mild fatty liver (66) , metabolic dysfunction–associated steatotic liver disease (67) and secondary sclerosing cholangitis in critically ill patients (68). Most studies used serum samples, while one used stool samples (68). The study quality was mainly rated as “good”, with only one publication rated as “medium” (67).

Across the included studies, participants were overweight or obese.

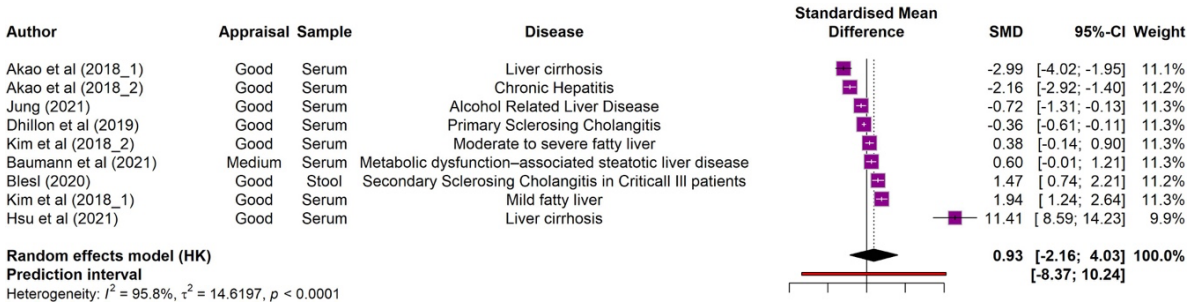

Figure 5: Forest plot of SMDs comparing patients with liver diseases and healthy controls. The pooled effect was calculated using a random effects model.

The random-effects model showed an overall SMD of 0.93 (-2.16; 10.24), indicating no significant overall effect. The SMDs varied widely, ranging from –2.99 to +11.41, indicating large variability in zonulin levels. Four analyses showed negative effect sizes, showing lower zonulin levels in patients compared to controls. In contrast, five analyses reported positive effect sizes, of which three were statistically

significant. The strongest positive effect was observed in Hsu et al., representing an outlier with a much larger effect than all other studies (63).
The between-study heterogeneity was extremely high (I^2 = 95.8%, τ^2 = 14.6197).
The prediction interval was very broad (–8.37 to +10.24).
The weighting of the studies was balanced, ranging from 9.9% to 11.3%.

No subgroup analysis were conducted because the groups were too small. In the meta-regression, age, gender, and BMI were included as continuous variables, while non applicable values were excluded. None of these variables accounted for the observed heterogeneity (R^2 = 0%), most likely due to the small overall sample size and the exclusion of studies with missing data.

3.2.4 Remaining studies ("others")

The group of remaining studies (Fig.6) covered 23 study arms from 19 studies published between 2014 and 2022. This group included a wide range of diseases, such as kidney transplant (69), anorexia nervosa (70, 71), multiple sclerosis (72), acute myocardial infarction (73), obstructive sleep apnoe (74), HIV infection (75), celiac disease (76), bipolar disorder (77), chronic kidney disease (with or without iron deficiency) (78), autism spectrum disorder (79), Parkinson's disease (80, 81), centenarians (73), chronic obstructive pulmonary disease (82), type 1 diabetes (83), maturity-onset diabetes of the young (83), coronary artery disease (84), pregnancy-induced hypertension (85), anxiety and depression disorder (86), and high blood pressure (87).
Except for two publications, which used stool samples (80, 81), all analyses were based on serum samples. The quality was mainly rated as good. Six studies were rated as "medium" (69, 75, 76, 78, 86, 87).
The heterogeneity between the studies is very high with an I^2 value of 96.0%.
No meta-analysis was conducted for this group because the studies were too heterogenous.

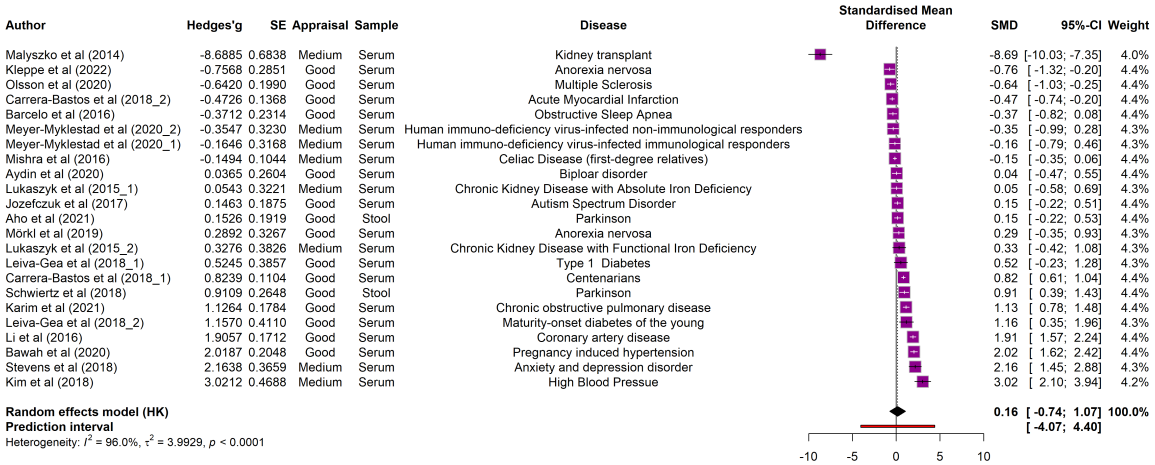

Figure 6: Forest plot of SMDs comparing patients with diseases that did not fit in any cluster and healthy controls. The pooled effect was calculated using a random effects model.

3.3 Comparison between different diseases

Table 2: Table showing the comparison of zonulin levels between different diseases.

Study Year Country	Samp-le type	Patient group 1					Patient group 2				
		Disease	Zo-nulin [ng/ml]	Age [years]	BMI [kg/m²]	Sex (m/f)	Disease	Zo-nulin [ng/ml]	Age [years]	BMI [kg/m²]	Sex (m/f)
Hunt 2014 USA	Serum	Human Immuno-deficien-cy Virus - Alive	5,48 ± 2,43	44	*NA*	NA n = 122	Human Immuno-defi ciency Virus - De-ceased	3,99 ± 2,69	47	*NA*	NA n = 61
Chwist 2014 Poland	Serum	Mod-erate-to-severe fibrosis	6.93 ± 1.76	54.2 ± 10.6	32.3 ± 3.89	NA n = 16	None-to-minimal fibrosis	6.69 ± 1.95	43.1 ± 13.07	30.7 ± 4.65	NA n = 54
Dschietzig 2016 Germany	Serum	Auto-matic implant-table cardio-verter defibril-lator	10,61 ± 2,9	67 ± 12	NA	NA n = 231	Con-gestive heart failure	129 ± 17,77	71 ± 12	NA	NA n = 129
Rivas 2019 USA	Serum	Kawa-saki Disease	27,4 ± 14,3	4 months	*NA*	23/11	Febrile controls	18,02 ± 10,31	1 month - 9 years	*NA*	9/16

				- 15 years							
Horvath 2019 Austria	Stool	Patients with liver cirrhosis with Proton Pump Inhibitor intake	70.8 ± 21.9	57.3 ± 9.1	*NA*	38 / 10	Patients with liver cirrhosis without Proton Pump Inhibitor intake	86.5 ± 38.4	56.9 ± 8.1	*NA*	24 / 15
Caviglia 2020 Italy	Serum	Inflam-matory Bowel Disease	44,4 ± 17.3	48	*NA*	62 /39	Irritable Bowel Syn-drome	42,4 ± 9,3	43	*NA*	6/13
Wang 2022 Sweden	Serum	Incident Inflam-matory Bowel Disease	61,2 ± 9,9	55,7 ± 2,9	24,7 ± 3,3	0/48	Preva-lent Inflam-matory Bowel Disease	63,2 ± 8,5	54,2 ± 2,3	24,3 ± 4,3	0/18

A total of seven studies published between 2014 and 2022 were included, covering a broad range of diseases: HIV infection (88), liver fibrosis and cirrhosis (89), heart failure (90), Kawasaki disease (91), IBD and irritable bowel syndrome (IBS) (57, 92). Most studies measured serum zonulin, while one used stool samples. The reported mean zonulin concentrations varied widely from 3.99 ng/ml to 129 ng/ml.

In the study by Hunt et al. (2014), serum zonulin levels were higher in HIV survivors (5.48 ± 2.43 ng/ml) than in deceased patients (3.99 ± 2.69 ng/ml), but statistical significance was not reported (88).

Chwist et al. (2014) examined serum zonulin in patients with liver fibrosis (89). Those with moderate to severe fibrosis had mean levels of 6.93 ± 1.76 ng/ml, compared to 6.69 ± 1.95 ng/ml in patients with none or minimal fibrosis, indicating only minor differences.

Horvath et al. (2019) analysed stool zonulin in patients with liver cirrhosis, showing higher concentrations in patients without proton pump inhibitor (PPI) intake (93).

Dschietzig et al. (2016) reported serum zonulin levels of 10.61 ± 2.9 ng/ml in patients with an implantable cardioverter-defibrillator (ICD), and a much higher mean of 129 ± 17.77 in patients with congestive heart failure (90).
Rivas et al. (2019) found elevated serum zonulin in children with Kawasaki disease (27.4 ± 14.3 ng/ml) compared to febrile controls (18.0 ± 10.3 ng/ml) (91).

Caviglia et al. (2020) observed similar serum zonulin concentrations in IBD (44.4 ± 17.3 ng/ml) and IBS (42.4 ± 9.3 ng/ml) patients, with only a small difference between both groups (92).
In contrast, Wang et al. (2022) reported higher overall serum zonulin levels in both incident IBD (diagnosed during follow-up) with 61.2 ± 9.9 ng/ml and prevalent IBD (diagnosed before baseline) with 63.2 ± 8.5 ng/ml, with minimal differences between disease stages (57).

3.4 Intervention studies

Table 3: Overview of intervention studies investigating the effects of various interventions on zonulin levels.

Study Year Country	**Sample type**	**Disease**	**Duration**	**Intervention**	**Characteristics**			**Baseline**	**End of treatment**
					Age [years]	BMI [kg/m²]	Sex (m/f)	Zonulin [ng/ml]	
Lamprecht 2012 Austria	Stool	Athletes	14 weeks	Probiotic	37.6 ± 4.7	23.7 ± 2.2	11 / 0	31.9 ± 14.9	**23.6 ± 9.9**
				Placebo	38.2 ± 4.4	23.9 ± 3.1	12 / 0	31.5 ± 13.1	**33.2 ± 11.8**
Lamprecht 2015 Austria	Stool	Athletes	12 weeks	Zeolite	33.6 ± 5.3	22.7 ± 2.2	22 / 5	**61.2 ± 11**	**43.8 ± 14.3**
				Placebo	37.2 ± 4.9	23.2 ± 2.1	21 / 4	56.1 ± 13.2	59.6 ± 12.3
Stadlbauer 2015 Austria	Stool	Metabolic Syndrome	12 weeks	Probiotic	51 ± 11	35 ± 5	9 / 4	85.93 ± 45.62	78.15 ± 44.95
								42.77 ± 8.19	51.23 ± 13.7

	Serum			Placebo	55 ± 9	32 ± 4	9 / 6	87 ± 51.85	68.67 ± 26.42
								46 ± 8.59	46 ± 14.4
Stenman 2016 Finland	Serum	Obesity	6 months	Dietary fiber + Probiotic	*NA*	*NA*	*NA* n = 35-36	64.6 ± 14.2	63.4 ± 14.2
				Probiotic	*NA*	*NA*	*NA* n = 25	58.4 ± 11.4	57.1 ± 8.3
				Dietary fiber	*NA*	*NA*	*NA* n = 35 - 36	55.5 ± 9.1	58.4 ± 12
				Placebo	*NA*	*NA*	*NA* n = 37	56.5 ± 12.6	59.7 ± 10.9
Horvath 2019 Austria	Stool	Diabesity	6 months	Synbiotic	61 (56; 65)	33 (31:34)	11 / 1	88.2 ± 43.8	102.3 ± 91.7
								2.4 ± 0.6	2.4 ± 0.7
	Serum			Placebo	59 (54;63)	34 (32; 36)	8 / 6	103.4 ± 65.4	86.1 ± 45.1
								2.1 ± 0.7	2.3 ± 0.5
Bloomer 2020 USA	Serum	Health	8 weeks	Ambrotose (A) 4g	26.9 ± 5.4	24.9 ± 4.5	NA n = 15	39.4 ± 7	37.6 ± 9.4
				Ambrotose (A) 2g	26.3 ± 5.7	24.2 ± 4.1	NA n = 15	37 ± 6.6	35.3 ± 7.4
				Ambrotose (L) 4g	30.2 ± 11.4	27.1 ± 4.8	NA n = 15	34.9 ± 6	38.2 ± 6.3
				Ambrotose (L) 2g	28.1 ± 7.4	24.4 ± 4	NA n = 15	34.1 ± 7.7	36 ± 6.5
				Placebo	29.5 ± 11.6	26.4 ± 4.3	NA n = 15	37.8 ± 7.8	38.3 ± 7.9
Horvath 2020 Austria	Stool	Liver cirrhosis	6 months	Probiotic	60 (54; 64)	27.4 ± 3.7	32 / 12	87.6 ± 37.1	91.6 ± 75
								38.5 ± 18	37.7 ± 18.9
	Serum			Placebo	56 (50;63)	27.1 ± 4.6	26 / 10	72.7 ± 30.3	74.2 ± 23.8
								46.6 ± 24.7	45.5 ± 20.8
Reining-haus 2020	Serum	Depres-sion	28 days	Probiotic	43 (14.31)	26.29 (5.78)	8 / 20	48.8 ± 15.96	50.16 ± 15.91

Austria				Placebo	40.11 (11.45)	25.74 (7.29)	6 / 27	52.01 ± 10.91	52.24 ± 13.83
Chai-yasut 2021 Thailand	Serum	Obesity	12 weeks	Synbiotic	54.78 ± 1.92	28.97 ± 0.77	*NA* n = 36	**1.37 ± 0.17**	**0.98 ± 0.18**
				Placebo	59.94 ± 1.32	30.01 ± 0.47	*NA* n = 36	1.42 ± 0.16	1.41 ± 0.16
Ney-rinck 2021 Belgium	Stool	Obesity	3 months	Prebiotic	*NA*	*NA*	*NA* n = 11	244 ± 234.1	224.8 ± 331.8
				Placebo	*NA*	*NA*	*NA* n = 9	228.7 ± 161.2	239.9 ± 215.8

A total of ten intervention studies between 2012 and 2021 were included, investigating the effect of various interventions on zonulin levels, measured either in serum or stool.

The studies covered a wide range of populations, including healthy subjects (94-96), individuals with metabolic syndrome (49), obesity (97-99), diabesity (100), liver cirrhosis (101), or depression (102). Study durations ranged from 28 days to 6 months. Interventions included probiotics, synbiotics, prebiotics, zeolite, Ambrotose, or dietary fiber.

Across the included studies, three publications demonstrated significant reductions in zonulin levels following intervention. Lamprecht et al. (2012) reported a decrease in faecal zonulin concentrations in athletes after 14 weeks of probiotic supplementation (31.9 ± 14.9 vs 23.6 ± 9.9 ng/ml), while the placebo group showed no improvement (94). In another study, Lamprecht et al. (2015) documented a significant decline in faecal zonulin levels after 12 weeks of zeolite supplementation (61.2 ± 11 vs 43.8 ± 14.3 ng/ml), whereas values in the placebo group slightly increased (56.1 ± 13.2 vs 59.6 ± 12.3 ng/ml) (95). Chaiyasut et al. (2021) observed a reduction in serum zonulin in obese individuals treated with synbiotics for 12 weeks (1.37 ± 0.17 vs 0.98 ± 0.18 ng/ml), with stable levels in the placebo group (98).

In contrast, most studies reported no effects of the tested interventions. Trials in patients with metabolic syndrome (49), obesity (97-99), diabesity (100), liver cirrhosis (101), and depression (102), as well as a trial testing Ambrotose in

healthy adults (96), did not demonstrate clear reductions in zonulin levels compared with placebo.

4 Discussion

Zonulin is widely used as a marker of intestinal permeability, although it is still unclear which proteins are actually detected by many commercial ELISA assays. This needs to be considered when interpreting published studies. This review therefore investigated zonulin as a potential biomarker in clinical practice by comparing its measured levels in patients with different diseases with those in healthy controls. Furthermore, it examined changes in zonulin levels in response to various therapeutic or lifestyle interventions.

Overall, the findings suggest that zonulin levels are more often elevated in metabolic and gastrointestinal disorders than in other disease groups. In particular, the case-control studies showed significant differences between patients with metabolic disorders and healthy controls. This fits well with current knowledge, as obesity and diabetes may lead to chronic inflammation and changes in the gut microbiota, which can affect the intestinal barrier and may increase zonulin levels (17-19).

A similar pattern can be seen in gastrointestinal diseases. Patients with gastrointestinal disorders also showed significantly higher zonulin levels compared to healthy controls. Diseases such as inflammatory bowel disease (IBD) are associated with a disrupted intestinal barrier (2, 18). This leads to increased intestinal permeability, which may explain the elevated zonulin concentrations observed in these patients.

In contrast, results for liver diseases were inconsistent, showing both decreased and increased zonulin concentrations across the studies. One possible explanation is that recent evidence suggests that most of the circulating zonulin is synthesized in the liver (103). It was observed that reduced serum zonulin levels in liver cirrhosis are likely a consequence of impaired liver synthesis rather than increased intestinal permeability, limiting its reliability as a marker of gut permeability in serum sample types. In contrast, faecal zonulin measurements are not affected

by hepatic synthesis and may be a better marker of intestinal permeability in patients with cirrhosis (103, 104).
This also highlights the importance of sample type. Faecal zonulin values were generally higher, whereas serum zonulin tended to be lower. This finding suggests that zonulin concentrations differ between serum and stool. Faecal zonulin has been linked to intestinal permeability, as zonulin released from the gut may enter the intestinal lumen. In contrast, serum zonulin has been suggested to reflect systemic processes, as it may originate from multiple tissues (105, 106).
Taken together, these differences between disease groups suggest that zonulin does not behave in the same way across all conditions. This may also explain why no clear pattern was found in the remaining disease group, which indicates that zonulin is not a general marker.

When diseases were compared directly, zonulin concentrations also varied between disease types and stages. Among all diseases analysed, HIV infection showed the lowest mean serum zonulin concentrations, even though the disease is characterized by strong immune activation. The reason for these low levels is currently unclear, as the specific role of zonulin in HIV infection has not been fully understood (107).
When comparing early versus advanced disease, for example incident versus prevalent IBD, zonulin values were similar. However, only the prevalent IBD group showed significantly higher zonulin levels compared with its controls, whereas no significant difference was observed for incident IBD. Accordingly, Wang et al. suggested that increased intestinal permeability is more likely a result of chronic intestinal inflammation than its cause (57).

Intervention studies showed also variable results. Most of these interventions aim to modulate the gut microbiota, strengthen intestinal barrier function and reduce inflammation. Probiotics may stabilize TJs between epithelial cells by competing with pathogenic microbes, stimulating mucus secretion, and increasing SCFAs and therefore reducing intestinal permeability (108-110). Prebiotics promote the growth of beneficial gut bacteria. Synbiotics, which combine pro- and prebiotics, may improve these effects (108). Some studies investigating these interventions

showed significant effects on zonulin levels. Lamprecht et al. (2012) reported a significant reduction in faecal zonulin following probiotic supplementation in athletes (94). Similarly, Chaiyasut (2021) et al. observed a significant decrease in serum zonulin levels after synbiotic supplementation in individuals with obesity (98). These findings suggest that microbiota targeting interventions may improve intestinal barrier integrity and reduce permeability.

Zeolite supplementation has also been investigated. Zeolites are mineral supplements that can bind and remove harmful substances like toxins and free radicals, which may help lower zonulin levels. One study using zeolite supplementation (95) demonstrated clear reductions in faecal zonulin among athletes, suggesting a stabilizing effect on the gut barrier. However, it's still unclear how zeolites affect inflammation and gut barrier health (95).

Despite these positive findings, most intervention studies did not show clear changes in zonulin concentrations. This variability is also reflected in recent meta-analyses. Several systematic reviews and meta-analyses have evaluated the impact of these supplements on intestinal permeability. One review reported that probiotic and synbiotic interventions significantly reduced serum zonulin levels, although study heterogeneity was high (111).

A more recent meta-analysis, which evaluated the effect of prebiotics, probiotics and synbiotics on intestinal health, also observed a significant decrease in zonulin following supplementation. Subgroup analyses showed that probiotics alone are most effective in reducing zonulin. However, the review notes that results vary between different populations and study (108). A similar pattern was observed in the present review, where some studies reported reductions in zonulin concentrations following interventions, which may reflect potential improvement in gut barrier function. On the other hand, most studies reported no, minimal or even paradoxical effects of interventions on zonulin.

It seems that zonulin reduction are more likely in healthy or mildly metabolically affected individuals, whereas effects in chronic diseases are less consistent. This may be explained by the fact that healthy participants show a more resilient microbiota and an intact barrier, while chronically ill patients show persistent

dysbiosis and inflammatory activation (112). As a result, some interventions showed positive effects in healthy subjects but limited or absent effects in chronic diseases. Individual intervention studies support these findings. In healthy athletes, a 14-week multispecies probiotic supplementation reduced faecal zonulin, while placebo remained unchanged (94). Similarly, a 12-week zeolite supplementation decreased faecal zonulin significantly, whereas the placebo group experienced a slight increase (95). In obese participants, a 12-week synbiotic intervention reduced serum zonulin (98). Together, these results suggest that nutrition-based interventions such as probiotics, synbiotics or zeolite can reduce intestinal permeability in relatively healthy or prediabetic populations. However, the intestinal barrier is a dynamic system and biomarker levels may vary over time. Evidence from studies on LBP suggests, that single measurements may not adequately reflect intestinal permeability and repeated measurements may be necessary for reliable results (113).

Regarding the interpretation of these results, one limitation should be considered. Heterogeneity was very high (90%-96%), which is expected when analysing a biomarker across different diseases rather than a single, specific condition or treatment. In addition, the included studies covered a long time period (2012 – 2022), and differences in assay lots and antibody performance may have further increased heterogeneity. The wide prediction ranges show that future results are quite uncertain. This large variation possibly comes from several factors, such as the complex regulation of zonulin, differences in disease mechanisms, and variations in study design, participant groups, and analysis methods. A few studies with very strong results (63, 69) also had a big influence, so the results should be interpreted carefully.
Subgroup analyses confirmed that disease type explained most of the variance, while factors such as BMI, age, or sample type were not significant moderators.

Considering all these points, zonulin findings should be interpreted carefully. Elevated zonulin-like proteins in metabolic and gastrointestinal disorders may show an impaired gut barrier. In liver disease, low zonulin levels might indicate reduced hepatic protein synthesis, and stool might be the preferable sample

material. However, more studies comparing serum and stool samples in liver diseases need to be carried out. The clinical relevance of zonulin detected by the antibody raised against the octapeptide GGVLVQPG cannot be fully exploited until the target protein is identified.

5 Conclusion and outlook

Zonulin is commonly used as biomarker for intestinal permeability. However, different antibodies are produced by different vendors. One antibody (Immundiagnostik, Bensheim, Germany) was raised against the octapeptide GGVLVQPG, but its actual target is still unknown. Here the clinical effect of the antibody was investigated to assess the relevance of the unknown protein. This meta-analysis shows that zonulin values measured by the antibody raised against the octapeptide are significantly increased in metabolic and gastrointestinal disorders and may be influenced by interventions, supporting the potential clinical relevance of this marker. However, high heterogeneity should be considered when interpreting the results. At the same time, these findings highlight the need to identify the actual target protein.

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Notice regarding the use of artificial intelligence:

In this thesis, ChatGPT (versions GPT-4, GPT-4o, GPT-5, GPT-5.3), provided by OpenAI, was used solely to optimise the language (grammar, spelling and wording suggestions) between March 2024 and June 2026; available at https://chat.openai.com. All text in this thesis was written on my own.