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REVIEW

Intestinal Permeability in Relapsing-Remitting Multiple Sclerosis

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Abstract Changes of intestinal permeability (IP) have been extensively investigated in inflammatory bowel diseases (IBD) and celiac disease (CD), underpinned by a known unbalance between microbiota, IP and immune responses in the gut. Recently the influence of IP on brain function has greatly been appreciated. Previous works showed an increased IP that preceded experimental autoimmune encephalomyelitis development and worsened during disease with disruption of TJ. Moreover, studying co-morbidity between Crohn's disease and MS, a report described increased IP in a minority of cases with MS. In a recent work we found that an alteration of IP is a relatively frequent event in relapsing-remitting MS, with a possible genetic influence on the determinants of IP changes (as inferable from data on twins); IP changes included a deficit of the active mechanism of absorption from intestinal lumen. The results led us to hypothesize that gut may contribute to the development of MS, as suggested by another previous work of our group: a population of CD8+CD161high T cells, belonging to the mucosal-associated invariant T (MAIT) cells, a gut- and liver-homing subset, proved to be of relevance for MS pathogenesis. We eventually suggest future lines of research on IP in MS: studies on IP changes in patients under first-line oral drugs may result useful to improve their therapeutic index; correlating IP and microbiota changes, or IP and blood-brain barrier changes may help clarify disease pathogenesis; exploiting the IP data to disclose co-morbidities in MS, especially with CD and IBD, may be important for patient care.

Keywords Multiple sclerosis · Intestinal permeability · Mucosal-associated invariant T (MAIT) cells · Autoimmune comorbidity · Celiac disease · Crohn' disease

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Introduction

The gastro-intestinal surface is the main place of interaction between external and internal stimuli because of its quantitative (approximately 400 m^2 of extension, the largest one in the body) and qualitative peculiarities. It serves as barrier, microbiota harbor, surface of nutrients absorption, and site of crucial interaction between immune, endocrine and nervous system. The structural components of this complex surface are at least three: the mucus layer, a physical barrier covering the epithelium, made of mucins and anti-bacterial molecules that contribute to spatially segregate the microbiota from the epithelium top [1]; the epithelial cells, connected by tight junction (TJ) proteins that regulate the paracellular permeability; an immunological barrier, mainly constituted by M cells and elongations of "antigen presenting cells" dwelling in the lamina propria, that scan the luminal antigens and elicit tolerogenic or pro-inflammatory programs according to the friendly or harmful nature of the trigger, as well as by intraepithelial

lymphocytes and Paneth cells secreting anti-bacterial peptides. In the context of these barrier functions there are several notable differences between large and small intestines. Few enzyme-secreting cells are found in the wall of the large intestine, and there are no circular folds or villi; the large intestine has instead far more intestinal glands, which contain a vast population of goblet cells secreting mucus favoring the movement of feces and protecting the intestine from the effects of enteric bacteria. Microbes are more abundant in the colon than in the small intestine, which accounts for the thicker mucous layer standing above the layer of colonocytes. The small intestine is characterized by the presence of M cells and Paneth cells that 'sense' microbes and produce antimicrobial peptides. The lamina propria, underlying the epithelium barrier, is another place of complex interactions harboring large amounts of cells particularly from the innate immunity, that are often typically or solely present in the gastro-intestinal tract, as well as of cell and circuits connected to the enteric and central nervous system (the so-called "gut-brain axis").

The crossing of gastro-enteric barriers occurs by transcellular transport or para-cellular pathway—the former is largely dependent on selective carriers especially involved in nutrient absorption; the latter takes place in the intercellular space and tend to be low, under the strict control of the TJ proteins, such as occludin, claudin, and zonulin-1. TJ proteins are the main players in the regulation of intestinal permeability (IP), in specific, occludin and claudin interact with zonula occludens proteins, that in turn bind cytoskeleton, regulating cell cycle, cell polarity and permeability functions. TJ assembly and disassembly occur under the influence of several cues, coming from intra- and extra-cellular triggers, such as dietary factors, microbiota components, molecular signals from cytokines, growth factors and proteases, intra-cellular kinases and level of cell metabolic status [2].

Changes of IP have been extensively investigated in inflammatory bowel diseases (IBD) and celiac disease, two autoimmune disorders with a known unbalance between microbiota, IP and immune responses in gut [3, 4]. Most recently the influence of IP changes on organs far from gut has been appreciated, with special attention to how it affects brain function. An altered physiology and/or immune function of gut mucosa may impact IP leading to an increase of epithelial paracellular space. A change in intestinal permeability may be considered as a biomarker for local or even distant immune-mediated disorders. In fact, increased gut permeability allows the passage of macromolecules, toxin, and bacterial species, both pathogenic and commensal, through the intestinal epithelial layer. This event may trigger immune-mediated illnesses in different systems, even distant from the gastrointestinal tract, such as central nervous system [5, 6]. Following this hypothesis, Fasano et al. measured zonulin levels in patients with multiple sclerosis (MS) and healthy controls, finding higher levels in diseased subjects with active disease, while patients in the remitting phase showed values comparable with healthy controls [7]. In this context, recent studies have pointed out the role of gut and microbiome dysfunctions and also a potential role of commensal microbiota in the pathogenic process that underpins the development of experimental autoimmune encephalomyelitis (EAE), the animal model of MS [8–10]. These investigations, among others, led to the current concept of "microbiota-gut-brain axis", including multiple possible ways of communication such as the gut-brain's neural network, the neuroendocrine-hypothalamic pituitary adrenal axis, the gut immune system, some neurotransmitters and neural regulators synthesized by gut bacteria, and barriers including intestinal mucosal barrier and blood-brain barrier.

Intestinal Permeability in Mmultiple Sclerosis: the Past

Very few studies on IP changes in MS and EAE are present in literature. The first report of increased IP in a minority of cases with MS was described approximately 20 years ago, studying co-morbidity between Crohn's disease and MS [11]. Most recently, the speculation regarding the potential role of the microbiota-gut-brain axis in the pathogenesis of demyelinating diseases prompted studies on IP changes in EAE and MS. In 2014, Nouri et al. showed that an increased IP precedes EAE development and it is worsened during disease with disruption of TJ. These changes seem to be associated with unbalance of mucosal immunity with prevalence of pro-inflammatory Th1−Th17 subsets over T regulatory cells. Moreover, they showed that similar alterations of intestinal barrier occur in the passive model of EAE after transfer of encephalitogenic T cells [12]

Along this line of thinking our group recently found changes of gut barrier in patients with relapsing-remitting disease [13]. Our initial hypothesis consisted in verifying whether a dysfunction of gut permeability may contribute to MS immune-pathogenesis. Intestinal dysfunction may be quantified using a gastrointestinal test that directly measures the ability of lactulose and mannitol, two non-metabolized sugar molecules, to permeate the intestinal mucosa. The urinary levels and the ratio of the two sugars reflect the degree of IP and of mucosal abnormalities of the small intestine. We analyzed IP in MS patients compared to healthy donors, including also couples of disease-discordant monozygotic twins, in order to assess how genetic factors may influence this data.

We enrolled patients with relapsing-remitting MS [14] aged between 25 and 55 years. The exclusion criteria were the presence of serious medical or psychiatric illnesses, conditions possibly interfering with the IP test, with special attention to gastrointestinal disorders (i.e. dyspeptic disturbances, dysfunction of gastro-intestinal motility and any other relevant gastrointestinal disease), renal function (as indicated by serum

creatinine level), and bladder dysfunction (when indicated, a negative ultrasound and/or urodynamic examination was obtained), pregnancy and breast-feeding. Age- and sex-matched healthy individuals were enrolled as controls. All participants followed a lactose-, lactulose-, and mannitol-free diet for 72 h before the test. A list of forbidden food was provided. A contrast-enhanced MRI was obtained from MS patients within 3 months from sampling. The urine concentrations of lactulose and mannitol were measured using a method based on liquid chromatography combined with mass spectrometry [15]. Results are expressed as a ratio of the fractional excretion of lactulose to the fractional excretion of mannitol (L/M ratio), which quantifies the IP. The value of urinary mannitol concentration reflects the surface of intestinal wall able to actively absorb substances from the lumen. The permeability was considered altered when the L/M ratio was > 0.03, while normal concentration of urinary mannitol was <900 mg/L. These values were obtained from a group of historical controls that were in line with published data [16].

Twenty-two patients (mean age 37.5 ± 9.93; F/M ratio 15/7) and 18 healthy donors (HD; mean age 38.5 ± 10.66; F/M ratio 15/3) were studied. We enrolled five twin pairs: one was concordant and 4 were discordant for disease. Among affected individuals, 18 were treatment naive, while 4 had stopped disease-modifying therapies at least 6 months before study enrolment. All the investigations were performed at least 4 months after the last steroid therapy. A significant difference in the proportion of participants with increased IP was observed (16/22 (73%) patients vs 5/18 (28%) HD; p=0.005). The difference was even more significant without considering twin couples, suggesting a genetic influence on IP determinants (13/16 (81%) patients vs 4/14 (28%) HD; p<0.001) (Fig. 1). Accordingly, the continuous variable of L/M ratio showed significantly higher values in patients compared to controls (p=0.0284 and p=0.0176 considering only non-twin patients). Urinary mannitol concentration was significantly lower in patients compared to controls (Fig. 2; p=0.022 for all participants, and p=0.0168 without considering twins), suggesting a deficit of the active mechanism of absorption from intestinal lumen. We found no significant correlations between IP changes and the main clinical/radiological parameters of the patients (EDSS, disease duration, relapses occurring 6 months before and 6 months after the IP test, disease activity at MRI); though, since our study is a pilot one, with a small sample size, it was likely not sufficient to find such correlations.

Our study suggests the following points: an alteration of IP is a relatively frequent event in MS; there is a genetic influence on the determinants of IP changes (as inferable from data on twins); IP changes include a deficit of the active mechanism of absorption from intestinal lumen, as suggested by the significant decrease of urinary mannitol concentration in MS patients.

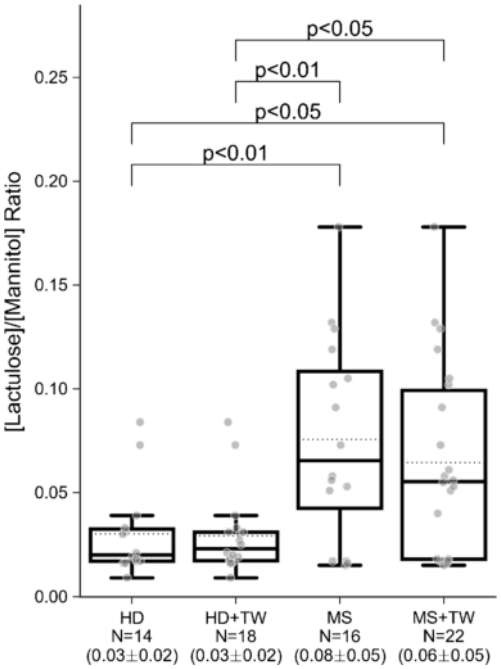

Fig. 1 Evaluation of the intestinal permeability by urinary lactulose (Lac) / mannitol (Man) ratio. Values in healthy controls (HD) and MS patients, respectively, without and with twin pairs (TW)

The above-mentioned study, aimed at identifying an association between Crohn's disease and MS, used peripheral blood CD45 isoform expression and IP as biomarkers and reported abnormalities in a minority (approximately 20%) of patients (10). Our study supports the conjecture regarding the role of gut in the disease development showing an unprecedented proportion of IP changes in patients with relapsing-remitting MS. To understand whether IP changes are associated with neuroinflammation as an epiphenomenon or are part of causative factors, we need future studies that correlate IP changes with alterations of microbiota and their systemic effects on immune regulation.

Current clinical and experimental data do not allow drawing conclusion about the role of IP changes in MS pathogenesis. The above cited EAE paper showed that the circulating autoreactive T cells play an essential role for morphological and functional changes of gut barrier [12]. However, in a recent study that showed a correlation between microbiota alterations and Th17 subset expansion in gut of MS patients during disease activity, no data on IP were obtained [17]. Hence it is currently not known whether and to what extent a relationship between increased intestinal permeability and resultant effects on gut dysbiosis and systemic immune dysregulation can be considered as a trigger for MS development.

Another recent study from our group supported again the hypothesis that gut is somehow involved in MS pathogenesis. $CD161^{high}CD8+$ T cells that overlap with mucosal-associated invariant T (MAIT) cells, seem to contribute to the pathogenesis of MS [18]. MAIT cells have recently received much

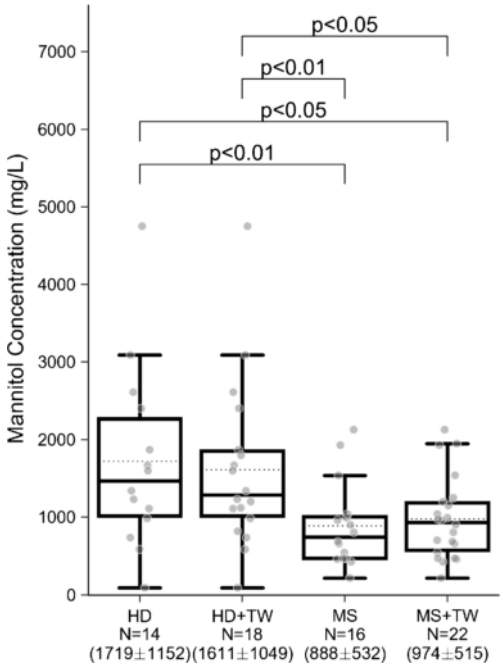

Fig. 2 Urinary concentration of mannitol (expressed as mg/L), reflecting the surface of intestinal wall able to actively absorb substances. Values in healthy controls (HD) and MS patients, respectively, without and with twin pairs (TW)

attention as a gut- and liver-homing subset, responding to microbes and capable of several effector functions, shared by both innate-like and adaptive arm of T cell immune responses. Furthermore, MAIT cells constitute a subset of αβ T lymphocytes characterized by a semi-invariant T cell receptor alpha chain. The xMHC class I-like protein is responsible for presenting bacterially-produced vitamin B metabolites to MAIT cells, which become activated and go through clonal expansion, memory, and an array of antimicrobial responses. In humans, MAIT cells express high levels of CD161, interleukin-18 receptor, and chemokine receptors, such as CCR6 on their cell surface [19].

Using different approaches including microarray technology on circulating CD8+ T cells from monozygotic twins discordant for MS, flow cytometric analysis, and post-mortem examination of MS brains we obtained concordant results. Moreover, peripheral CD8+ T cells expressing at high density CD161+ (NKR-P1A) (bio)-marks MS compared to healthy status (Table 1). Also, the settlement of this subset in brain lesions as well as other functional evidence, such as the production of interleukin 17 and expression of CCR6, strongly suggests that CD161+ CD8+ T cells participate in the pathogenesis of MS by acting as effectors and targeting the CNS. MAIT cells are also activated in IBD, and this results in an increased recruitment towards the inflamed tissues and a switch in the pattern of cytokine secretion toward a pro-inflammatory profile [20]. Similar findings are seen in psoriatic arthritis

Table 1 Flow cytometric analysis of peripheral blood CD8+ T cells expressing CD161. Values are the mean (SD) percentages of CD8 + T cells belonging to each subset ($CD161^-$, $CD161^{low}$ and $CD161^{high}$)

	MS (*n*=20)	HD (*n*=47)	MS vs HD
$CD\ 161^{high}$	15.4 ± 8.2	8.3 ± 8.2	p =0.003
$CD161^{low}$	7.9 ± 3.1	9.1 ± 5.7	ns
$CD161^-$	76.6 ± 10.2	79.7 ± 13.7	ns

MS Multiple Sclerosis, *HD* healthy donors, *ns* not significant

where IL-17-producing CD8+ T cells are found in the joints and might contribute to the disease process [21].

Intestinal Permeability in Multiple Sclerosis: the Future

The IP role in MS is currently a very interesting topic for at least two reasons. First, it may have relevance for oral treatments that have been recently introduced in clinical practice as first-line disease modifying therapies. As a matter of fact, these drugs are metabolized to the active form or excreted through the bile in the intestine, and are not rarely associated with gastrointestinal adverse effects, that may even lead to their discontinuation. Studies on IP changes in patients under first-line oral drugs may prove to be useful in improving their therapeutic index and to allow a personalized medical approach in the care of patients with relapsing-remitting disease. Secondly, the complex interplays occurring at the gastrointestinal level suggest future investigations where the IP status should be linked to other key variables, such as MAIT cells and microbiota, in order to deepen the role of gastro-enteric alterations in MS development. In this context, the possible relationship between blood-brain barrier and IP may be another intriguing subject for future research. A recent work from Rescigno's group on how gut-vascular barrier controls systemic dissemination of bacteria may support this relationship [22]. Future studies might analyze whether and how changes of blood-brain and blood-gut barrier can interact with each other.

The above issues may require methodological improvements of future research on IP changes in MS patients. Our results need to be confirmed on a larger population of patients, possibly by serial determinations, to complete our understanding of whether and how IP changes affect disease course. Further studies on barrier dysfunctions in large intestine, through oral chromium (51 Cr)-EDTA, may integrate the work on small intestine and help clarify this point. Recent methods, using multi-sugar tests, allow to assay different segments of gastro-intestinal tract and may be more informative to help understand which level of the intestine is particularly involved in the MS pathogenesis [23].

Among the peripheral markers of IP, the role of zonulin has been recently recognized. This modulator of the tight junctions has been investigated in autoimmune disorders associated with an IP dysfunction, such as celiac disease and type 1 diabetes, and its increased expression proved to correlate with increased IP [24]. Furthermore, calprotectin appears to be another good marker of altered permeability and inflammation in the colon. This protein is a heterodimer consisting of two calcium binding proteins, MRP8 and MRP14 (myeloid related proteins), which may be studied in serum or faeces of MS patients [25]. Faecal calprotectin has recently been reported as an informative and handy biomarker by recent studies in inflammatory bowel diseases [26].

Another field of interest for the IP status in MS includes its comorbidities. These have recently received much attention in the management of MS patients and are considered an important determinant of disease outcome [27, 28] (Fig. 3). Comorbidities may have implications for both clinical management and pharmacological treatment, particularly considering that existing and novel therapies for MS may influence the risk of autoimmune comorbidities, which add to known risks of spontaneous co-occurrence of autoimmune disorders in MS [29] (Fig. 4 a,b). In particular, celiac disease (CD) and IBD are among those diseases of main importance where IP changes are known to underlie pathogenic loops, supporting the hypothesis of possible relationship between gastro-enteric barrier dysfunction and MS.

CD is one of the many autoimmune diseases whose association with MS has been the subject of some, albeit limited, investigations [27]. In Europe and the United States, the mean frequency of CD in the general population is approximately 1%, with some regional differences. Although

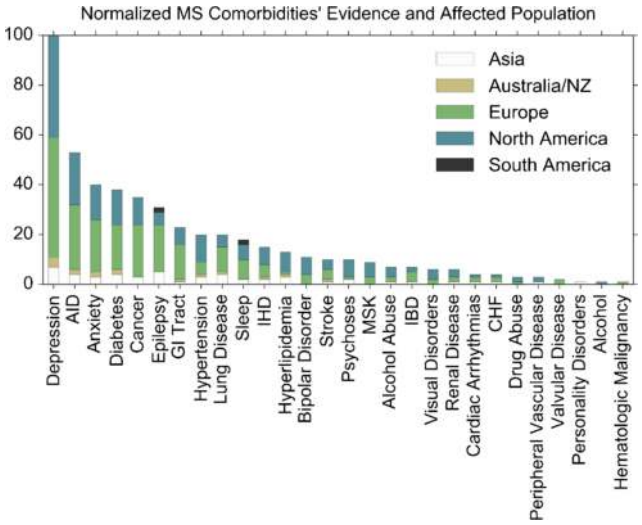

Fig. 3 Number of studies (y-axis) that reported co-morbidities in MS. Data were obtained combining all 249 studies cited in reference 36. Data was aggregated by reported co-morbidity, and then stratified by affected population

the incidence of clinically diagnosed CD cases is increasing, still a larger part of the CD cases remains undetected, with a ratio of 1:3 to 1:5, respectively, of diagnosed and undiagnosed cases [30]. Therefore, it is highly possible that a number of MS patients have simultaneously an undiagnosed CD or receive a late diagnosis of CD. The real prevalence of CD in MS is still unclear because of limited population studies and different diagnostic assessments. In different papers [31–34], the prevalence of CD ranges from 0 to 11%, with only one study showing an increased prevalence of CD in MS patients and in their first-degree relatives compared to healthy individuals [34]. Our recent work showed ten times

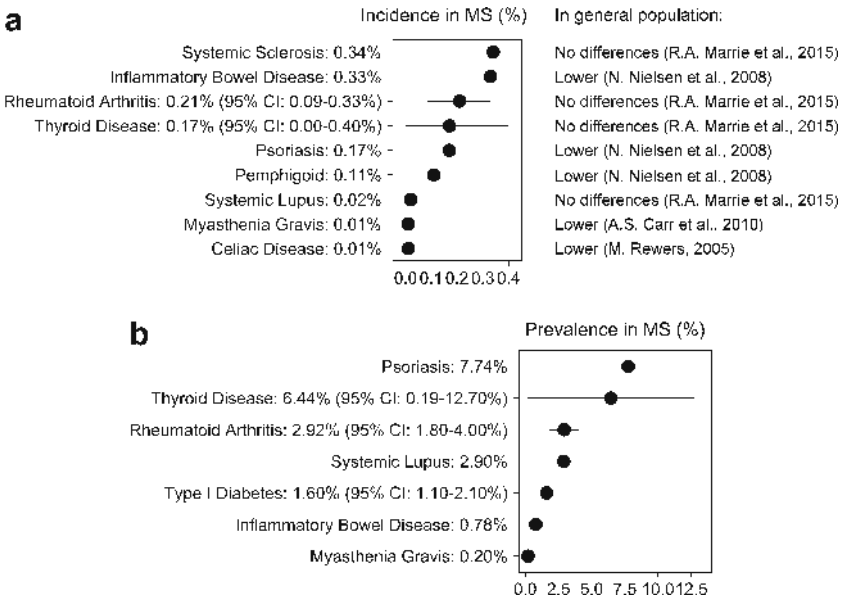

Fig. 4 Incidence (**a**) and prevalence (**b**) of autoimmune disorders co-occurring with multiple sclerosis, as reported in a recent informative review [29]

higher prevalence of MS cases in the CD population followed at a tertiary academic referral center of gastroenterology, in comparison to that reported in the general European population (submitted). Delay or failure to diagnose CD in MS patients may have several implications for MS clinical course and choice of therapeutic options. Early recognition of CD in MS patients is critical to avoid malabsorption and adverse influences on immune responses, neurological status and the absorption of orally administered drugs.

The relationship between MS, IBD and IP changes has been historically recognized, as demonstrated by the above-reported work published in 1996 [11]. The authors reported for the first time the presence of biomarkers related to Crohn's disease in MS patients, such as IP changes and high CD45RO expression in peripheral blood B cells. Another systematic review and meta-analysis focused on the association between IBD and MS and analyzed 1,086,430 patients and found concurrent diseases in 0.08% of cases; the relative risk for IBD/MS comorbidity was 1.54, without significant difference between MS or IBD registries and Crohn's disease or ulcerative colitis [35]. The significant increased risk of IBD in MS patients suggests that regular gastroenterology monitoring and possible IP studies can be used to optimize clinical practice in specific situations such as comorbidities, oral therapy, and treatment that raise the risk of autoimmune disorders in susceptible subjects. Moreover, future studies on IP status in MS and on its comorbidities such as CD or IBD may be of interest not only to assist clinical management and pharmacological treatment of patients but also to directly target IP changes. Drugs designed to counteract dysfunctions of gastro-enteric barrier may help ameliorate or normalize IP, and can also be beneficial on the immune-pathogenic loop. An interesting example of which is a work with lazarotide, a 8-mer peptide with activity as TJ regulator tested in a model of CD, which inhibits gliadin-induced macrophage accumulation in the intestine and preserves TJ structure [34]. Hopefully, future treatment focused on IP changes might be repurposed in MS field to treat patients with gut barrier dysfunction and possibly impact on the immune-pathogenic mechanisms mediating the progression of the disease.

Required Author Forms Disclosure forms provided by the authors are available with the online version of this article.

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