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Intestinal permeability in physiological and pathological conditions: major determinants and assessment modalities

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Abstract: Intestinal permeability is the property that allows solute and fluid exchange between intestinal lumen and intestinal mucosa. Many factors could have major impact on its regulation, including gut microbiota, mucus layer, epithelial cell integrity, epithelial junction, immune responses, intestinal vasculature, and intestinal motility. Any change among these factors could have an impact on intestinal homeostasis and gut permeability. Healthy condition is associated to normal intestinal permeability whereas several intestinal and extra intestinal disease, like inflammatory bowel disease, irritable bowel syndrome, non-alcoholic fatty liver disease among others, are associated to increased intestinal permeability.

This review aims to synthesize determinants on intestinal permeability and to report methodologies useful to the measurement of intestinal permeability in clinical practice as well as in research settings.

Key Words
Intestinal permeability, Microbiota, Gut barrier, Mucosal immunology, Barrier protector, Ibd, Lactulose/mannitol ratio, Cr51edta, Leaky gut.

Introduction

The gastrointestinal (GI) tract accounts for a global surface of more than 200-meter square, being perhaps the most exposed system to the outside world of our body, comprehending thousands of compounds from foods and associated microorganisms[1]. This condition requires a complex defensive system that separates intestinal content from the host tissues, and regulates nutrients adsorption, allowing interactions between the resident microbiota and intestinal immune system, ruling intestinal translocation of bacterial compounds from external to the internal world: this is the functional unit called "Gut Barrier", which is composed by the epithelial/intestinal mucosal barrier, the Gut Microbiota, the intestinal mucus layers, the innate and adaptive immune system associated to gut mucosa, the intestinal vascular/lymphatic system, the intestinal endocrine and neuroenteric system, the enzymatic system[2] (Figure 1).

The outer layer is composed by gut microbiota that competes with pathogens for space and resources, elaborates molecules required for mucosal integrity, and modulates the immunological patterns of lower barrier. Intestinal microbiota refers to the entire population of microorganisms colonizing the gastrointestinal tract[3], displaying great biodiversity[4]. It includes not just bacteria, but also fungi, archaea, yeast and viruses, that have a mutualistic relationship with bacteria, within themselves and with their host, co-habiting with enterocytes in a symbiotic relationship[3]. Bacteria up to know are the most studied and characterized: the majority of them belongs to two main phyla, *Bacteroidetes* and *Firmicutes* followed by *Proteobacteria, Actinobacteria and Fusobacteria*[4]. Its qualitative and quantitative composition varies according to the

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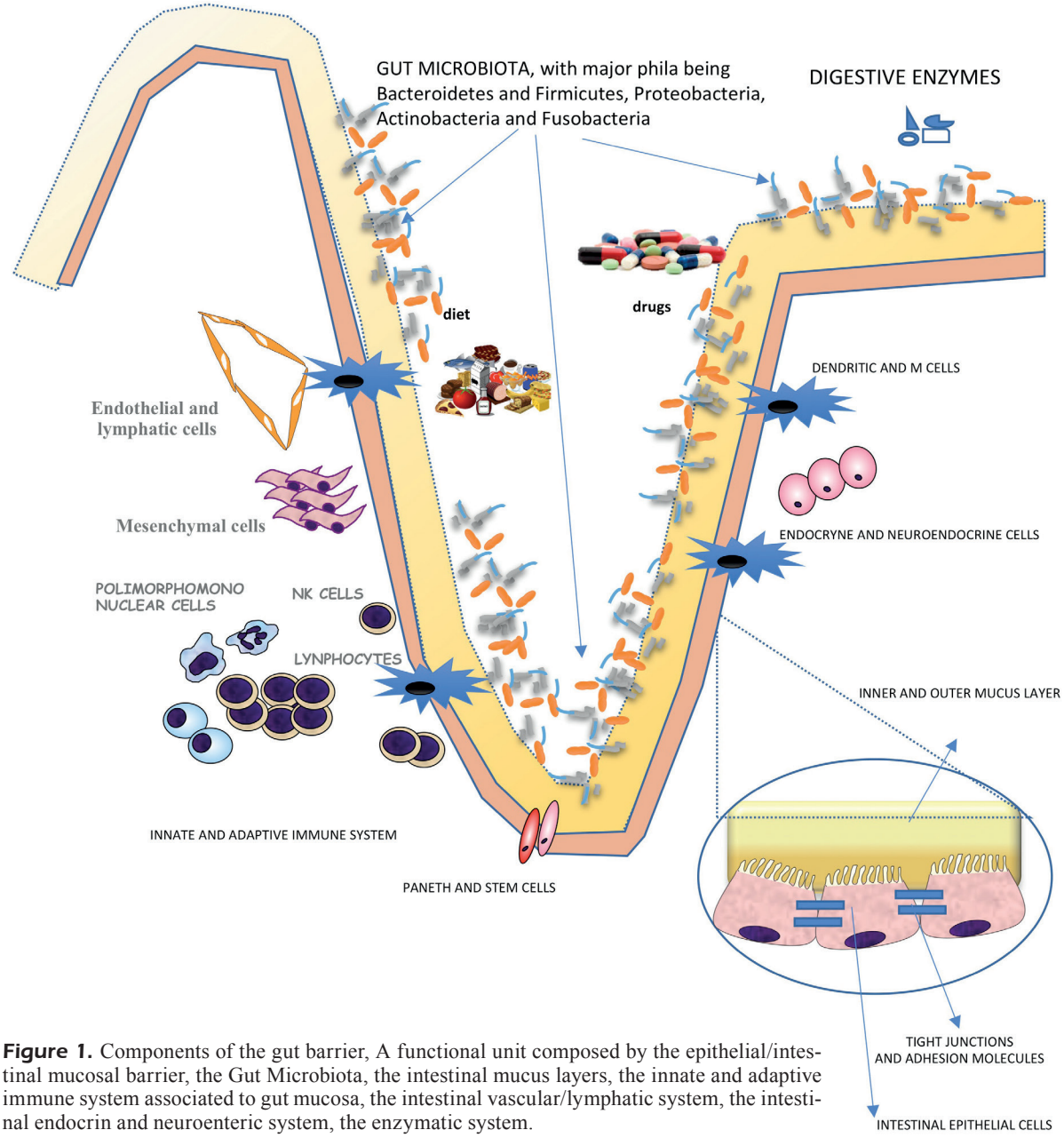

Figure 1. Components of the gut barrier, A functional unit composed by the epithelial/intestinal mucosal barrier, the Gut Microbiota, the intestinal mucus layers, the innate and adaptive immune system associated to gut mucosa, the intestinal vascular/lymphatic system, the intestinal endocrin and neuroenteric system, the enzymatic system.

age, host genetics, diet and the local environment, like pH and oxygen content[3,4].

Gut microbiota displays different functions, including metabolic, immunological and gut protection in the regulation of barrier function, metabolism of nutrients, trophic function of the mucosa, drug metabolism, and toxin metabolism. It assists in the digestion of energy substrates, in producing vitamins and hormones and in protecting the host from pathogen species[2]. Gut microbiota is a key element in balancing innate and adaptive immune systems within the gut[3].

Gut microbiota is in close contact with another simple mechanism of antimicrobial protection: the intestinal mucus, the first physical barrier that bacteria meet in the intestinal tract. It separates the endoluminal contents from inner layer of the gut barrier and contains antimicrobial products and secretory IgA. The goblet cells produce factors like trefoil-factor and the resistin-like molecule-β that can stabilize mucin polymers and thereby maintain barrier integrity[3]. The mucus is composed of two layers: an inner layer firmly attached to the epithelial cells, which is imper-

vious to bacteria and functions as a protective barrier for the epithelial cell surface, and an outer layer that is less sticky. Both layers are organized around the highly glycosylated mucin MUC2, which forms an amorphous polymer-like cover and is secreted by goblet cells[5].

The inner layer consists of a complex network of other human cells. The epithelial/intestinal mucosa barrier is composed by epithelial cells (enterocytes), covering the entire intestinal surface organized in villi and circular folds[2]. Enterocytes display on the apical surface microvilli or brush border, site of several intestinal enzymes. The junction among enterocytes is ruled by adherens junctions (AJs) and tight junctions (TJs), including cadherins, claudins, occludin, and junctional adhesion molecules (JAM) proteins, intercellular proteins making bridges among cells and which seal adjacent cells together, making them a phisical barrier not permeable to bacteria or other substances. TJ is dynamic gates, whose function is regulated by several factors, including alcohol, food components, bacterial products, inflammatory molecules and drugs[2].

Immune cells of the intestinal mucosa are organized in a specialized and compartmentalized system known as "gut-associated lymphoid tissue" or GALT. It is one of the largest lymphoid organs, which determine the immune responses to pathogenic microorganisms and immune tolerance to commensal bacteria. This ability is mediated by dendritic cells and M-cells in Peyer's patches. These cells are able to internalize microorganisms and macromolecules, presenting the antigens to naive T lymphocytes, which differentiate and are responsible for immune responses, including the production of several types of cytokines[2,6].

Physiological Regulators of Intestinal Permeability

The homeostasis of the intestinal epithelium and the regulation of intestinal epithelial cell polarity are maintained by a complex interplay of multiple regulatory mechanisms as Wnt, Notch, Hippo, transforming growth factor-β (TGF-β)/bone morphogenetic protein (BMP) and Hedgehog pathways. These pathways maintain the crypt-villus architecture and regulate multiple self-renewing organs[7]. The Adhesion Junction and Tight Junction complexes also play a crucial role in the regulation of cellular polarization, proliferation, and differentiation[7]. The intracellular domains of these transmembrane proteins interact with cytosolic scaffold proteins, such as zonula occludens proteins, which in turn anchor the transmembrane proteins to the actin cytoskeleton. The interaction of Tight Junction proteins with the actin cytoskeleton contributes to barrier integrity[8]. In this setting, gut microbiota plays a crucial role in physiologic conditions[3,4], being a positive stimulus. Nutrients also play an important role. Dietary components, such as omega-6 polyunsaturated fatty acids (v6-PUFAs), long-chain saturated fatty acids, protein, and digestible carbohydrates, are associated to intestinal inflammation and increased intestinal permeability. In contrast, omega-3 polyunsaturated fatty acids (v3-PUFAs), vitamin D, medium chain triglycerides, bioactive food-derived peptides, some probiotics and prebiotics and non-digestible carbohydrates were described to reduce intestinal permeability and improve intestinal health. SCFAs produced from butyrate fermentation, when administered orally to animal models of IBD, protect against mucus layer alterations[9]. Other factors are also associated to a physiologic modification of gut barrier, however they are not considered in the present review.

Pathological Regulators of Intestinal Permeability

Intestinal permeability can be altered by cytokine-mediated dysfunction, resulting in immune activation and tissue inflammation. In particular, IFN-γ, increases paracellular permeability in intestinal epithelial cells through the redistribution and expression of Tight Junction proteins and the rearrangement of the actin cytoskeleton. Other powerful and pro-inflammatory cytokines were associated to Tight Junction impairment through several mechanisms: TNF-α, by inducing apoptosis of intestinal epithelial cells, IL-1β, among others. Interleukin-10 (IL-10), a well-defined anti-inflammatory cytokine, has, on the other hand, protective effect on Tigh Junction i*n vitro* as well as *in vivo* experimental system, contra-balancing the pro-inflammatory effect of TNF-α and IFN-γ[8]. Aging is a powerful inducer of gut barrier dysfunction, following several mechanisms. Aging in fact alters intestinal smooth muscle contractility, as well as the neural innervations of the GI tract musculature and sensory signaling[6]. Also stress, directly and indirectly increases intestinal permeability as suggested by experimental models[10,12]. Abnormal physical exercise as well as the use of drugs like NSAIDS is associated to intestinal hyperpermeability[13,14]. Other factors are also associated to a pathologic modification

of gut barrier, however they are not considered in the present review.

Diseases Associated to Increased Intestinal Permeability

Increased intestinal permeability is an indicator of intestinal barrier dysfunction. Increased intestinal permeability is widely recognized as an underlying pathogenic factor, not only in IBD, but also in other gastrointestinal and non-gastrointestinal diseases[15]. In particular, associated diseases, more or less associated to "the leaky gut syndrome", include type I diabetes, immunodeficiency, multiple sclerosis, rheumatoid arthritis, behavioral disorders, but also Irritable Bowel Syndrome (IBS), Intestinal Bowel Disease (IBD), celiac disease (CD), infectious enterocolitis, small intestinal bacterial overgrowth (SIBO), food allergies and intolerances and behavioral disorders[16].

IBD

It is still not clear whether inflammation in IBD precedes epithelial barrier dysfunction, like reported in some papers or whether, conversely, the barrier dysfunction follows chronic inflammation[17]. Regardless of this aspects, molecular interactions of adherent invasive bacteria, like *E. Coli*, with intestinal epithelial cells induce inflammatory responses leading to the overproduction of proinflammatory cytokines[18], which in turn in increase local intestinal injury, induce NF-kB gene expression within epithelial cells, potentiating the local inflammatory cascade circle with newer production of pro-inflammatory cytokines such as interleukin IL-1b, TNF-α and IFN-γ. This cytokine profile also promotes tight-junction dysregulation and enhances intestinal permeability following a reorganization of tight junction proteins such as zonulin-1, JAM-A, occludin, claudin-1, and claudin-4[2,18]. A consequence of such multipronged disruption to barrier integrity is heightened bacterial translocation with elevated circulatory bacterial endotoxins (such as lipopolysaccharide)[18,19]. In clinical practice, an increased intestinal permeability precedes clinical relapse in IBD, suggesting that a permeability defect is an early event in disease exacerbation[20].

Irritable Bowel Syndrome (IBS)

IBS patients have been associated to an increased intestinal permeability, especially diarrhea-predominant subgroup, in the post infectious IBS-subgroup, anxiety, and depression and also in pediatric population[21]. A significant reduction in Tight junction proteins and in zonulin-1 mRNA expression in experimental models of IBS[2] together with a possible involvement of Zonulin signaling through PAR2 in IBS-Diarrhea type[16].

Celiac Disease

Numerous *in vitro, ex vivo,* and *in vivo* studies have confirmed that gliadin, the main antigen of gluten, increases intestinal permeability. *In vitro,* following binding to its receptor CXCR3 on epithelial cells, gliadin activates MyD88-pathway, determining the release of zonulin[22]; on the other site, human studies showed that CXCR3 is overexpressed in celiac disease patients, co-localizing with specific gliadin peptides, suggesting that this pathway is clearly involved also *in vivo* to explain the increased intestinal permeability observed in this patients[23]. Moreover, abnormal intestinal permeability could be an early signature of celiac disease, prior to antibodies detection. Finally, intestinal permeability measurements seem more sensitive than antibody testing for detecting gluten exposure[24,25]. Gluten-free diet associate to significant decrease of permeability[24], together with serum zonulin levels decrease and autoantibody titers normalization[23]. This observation has been confirmed in another study measuring intestinal permeability by the lactulose/mannitol ratio, following 1 year from starting gluten-free diet[24].

Type-1 Diabetes (T1D)

Recent studies have shown that altered intestinal permeability is also involved in T1D prior to the onset of complications. This hypothesis is supported by studies performed in BioBreeding diabetic-prone (BBDP) rats that develop T1D spontaneously. In this animal model, an increased permeability of the small intestine (but not of the colon) preceded the onset of diabetes mellitus by at least a month. Histological evidence of pancreatic islet destruction was absent at the time of increased permeability but was clearly present at a later time[20]. Furthermore, diabetes is associated with increased lipopolysaccharides levels (LPS), causing the so called "metabolic endotoxemia", triggering pro-inflammatory cytokine secretion and inducing insulin resistance[26-28]. Other studies confirmed these findings, suggesting a zonulin-dependent mechanism[29], in fact oral administration of the zonulin inhibitor AT1001 (larazotide acetate) to BBDP rats blocked autoantibody formation, increased intestinal permeability and reduced the incidence of diabetes[20].

Obesity

Circulating zonulin seems to increase with body mass index, waist to hip ratio, fasting insulin, fasting triglycerides, uric acid and IL-6 perhaps following STAT3 activation[23,30]. Evidence has also been provided suggesting that increased zonulin levels not only is associated with obesity, but also with its metabolic complications[16].

Brain-Gut Axis Alterations and Behavioral Disorders

A bidirectional communication exists between gut and brain, through the spinal cord, the enteric nervous system, the hypothalamic pituitary adrenal axis, and the central nervous system[31]. Leaky gut and gut microbiota alterations have been associated to brain alterations resulting in behavioral alterations[31,32]. Highly sensitive gastrointestinal tract, responsible for visceral hypersensitivity, could activate amygdala, a key component of the central nervous system, responsible for pain processing and modulation of pain-related emotional affective dimension; its activation in turn could lead to activation of the hypothalamus-pituitary-adrenal (HPA) axis[32]. Preclinical evidence demonstrates that stress can contribute to gut alterations, especially in relation to barrier function. Meddings and Swain[33], in particular, showed that 24 h after rats were subjected to a 20-min swim stress, they had higher urinary excretion of sucrose, lactulose/mannitol, and sucralose than no stressed control rats, which suggests an increased gastrointestinal permeability. Interestingly, these effects were absent in adrenalectomized rats subjected to the same stress procedure. Further, this was confirmed by pharmacological antagonism of glucocorticoid receptors by RU-486, which also prevented the effects of the swim stress on gastrointestinal permeability[33]. On the other hand, there is evidence that glucocorticoid secretion is stimulated independently of ACTH (Adren Cortico Tropic Hormone). For example, intraperitoneal exposure to live bacteria or bacterial LPS leads to an enhanced glucocorticoid secretion that is mediated by bacterially stimulated prostaglandin secretion and not by ACTH[34,35]. Although this effect may arise from bacteria and/or bacterial cell wall components coming from any source, it suggests that enhanced permeability might stimulate the secretion of cortical steroids, which could contribute to alterations in neuronal plasticity and therefore induce behavioral responses to stressful situations[36,32]. Alcohol dependence has traditionally been considered a brain disorder. In the context of alcohol abuse, a relationship between the microbiota, barrier function and comorbid depression has recently been reported. Microbiota-derived LPS and peptidoglycans were demonstrated to cross the gut barrier and activate the irrespective receptors, TLR4 and TLR2 in peripheral blood mononuclear cells. In contrast, short term alcohol withdrawal was associated with the recovery of TLR4 receptors. The same group also demonstrated that increased intestinal permeability occurred in a subgroup of alcohol dependent subjects which were associated with higher depression and anxiety scores as well as unaltered gut microbiota profile[37].

Other Diseases

Other intestinal and extra-intestinal diseases were associated to increased gut permeability and "leaky gut syndrome", including and not limited to alcoholic liver disease, nonalcoholic steatohepatitis, liver cirrhosis, primary biliary cholangitis, obstructive jaundice, severe acute pancreatitis, chronic heart failure, depression, endotoxemia, proinflammatory[38].

Therapeutic Intervention for Intestinal Permeability Dysfunction

A unique cure for intestinal permeability dysfunction is currently not available, however different approaches targeting major determinants of gut permeability can be utilized. Among them we will discuss mainly and briefly about modifiers of gut microbiota (antibiotics and probiotics. probiotics and diet), drugs affecting immune system (steroids, aminosalicylates, anti-TNF agents) and drugs affecting mucosal barrier (barrier protectors).

Modifiers of Gut Microbiota: Antibiotics

A strong body of evidence has now clearly demonstrated that the use of antibiotics has several short and long-term implications in the ecology of the normal gut microbiota, including a disruption of the competitive exclusion machinery that predispose to infections, including for instance *Cl. Difficile* infection or *Salmonella* infection. For example, it was shown that the effect of even short-term use of broad-spectrum antibiotics with predominant anaerobic coverage like clindamycin could last up to 2 years, with a persistent non-recovery of the diversity of *Bacteroides*. The effect of ciprofloxacin is relatively short-lived with abrupt reduction of *Ruminococcus* sps. Another study[3] showed that ciprofloxacin and beta-lactams reduce microbial diversity by 25% and the core taxa from 29 to

12 with an increase in the *Bacteroidetes*: *Firmicutes* ratio. Gut microbiota alteration associates to impaired metabolic function of gut microbiota, particularly the formation of SCFAs from nutritional carbohydrates (resistant starch, indigestible polysaccharides and other dietary fiber) and endogenous carbohydrates[39]. Among antibiotics, the poorly absorbed antibiotic rifaximin display a peculiar role, not exerting non-traditional effects additional to the bactericidal/bacteriostatic activity on the gut microbiota: rifaximin was shown to reduce bacterial virulence and translocation, modulate gut microbial composition increasing *Bifidobacteria*, *Faecalibacterium prausnitzii* and *Lactobacilli*, which usually exert beneficial effects to the gut[40].

Modifiers of Gut Microbiota: Probiotics

Several studies demonstrated the role of probiotics in reducing intestinal permeability: for example, in a doble-blinded, placebo controlled, cross-over study, *Lactobacillus rhamnosus* and *Lactobacillus reuteri* were administered for 6 weeks to 41 children suffering from atopic dermatitis, resulting in a decrease of intestinal permeability as documented by Lactulose/mannitol urine ratio[41]. Moreover, *L. rhamnosus GG* accelerates intestinal barrier maturation and induces claudin 3 expression in animal models, while *Lactobacillus casei* increases the expression of zonulin genes in Caco-2 cells[2]. *Bacillus subtilis* and *Bacillus clausii,* by adhering to intestinal walls, reduce intestinal permeability and increase secretory immunoglobulin IgA[42,43]. The probiotic *Escherichia Coli (E. coli) Nissle 1917* was shown to positively modulate intestinal epithelial barrier through increased expression antimicrobial like b-defensin-2[44,45] and upregulation and redistribution of the TJ proteins ZO-1[46], ZO-2[47] and claudin-14[47-50]. In ulcerative colitis *E. coli Nissle 1917* is effective as mesalazine in maintenance of remission in UC patients, suggesting direct immunomodulatory property to control intestinal inflammation[49]. Furthermore, Barbaro et al[51] demonstrated that *E. coli Nissle 1917* increases intestinal integrity and paracellular permeability using Caco-2 cells as *in vitro* model of intestinal permeability with biologic samples taken from IBS patients exposed or not to this probiotic. *In vitro* positive effect paralleled to clinical efficacy of the probiotic. The probiotic compound VSL#3, composed by *Lactobacillus*, *Bifidobacteria*, and *Streptococci*, protected the intestinal epithelial barrier in a murine model of colitis by maintaining TJ protein expression and preventing apoptosis[52]. Other studies[53] showed that *Bifidobacteria* and *Lactobacillus*, but not *Streptococci*, recovered intestinal barrier function correlated with a modulation of claudin-1 and occludin in a mouse model of post-infectious irritable bowel syndrome, and the mixture of 3 strains was superior to any single one. Finally, the secreted metabolites of probiotics are cytoprotective to intestinal epithelium and have been shown to attenuate inflammation and reduce gut permeability. An *in vitro* study has demonstrated that probiotic conditioned media (PCM) from *Bifidobacterium infantis* and *Lactobacillus acidophilus* treatment improved Caco-2 barrier function in a dose-dependent manner within a specific period of incubation and prevented the barrier compromise due to IL-1b stimulation, by normalizing the expression of TJ proteins, occludin and claudin-1[54].

Modifiers of Gut Microbiota: Prebiotics

Prebiotics, defined as non-digestible carbohydrates that act as a fermentation substrate within the colon conferring health benefits on the host[57] and including inulin-type fructans (inulin, oligofructose and fructooligosaccharides) and galactans (galacto-oligosaccharides)[56], are known to promote the proliferation of beneficial lactic acid producing species such as *Bifidobacteria* and *Lactobacilli*[57]. Saccharides such as inulin and other fructo-oligosaccharides, galactooligosaccharides, and polydextrose are widely used to improve gastrointestinal outcomes and display positive effect on intestinal permeability[58]. Animal studies showed that prebiotic treatment dose-dependently increases *Bifidobacteria*[49,59], reduces gut permeability and endotoxemia[60,61] and improves glucose tolerance[62]. Prebiotics and their fermentation products have been shown to reduce gastrointestinal permeability by a variety of mechanisms: direct effect of the SCFA butyrate on gut epithelial cells integrity[63], indirectly potentiating the local overgrowth of symbionts and mucin production[55,56,64,65,66,67,68].

Drugs Affecting Immune System

In Crohn's diseases, corticosteroids induce clinical remission of the disease together with a clear reduction of the intestinal permeability in an approximately 50% of patients as measured by the lactulose/mannitol ratio[69]. Similar data were shown in active UC as well as in children

and adolescent patients. This effect is related primarily to anti-inflammatory properties of corticosteroids, including the capacity to inhibit the expression of proinflammatory cytokines such as TNF-α and NF-kB[2].

Also 5-aminosalicylic acid (5-ASA), more relevant for ulcerative colitis patients and mild diseases, display positive effect on intestinal permeability as well as inducing the reestablishment of mucosal integrity through TGF pathway and the P-PAR-_ pathway[2,70]. Anti-TNF-α agents are standard of care in IBD since at least 10 years, inhibiting the TNF-α pathway, reducing inflammation and restoring mucosal integrity. An interesting study on twenty-three patients with active Crohn's disease, using 51CrEDTA test to evaluate intestinal permeability 4 weeks before and after a single infusion of 5 mg/kg infliximab, demonstrated that the effect of this drug was also associated to an important reduction in small intestine permeability and overall permeability. This reduction was proportional to disease activity index and mucosal healing[71].

Drugs Affecting Mucosal Barrier

Mucosal protectors, like sucralfate and bismuth, has been used for a long time in the treatment of peptic disease. These compounds protect the epithelial cells from gastric acids and digestive enzymes. Mucosal protectors also seem to be effective in reducing intestinal inflammation in infectious diarrheas: for example, gelatin tannate has been found to be capable of forming a protective mucoadhesive film in the intestine, reducing inflammation of the wall and bacterial fermentations in children with acute diarrhea[2]. Gelatin tannate, by reducing the clinical activity of acute colitis and the proinflammatory effects of lipopolysaccharide (LPS), is emerging as a mucosal barrier protector, for its property of intestinal barrier modulator. It is a combination of tannic and gelatin, and may act by creating a protective film forming bonds with the mucin, thereby protecting the gut from the aggressive penetration of commensal bacteria (barrier protector)[5], alone or in combination with probiotics[49,72,73].

Assessing Intestinal Permeability

Several techniques have been developed to study intestinal permeability, mostly including indirect methods which allow, using specific probes, to measure intestinal permeability trough urinary or blood samples analysis (Table I). Probes could be metabolic active or not active, radiolabeled or not, selective or non-selective for differential segment of the intestine. Usually these probes are differentially transported across the intestinal epithelium, or by trans-cellular or paracellular routes. The paracellular route is more similar to the diffusion and it is not carrier-mediated. Intact intestinal epithelial barrier is essential for preventing penetration of these molecules. An increase intestinal permeability can be measured with the increased concentration in the blood or urine of such probes. Most of the probes used to measure intestinal permeability are water-soluble, and therefore, incapable to penetrate the lipid bilayer of enterocytes membranes: their concentration within the body is therefore more dependent on paracellular route through the tight junctions. The smaller probes can easily pass through the small, more numerous and more accessible tight junctions of the villous tips, whereas the larger probes have to make use of the larger, less accessible and less numerous pores at the crypt base. Usually small proportions of the utilized probes get through the intestinal mucosa, reach the circulation, get filtered by the kidney and get measured in the urine. However, the urinary excretion of a test probe could be dependent on several non-mucosal factors (such as gastric emptying, intestinal transit, renal clearance and incomplete urine recovery) other than the mucosal integrity itself. Combining at least 2 probes was proposed to minimize confounding factors[74,75]. Finally, direct measurement of gut barrier integrity uses the Confocal Laser Endomicroscopy (CLE) technology[2]. Of interest, although their use is more limited to research only, are the *in vitro* techniques of gut permeability measurement. Here is a summary of principal techniques and their clinical or research use (Table I).

Lactulose/Mannitol (L/M) for the Measurement of Gastro-Intestinal Permeability

It is commonly used to measure intestinal permeability. This noninvasive test has been used in clinical practice for the estimation of intestinal permeability in patients with atopic dermatitis[76,77], cow's milk protein intolerance[78,79], celiac disease[80,81.82.83], cystic fibrosis[84], Crohn's disease[85,86], acute and chronic diarrhea[87-89] and other diseases[90,91,92]. This procedure is based on the oral administration of two compounds of different molecular size and absorption route, and on the measurement of their urinary excretion. Monosaccharides, such as mannitol (M), pass through the

Table I. Techniques available to measure intestinal permeability in humans.

Procedure	Type	Suitability for clinical practice	GI tract segment of analysis	Main references
Caco-2 coculture system	*In vitro*		na	
TEER	*In vitro*		na	
Lactulose/Mannitol RATIO in urinary excretion	*In vivo*	*X*	Mainly small intestine	69-85,86
Sucrose in urinary excretion	*In vivo*	*X*	Mainly gastric	90,91
Sucralose in urinary excretion	*In vivo*	*X*	Mainly colonic	93,94
CrEDTA test	*In vivo*	*X*	Whole gut	96,97
Studies on intestinal human biopsies	*In vivo*		*Depending on site of the biopsy*	
Serological markers of intestinal permeability (zonulin, LPS)	*In vivo*	*X*	*Whole gut*	*24,104-111, 115-117*
Confocal Laser Endomicroscopy (CLE) technique	*In vitro*	X, depending on availability of endoscopic confocal facility	Depending on site of the biopsy	

transcellular routes of aqueous pores, reflecting the degree of absorption of small molecules. Disaccharides, such as lactulose (L), pass through the intercellular junction complex, reflecting the permeability to large molecules. In disorders of the small intestine, transcellular permeability tends to decrease, reflecting a diminished number of mucosal cells, whereas paracellular permeability tends to increase, reflecting damaged tight junctions. The permeability of mono- and disaccharides is compared and expressed as the ratio L/M. The ratio of the excretion percentage of lactulose and mannitol in urine is a sensitive, direct, accurate and non-invasive indicator of intestinal permeability. The lactulose/mannitol test is performed after an overnight fasting and a pre-established diet, to minimize confounding factors like ingestion of high dosage of mannitol (chewing gum, sweeteners, etc.). The solution contains a standard dose of lactulose and mannitol (a consensus is not available, usually 5 g of mannitol in 250 ml of water and 10 g of lactulose in 250 ml of water are considered an average dosage). The total urine volume collected is measured after 6 hours of collection (it is possible to cryopreserve the sample at -20°C until analysis). Several procedures have been reported for urinary quantification of Lactulose and Mannitol, but to date, the most used is the HPLC-MS/MS (high performance liquid chromatography-mass spectrometry), a sensitive and specific assay. The fractional excretion of lactulose is usually calculated from the ratio lactulose excreted (mg)/ lactulose ingested (mg). The amount lactulose excreted is obtained from mg/L lactulose per liter of urine. The same is for mannitol. The values of lactulose and mannitol calculated in the pre-test urine as mg/L are subtracted from the same value obtained in the 6 h collected urine. Results are expressed as ratio of the fractional excretion of lactulose to the fractional excretion of mannitol (L/M ratio). Usually, L/M ratio >0,030 has the meaning of increased intestinal permeability[93]. Lactulose and mannitol represent ideal compounds for measuring differential sugar absorption because they are passively absorbed and not metabolized by human cells before urine excretion. Lactulose and mannitol, however, are degraded by colonic bacteria: for this reason this test is more influenced by gastroduodenal and small intestinal permeability[75]. With this analysis, the intra-individual differences in gastric emptying, small intestinal transit, and urinary excretion are therefore eliminated[94]. Furthermore, the L/M urinary test is widely accepted as a reliable method for assessing small intestinal permeability, because nontoxic, non-invasive, simple to perform, relatively inexpensive, and reproducible[95]. It is currently used also in pediatric population. The contemporary analysis of intestinal gases hydrogen and methane could be of some help in gastrointestinal disorders, however dedicated study are encouraged.

Sucrose (SAC) for the Measurement of Gastric Permeability

Sucrose is a disaccharide that has been demonstrated to indicate gastroduodenal permeability when measured by 5 hours from ingestion in urine[96] (when the SAC urinary excretion was >

0,23% at 5 h post dose administration, it was considered value to classify increased permeability)[75]. It is degraded in the first three hours after ingestion and it is hydrolyzed by the enzyme sucrose-isomaltase, which is well expressed in the duodenum. As hydrolysis of sucrose is very fast, it has been shown that measurement of sucrose in the urine is dependent mainly on the gastric permeability[97]. Sucrose permeability is simple, cheap and readily accepted by patients. This test was proposed as possible not invasive technique to follow up patients at risk of upper GI disease, like those exposed chronically to oral NSAID[98].

Sucralose for the Measure of Colonic Permeability

Sucralose is an artificial sweetener formed by the chlorination of sucrose and is a unique disaccharide probe which is stable in the colon, since it is not fermented by the action of gastrointestinal bacteria and can therefore be used as a measure of whole gut permeability[99]. Sucralose is often administered concomitantly with other sugars (triple or quadruple sugar test) for the study of the entire intestinal tract. For example, a 'triple-sugar' test, with lactulose, mannitol and sucralose had been used in humans to assess gastrointestinal damage caused by non-steroidal anti-inflammatory drugs (NSAIDs)[100] and nicotine patches[101]. In other studies, which used a four probe based solution (sucrose, lactulose, mannitol, sucralose) urine were analyzed at 0 to 3, 3 to 5, and 5 to 24/26 hours to estimate permeability of the gastric and proximal small intestine, distal small intestine, and colon, respectively[2,102]. IBS patients display a positive test, indicating increased intestinal mucosa permeability[97].

51 Cr-EDTA, the Radiolabeled Probe to Measure

Intestinal Permeability

A common non-degraded radiolabeled chelate used in the assessment of mucosal permeability is 51-chromium labelled ethylene-diamine-tetra-acetate (51Cr-EDTA). It is a chemically stable hydrophilic chelate with a molecular weight of 360 and a radius of 7A. 51Cr-EDTA is not metabolized in the tissue and found non-toxic even at high plasma concentrations[103]. The test consists in the measurement of radioactive labelled molecules in the urine after an oral administration, or assessment of plasma clearances of tracer. 51Cr-EDTA is thought to cross the intestinal epithelium via para-cellular route. This test is used to study the intestinal permeability index in both humans and animals[103]. In particular, after an overnight fasting and pretest dietary restrictions, as for the triple sugar test, patients ingest 1.85 MBq of 100 μL of 51chromium labelled ethylene-diamine-tetra-acetate (51Cr-EDTA), in 100/120 mL of water followed by 200 mL (300 kcal) of a nutritional supplement). The composition of the test meal is standard (protein 12 g, carbohydrate 36.8 g, and fat 11.6 g) Eating and drinking is not allowed for the first 3-4 h. Subjects collect their urine in three containers with 0.5 mL 20% chlorhexidine for time periods 0-3 h, 3-5 h, and 5-24 h, to relate to permeability within the proximal small intestine, distal small intestine, and large intestine[104]. Other groups proposed urine collection from 0 to 6 h[105] and from 0 to 24 h[106]. Collected urine is counted for radioactivity in a γ – scintillation counter in triplicate. Results are expressed as the percent urinary excretion of the orally administered dose of 51Cr-EDTA[104].

Other tests

Other probes used seldom and mostly in experimental settings including iohexol test and PEG test[54,70]. Briefly iohexol is contrast agent (large molecule of 821 Dalton) with a low absorption under normal conditions, not binding serum proteins and filtered through the glomerulus without indications of tubular secretion or reabsorption. In IBD patients (50% of Crohn's patients and 31% of ulcerative colitis patients), iohexol was increased within serum by 3 and 6 h following oral ingestion[107]. On the other hand orally administered polyethylene glycole urinary recovery was increased in obstructive jaundice and severe pancreatitis[108,109].

Serological Markers of Intestinal Permeability: Serum Zonulin and Others

Research during the development of a vaccine for Vibrio cholera led to the discovery of zonula occludens toxins, an enterotoxin which is able to reversibly open intracellular tight junctions[110]. The discovery of zonula occludens toxins has shed light on the intricate mechanisms involved in the modulation of the intestinal paracellular pathway[20]: zonula occludens toxins causes polymerization of actin of targeted cells leading to disassembly of tight junction complexes through a protein kinase C (PKC)-dependent mechanism[111,112] because it causes the inactivation and cleavage of zonulin, determining a consequent increase of jejunum and ileum permeability[23,113].

The cleaved form of zonulin is released from the intestine and it circulates in the peripheral blood, being easily measured by ELISA kit[30]. In humans serum zonulin strongly correlated with the lactulose/mannitol urinary ratio[24]. Other studies[114] suggested that Intestinal permeability can also be detected indirectly by assessing serum lipopolysaccharide levels (LPS) using ELISA kits. LPS is the major component of the outer membrane of Gram-negative bacteria and is composed of a hydrophobic lipid (lipid A), a hydrophilic core oligosaccharide and a repeating hydrophilic polysaccharide side chain (O-antigen)[115]. Under physiological conditions, an intact intestinal lining not only protects the host from direct interaction with pathogenic gut bacteria (likely to increase during dysbiosis) but also prevents the translocation of bacteria and bacterial endotoxin (e.g., Lipopolysaccharide, LPS) to systemic circulation[116]. An injured intestinal barrier allows LPS to go through intestinal mucosa and enter blood circulation, prior then a real bacteria translocation because of a lower molecular weight[114]. Increased LPS has been associated to a high-fat diet, resulting in "metabolic endotoxemia"[115], leading to insulin resistance development[117,118], T2DM and atherosclerosis[116,119-121]. Local intestinal and systemic inflammation lead to overexpression of proinflammatory cytokines[122] that in turn increase gut permeability[123] and further increase in LPS translocation[27] leading to a vicious cycle[116,124]. Consistently, patients with obesity, diabetes, CVD, and NAFLD have higher circulating LPS levels than healthy individuals[116]. Additionally, measurement of d-lactate (a product of anaerobic metabolism from intestinal bacteria) concentration in the circulation may reflect colonic absorption of bacterial metabolism products. d-lactate and bacterial endotoxins are considered primarily as markers of colon absorption, partially reflecting the permeability of the intestinal wall[125-127].

Direct Methods to Measure Intestinal Permeability: Confocal Laser Endomicroscopy (CLE) Techniques

Combining endoscopy and histology is reality nowadays, with the consequent possibility to evaluate intestinal permeability *in vivo*[15]. Briefly, patients undergone to endoscope-based Confocal Laser Endomicroscopy (eCLE) can be assessed with 1000-folds magnification of the intestinal mucosa with a lateral resolution of 0.7 μm. The intravenous injection of fluorescein sodium at standard intervals, allow the detection of the "fluorescein leakage", the direct evidence and measure of a pathological "leakage" of the gut barrier[15]. Very recently, to quantify the severity of the barrier dysfunction, a new quantitative numerical score, the Confocal Leak Score (CLS), has been developed[15]. Previous classifications of the barrier dysfunction by CLE included the Watson grade and the epithelial gap counts[128]. Mainly for colonic permeability measure, "Epithelial gap density" has been proposed as a surrogate marker of intestinal permeability. It is defined as the number of intestinal epithelial gaps normalized to total epithelial cells counted on CLE images. It is a reproducible semi-quantitative measure and is significantly increased in IBD patients[129]. The intestinal epithelial gaps can be observed by using confocal laser endomicroscopy (CLE)[130].

In vitro Modality of Measurement of Intestinal Permeability

TEER

Transepithelial/transendothelial electrical resistance (TEER) is the measurement of electrical resistance across a cellular monolayer and is a very sensitive and reliable quantitative method to confirm the integrity of tight junctions in monolayers of epithelial and endothelial cells. TEER reflects the ionic conductance of the paracellular pathway in the epithelial monolayer[131] and therefore has been used in studies on the transport of drugs, chemicals, dyes, and general membrane leakage[132]. The electrical resistance of a cellular monolayer, reported in units of $\Omega.cm^2$[133], is a quantitative measure of the barrier integrity[134]. The classical setup for measurement of TEER consists of a cellular monolayer cultured on a semipermeable filter insert which defines a partition for apical (or upper) and basolateral (or lower) compartments. An alternating current (AC) voltage signal is applied at a frequency of 12.5 Hz to avoid any charging effects on the electrodes and the cell layer[133] and with a current of 10 Ma[135]. Ohm's law is used to calculate the electrical resistance of the system[136]. Intestinal cells derive from stem cell-derived intestinal organoid cultures, which in turn use media containing canonical Wnt ligand, Responding, and Noggin to support intestinal epithelial stem cell growth. Once enough intestinal cells from these 3D spheroid/ organoid cultures are generated, 2D intestinal epithelial monolayers can be created on Transwell membranes for assays[137]. At present, there are numerous cell lines and their co-cultures being studied for *in vitro* models of

the GI tract. The most widely used cell line for developing human GI tract *in vitro* models is the Caco-2 line, which can be maintained easily in cell culture for many weeks and are capable of establishing tight junctions in culture[133]. The addition of fibroblast co-cultures seems not to alter TEER readings, but provide a more heterogeneous monolayer with prismatic cells and luminal cystic structures in the epithelium, as shown by hematoxylin and eosin staining. There also has been research on the co-culture of intestinal epithelial monolayers with human monocyte-derived macrophages to investigate the importance of the interaction of the intestinal epithelium with the mucosal immune system. For example, it was found that the presence of monocyte derived macrophages with intestinal epithelial cells derived from differentiated enteroids increased TEER and barrier function from approximately 800 $\Omega.cm^2$ to approximately 1000 $\Omega.cm^2$[138], suggesting a potential role of the macrophages in enhancing maturation of the intestinal epithelium and thickening the physical barrier[132].

TEER measurements and dye flux assays, such as the FITC-DEX assay[132], frequently are performed together to provide a thorough characterization of the barrier function of cell monolayers[139]. Another more recent study showed that Aflatoxin M1 (AFM1) and ochratoxin A (OTA), mycotoxins commonly found in milk (but also in cereals and beans) individually or collectively increased the paracellular flux of lucifer yellow and fluorescein isothiocyanate (FITC)-dextrans and decreased transepithelial electrical resistance values in differentiated Caco-2 cells after 48 h of exposure, indicating increased epithelial permeability[140]. There are also many other advantages in the use of TEER: it is in real-time, is nondestructive, often noninvasive, can be applied to monitor live cells during their various stages of growth and differentiation and allows cell cultures to be re-used for additional studies[133].

Conclusions

Intestinal permeability is a clinical entity associated to intestinal and extra-intestinal diseases. It is an overall measure of intestinal homeostasis and gut barrier integrity. Different drugs can affect intestinal permeability in healthy and disease as shown by several publications or trials, dealing with gut microbiota modulation (antibiotics, probiotics, food and diet), gut barrier protection and/or intestinal mucosa immunity. The measure of intestinal permeability is a major challenge to increase use and utility of this measure in clinical practice. Even if a standardized measure has not been developed, reproducible methods have been described and summarized. The use of such old or new and emerging methodologies, perhaps standardized with dedicated studies, could force the clinician to develop personalized approaches to difficult disease like the one associated to increase gut permeability.

Conflict of Interests

No potential conflicts of interest. No financial support.

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