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Recognizing the leaky gut as a trans-diagnostic target for neuro-immune disorders using clinical chemistry and molecular immunology assays

* Denitsa Simeonova ^a, Mariya Ivanovska ^b, Mariana Murdjeva ^b, Andre F. Carvalho ^{c,d}, Michael Maes ^{a,e}

^a Department of Psychiatry and Medical Psychology, Medical Faculty, Medical University of Plovdiv, Plovdiv, Bulgaria

^b Department of Microbiology and Immunology, Faculty of Pharmacy, Medical University of Plovdiv, Plovdiv, Bulgaria

^c Department of Psychiatry, University of Toronto, Toronto, ON, Canada

^d Centre for Addiction and Mental Health (CAMH), Toronto, ON, Canada

^e Department of Psychiatry, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand

Corresponding author:

Dr. Denitsa Simeonova

Abstract

Background: Increased intestinal permeability with heightened translocation of Gram-negative bacteria, also known as “leaky gut”, is associated with the pathophysiology of neuroimmune disorders, such as major depressive disorder (MDD), chronic fatigue syndrome (CSF) and (deficit) schizophrenia, as well as with general medical disorders, including irritable bowel syndrome. This review aims to summarize clinical biochemistry and molecular immunology tests that may aid in the recognition of leaky gut in clinical practice.

Methods: We searched online libraries, including PubMed/MEDLINE, Google Scholar and Scopus, with the key words “diagnosis” or “biomarkers” and “leaky gut”, “bacterial translocation”, and “intestinal permeability” and focused on papers describing tests that may aid in the clinical recognition of leaky gut.

Results: To evaluate tight junction barrier integrity, serum IgG/IgA/IgM responses to occludin and zonulin and IgA responses to actomyosin should be evaluated. The presence of cytotoxic bacterial products in serum can be evaluated using IgA/IgM responses to sonicated samples of common Gram-negative gut commensal bacteria and assays of serum lipopolysaccharides (LPSs) and other bacterial toxins, including cytolethal distending toxin, subunit B. Major factors associated with increased gut permeability, including gut dysbiosis and yeast overgrowth, use of NSAIDs and alcohol, food hypersensitivities (IgE-mediated), food intolerances (IgG-mediated), small bacterial overgrowth (SIBO), systemic inflammation, psychosocial stressors, some infections (e.g., HIV) and dietary patterns, should be assessed. Stool samples can be used to assay gut dysbiosis, gut

inflammation and decreased mucosal defenses using assays of fecal growth of bacteria, yeast and fungi and stool assays of calprotectin, secretory IgA, β -defensin, α -antitrypsin, lysozyme and lactoferrin. Blood and breath tests should be used to exclude common causes of increased gut permeability, namely, food hypersensitivities and intolerances, SIBO, lactose intolerance and fructose malabsorption.

Discussion: Here, we propose strategies to recognize “leaky gut” in a clinical setting using the most adequate clinical chemistry and molecular immunology assays.

Key words: depression, chronic fatigue syndrome, inflammation, immune, gut, bacteria

1. Introduction

The gastrointestinal system, and specifically the gut barrier of the gastrointestinal tract, is one of the largest immune system organs, serving as not only a tool for maintaining the digestion and absorption of nutrients but also as a complex mechanism protecting organisms from the external environment [1]. The mucosal barrier contacts a vast number of external agents and must both maintain nutrient absorption and stop potentially toxic noxa, such as food antigens, commensal bacteria, toxins and pathogens, from translocating into peripheral blood. Disturbance of the intestinal barrier function may increase its permeability, potentially leading to the translocation of external pathogens, including Gram-negative bacteria, unprocessed food, particles and toxins, which enter the host organism and may facilitate local and systemic inflammation. Hence, the gut wall barrier functions as a gatekeeper.

Increased intestinal permeability with heightened translocation of Gram-negative bacteria, also known as “leaky gut”, may be involved in the pathophysiology of many neuroimmune diseases, such as major depressive disorder (MDD), chronic fatigue syndrome (CSF), deficit schizophrenia, and multiple sclerosis [2–5]. Increased translocation of Gram-negative bacteria from the gut to the blood is an important factor linking gut microbiota to peripheral immune-inflammatory responses, which may eventually contribute to neuroinflammation and hence to a myriad of neuropsychiatric disorders.

The human intestinal microbiota comprises 100 trillion organisms [6], and its composition may influence the permeability and integrity of the intestinal barrier and thus also influence bacterial translocation [7]. Disturbance of the gut barrier allows commensal Gram-

negative bacteria to potentially translocate into the lamina propria and mesenteric lymph nodes (MLNs) [8] [9]. Translocation of these Gram-negative bacteria may activate the CD14-Toll-like receptor-4 (TLR4) complex, which recognizes bacterial lipopolysaccharides (LPSs) [10]. Binding of bacterial LPSs to the TLR4 complex in MLNs activates the production of inflammatory cytokines by the latter. Moreover, in people with deregulated immune defenses, these Gram-negative bacteria may migrate to the periphery and thus drive systemic immune-inflammatory responses. The pathways mentioned above may explain why a pathological increase in gut permeability appears to be involved in the pathophysiology of several inflammation-related conditions, such as mood disorders, deficit schizophrenia, rheumatoid arthritis, multiple sclerosis, Alzheimer's disease (AD), and diabetes [11–15]. Moreover, the effects of common environmental factors, including poor diet, alcohol intake and chronic nonsteroidal anti-inflammatory drug (NSAID) use, may disturb immune-inflammatory pathways that are at least partly mediated by increased gut permeability [16][17].

Clinical chemistry and molecular immunology tests have been developed by different laboratories to screen for the presence of leaky gut and its predisposing, precipitating and maintaining factors. Nevertheless, this accumulating evidence has not been reviewed as an application for the clinical recognition of leaky gut in the context of neuropsychiatric disorders. Thus, this paper aims to review the literature on the clinical chemistry and molecular immunology assays for assessing leaky gut and related conditions.

2. Methods of the review

We searched the Pubmed/MEDLINE, Google Scholar and Scopus online databases using combinations of the key words “diagnosis” or “causes” and “leaky gut”, “bacterial translocation”, “intestinal permeability”, “irritable bowel syndrome”, and “dysbiosis” from 1990 to July 2018, focusing on papers that described potential clinical biochemistry or molecular immunology tests that may aid in the recognition of leaky gut. Before describing the findings (section 5), we provide a concise summary of leaky gut (section 3) and describe common predisposing, precipitating and maintaining factors of leaky gut (section 4).

3. Pathophysiology of leaky gut

3.1. Intestinal barrier

The intestinal barrier consists of a single layer of epithelial cells and two layers of mucus and covers a surface of 400 m², thereby serving as a protective physical barrier isolating the host organism from the external environment [1]. Several subtypes of epithelial cells are distinguishable, including enterocytes, goblet cells, Paneth cells, microfold cells (M cells), and enteroendocrine cells [18]. The two mucosal layers, namely, the inner and outer layers, cover the entire surface of the epithelium and protect it from external microorganisms. The mucus consists mostly of MUC2 and other compounds, such as sIgA, enzymes and proteins, including lactoferrin [19]. This population of commensal gut bacteria has been described as part of the intestinal barrier, mostly because these bacteria can influence gut permeability, are involved in the absorption and digestion of nutrients, and regulate gut colonization by pathogenic bacterial strains [20].

3.2. Paracellular transport

Intestinal epithelial cells, which comprise the intestinal barrier, are continuously linked to tight junctions [21]. The latter regulates hydroelectrolytic flow and the paracellular translocation of small molecules and leukocytes bidirectionally from the lumen into the blood stream and vice-versa. Tight junctions regulate paracellular transport, and many factors can influence their permeability [16, 22]. Given the vast amount of clinical disorders that are associated with gut leakiness, the pathophysiology of tight junctions has become a focus of increased clinical research. The complex network of gut-associated lymphoid tissue and the intestinal epithelial barrier with its intercellular tight junctions control the balance between tolerance and immunity to nonself antigens. Zonulin is known to be an important mediator that increases gut leakiness [23, 24] and is thus a relevant biomarker of intestinal hyperpermeability [25–27]. Gliadin, a glycoprotein present in wheat, is another factor that may contribute to the occurrence of leaky gut by altering zonulin signaling and thereby increasing the intestinal permeability to macromolecules and Gram-negative bacteria [28, 29]. Moreover, increased permeability of the gut tight junction barrier may be promoted or aggravated by oxidative processes and by increased interleukin-1 β (IL-1 β), IL-6 and tumor necrosis factor- α (TNF- α) signaling [3, 30–32]. NF- κ B partly mediates IL-1 β [33] and TNF- α -induced increases in intestinal epithelial tight junction permeability. For example, TNF- α signaling exerts its effects on gut permeability via p50/p65 binding upon activation of the myosin light chain kinase (MLCK) promoter, which eventually leads to the MLCK-mediated opening of the intestinal tight junction barrier [34].

Increased bacterial translocation is accompanied by elevated peripheral levels of LPSs and other toxins derived from Gram-negative bacteria with subsequent activation of the TLR2 and TLR4 complexes, which are triggered by different pathogen-associated molecular patterns (PAMPs), including LPSs. Activation of the TLR complex may activate intracellular signaling networks, such as NF- κ B and mitogen-activated protein kinases (MAPK), leading to elevated levels of proinflammatory cytokines, reactive oxygen species (ROS) and reactive nitrogen species (RNS). The latter may react with membrane fatty acids, leading to structural changes in the cell membranes and lipid macromolecules and thereby forming oxidative-specific epitopes (OSEs), including malondialdehyde (MDA) [35][36]. Moreover, LPSs may also induce the production of lysozyme and other inflammatory mediators [37, 38]. Therefore, leaky gut appears to be closely associated with increased bacterial translocation, which may drive systemic immune-inflammatory and oxidative processes, termed “gut-derived inflammation”, and eventually deregulate brain functions [2–5]. As such, leaky gut is an important mediator of aberrations of the gut-brain or microbiome-brain axis. Increasing evidence suggests that gut dysbiosis is associated with various neuropsychiatric disorders, including major depression, CFS, deficit schizophrenia and anxiety [2–5, 39–41].

4. Common causes of increased gut permeability

4.1 Diet

Western-type diets, which have high relative contents of fats and refined sugars, may promote gut dysbiosis and hence increase gut permeability and gut-derived inflammation [42–44]. The detrimental effects of Western-type diets may partially result from the wide use of processed

industrial foods and additives as well as from lower relative contents of omega-3 polyunsaturated fatty acids (PUFAs). Several additives have been shown to increase gut leakiness by affecting tight junctions and thereby promote the translocation of immunogenic agents [45], while diets rich in omega-3 PUFAs are protective in experimental models of gut disease and may restore normal permeability [46]

In Western countries, there are increased incidences of allergic diseases, such as asthma, eczema and food allergies, which may be partially explained by the “hygiene hypothesis”, i.e., absence of early exposure to infectious factors and parasites increases the predisposition to allergies via inadequate Th1 cell stimulation, resulting in the effects of Th2 cells not being neutralized and increased susceptibility to allergens [47, 48]. Moreover, intestinal changes in the gut microbiome can lead to different immunological changes that result in development of allergies [49]. In this respect, the newly revised “microflora hypothesis” suggests that changes in gastrointestinal microbiota and reduced microbial exposure are due to changes in diet and antibiotic use. These changes can lead to an underdeveloped microbiome [50], which may disrupt proper immune system maturation and development of immunological tolerance, thereby increasing allergic hypersensitivity [50]. Several prospective studies using culture-based methods of microbiota analysis have shown that significant reductions in certain groups of bacteria are associated with allergic symptoms early in life. For example, levels of *Bifidobacterium sp.* and *Enterococcus spp.* correlate with allergic symptoms in the first month of life [51], and higher *Bacteroidetes*-to-*Bifidobacterium spp.* ratios have been observed in infants (2 years of age) with symptoms of atopy [52].

4.2. Food hypersensitivities

Hypersensitive reactions to food may be mediated by immune (type 1 allergies and type 3/4 food intolerances) or nonimmune (lactose intolerance and fructose malabsorption) factors [53]. These adverse reactions to food, including different types of food allergies, may cause gastrointestinal problems, such as increased gut permeability. For example, type 1 food allergies (IgE-associated) occur in 3% of the population and may affect not only the gastrointestinal tract but also other target organs [53]. These effects are mediated by mast cells as well as by basophil IgE and inflammatory products, including those produced during chronic allergic inflammation, and the changes induced are accompanied by increased gut permeability [53, 54]. Common type 1 food allergens are tree nuts, peanuts, fish, shellfish, peas, beans, eggs, and cow milk. In one study on three different types of BALB/c mice (germ-free, specific pathogen-free, and SOPF-specific and opportunistic pathogen-free), the three groups of mice showed different responses to beta-lactoglobulin sensitization, with higher beta-lactoglobulin-specific IgG1 and IgE concentrations being observed in germ-free mice [55]. In some human clinical trials, probiotics were used to examine the prevention or treatment of allergic diseases.

Non-IgE-mediated food allergies and food intolerances occur more frequently in the population and may be T lymphocyte-mediated (e.g., delayed hypersensitivity or type 4) or IgG-mediated (type 3 food intolerances), whereas IgG antibodies bind food allergens and form immune complexes [56]. Food sensitivities and increased intestinal permeability are often associated

conditions [57, 58]. Mucosal leakiness may lead to translocation of antigens across the gastrointestinal mucosal barrier, potentially playing a role in sensitization to food allergens and food allergies in susceptible individuals [59]

4.3. Irritable bowel syndrome (IBS)

The definition of irritable bowel syndrome (IBS) is based on Rome IV criteria [60], namely, recurrent abdominal pain at least 1 day/week on average in the last 3 months associated with 2 or more of the following: 1) can increase related to defecation, 2) is associated with a change in frequency of stool, and/or 3) is associated with a change in the form (appearance) of stool [60]. IBS may be associated with increased gut leakiness and an impaired gut-brain axis [61–65]. Interestingly, immune activation in the distal bowel and peripheral low-grade inflammation appear to play major roles in the pathophysiology of IBS. Furthermore, IBS is associated with gut dysbiosis, which may induce increased gut permeability and alterations of intestinal motility and nociception [66]. Compared to healthy controls, patients with IBS have a different microbiotic composition, as they exhibit a 2:1 ratio of *Firmicutes* to *Bacteroidetes*, increases in *Dorea spp.*, *Ruminococcus spp.* and *Clostridium spp.*, and decreases in *Bifidobacterium spp.* and *Faecalibacterium spp.* [67]. In IBS, mast cells are upregulated, which may be associated with increased production of nitric oxide (NO), thereby promoting the production of various cytokines, such as IL-1 β , IL-1, IL-13 and TNF- α . Peripheral levels of cytokines, including IL-6, TNF- α , and

IL-1 β , are increased in patients with IBS [68], while monocyte chemotactic protein-1 is elevated and may be involved in inflammation sustainment [69].

4.4. Chronic alcohol use

Chronic alcohol consumption has negative effects on vitamin and nutrient transport and is associated with impaired water and salt absorption and increased gut permeability [70, 71]. As byproducts of alcohol metabolism, acetaldehyde and NO are involved in alcohol-induced barrier dysfunction, and acetaldehyde is associated with gut leakiness and increased endotoxin translocation [72]. In Caco2 cells, acetaldehyde increases epithelial permeability, which is linked to higher tyrosine phosphorylation of zonulin, E-cadherin, and β -catenin [73]. Furthermore, exposure of Caco2 cells to ethanol enhances the expression of NO synthase, which leads to release of NO and increased gut permeability [74]. Alcohol mediates mucosal impairment and barrier dysfunction. Alcohol-induced permeability is accompanied by higher translocation of LPSs in the host, which may induce local and systemic inflammation and damage tissues, including the liver [75–77].

4.5. Nonsteroidal anti-inflammatory drugs (NSAIDs)

Gastrointestinal side effects are commonly observed in patients who use NSAIDs, and numerous data suggest that long-term NSAID use may alter gut barrier function, resulting in exacerbations of inflammatory bowel disease (IBD) [78–85]. In studies comparing short- and long-term NSAID use, ingestion of NSAIDs was shown to be associated with intestinal barrier

dysfunction in both healthy controls and patients [80, 86, 87]. NSAID-induced gastrointestinal damage was initially thought to result from cyclooxygenase inhibition and reduced prostaglandin synthesis, but gastrointestinal injury was later found to be a complex process with multiple parallel mechanisms [79, 86, 88] involving neutrophils, microcirculatory disturbances, ROS and bile acids [79, 89, 90]. One mechanism by which NSAIDs may increase gut leakiness is by elevating the expression and activity of intestinal NO-producing enzymes [90]. NSAIDs have also been shown to alter the energy production needed for proper functioning of the tight junction complex, and their use is therefore associated with increased gut leakiness and inflammation [91].

4.6. Systemic inflammation

Systemic inflammation increases gut permeability, especially that of the paracellular variety [92]. In response to various cellular stimuli, NF- κ B is activated in different immune-inflammatory disorders, such as CFS, IBD, multiple sclerosis, atherosclerosis, systemic lupus erythematosus, type I diabetes, chronic obstructive pulmonary disease and asthma [2, 93]. NF- κ B is a family of transcription factors that regulate genes involved in different processes of immune-inflammatory and oxidative stress responses [94]. Activation of NF- κ B involves two signaling pathways, the canonical and alternative pathways, both of which are important for regulating inflammatory and immune responses [95–97]. NF- κ B is a key transcription factor of M1 (classically activated) macrophages and is required for the induction of numerous immune-inflammatory genes, including those encoding TNF- α , IL-1 β , IL-6, IL-12 and cyclooxygenase-2 [98]. M1 macrophages are characterized by the production of proinflammatory cytokines, such as IL-1, IL-6, IL-12, TNF-

α and chemokines, involved in various inflammatory processes and promote the differentiation of T helper (Th) cells, including Th1 and Th17 cells, which in turn, mediate inflammation and autoimmune responses [98, 99]. M2 (alternatively activated) macrophages produce anti-inflammatory cytokines (IL-10 and IL-13) and are important for the resolution of inflammation [100]. Thus, increased levels of IL-1 β , IL-6, and TNF α partially mediated by NF- κ B can damage the protective barrier function and thereby enlarge the spaces between gut wall cells, which results in increased permeability of the intestinal epithelial tight junction barrier [3, 30–32]. Interestingly, genetic mutations of NF- κ B-stimulating receptors and NF- κ B target genes may be associated with IBD [101]. Moreover, deletion of NF- κ B essential modulators from intestinal epithelial cells can induce spontaneous chronic inflammation in mice [102].

4.7. Yeast overgrowth and dysbiosis

The human intestine is colonized by numerous bacterial species and phyla as well as by fungal and viral communities; as a whole (termed the microbiota), these organisms and their collective genomes constitute the gut microbiome [6, 103]. The gut microbiome, and particularly commensal bacteria, may benefit the host [104, 105], while changes in the microbiome composition may induce specific metabolic processes in the gut and cause pathogenic conditions. Imbalances in gut microbiota or gut dysbiosis may cause leaky gut and bacterial translocation, a process observed in various pathological conditions, such as Crohn's disease (CD), ulcerative colitis, IBS, obesity, type 1 diabetes, major depression, and CFS. For example, *Candida albicans* is a normal component of the human microbiome. In the presence of dysbiosis, however, yeast overgrowth can

occur, which could adversely affect the host's health. A study on gastrointestinal colonization with *Candida* showed sensitization to food allergens, partially resulting from mast cell-mediated hyperpermeability in gut mucosa [106].

4.8. Psychological stressors

Stress, according to the definition given by Selye, is an acute threat to homeostasis and has both short- and long-term effects on the physiology of the gastrointestinal tract. For example, exposure to stressors may result in dyspepsia, diarrhea and acid reflux. Stress-induced effects on gut physiology include 1) various changes in gastrointestinal motility; 2) increased gut leakiness [107]; 3) altered gastrointestinal secretion; 4) higher visceral perception; 5) impaired blood circulation in the mucosa; [108] and 6) changes in the gut microbiome composition and virulence [109]. Mast cells are strongly involved in the function of the brain-gut axis and affect intestinal function not only by increasing gut permeability [107] but also by translating stress signals to the intestines, thus promoting the release of neurotransmitters and proinflammatory cytokines [110, 111].

4.9. Systemic infections, including HIV

Gut permeability is up to 5 times higher in HIV-infected patients than in healthy controls, and higher gut permeability in HIV patients partially accounts for the so-called HIV enteropathy syndrome, which describes various gastrointestinal changes in HIV patients, such as diarrhea, vitamin malabsorption and gut inflammation. Interestingly, histological changes in HIV

enteropathy patients, such as lymphocyte infiltration and impairment of the epithelial layer, presented as blunting and atrophy of villi and hyperplasia of crypts, are present in the absence of enteropathogens, which are normally associated with enteropathy [112, 113]. The pathophysiology of HIV enteropathy is not completely understood, but two major explanatory hypotheses exist: 1) direct virotoxic damage on enterocytes induced by HIV [113–115], and 2) local response of the gastrointestinal immune system, which is correlated with viral replication and gut inflammation [116–118].

5. Diagnostic tests in clinical practice

Table 1 shows a list of probes that may help diagnose leaky gut and comprises serum, stool, urine and breath tests screening for leaky gut and gastrointestinal conditions that are frequently associated with leaky gut. This list comprises tests to detect leaky gut (e.g., antibodies directed at zonulin and IgA/IgM levels of Gram-negative bacteria), tests that may indirectly suggest a leaky gut (e.g., gut inflammation) and tests probing conditions that predispose or maintain leaky gut (e.g., food hypersensitivities and intolerances). In section 5.1., we discuss these stool, serum, urine and breath tests.

5.1. Stool tests to detect gut inflammation

5.1.1. Fecal calprotectin

Fecal calprotectin is a marker used to measure the severity of ulcerative colitis and CD (158). Calprotectin is a heterodimer consisting of two proinflammatory proteins, S100A8 and

S100A9, the latter of which is a biomarker for assessing AD and dementia [119]. A study on AD patients showed that the majority of AD patients had elevated fecal calprotectin concentrations (>50 mg/kg) [120], and increased calprotectin levels indicate gut inflammation. In the presence of inflammation in the intestinal immune system, calprotectin is released as a result of neutrophil granulocyte degranulation [121, 122], and the released calprotectin translocates through the gut barrier, enters the lumen and is absorbed by fecal material. Higher stool levels of calprotectin indicate higher neutrophil numbers in the gut mucosa and mucosal damage; therefore, the assay of calprotectin may be used as an indirect biomarker of gut inflammation.

In clinical practice, fecal calprotectin testing can be performed to differentiate organic from functional bowel diseases [123]. While stool calprotectin is significantly elevated in IBD patients, it is also elevated in IBS patients compared to that in healthy people but not to the extent observed in IBD patients. Normal values of fecal calprotectin are $< \text{or } = 50.0$ mcg/g, suggesting an absence of inflammatory processes in the gut. Measurements between 50.1-120.0 mcg/g are considered borderline and might be indicative of a mild inflammatory process or suggest use of drugs, such as NSAIDs and aspirin. Results $> \text{or } = 120.1$ mcg/g are considered abnormal and suggest active gut inflammation.

5.1.2. Fecal lysozyme

Lysozyme, an enzyme that catalyzes the hydrolysis of Gram-positive bacterial cell walls, exerts an antimicrobial activity that helps prevent interactions between bacteria and the intestinal barrier cell wall [124]. Moderate elevation is suggestive of pathogenic overgrowth of bacteria, yeast

and *Candida albicans*. Fecal lysozyme levels are elevated in IBD patients compared with those in healthy controls [125, 126]. Moderate to high levels of lysozyme may indicate an active inflammatory bowel condition, which often requires further testing, including colonoscopy.

5.1.3. Fecal β -defensin 2

Beta-defensins have antimicrobial effects and support the barrier function of intestinal epithelial cells [127]. These proteins are vital components of the intestinal wall barrier and exert their protective effect by binding to microbial membranes and attracting immune cells [128]. The levels of β -defensin-2 in patients with ulcerative colitis are higher than those in healthy controls, whereas they may be reduced in CD patients [129]. β -defensin 2 is a useful marker for differentiating between both IBD and IBS and between IBD and CD. By inference, elevated levels of β -defensin in IBS implicate activation of mucosal innate immunity with a proinflammatory response even without structural signs of inflammation.

5.1.4. Fecal α 1-antitrypsin

Inflammatory bowel conditions are linked to development of protein-losing enteropathy, during which increased loss of plasma proteins in the gut is observed [130]. The levels of α 1-antitrypsin in feces may be used to indicate the amount of protein loss in the gut; therefore, high α 1-antitrypsin levels may be helpful for the diagnosis of protein-losing enteropathy (protein leakage). Measurements of α 1-antitrypsin in feces is another important biomarker for IBD and IBS differentiation.

5.1.5. Fecal secretory IgA (sIgA)

Secretory IgA (sIgA) plays a major role in protecting the gut epithelium from pathogens and enterotoxins and is excessively produced by the intestinal mucosa and secreted into the intestines in response to presentation of external antigens to gut-associated lymphoid tissue. Through immune exclusion, sIgA prevents contact between pathogenic microorganisms and the epithelium and promotes the removal of bacteria by peristaltic movements [131]. On the other hand, sIgA plays role in the gut microbiota composition. On average, 25%-75% of intestinal bacteria are covered with sIgA, suggesting that sIgA may be an important indicator of gut health. Measurements of sIgA in stool are low in patients with IBD and celiac disease, and in immunocompromised patients, food allergies, SIBO, and yeast overgrowth are observed. Psychological stressors and negative emotions may result in low sIgA levels [132]. High levels of sIgA are associated with chronic infection and some viral infections, such as Epstein-Barr virus (EBV), cytomegalovirus (CMV), HIV and inflammation. Since gut inflammation is a leading cause of increased gut permeability, measuring stool sIgA offers important diagnostic information. The reference range of sIgA is between 51–204 mg/dL (Genova Laboratory range).

5.1.6. Fecal lactoferrin

Lactoferrin is a glycoprotein released by mucosal membranes and activated leukocytes [133]. In the presence of gut inflammation, recruitment of neutrophils to the area of inflammation leads to release of lactoferrin and, consequently, increased lactoferrin levels in stool [134].

Increased stool levels of lactoferrin show that this assay can be used as a marker of gut inflammation with a 90% specificity and to rule out IBS [135]. A meta-analysis of 7 studies evaluating the diagnostic value of fecal lactoferrin to distinguish between IBS and IBD showed that fecal lactoferrin is a sensitive and specific marker for differentiating between the two conditions [136]. Moreover, in pediatric populations, increased fecal lactoferrin is a sensitive marker of intestinal inflammation [137].

5.1.7. Stool cultures and stool microscopy

Different laboratories offer the possibility to assay comprehensive stool profiles of gut microbes with bacterial (*Bifidobacter* sp., *Lactobacillus* sp., *Enterococcus* spp., *Bacteroides* sp., *Prevotella* sp., *Fusobacteria* s., *Mycoplasma* sp., *Klebsiella oxytoca*, *Escherichia coli*, *Campylobacter*, *Clostridium difficile*, *Citrobacter freundii*, *Helicobacter pylori*) and mycological (*Candida albicans*) cultures and parasite (worms) microscopy. These tests allow the identification of dysbalances between beneficial (protective) and dysbiotic flora and detection of yeast or fungal overgrowth (e.g., opportunistic fungi including *Aspergillus* and *Candida*), which may be involved in SIBO and gut dysbiosis and lead to increased gut permeability. These tests may also indicate the presence of *Giardia lamblia*, *Entamoeba* and *cryptosporidium* infections.

One of the main consequences of gut dysbiosis is yeast overgrowth. *Candida* overgrowth has a negative effect on gut health, as excess yeast results in the secretion of toxins throughout the body, which can cause a variety of symptoms that are sometimes seemingly unrelated to the gut, such as brain fog, fatigue, rashes and anxiety [138, 139]. Simple stool cultures and stool PCR,

which is highly sensitive for candidiasis, are options for assaying fungi. Moreover, IgG, IgM and IgA antibodies against *Candida* can be tested in serum.

5.2. Serum measurements

5.2.1. Serum zonulin and zonulin antibodies

Zonulin is a physiological mediator that can reversibly regulate tight junction leakiness [26, 140]. A quantitative sandwich enzyme-linked immunosorbent assay (ELISA) has been developed to measure serum zonulin as a biomarker of intestinal barrier integrity in several inflammatory diseases [26, 27]. Interestingly, in many autoimmune diseases, including celiac disease [141] and type 1 diabetes [142], the onset of the disease may be preceded by an upregulation of zonulin levels. Serum zonulin measurements have been validated with the lactulose/mannitol (L/M) test (see below), as significant associations between serum zonulin and the test outcomes have been observed [142]. Nevertheless, the association between both test results was only $r=0.36$, which is insufficient for applications in clinical practice. Another problem is that zonulin has a short half-life, and a single measurement of serum zonulin is therefore not very reliable [143]. Recently, the authors of the latter manuscript reported that serum zonulin levels were quite stable and significantly associated with anti-zonulin IgG and IgA levels, especially with those of IgA, suggesting that assaying zonulin antibodies may be more sensitive for detecting leaky gut than assaying serum concentrations. Therefore, detection of antibodies against zonulin indicates that the normal regulation of tight junctions is compromised and that tight junctions break down due to an

autoimmune mechanism initiated by environmental triggers, such as infections, toxic chemicals, and some dietary proteins and peptides [144].

5.2.2. Bacterial cytotoxins

Directly measuring LPS levels in serum is not an easy task. One popular test is the limulus amoebocyte lysate (LAL) assay, although several factors might compromise this test. On the one hand, the blood sampling itself could be problematic since bacteria and their products are present everywhere in the environment and avoiding contamination during sampling and testing is therefore not easy [145]. For example, the widely used heparinized tubes are sometimes contaminated with endotoxin [145]. LAL reaction kinetics is another factor that should be considered, as different manufacturers provide LAL tests with differing sensitivities.

The assay of IgA and IgM responses to sonicated samples of Gram-negative bacteria provides a more adequate method to measure the presence of Gram-negative bacteria in the peripheral blood and, by inference, increased bacterial translocation. For example, major depression, CFS and deficit schizophrenia are characterized by increased IgA and IgM responses to sonicated samples of gut commensal Gram-negative bacteria [2-4]. These bacteria include *Hafnia Alvei*, *Pseudomonas aeruginosa*, *Morganella morganii*, *Pseudomonas putida*, *Citrobacter koseri*, and *Klebsiella pneumoniae*. Increased levels of these IgA/IgM responses indicate increased translocation of Gram-negative bacteria due to increased gut permeability [2, 3]. Apart from major depression, other disorders characterized by increased antibodies to LPS include infections with

Gram-negative bacteria, IBS [62], increased intestinal permeability [2, 3], Miller Fisher syndrome [146], and short bowel syndrome.

Bacteria such as *Salmonella*, *Shigella*, *Escherichia coli* and *Campylobacter jejuni* release cytotoxins, including cytolethal distending toxin, subunit B (CdtB), which can penetrate cells, inducing cytoskeletal damage and apoptosis and thereby inducing epithelial cell damage. Subsequently, bacteria may gain access to connective tissue surrounding the gut wall, which damages the gut barrier. IgG, IgA and IgM antibodies to CdtB are measurable in serum and, when present, indicate gut dysbiosis, as detected in IBS patients [147–149].

5.2.3. Ig responses to zonulin, occludin and Gram-negative bacteria

For a more comprehensive clinical evaluation, antibodies against the tight junction proteins occludin and zonulin and epithelial structure proteins from the actomyosin network should be analyzed in combination with antibody levels against Gram-negative bacteria [143].

When zonulin antibody levels are measured in conjunction with levels of LPS and actomyosin, the resulting information can provide a more accurate recognition. The detection of positive zonulin antibody levels indicates a paracellular breakdown of the intestinal barrier that is triggered by factors other than bacterial antigen infiltration. If antibodies against Gram-negative bacteria alone are elevated while antibody levels against occludin/zonulin and actomyosin are negative, the patient may have gut flora dysbiosis. When antibodies against both Gram-negative bacteria and occludin/zonulin are present but antibodies against actomyosin are not, breakdown of the intestinal barrier caused by infiltration of bacterial antigens via the paracellular pathway is

probable. Elevated antibody levels against Gram-negative bacteria and actomyosin but not against occludin/zonulin indicate a breakdown in the intestinal barrier integrity caused by transcellular bacterial antigen translocation. Elevated antibody titers against zonulin and Gram-negative bacteria as well as actomyosin may indicate translocation via paracellular and transcellular pathways.

5.2.5. Food hypersensitivity tests

5.2.5.1 Type 1 food allergies

IgE-mediated food allergies are immediate type 1 hypersensitivity responses to food substances and may present as difficulty breathing, swelling and even anaphylactic shock [150, 151]. One of the most common IgE allergens is peanuts. When an allergic person is exposed to peanuts for the first time, IgE antibodies against the “toxic” substance, peanuts, are produced. IgE antibodies bind to the peanut allergens and attach to mast cells, which initiates histamine-inducing symptoms, such as itching and inflammation, upon rechallenge. IgEs against common food allergens (tree nuts, peanuts, fish, shellfish, peas, beans, eggs, and milk) in serum should be measured in patients with a history and clinical presentation of type 1 allergies.

5.2.5.2. Type 3-4 food sensitivities

As reviewed in section 4.2., food intolerances can be either T lymphocyte- or IgG-mediated. Blood tests were developed to measure IgG (type 3)- and delayed hypersensitivity (type 4)-mediated food intolerances to a panel of food constituents (e.g., between 50 and 300 common food

constituents). The tests are carried out with fluorescence analysis, ELISAs, or lymphocyte transformation. Nevertheless, these food-specific immune tests are not recognized as probes for food allergies and are not always reliable [152]. Moreover, the assays of food-specific IgG4 levels may be more of a biomarker of exposure to the foods tested and perhaps even tolerance [152]. The European Academy of Allergy and Clinical Immunology (EAACI) task force concluded that IgG4 testing for food intolerances is probably irrelevant for the laboratory work-up of intolerance and has no clinical diagnostic validity in cases of food-related complaints [153]. Nevertheless, the assay of total IgG food intolerances with an exclusion diet based on the test results may be useful for the diagnosis of IBS, CU, bipolar disorder, schizophrenia, autism, obesity and migraines [154-158].

5.2.6. Transglutaminases and gliadin

Celiac disease is an enteropathy caused by immune inflammation in the gut mucosa following ingestion of gluten-containing proteins in genetically predisposed patients [159]. Because mucosal inflammation in the small intestine leads to villous atrophy, the definitive tool for diagnosing celiac disease is jejunal biopsy. An easier, noninvasive and highly sensitive test for celiac disease involves the detection of disease-associated antibodies, such as tissue transglutaminase (tTG) and anti-gliadin antibodies. The assay of tTG-IgA in serum is a specific test for celiac disease [160], although confirmatory diagnosis with jejunal biopsy is advised. The assay of anti-gliadin antibodies (IgA-IgG) is another comparable test that is useful for screening patients with suspected celiac disease [161]. The combination of both tTG-IgA and anti-gliadin antibodies may improve the sensitivity of detecting celiac disease [162]. Celiac disease is characterized by loss

of intestinal barrier function, which is at least partially induced by gliadin-induced zonulin release [163, 164] Anti-gliadin antibodies may also appear in subjects with nonceliac gluten sensitivity and be helpful for diagnosing this condition [165].

5.3. Breath tests

A number of hydrogen breath tests can be used to detect conditions that are etiologically associated with leaky gut, including lactose and fructose intolerance and SIBO [166]. Hydrogen breath tests are probes that measure the amount of exhaled hydrogen collected in a container after swallowing sugar solutions (including lactulose, lactose and fructose). Upon shortage of sugar-metabolizing enzymes, sugars pass undigested into the intestines, with larger amounts of undigested sugars accumulating in the colon. Consequently, the undigested sugars are digested by bacteria, thereby forming hydrogen (and other gases), which is absorbed by the intestinal mucosa, transported to the lungs and then exhaled. Normally, no or small amounts of hydrogen are measurable; however, exhaled hydrogen levels increase upon shortage of sugar-related enzymes and the metabolization of sugars by bacteria. These tests therefore measure the bacterial metabolism of undigested sugars.

5.3.1. Hydrogen breath test following lactose ingestion

The lactose tolerance test is performed by drinking a high-lactose solution and measuring hydrogen levels approximately 2 hours later. Lactose intolerance is the consequence of a shortage of lactase, the enzyme that breaks down lactose, in the small intestine. Consequently, lactose cannot

be broken down to glucose and galactose and therefore passes into the colon to be metabolized by bacteria into hydrogen [167]. People with lactose intolerance often show symptoms of IBS, including bloating, gas, flatus, cramps, diarrhea, nausea and vomiting, when they consume milk or milk products [168]. Diseases of the brush border (e.g., celiac disease, SIBO) are often accompanied by secondary lactose intolerance. Lactose-intolerant individuals often show nutrient deficiencies, including low levels of calcium and vitamin D, which have anti-inflammatory properties and may thus have a strong impact on many inflammatory disorders [167]. Diets high in sugar are known to induce gut dysbiosis, and refined sugars mediate overgrowth of opportunistic bacteria [169].

5.3.2. Fructose malabsorption

People with deficient fructose carriers in their small intestines show impairments in fructose absorption, causing fructose contents to be increased in the intestinal tract, including the colon [170], wherein fructose is fermented by enterobacteria into hydrogen and other gases. The increased production of hydrogen can be detected using a hydrogen breath test after the ingestion of fructose. Fructose malabsorption produces symptoms reminiscent of IBS [171]. Importantly, some evidence indicates that fructose malabsorption is associated with changes in the gut microbiome composition, the tight junctions barrier and increased bacterial translocation [172].

5.3.3. Lactulose breath test and small intestine bacterial overgrowth (SIBO)

The hydrogen breath test following ingestion of lactulose, a nonabsorbable sugar, may be used for the diagnosis of SIBO, a condition characterized by increased bacterial populations in the

small intestines [173]. As lactulose passes through the small intestines, it is digested by increased bacterial populations, thereby forming hydrogen, which is measurable by the breath test. An elevation of breath hydrogen 20 ppm above the initial level within 90 minutes after swallowing lactulose is indicative of SIBO [166]. Significant associations exist between small intestinal overgrowth and IBS and between gut dysbiosis and IBS [174, 175].

5.4. Urine tests

5.4.1. Lactulose/mannitol test

The L/M test is often employed to probe leaky gut, as it evaluates small intestinal permeability by measuring the urinary excretion of lactulose and mannitol after oral administration of both test substances. Lactulose is a large molecule that is not normally transported through the intestinal barrier, and its absorption signals increased permeability of the tight junctions barrier, while mannitol can normally cross the gut barrier. The excreted amounts of both test substances in the urine over a 5 h period are used to identify leaky gut [176]. Factors such as renal function, gastrointestinal motility and dilution affect both probes equally, which allows correct measurements of the ratio in patients [17]. This noninvasive test can be used in clinical practice for the identification of increased gut permeability, albeit with lower sensitivity [177–180].

5.4.2. Urinary markers of gut dysbiosis

Some laboratories offer urinary dysbiosis tests to measure byproducts of gut bacterial and yeast metabolites or organic acids, which are transported to the urine and can thus be measured in

the urine. Changes in sets of these byproducts may indicate gut dysbiosis. These tests are not highly specific, as changes in diet, medications and metabolic disease can induce similar changes. These byproducts include benzoate, hippurate, phenylacetate, arabinose, arabinitol, indican, tartrate and ketoglutarate [181]. Another possible urinary biomarker of leaky gut is D-lactate, a product of bacterial metabolism [182]. A recent publication showed that in patients with myocardial infarction, increased plasma levels of D-lactate and LPSs were associated with peripheral inflammatory responses and cardiovascular events [183]. Bacterial DNA blood levels were observed in many patients with end-stage renal disease and associated with increased D-lactate as well as inflammatory biomarkers [184]. In rodent models, increased D-lactate levels in blood are associated with increased LPS levels [185].

5.5. Confocal laser endomicroscopy

Confocal laser endomicroscopy (CLE) is a method used for the diagnosis of gastrointestinal tumors [186, 187], celiac disease, collagenous colitis, and IBS [188]. Achieving up to 1000-fold magnification, CLE allows detection of cellular and subcellular alterations, such as cell shedding, in real time. Therefore, CLE is a valuable technique for visualization of mucosal barrier defects, and with the use of contrast agents, CLE allows direct observation of immune processes in the gut epithelium. A study using CLE to detect barrier dysfunction in IBD patients showed that this test may predict relapses of IBD [189]. CLE can also be used to predict future hospitalizations, as one study showed that increased epithelial gaps are positively correlated with future exacerbations and require surgery [190]. Another study showed that CLE can be used to detect specific foods causing

epithelial impairment and loss of barrier function and could therefore be developed to test for food allergies and, consequentially, “exclusion diets” [188].

6. Conclusions

Table 2 shows a list of different tests that are useful for diagnosing leaky gut and associated conditions and includes stool, serum, urine and breath tests. We recommend that this test battery (without the less reliable or sensitive tests shown in Table 2) be used for subjects with comorbid neuropsychiatric and IBS conditions, food hypersensitivities, lactose and fructose intolerance and other functional gastrointestinal symptoms. Even in the absence of gastrointestinal symptoms, many patients with neuropsychiatric disorders (and other medical disorders) suffer from leaky gut, which contributes to the immune-inflammatory and oxidative pathways in these neuropsychiatric and medical disorders. Nevertheless, increased gut permeability is almost always a consequence of other conditions, including gut dysbiosis, infections, psychosocial stressors, substance use (e.g., alcohol, NSAIDs), food hypersensitivities, SIBO, fructose intolerance, Western inflammatory or prooxidant diet, toxins, and diverse immune pathologies, and is especially a consequence of the primary neuroimmune disorders themselves. Therefore, we recommend assaying serum IgM/IgA responses to Gram-negative bacteria and antibodies against zonulin/occludin and actomyosin to exclude possible effects of leaky gut on neuroimmune disorders. If this assay is positive, further testing with the stool, serum, urine and breath tests shown in Table 2 is needed depending on the symptoms of the patient.

Figure 1 shows a flow chart of our proposed strategy to assess leaky gut and related conditions in patients with neuroimmune disorders. We propose assaying serum IgM/IgA/IgG antibody levels of zonulin, occludin and actomyosin and IgM/IgA levels of Gram-negative bacteria or LPSs in all patients with neuroimmune disorders, including those with major depression, bipolar disorder (especially type 1), CFS and (deficit) schizophrenia, because a considerable number of those patients may show biomarkers of leaky gut [2-4]. If these tests indicate leaky gut, of either the transcellular or paracellular variety, we recommend employing other stool and urine tests to assess for gut dysbiosis, gut inflammation, dysfunctional gut protection and bacterial metabolites; when needed, we recommend food hypersensitivity (type 1), food intolerance (total IgG), and hydrogen breath tests to assess for lactose intolerance, fructose malabsorption and SIBO. These tests are used to elucidate possible factors precipitating or maintaining leaky gut that can be subsequently treated successfully with glutamine, zinc, oral agents (targeting leaky gut) [191], short-term antibiotics (SIBO), exclusion diets (food intolerances and hypersensitivities, lactose intolerance, fructose intolerance), and probiotics (gut dysbiosis). Moreover, we advise that common tests be performed to measure components activated in the immune-inflammatory (e.g., some cytokines and acute-phase proteins) and nitro-oxidative (e.g., lipid hydroperoxides, aldehyde formation) pathways that may affect blood-brain barrier permeability and, consequently, neuroprogression, as they promote the process by which immune-inflammatory products induce neural dysfunctions, including lowered neurogenesis and alterations in neuroplasticity, neurotoxicity and excitotoxicity [192].

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DS and MM designed the study. DS conducted data base searches and made a first draft. All authors contributed equally to the writing up of the paper. All authors agreed upon the final version of the paper.

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Conflict of interest

The authors have no conflict of interest with any commercial or other association in connection with the submitted article.

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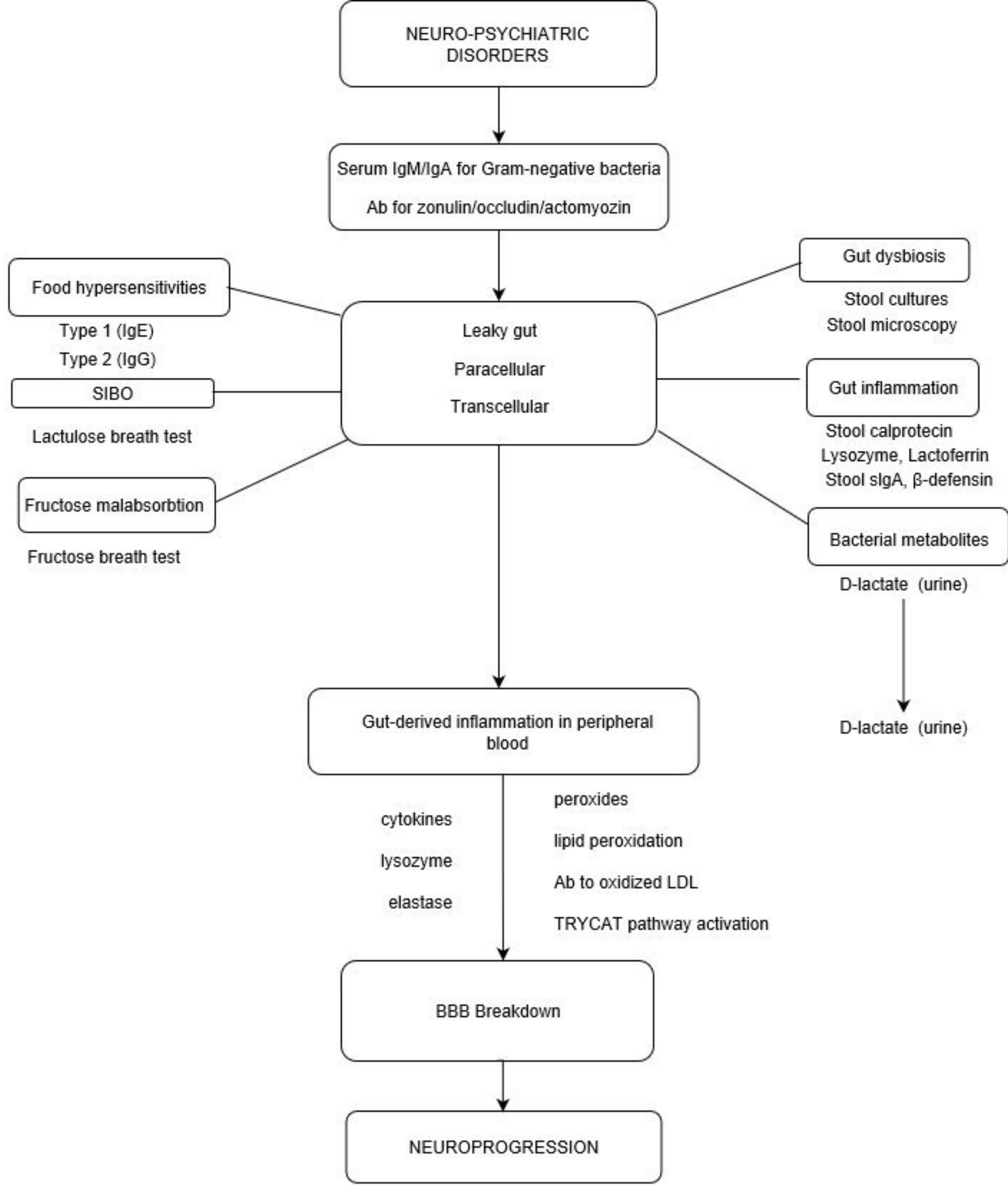


Table 1. Minimum panel to examine leaky gut using blood, stool, urine and breath tests

A. More direct serum tests for leaky gut
1. Serum zonulin / occludin antibodies
2. Serum IgA/IgM responses to Gram-negative bacteria
3. Serum antibodies to zonulin / occludin / actomyosinin and Gram-negative bacteria
4. Serum antibodies to cytolethal distending toxin
5. Lactulose / mannitol test (urine excretion)
B. Stool and urine tests indirectly indicating leaky gut
1. Gut inflammation
2. Decreased mucosal defenses
3. Stool bacteria cultures (gut dysbiosis)
4. Yeast or fungi overgrowth (gut dysbiosis)
5. D-lactate in urine/plasma (gut dysbiosis)
C. Tests probing common factors underpinning or maintaining leaky gut & dysbiosis
1. Type 1 food hypersensitivities, serum IgE to common food constituents
2. Type 3 food intolerances, serum total IgG to common food constituents
3. Lactose intolerance, breath test
4. Fructose malabsorption, breath test
5. Small intestine bacterial overgrowth, breath test
6. Microscopic parasite detection in stool

Table 2. A clinical guide of useful tests to diagnose leaky gut

Test material	Assays	Indicates
1. Stool	Calprotectin	Gut inflammation
	Lysozyme	Gut inflammation
	Lactoferrin	Gut inflammation
	β -defensin	Gastro-intestinal immunity
	α -antitrypsin	Protein leakage
	Secretory IgA	Mucosal defenses, lowered protection or gut inflammation
	Stool bacteria cultures	Gut dysbiosis
	Stool yeast and fungi cultures	Yeast and fungi overgrowth
	Stool parasites microscopy	Infection with parasites
2. Serum	Serum zonulin levels	Gut permeability (less reliable)
	Direct assays of LPS	Translocation Gram-negative bacteria (less reliable)
	IgA/IgM Gram-negative bacteria	Translocation Gram-negative bacteria
	Ab to zonulin/occludin, Gram-negative bacteria and actomyosin	Bacterial translocation through paracellular and/or transcellular pathways.
	Cytolethal distending toxin	Gut dysbiosis
	IgE to common food allergens	Type 1 food allergy
	IgG to common food constituents	Type 3 / 4 food intolerances
	Transglutaminase and gliadin antibodies	Celiac disease, non-celiac gluten sensitivity (loss of tolerance to gluten), abnormal mucosal immunity
3. Breath tests	Lactose	Lactose intolerance
	Fructose	Fructose malabsorption

	Lactulose	Small intestine bacterial overgrowth
4. Urine tests	Lactulose/mannitol test	Leaky gut (low sensitivity)
	Urinary markers of gut dysbiosis	Gut dysbiosis (not yet validated)
	D-lactate in urine or plasma	Leaky gut
5. Endoscopy	Confocal laser endomicroscopy	Leaky gut

