Review

Mucus perspective to conduct the molecular concertation in inflammatory bowel diseases

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Abstract: The inflammatory bowels diseases (IBD) are autoimmune diseases that deeply impact the patients' quality of life. The IBD pathogenesis is not yet defined, but evidence demonstrated that the IBD chronic inflammation is related to an impaired intestinal barrier. Traditionally, two actors were considered for their contribution to this disfunction: the gut microbiota and intestinal epithelium. However, a third element, which is the intestinal mucus, should be considered as peer of the epithelium and microbiota. Indeed, mucus represents the biological interface between bacteria and cells, filtering molecules or toxins and preventing bacteria penetration exploiting both structural and compositional properties. The boosting effect of the mucus characterization towards IBD comprehension is far too underestimated, although some mucus-oriented studies are already reported in literature. This work reviews the intestinal barrier features, describing each component of the gut mucosa (*i.e.*, epithelium, microbiota, and mucus) in a mucus-oriented perspective**.**

Keywords: dysbiosis, rheology, mucosal barrier; leaky gut, autoimmune,

1. Introduction

In the last years, the prevalence of autoimmune diseases increased worldwide[1]–[3]. More than 60 different pathologies are grouped as autoimmune diseases by the World Health Organization, (WHO) without stressing the differences, but focusing on the common aspects: immune cells (or their products) react abnormally to the presence of the body self-antigens, triggering non-physiological bio-mechanisms that lead to inflammation and other dysfunctions [4], [5].

The prevalence of autoimmune diseases is growing worldwide particularly considering pathologies related to the gastrointestinal tract with a net increase of ~ 6% per year [3]. The severity and the impact on patients' life depend on the specific gastrointestinal pathology. For instance, diseases such as celiac disease can be easily controlled and managed through gluten-free diets, while the inflammatory bowel diseases (IBD), such as Crohn's disease (CD) and ulcerative colitis (UC), maybe deeply debilitating and lacking a resolutive drug therapy heading for the remission of symptoms and the prevention of relapse [6].
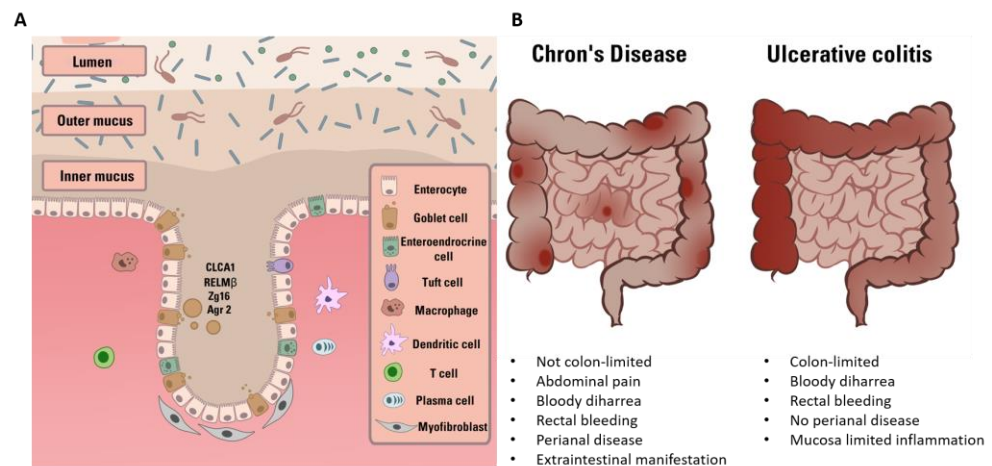
Figure 1 A) Graphical representation of the gut epithelial barrier. From the lumen to the lamina propria, the structure of the barrier is composed by: (1) outer mucus enriched of bacteria; (2) inner mucus; (3) cell layer; (4) lamina propria enriched of immune cells. B) Differences between the CD and UC characteristics in terms of inflammation distribution (darker areas in the images) and commonly related symptoms [15].

In healthy condition, the gut protects the human body as a shield against pathogens and toxins, while governing the communication between cells and the overwhelming number of microorganisms, collectively called microbiota, that symbiotically grow in the human intestine[7]. Even if the microbiota and gut-tissue crosstalk are not yet completely explored, their potential in modulating the human health are leading to new therapeutic strategies, which are well-represented by the so-called pro-, pre- and post-biotic approaches [8]–[10].

Although the microbiota and gut epithelial cells are unanimously recognized to be strictly dependent [11], [12], they do not directly act on each other but by means of a third, cell-free, intermediator: the intestinal mucus.

The intestinal mucus is a biological hydrogel covering the entire gastrointestinal tract [13]. It supports the microbiota growth and modulates, through specific physico-chemical and structural properties, the communication between bacteria and cells [13] by selectively filtering signal molecules or altering their absorption profile. Despite this, the mucus is rarely addressed with the dignity that it deserves [14]–[16] as a peer of microbiota and cells on the gut barrier properties in the study of IBD. Independently from the specific aim of the research, when a complex biological phenomenon is studied regarding the intestinal barrier, a mucus-including perspective, other than a purely cellular/microbiological one, could bring new insights in the etiology of these diseases by providing a "microbiota-mucus-epithelium" triad generating the final pathological scenario [17].

In this review, we present each component of the "microbiota-mucus-epithelium" triad separately. We particularly focus on how the mucus properties, under a materialistic point of view, may contribute as a cell-free intermediator of the biological actors in the pathological scenario of the IBDs.

2. General aspect and relevance of Inflammatory Bowel Disease

From 1990 to 2017, the number of patients diagnosed with autoimmune IBD doubled from ~ 4 to ~ 8 millions, with outstanding increase in respect to the past decades [18].

The diagnosis of CD and UC is not a simple process (Figure 1), as they are studied with techniques developed to highlight the shared molecular mechanisms that trigger the inflammatory response of cells. Abdominal pain, severe incontinence (more than 10 evacuations per day), colon dilation, continuous bleeding and weight loss, are specific indicators of UC but not of CD [19]. In this last case, highly variable symptomatology combined with other techniques as endoscopy, radiology and histology, makes the analysis of the

patient's history the preferential approach to the disease management [1], [15]. In case of CD, the inflammation can be patchy extend to different districts of the gastrointestinal (GI) tract, with frequency in the ileum and/or colon (95-98%), and, in just 2-5% of cases, in the upper GI tract [16], [17]. Differently, the UC is a disease located in the colon, which not usually involve the whole thickness of mucosa (on the opposite of CD)[20]. According to the disease severity, the UC inflammation could extend until to involve the entire colon (*i.e.*, pancolitis)[22].

CD and UC they are commonly recognized as relapsing inflammatory diseases developed in patients with genetic predisposition combined with environmental factors (*e.g.*, diet, smoke, sanitation and socio-economic status)[23]. Intense industrial and traffic-derived pollutants (*i.e.*, NO_2 and sulphur dioxide, respectively) were associated to the IBD disease development in a still unknown age-dependent mechanism. Moreover, higher cumulative smoking (*i.e.* the number of cigarettes packages per year) was associated to a higher risk of CD development [24]. Interestingly, smoking cessation decreases the probability to incur in CD but increases the probability to incur in UC [25]. These different effects suggest that environmental factors should not be undervalued in the study of IBD, but instead should be considered as a contributing cause with the genetic predisposition to the IBD development.

Although the specific causes of CD and UC remain unknown [26] UC and CD have historically been considered as two distinct diseases, the hypothesis of a common origin has been proposed in recent years [27], [28]. Regardless the experimental evidence that support this hypothesis or not, the scientific community is unanimous in identifying the changes in either gut microbiota or mucus or cellular barrier dysfunctions as fundamental phenomena to be considered, whether they were interpreted in terms of causes or symptoms [29]–[31]. The synergy with which the components of the "microbiota-mucus-epithelium" triad generates the final phenomenon of the leaky-gut is underestimated, independently from how the biological events are considered. It is therefore essential to optimize the traditional methods used for IBD studies - and in general mucosal-associated diseases - developing new technologies and tools that are able not only to the investigate the triad as a whole, but also to distinguish one by one the contribution that each actor performs towards the final result [11], [32]–[35]
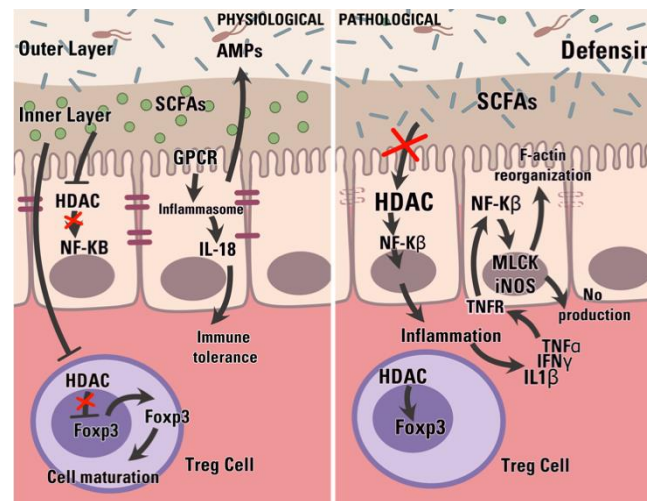
Figure 2 Graphical representation of molecular mechanisms involved in barrier impairment in a physiological (left) or IBD condition (right). (Left) SCFA-producing bacteria are abundant and release large quantity of SCFAs (green particles). These molecules interact with epithelium cells and immune cells leading to AMPs production, immune tolerance and Treg cells maturation. (Right) in IBD pathological condition, SCFA-producing bacteria together with the anti-inflammatory effect and immune tolerance decrease. Consequently, NF-kB-mediated pathway leads to pro-inflammatory factor production and immune response. All together, these mechanisms cause TJs disruption, gut barrier impairment and increase of permeability to pro-inflammatory bacterial molecules.

3. Intestinal epithelial cells in Inflammatory Bowel Disease

The epithelial barrier is a complex biological structure made of different type of highly specialized cells, which is defective in both CD and UC [36], [37]. Dysbiosis and inflammation are the two main factors studied for their effect on the cellular barrier integrity, metabolism and functionality. Butyrate-producing bacteria influence several barrier-related molecular pathways (Figure 2). Colonocytes (enterocytes of colon region) metabolize butyrate through β-oxidation process, consuming free-oxygen and establishing an anaerobic environment thus promoting obligate anaerobes over facultative anaerobes proliferation (*i.e. Bacteroides fragilis* over *Escherichia coli*) [31], [37]. Furthermore, butyrate regulates Treg cells by promoting the expression of *Foxp3* gene [38], which is one of the key transcription factors triggering the Treg cell maturation [39]. A dysbiotic condition could lead not only to a reduction of butyrate-producing bacteria, but also to the shift of colonocyte metabolism from β-oxidation to anaerobic glycolysis. This change increases levels of oxygen and nitrates and promotes the facultative anaerobes proliferation but also pathogens colonization [40]. Similarly, the butyrate depletion leads to a decrease in Treg cell maturation promoting inflammation and pro-inflammatory molecules, such as defensins and bacteriocin [41], [42] (Figure 2).

Gut dysbiosis alters also intestinal epithelium function by impairing the cellular barrier permeability. The maintenance of the epithelium integrity allows a regulated flux of nutrients, water and ions and avoids the passage of bacterial molecules or microorganisms. This regulation mechanism is mainly due to the presence of tight junctions (TJ) between epithelium cells [43]. TJs are composed by different proteins, such as claudins, occludin and others (e.g. zonula occludens-1, ZO-1 protein)[44], [45], which are sensitive to inflammatory mediators and in particular the tumor necrosis factor-α (TNFα), among others [46]–[49]. TNFα is one of the main pro-inflammatory factors produced by T cells, macrophages and monocytes during inflammation in both CD and UC [50]. This cytokine activates different and complex signaling pathways that lead to the endocytosis of occludins and to the promotion of claudin-2 expression, which forms channels for the flux of small

cations and water and is associated to leaky epithelia and intestinal barrier integrity impairment in both CD and UC   [43], [49], [51], [52].

Inflammatory mediators showed a role in epithelium integrity regulation also by triggering the nitric oxide synthase (NOS) pathway [53]. Nitric oxide (NO) is a reactive molecule synthesized by the inducible NOS (iNOS) through the oxidation of L-arginine, leading to the production of citrullin and NO. In physiological conditions, NO protects the epithelium integrity from oxidative stress-mediated by reactive oxygen species [54]. Instead, in pathological conditions, iNOS activity seems to have an opposite role as it contributes to an increase in intestinal permeability and correlates with prolonged gut inflammation [55]. Indeed, iNOS activity was found significantly higher in colonic biopsies of UC patients respect to control subjects. They showed an increasing colonic level of citrullin and thus, of NO. TNFα or the combination of IL-1α and IFN-γ are the key mediators of iNOS expression upregulation [53].

Interestingly, studies on the use of exogenous NO to treat a colonic epithelium model showed a protective role also at higher doses, in contradiction with what observed with native NO [54]. The presence of conflicting results in the literature may be indicative of the fact that cellular models, although useful tools for the decrypt the complex molecular pathways, are currently unable to model the biological phenomenon as a whole. *In vitro* models including non-biological components, such as advanced mucus models, could allow to understand whether the contradictions in the literature describes a real physio-pathological phenomenon or a bias, born by purely biological-oriented approaches without synergy with materialistic-oriented ones.

4. Intestinal microbiota and dysbiosis in Inflammatory Bowel Disease

More than 100 trillion of microorganisms inhabit the human intestine and compose the gut microbiota, including fungi, bacteria, and yeast. In a physiological condition, bacterial population is highly diversified and mainly characterized by the *phyla* Firmicutes (i.e. *Lactobacillus* and *Clostridium*), Actinobacteria (i.e. *Bifidobacterium*), Bacteroidetes (i.e. *Bacteroides*), Proteobacteria (i.e. *Escherichia*), Fusobacteria (i.e. *Helicobacter*) [56] with Firmicutes and Bacteroidetes representing the 90% of the whole microbiota composition. The gut anatomical region, the human age and the state of health profoundly influence the biodiversity and the bacterial abundance. For examples, infants of the first year of life have significant biodiversity in Firmicutes and Proteobacteria respect to adulthood and even more to old age (> 70 years old) [7], while pathologies like the irritable inflammation syndrome are associated with a reduction in aerobic bacteria – such as *Lactobacillus* – respect to the healthy condition [57].

The intestinal flora contributes to the intestinal barrier homeostasis mainly by inhibiting pathogen infection and regulating the nutrients extraction, synthesis and absorption [56].

The gut microbiota can interfere with pathogens adhesion and infection by physically protecting the intestinal mucosa in a prey-predator mechanism or altering intestinal physicochemical features (*e.g.* pH) [58]. Furthermore, the microbiota can promote the maturation of the gut-associated lymphoid tissue (GALT) and stimulate immune cells to be responsive [59]. For instance, *Bacteroides fragilis* produces the polysaccharide A able to stimulate the maturation of regulatory T cells (Treg), thus promoting a state of immune tolerance and avoiding uncontrolled inflammation [60]. Simultaneously, bacteria can contribute to host defense from pathogen infection by stimulating specific intestinal epithelium cells to produce antimicrobial peptides (AMPs). Among the AMPs, defensins are molecules able to impair the bacterial cell wall integrity and prevent the pathogens colonization [61]–[63].

Gut microbiota is also a key regulator of nutrients metabolism as bacteria can process non-digestible fibers into carbon dioxide, hydrogen and short-chain fatty acids (SCFA)[64], [65]. SCFAs – such as acetate, propionate, and butyrate– are a fundamental energy source for the host organism since they represent metabolites for lipogenesis and

gluconeogenesis [66]. Each type of SCFA specifically contributes to the host tissue and organs. For instance, butyrate produced by Firmicutes is an important energy source for colonic cells, a protective element for intestinal barrier integrity and an inhibitor of inflammation [67]. Although the process of SCFAs fermentation gained the researchers attention in different microbiota-related studies, it has not yet been included in an overall view of the gut, especially comprehensive of mucus. Indeed, it is well defined either the impact of SCFAs on cells metabolism or their effect on the mucus viscoelastic properties, especially in viscosity, which is one of the leading parameters influencing the molecules diffusion in gut mucosa [68]. However, no studies investigated the possible synergic machinery of pathological microbiota composition (dysbiosis) and variation in SCFAs fermentation with the subsequent modification of the mucus properties, varied diffusion of molecules towards epithelium, and therefore altered cellular metabolism.

The intestinal microbiota composition is influenced by a multitude of factors rather than SCFAs – such as diet, antibiotic drugs and disease development – and its alteration is commonly defined as dysbiosis. Inflammatory bowel disease is one of the pathological conditions found to affect both the gut microbiota stability and the intestinal barrier homeostasis. In particular, IBD is related to a reduced immune tolerance to the microbiota-associated molecular patterns (MAMP) and an increase in inflammation of the intestinal barrier [62].

CD and UC have a different impact on gut systems (Table 1)[69]. Each inflammatory condition alters the bacterial composition reducing the biodiversity and thus, the abundance of key regulators of gut homeostasis [69]. For instance, *Fecalibacterium Praustnizii* is one of the main butyrate-producing bacteria and was found to be lower in both CD and UD patients respect to healthy patients[70]. A reduction in butyrate, as in other SCFAs, causes impairment in SCFA-associated mechanisms, which regulate the immune response by promoting AMP production and by modulating inflammation through the inhibition of NF-κB signaling pathway [67]. Changes in these SCFA-associated processes lead to a decreasing level of defensins production, a lower level of GALT stimulation and an increase in gut inflammation [62], [67].

Although the cause-effect relationship between IBD and dysbiosis is still not well defined [71]–[73], the importance of microbiota in IBD progression is an undeniable phenomenon [74], [75]. For this reason, the gut microbiota manipulation of CD and UC patients represent a promising therapeutic approach to reduce pathological symptoms, such as diarrhea and gut inflammation. In this regard, probiotics and fecal microbiota transplantation (FMT) are promising strategies[76]–[78]. While probiotics approach appears poorly standardized and confuses in its efficacy, FMT is a clinical reality with significant dysbiosis and symptoms remission data [79].

FMT requires the transplantation of fecal microbiota of a healthy donor into an intestinal tract of a recipient [80]. The transplantation success depends on the type of dysbiosis, which can be defined as primary or secondary. The primary condition is generally acute and transient and is triggered by antibiotics, malnutrition or pathogen infection. Instead, the secondary condition is commonly chronic and recurrent due to side effects of therapies (such as chemotherapy) or complex diseases, such as IBD, irritable bowel syndrome and autism [80]. Many aspects influence the success of FMT and thus of the clinical remission: (1) mucosal inflammation; (2) fecal sample manipulation; (3) donor and recipient compatibility; (4) administration method; (5) frequency of transplants; (6) pre-treatment with antibiotics [81], [82].

The percentage of clinical remission is different for CD and UC: about 25% in UC and over 55% in CD. FMT success is featured by increasing biodiversity, rescue of Treg cells at the intestinal barrier, immune tolerance enhancement, SCFAs levels increase and reduction of barrier permeability to pathogens and bacterial molecules [79], [83], [84].

Table 1. Gut microbiota composition versus CD and UC. Summary of CD and UC impact on gut microbiota composition and function respect to healthy conditions.

	Decrease	Increase	Dysbiosis impact	Ref.
Chron's disease	Actinobacteria *Bifidobacterium adolescentis* Bacteroidetes *Bacteroides fragilis* Firmicutes *Fecalibacterium Praustnizii* *Eubacterium spp.* *Lachnospiraceae* *Clostridium prausnitzii* *Roseburia spp.* *Ruminococcus spp.* *Dialister invisus*	Actinobacteria Bifidobacteriaceae Coriobacteriaceae Firmicutes *Ruminococcus gnavus* *Clostridium difficile* Proteobacteria *Escherichia coli*	Reduction of defensins production by Paneth cells Lower level of SCFAs Gut inflammation	[62], [69], [70], [82], [85]–[88]
Ulcerative colitis	Firmicutes *Roseburia hominis* *Fecalibacterium Praustnizii* *Clostridium* *Enterococcus* Proteobacteria *Escherichia coli*	Firmicutes *Clostridium difficile*	Reduction of defensins production by Paneth cells Lower level of SCFAs Gut inflammation	[81], [86], [89], [90]

5. Intestinal mucus Barrier in IBD

The intestinal mucus is a biological hydrogel covering the whole intestinal tract [13]. It has a complex bi-layered architecture that allows for symbiotic microbiota growth, on the one hand, the preservation of the sterility of the gut epithelium, on the other, while modulating the passages of molecules from bacteria to cells and vice versa [14]. The polymeric network is made of trimers of the gel-forming mucins (MUC2 in the intestine and MUC5A in the stomach) [13], [91] and is further stabilized by a set of specific structural protein, such as FCGBP and trefoil factor 3 (TFF3) proteins among others [92]–[94]. The gel-forming mucins are composed by many amino acids that is specific for the intestinal tract considered, but maintain a very conservative structure: a linear core with highly O-glycosylated branches, which increased the molecular weight of the mucin up to 50 kDa [13], [95]–[97]. Moreover, the gel-forming mucins are supported by other mucins, named transcellular mucins (*e.g.* MUC1, MUC3 and MUC4) that do not form the polymeric network of mucus but are involved in different biological functions, such as lubrication, bacterial adhesion and inflammation signaling [98], [99].

During disease, and in particular IBD, the homeostasis of the mucus ecosystem is impaired from a chemical and/or a physical point of view [100], altering the critical equilibrium between the microbiota (which resides in the mucus), mucin-secreting cells, and the biomolecules that diffuse in the network (at the base of microbiota-host communication) (Figure 3).
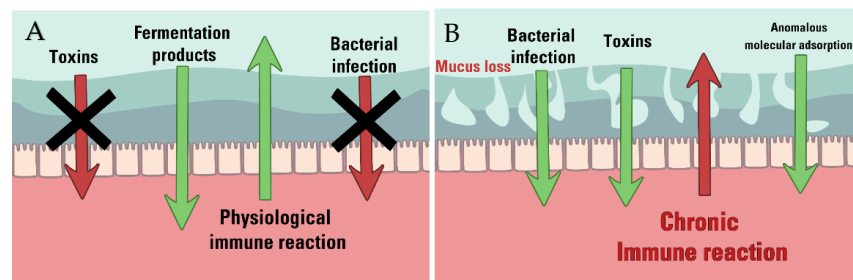
5.1 Compositional variation

Figure 3 In physiological condition (A), the mucus acts as a barrier for either toxins or bacteria adhesion (red arrows), modulating the immune response of the epithelial cell while filtering the nutrients and other molecules. During IBD (B) compositional changes and structural weakening impair these functions; bacteria are free to adhere to the epithelium and toxin diffusion is facilitate. These events contribute to the maintenance of the pathological state of inflammation.

During UC, the quantity and quality of the mucus is reduced considering the either whole mucus produced in bulk by the entire gut tissue either the mucus secreted at the single-cell level. Indeed, the number of mucin-secreting goblet cells is depleted[101], [102] [87], [88]. Proteomic analysis revealed alteration in the mucus protein-core of UC patients. In particular, the decrease of structural protein, both gel-forming and transmembrane, (MUC2, MUC4, MUC3, FCGBP and CLCA1) was combined with an increase of inflammatory-related protein[103]–[105] [87], [89], [90]. Interestingly, the reduction of [103] [89][104] [106], the increase of these proteins suggests an abnormal mucus production concomitant to an upregulated epithelial healing process.

IBDs were associated in changes of not only mucins production, but also composition. Physiologically, mucins can be classified as negative or neutral accordingly to the charge of glycosylated branches [107], [108]. The intestinal MUC2 is a negative mucin composed by a high quantity of sulfonate ($R-SO_3^-$) group, which is involved in the MUC2 resistance to the bacteria enzymatic activity[109], [110]. Histological results on IBD *ex vivo* models showed an outstanding reduction of the sulfation degree (more than 50%) [111], [112], suggesting that the pathological mucus has only a limited defensive potential concerning physiology as also demonstrated by other studies (described below).

The chronic inflammation of IBD pathologies resides in a defective immune response triggered by unbalance between pro- and anti-inflammatory signals [113]. Phospholipids play a key role in this process, as they greatly contribute, together with bacteria, in modulating the mucus function of filtering [114]–[117]. In physiological condition, phosphatidylcholine (PC) and lysophosphatidylcholine (LPC) are the two main phospholipid classes that are present in mucus, accounting together for more than 80% of total mucus phospholipids [114]. However, in the case of UC, a strong depletion of PC and LPC was observed. Differently, CD patients only expressed a decrease of LPC, while maintaining PC concentration similar to control (Table 2). In healthy condition, the phospholipids grant hydrophobicity to mucus [118], [119], which is one of the main physical properties contrasting bacteria adhesion to substrates [120]. For this reason, the phospholipids reduction occurring during IBD favoured the bacteria penetration in the gut tissue [121], [122]. Interestingly, the LPC/PC ratio was comparable to control in case of CD but increased in case of UC [123]. As LPC is commonly associated to phospholipase activity rather than synthesis [124]–[126], these results suggest two different mechanisms involved in IBD: limited availability of PC but downregulation of phospholipase (*i.e.* an increase of LPC/PC ratio) in case of UC and only a slight promotion of the phospholipase activity in case of CD.

Compositional variations are not only important as a cause of the changes in the physiological feature of mucus *per se*, but also because they can directly modify the chemical affinity of mucus with bacterial, cellular or other molecules, facilitating and impairing their diffusion towards epithelium [14], [127].

Table 2. Compositional variation in the human colonic mucus in IBD extracted from [119] and [114]. The mean values obt $\sigma_{mean} = \left(\sum \sigma_i^{-2} \right)^{-1/2}$ ith reference to the standard deviation (s.d.) and the mean s.d. obtained by the formula: .

	HEALTHY	CD	UC
LPC mean value [pmol/100 μg protein]	4371 ± 487	2961 ± 287	1738 ± 288
PC mean value [pmol/100 μg protein]	2983 ± 321	2508 ± 415	676 ± 104
% of LPC / protein	2.22% ± 0.25%	1.50% ± 0.15%	0.88% ± 0.15%
% of PC / protein	2.30% ± 0.25%	1.94% ± 0.32%	0.52% ± 0.08%
LPC/PC	1.45 ± 0.32	1.18 ± 0.31	2.57 ± 0.84

5.2 Structural weakening

Acting as a selective filter, the mucus behaves like a physical barrier between human cells and the environment, preserving the sterility of the epithelium while allowing the passage of nutrients and other molecules [13]. One of the main structural parameters of this barrier is its thickness since it is closely related to the function of the specific intestinal tract. For example, in the small intestine, where the nutraceutical molecules are absorbed, the mucus thickness is minimal thus facilitating the diffusion of the molecules from the lumen to the tissue. Conversely, in the colon, where the majority of the intestinal microbiota is hosted, the thick mucus layer acts as a protection against infections (Table 3). For this reason, the colonic thickness (*i.e.* ~150 vs ~50 μm) is significantly higher than small intestine [68], [128], [129].

The IBD mucus is usually investigated after identification of the inflammation degree by standard clinical criteria, expressed by numbers from 0 to 4 in proportion to the severity. In UC patients, the mucus layer was thinner than controls (especially in the descending colon and rectum) [130]. Moreover, the thickness reduction was exacerbated with proportionality to the severity of inflammation, showing a complete absence of mucus with a severe state of inflammation [131], [132].

The measurements of the CD mucus thickness are not homogeneous in the literature. Indeed, this structural parameter was found to be either higher [132], or lower [133], or comparable [134] to the healthy controls. This variability can be linked to the intrinsic heterogeneity in the distribution of the lesions in the case of CD compared to UC [20], [22], but also in variations of the mucus staining protocols and preservation as well as the not-standard definition of "controls", which often suffer from other intestinal pathologies. Interestingly, independently from the data obtained in the inflamed areas, the mucus thickness in undamaged colon portions of both UC and CD patients is not significantly different from healthy controls [135], suggesting the localized nature and impact of these pathologies.

Moreover, the mucus discontinuity, measured as the length of mucus-free mucosa normalized to the total specimen length, was significantly higher only in groups with severe active UC (25.7 %) than controls (1.0 %) [136]. No significant difference was found between controls and either quiescent UC or CD samples, as the adherent mucus layer was essentially continuous [136] similarly to the physiological condition [137].

In physiology, the mucus thickness is not the only structural factor affecting the mucus properties. Indeed, this parameter must be always coupled with the peculiar double-layered architecture, where the adherent mucus layer acts as the real barrier for the epithelium [138]. *In vivo* animal models with induced moderate UC showed a limited variation of the mucus thickness but a substantial modification of the mucus architecture. In particular, the adherent layer was much thicker than the controls [139], [140]. These results suggest that not only the amount of mucus is a pivotal element in IBDs, but also its quality. This consideration becomes more robust if the permeability of the mucus is considered, as an index of the efficacy in maintenance of the barrier properties. Microbeads with dimension comparable to bacteria (0.5-2 μm) were able to penetrate the mucus and reach the epithelium in both *ex vivo* human biopsies [103] and animal models [140]. Similarly to the thickness, the mucus weakening increased with the disease severity. Indeed, the percentage of beads reaching the epithelium was 36% and 20% in the case of acute disease and remission state, but only 10% in controls [103]. As structural properties correspond to chemical composition, the impaired barrier properties were as related not only to the decrease of the gel-forming mucin MUC2 but also of the support protein FCGBP [103]. This suggests the presence of two combined effects in UC: a reduced mucus production with a structural instability of the network.

Table 3. Human mucus thickness as indicator of the intestinal barrier weakening occurring during either Crohn's disease (CD) or ulcerative colitis (UC). N/A = not available.

	Right colon/caecum	Left colon/ Sigmoid	Rectum	Ref.
Controls	107 ± 48	134 ± 68	155 ± 54	[141]
	N/A	218 ± 81.07	N/A	[142]
	N/A	450 ± 70	N/A	[143]
UC	N/A	83 ± 49.93	N/A	[142]
	90 ± 79	43 ± 45	60 ± 86	[141]
CD	190 ± 83	232 ± 40	294 ± 45	[141]
	N/A	74 ± 40	N/A	[142]

6. Barrier integrity and permeability assays

The intestinal barrier integrity and permeability are standard parameters used in clinics to identify and define the severity of IBD. The measurement of these functional aspects is currently possible using different well-established methods [144], [145]:

- *Active and passive permeability assays:* Active permeability assays require the oral administration of sugars or polymers and the measurement of urinary concentrations at different time points from 30 min to 24 hours [146]. The higher is the molecule concentration, the leakier the epithelial barrier.   A common method is based on the administration of different molecules (e.g. lactulose, mannitol, PEG (polyethylene glycol) molecules and sucrose among others) and the measurement of their concentration in urine, thus obtaining information about the permeability of the mucosa in different region of the gut, which is in dependence of the size and the nature of the administered substance [146], [147][148][149][150]. The use of the radioactive 51Cr-EDTA were not only able to describe an increased permeability in CD patients (20% higher than healthy controls) but also to correlate this permeability variation with a reduction in specific bacteria abundance (i.e. Faecalibacterium prausnitzii) [151]. No consideration was proposed in terms of mucus properties, whose contribution remained clouded under the permeability characterization. The principle of passive permeability assays is similar to the active methods described

above. The main difference is the nature of the detected molecules, which are usually products of the individual metabolism (e.g. bacteria-derived molecules, intestinal integrity-related or immunological biomarkers) measured in plasma, serum and biopsies [149]. Among the bacterial-derived molecules, lipopolysaccharides (LPS) and butyrate are of primary importance, as they are the major components of the outer membrane of Gram-negative bacteria and biomarker of intestinal barrier integrity, respectively [152]–[154] [140], [155]. Epithelial and immune biomarkers detection in plasma and urine is another effective method to define the intestinal barrier integrity (i.e. citrullin and claudin-3) [53] [156].[157]. The inflammatory marker mainly investigated in UC and CD is calprotectin, a product of active and infiltrated neutrophils in the intestinal mucosa. High levels of fecal calprotectin (>100 µg/g) were correlated with higher levels of intestinal permeability indicators and give an indirect measure of IBD severity [151]. Similarly to active permeability assay, the passive permeability assays have the possibility to correlate bacterial products to dysbiosis and intestinal barrier impairment, but do not provide information about the relative contribution of mucus within the epithelial barrier. The inclusion of a more detailed characterisation of mucus could provide a deeper understanding of these experimental evidence. For instance, the study of the variation of the mucus network microstructure can elucidate if the diffusion of molecules from lumen to epithelium is facilitate or impaired. Similarly, compositional changes can provide information of the dye/molecules affinity with the mucus matrix.

- *Confocal laser endomicroscopy.* Confocal laser endomicroscopy allows the acquisition of confocal images during endoscopic procedures [158]. It provides information about the epithelial barrier morphology. This method requires the intravenous administration of a fluorescent dye – such as fluorescein – that could be easily excited by the laser of the endomicroscope. A detector then transforms the emitted light signal into an electrical input for computational recording. This method generates accurate images giving evident information on the barrier morphology and the epithelial crypts architecture [159]. These analyses highlighted frequent morphological features of IBD epithelia, such as intra- and inter-crypt distance increase, irregular crypt organization, micro- and macro-lesions leading to cell and molecule infiltrates increase [149]. Overall, endomicroscopy not only provides critical data for the determination of the barrier impairment but also allows to limit the collection of biopsies to strictly necessary cases. Despite the epithelial barrier is well examined, the mucus barrier contribution is still poorly considered while information about gut dysbiosis is not provided at all.

- *Ussing chamber.* The Ussing chamber-based method is an invasive assay used to determine intestinal barrier integrity and permeability. It is performed on intestinal tissue biopsies and requires invasive procedures to collect ex vivo specimens. This technique is based on the measurement of a voltage difference (ΔV_{EP}) and a trans-epithelial (o trans-mucosal) electric resistance (TEER o TER, respectively) between the apical and the basolateral side of the barrier. It requires the application of active ion transport through the epithelium and the measurement of the ΔV_{EP} and TER. The ion transport is correlated to the barrier integrity. The weaker the epithelium, the higher will be the transport and the lower the TER [149], [160] Like previous well-established clinical assays, the Ussing chamber-based method is a valuable approach to study the intestinal barrier function in IBD, as it was demonstrated for instance that IBD patients showed a decrease of TER near the 39% [161]. However, it cannot provide information about the relative contribution of the elements modulating barrier disruption.

Even though these methods allowed clinicians to evaluate functional aspects – such as intestinal barrier permeability, integrity and morphology – they do not discriminate the relative contribution of all the modulating elements (i.e. gut dysbiosis, epithelial barrier and mucus barrier). In particular, the assays that are commonly used for studying the IBD features rarely focused on the impact of mucus physicochemical and mechanical properties, which is usually simply considered as the stage, without active and/or substantial role, in the scene where the biological actors (i.e. epithelium and microbiota) perform their playscript. The inclusion of a more refined mechanical and compositional characterization of the mucus with the well-defined permeability assays would lead towards a synergistic comprehension of the IBD pathology from a biological and materialistic point of view, opening new horizons towards unexplored therapeutic strategies.

7. Conclusion

Autoimmune diseases of the intestinal tract are spreading worldwide. Researchers have proposed different hypotheses in their challenging task of identifying the origin of these diseases, but the pathophysiology of the inflammatory bowel disease (IBD) remains a not fully described process at the date. In this frame, the leaky intestinal barrier is unquestionable evidence, wheatear it is interpreted in the sense of trigger or symptom of the pathological states. Even if the leaky intestinal barrier in IBD is historically associated to the impaired intestinal cellular barrier and dysbiosis, it is a phenomenon derived by a complex play-script directed by another, not cellular, actor: the intestinal mucus. In this complex performance, the chemical and physical barrier of mucus properties cooperates in synergy with the bacterial and cellular metabolism, effecting the diffusion of bacterial- and cellular-derived molecules and modulating the inflammatory response. Although numerous advancements were made in the study of IBD thanks to the classic vision of these pathologies (such as the definition of standard analysis methods), the inclusion of a refined mucus characterization can open to new paths in the direction of the complete comprehension of the IBD condition. Indeed, the synergic approach between the biological- and mucus-oriented vision may boost towards new etiological and pathogenic insights, as well as alternative therapeutic strategies against IBD pathologies.

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