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New Aspect of Crohn's Disease Pathogenesis: Dysbiotic Gut Microbiome

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ABSTRACT

Crohn's disease is a chronic inflammatory disease of the intestine with an increasing incidence worldwide. It is a multifactorial disease where host genetic impairments and environmental triggers lead to the onset and maintenance of inappropriate host immune responses at the gut mucosa interface. The gut microflora regulates intestinal homeostasis including mucosal immunity and absorption of complex macromolecules. If the commensal microbiota is under "stress", a dysregulation of the innate immune function may be triggered and could elicit an inflammatory reaction that when it is perpetuated, may result in immune dysfunction as inflammatory bowel disease. This chapter provides an overview of the pathogenesis of Crohn's disease and their relation with the microbiota intestinal and reviews the literature regarding the associations between altered composition of intestinal bacteria, dysbiosis, to answer the question if dysbiosis is a cause or a consequence of intestinal inflammation

INTRODUCTION

The intestinal epithelium is the main interface between the immune system and the external environment. The development of a host's immune system is affected by continuous and dynamic interactions with the intestinal microbiota and its metabolites.

Bacteria are integral to the early development of the gut mucosal immune system, both in terms of its physical components and its function, and continue to play a role later in host life [1]

In the epithelium inhabits the microbial flora resident, whose role in innate immunity is based on the recognition and differentiation of enteric commensal bacteria from pathogenic, thus contributing to the maintenance of tolerance and intestinal homeostasis [2]. The interaction of genes that regulate immune functions is strongly affected by the environment, especially the intestinal microbial flora resident in susceptibility to develop CD. Although distinct microbial population inhabit all body surfaces exposed to the environment, the greatest and most varied microbial population resides in the intestine [3]. This microbial community consists of an array of bacteria, viruses, archaea, and microeukaryotes [4]. The intestinal microbiota is involved in a wide range of physiological and pathological processes in the host, playing an important role in human metabolism, immune system development, and pathogen regulation [5].

MICROBIOTA IN HEALTHY GUT

The density of microorganisms differs longitudinally along various points of the GI tract, with the lowest numbers in the stomach and the most in the large intestine [6]. The relatively lower density of microorganisms and their distinct composition may be related to a number of factors as pancreatic secretions, bile and gastric acid found in parts of the small intestine that collectively generate an environment unfavorable for most microorganisms [7]. Fecal samples are considered to be more representative of the luminal colonic microbiota than of the small intestine microbial residents. For this reason, the stool samples are poor determinants of the microbes associated with the bowel mucosa [8,9].

Swidsinski et al. reported that the bacterial composition varied distinctly among the fecal, mucus and epithelial crypt compartments [10]. While some bacterial species were present in all three gastrointestinal compartments, others were only presenting one or two of them. Bacteria found in all the compartments included Clostridium coccoides, Alphaproteobacteria, Coriobacterium, Lactobacillus, and Enterococcus group. Bacteria such as Ruminococcus and Bacteroides were found in the intestinal lumen and mucus layer. Some bacteria were only found in the feces, included Clostridium histolyticum and Clostridium lituseburense groups (including C. perfringens, C. botulinum, and C. difficile); Bifidobacterium, Veilonella, Streptococcus, and Enterobacteriaceae[10].

Many biochemical and physical factors combine to form a barrier overlaying the intestinal epithelium, with the extracellular mucus being the most important component [11]. The colonic

mucus layer is primarily comprised of MUC2 mucin glycoprotein protecting the epithelial cells layer [11]. Additionally, the mucin mucus layer functions is an attachment point and a source of nutrients for bacteria. The mucin mucus layer lining the GI tract consists of an outer and an inner layer firmly adherent to the epithelial glycocalyx. Normally, bacteria can be found in the outer layer, whereas the inner layer is devoid of microbes.

The normal GI mucosa is able to differentiate commensal microorganisms from pathogenic microbes due to the expression of pattern recognition receptors, including Nucleotide-Binding Oligomerization Domain-Containing proteins **(NOD)** found in the cytoplasm and Toll-Like Receptors **(TLRs)** located on the surface of cells [12].

MICROBIOTA COMPOSITION

Colonization by bacteria occurs within the first several hours of life. During infancy, variability in the composition of the gut microbiome among individuals depends on factors such as mode of delivery and type of infant feeding. Diversity increases rapidly in early childhood and this dynamic process leads to the development of the relatively more stable adult gut microbiome [13].

Normally, neonates are delivered vaginally and are thus subjected to their mothers' vaginal flora and may also be in contact with the fecal flora of the mother [13,14]. Infants who were delivered through cesarean section showed devoid of Bacteroides and contained significantly lower amounts of Escherichia–Shigellaat one month when compared with those who were delivered vaginally, although these differences do not remain detectable at six months of age [15].

During the first year of life, the composition of the gut microbiota is relatively simple and shows wide inter individual variations [16]. The infant's gut microbiota undergo a succession of changes that are correlated with a shift in feeding mode from breast- or formula-feeding to weaning and the introduction of solid food[16].

Ringel-Kulka et al. found that the microbiome of children was of considerably lower diversity than those of adults, but that the most abundant GI bacteria were similar in adults and children [17]. For example, Bifidobacteria were found in significantly higher amounts in children in comparison with adults [17,18].The human gut flora variations of specific genera have different capabilities, and different metabolic responses to diet or medication, giving a reason why different persons exhibit different responses to medical treatments.

Environmental factors, such as age, diet, stress, drugs, will strongly influence the composition of the human microbiota [19]. Both endogenous and exogenous factors will contribute to the microbiota composition. Several works have examined the changes in microbiota composition in different geographical areas, the faecal microbiota of subjects belonging to different ethnic groups, and continents [20]. Claesson et al. reported that Bacteroides species were found in higher numbers in fecal samples of subjects older than 65 years compared to the younger subjects and found specific differences in the proportion of certain Clostridium species between the two age groups [21].

Lin et al. showed differences in faecal microbiome structure and diversity according geographical location, between healthy children in Bangladesh and the United States **(US),** so the microbiome of US children were significantly less diverse than those of Bangladesh children [22]. The microbiotas of the US subjects were equally dominated by Bacteroides and Firmicutes, and in the Bangladesh children were more abundant in Firmicutes than in Bacteroides.

MICROBIOTA FUNCTION

The intestinal microbiota develops an important number of physiological processes, including metabolism, digestion and the immune response. The intestinal microbes convert certain carbohydrates such as resistant starch, cellulose, hemicellulose, pectins and gums through fermentation, generating metabolites favorable to the host, so the Short Chain Fatty Acids **(SCFAs)**, especially butyrate but also propionate and acetate, possess anti-inflammatory properties and may act as a source of energy [23].

Intestinal microbes synthesize all essential and non-essential amino acids [24] and are able to degrade proteins resulting in toxic metabolites. Gut microbes reduce serum cholesterol through conversion of bile salts, carry out biotransformation of bile [25]. The microbiota is able to produce a variety of vitamins (B group and K). The interaction of microbes with the host influences the development and maturation of the immune system.

The microbiota stimulates the host to produce Antimicrobial Peptides **(AMPs)**, and produces AMPs itself. Lactobacillus and Bacillus, produce antimicrobial substances active against a wide range of entero-pathogenic bacteria, both Gram positive, and Gram negative bacteria [26]. The AMPs have a different distribution in the Gastrointestinal Tract **(GIT)**, so the maximum antimicrobial activity level was found in the intestinal crypts and mucus layer, while at lumen level found a reduced activity [27].

Gut microbiota contributes to the maintenance of the integrity of the intestinal epithelial barrier through the maintenance of cell junctions, and the promotion of epithelial repair after damage [28]. Has an important role in the structural development of the gastrointestinal tract and immune system [29]. For these reasons, the composition of colonizing flora influences the immune individual variations [30].

There is a host-bacterial mutualism (both, human and microbes, have their benefit), the host contributes with essential nutrients for the survival of the microbiota and develops multiple roles in host nutrition, in protection against pathogens, and in activation and regulation of immune responses [31,32]. Although the more correct term to define this relation is *Amphibiosis* to define the relationship between humans and microbes that could be beneficial or pathological, depending on the context in which it occurs [33].

New scientific data confirm that is the bacterial function more important than bacterial composition [34]. In this sense, the presence of certain bacteria can functionally compensate for the absence of other species, and different bacterial groups can synergistically interact.

MICROBIOTA IN CROH'S DISEASE

Inflammatory Bowel Diseases **(IBD)** comprise a group of chronic relapsing inflammatory disorders involving the Gastrointestinal **(GI)** tract, which arise from the confluence of genetic, immunological, microbial, and environmental factors. IBD have traditionally been categorized into two main clinical phenotypes: Ulcerative Colitis **(UC)** and Crohn's Disease **(CD)** [35].

Crohn's disease **(CD)** is a chronic inflammatory disease of the intestine with an increasing incidence worldwide [36]. It is a multifactorial disease where host genetic impairments and environmental triggers lead to the onset and maintenance of inappropriate host immune responses at the gut mucosa interface. Crohn's disease may affect any part of the gastrointestinal tract from mouth to anus [37] Signs and symptoms often include abdominal pain, diarrhea (which may be bloody if inflammation is severe), fever, and weight loss. Other complications may occur outside the gastrointestinal tract and include anemia, skin rashes, arthritis and inflammation of the eye, between others.

The gut microflora regulates intestinal homeostasis including mucosal immunity and the absorption of complex macromolecules [38]. If the commensal microbiota is under "stress", a dysregulation of the innate immune function may be triggered and could elicit an inflammatory reaction that when it is perpetuated, may result in immune dysfunction as inflammatory bowel disease (Figure 1).

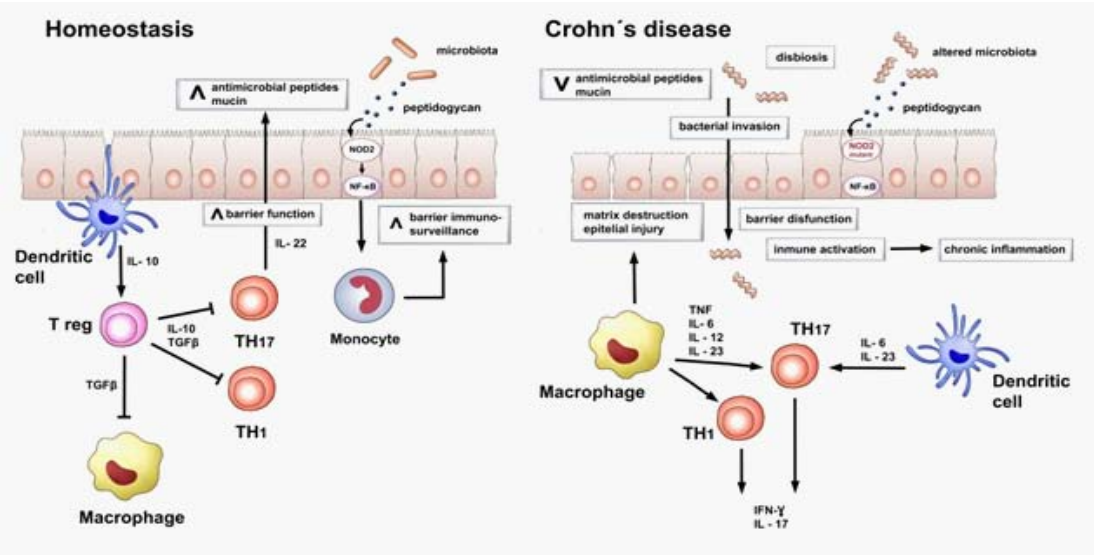

Figure 1: Mucosal homeostasis and immunological tolerance in healthy gut and activated inflammatory cascades in Crohn's disease. (Re-drawn of Sartor RB. Gastroenterology 2010; 139:1816-19).

Numerous studies have shown reduced diversity of the gut microbiota in IBD patients [39-42]. The most consistent observations of altered composition of the gut microbiota in IBD patients are a reduction in Firmicutes and an increase in Proteobacteria. To compare the results between different studies it is significant to consider the following factors: (1) sample source (biopsy or stool), (2) sampling location (inflammatory or non- inflammatory sites), (3) disease activity (active or quiescent), (4) medication, (5) diet, (6) age, (7) smoking, and (8) methods used to analyze the microbiota.

The recognition/ binding of the host receptor proteins to specific microbial molecules stimulates a signalling cascade that involves the nuclear factor NF-kb stimulating the expression of genes that encode pro-inflammatory molecules [43]. In a healthy host the "physiological inflammation", is controlled by specific mechanisms allowing a regulation of the pro-inflammatory signal, and the maintenance of homeostasis [44]. The control mechanism breakdown could lead to a persistent inflammatory state. The continuous stimulation of various commensal bacteria components produces a reduced expression of TLRs in the intestinal epithelium and a low production of inflammatory cytokines [45].

The microbial composition in the intestinal tract is considered a potential risk factor in patients with CD [46]. There are several lines of evidence for microbial involvement in IBD. For example, (i) inflammation occurs in regions with higher bacterial density, such as distal ileum and colon, (ii) germ-free animals do not develop ileitis [47] or colitis [48], (iii) antibiotics have shown therapeutic efficacy in IBD patients [49], (iv) the severity of the disease correlates with the bacterial density in the intestinal mucosa, and (v) a large number of studies reported an altered bacterial composition in IBD patients compared to healthy individuals, so-called dysbiosis [50].

GUT DYSBIOSIS: CAUSE OR EFFECT?

Dysbiosis has been defined by qualitative and quantitative changes in the intestinal flora, their metabolic activity and their local distribution [51]. Altered composition of intestinal bacteria—"dysbiosis"—is characteristic of Crohn's disease. The dysbiosis hypothesis considers that the modern diet, the lifestyle and the use of antibiotics have led to the disruption of the normal intestinal microflora as well as result in alterations in bacterial metabolism and the overgrowth of potentially pathogenic microorganisms that result in the release of potentially toxic products playing a role in many chronic and degenerative diseases [52,53].

Standards of gut microbiome dysbiosis were not uniform among all published works and maybe due to take into account a different phases of the disease (sometimes influenced by diets and therapies), different ages, populations and geographical areas, and different methodologies used for analysis. It is believed that early changes are likely to be more evident in new-onset and treatment-naive pediatric patients *vs* adults. Pediatric IBD is considered the "purest" form of disease without other influences of adult behavior, like smoking or disease comorbidity [54,55].

There is reduced faecal microbiome diversity in CD patients compared with healthy controls [56]. This decrease in diversity was found to be specific within phylum Firmicutes with an increase in Gammaproteobacteria [57] and proportions of the Clostridia altered [58]. The reduction in the numbers of Firmicutes is of particular interest because they are known producers of important Short Chain Fatty Acids **(SCFAs)**, such as acetate and butyrate that are known to have potent anti-inflammatory properties [59].

The decrease diversity has been associated with temporary instability in the dominant species in CD [60]. Microbiota of the non-inflamed gut in CD are more diverse than the microbiota of the inflamed gut, even within the same patient and with less bacterial load in inflamed regionsat distinct gut compartments [61]. This disproportion contribute to gut dysbiosis within the inflamed or non-inflamed mucosa.The intestinal dysbiosis and the loss of beneficial microbial products, can facilitate the proliferation of disease promoting bacteria that produce pro-inflammatory metabolites.

Advances in sequencing technology have allowed enablea correlation of inflammatory disease with a reduction in luminal microbiota diversity, including a decrease of Bifidobacterium and Lactobacillus and increase in pathogenic proteobacteria [62,63]. The gut microbiota in healthy shows little temporal change, while in IBD patients is unstable. The composition of the gut microbiota differs between active and quiescent stages of the disease. In CD patients, dysbiosis is observed even in patients with remission. Medication also affects the composition of the gut microbiota, for example, treatment with mesalazine reduces the total bacterial number to almost half [64].

To date remains unclear whether the dysbiosis observed in IBD is a cause or a consequence of intestinal inflammation. To answer this question, are necessary to examine longitudinal changes of the microbiota in a large number of IBD patients. Few information is available to know how dysbiosis regulates the gut immune system. It is important to understand the complex relationship between the gut immune system and the microbiota. Utilizing the gut microbiota as a diagnostic tool or biomarker should lead to further elucidation of the pathogenesis of IBD and development of therapies.

ENVIROMENTAL FACTORS AND MICROBIOTA IN CROHN`S DISEASE

Certain non-genetic factors are associated with the development of IBD due, in part, to their effects on the gut microbiome. Environmental factors that can disturb the balance of intestinal microbiotainclude diet, the use of antibiotics, and geographic location [65]. Disease treatments include dietary changes, immunosuppressive anti-TNFα antibodies and antibiotic therapy, and their effects on microbiota composition must be considered.

Antibiotic

Bacterial community was associated with intestinal inflammation, antibiotic use, and therapy. When faecal samples from a prospective cohort of pediatric Crohn's disease patients starting therapy with enteral nutrition or anti-TNFα antibodies reveal the full complement and dynamics of bacteria, fungi, archaea and viruses during treatment [66].

Antibiotic exposure was associated with increased dysbiosis, whereas decreased with reduced intestinal inflammation. The potential for antibiotics to determine the magnitude of impact on the intestinal florais related to its spectrum of activity [67] pharmacokinetics, dosage [68] and length of administration [69]. Regarding the spectrum of activity, an antimicrobial agent active against both gram-positive and -negative organisms will have a greater impact on the intestinal flora [67].

Antibiotics have been complained to provide benefits for CD patients as a first-line therapy and it is a factor affecting the gut microbial composition. So, the microbial network appears more dysbiotic in the context of antibiotic exposure in CD. Loss of protective microbes has the potential of triggering a proliferation of less beneficial species exacerbating the inflammation [70].

Diet

Other of the most important environmental factors affecting microbial composition is dietary choice, which has been shown to determine microbiome composition throughout mammalian evolution [71]. The composition of the diet has been shown to have a significant impact on the content and metabolic activities of the faecal flora.

Some diets promote the growth of beneficial microorganisms, while others promote activity of microflora that can be harmful to the host. It is known that a high-fat dietary intake have impact on the non-pathogenic microbiota remodeling radically the intestinal microbiota [72,73]. Moreover, there is evidence that non-absorbed carbohydrates (inulin and fructooligosaccharides) promote the growth of beneficial species, supplying a substrate for the production of short-chain fatty acids [74].

Changes in dietary patterns can affect the gut microbial composition [75], particularly in those individuals with reduced microbial [76]. For example, the vitamin D pathway is important in gut homeostasis and in signaling between the microbiota and the host immune system, and have implications for the development and severity of inflammation [77]. Dietary therapy had independent and rapid effects on microbiota composition distinct from other stressor-induced changes and effectively reduced inflammation.

Although no specific diet has been shown to directly cause, prevent, or treat IBD, it is important to take into account the interactions between nutrients and microbiota when studying the role of the microbiome in CD. Several studies evaluating the effect of diet modification in IBD have been performed, but there have been no large-scale randomized trials that have provided insights into the potential therapeutic actions of diet and dietary supplementation to date.

IBD SUSCEPTIBILITY GENES ASSOCIATION WITH MICROBIOTA

Genetic risk is not sufficient by itself in causing IBD and there is very likely a role for other factors in the development of these diseases [78]. Genetic studies provide strong evidence for the role of microbes in IBD pathogenesis. Genome-Wide Association Studies **(GWAS)** have already identified over 100 genetic risk loci, including 28 that are shared between Crohn's disease and ulcerative colitis [79]. Although host genetics play a critical role in disease pathogenesis, concordance rates in monozygotic twins of 35% for Crohn's disease indicate that non-genetic factors play a substantial role in the development of IBD [80].

Growing evidence suggests that many of the genetic risk alleles for IBD involve regulation of the epithelial barrier or innate immune responses to protect the host from bacterial invasion while others involve pathways that regulate the adaptive immune system [79]. These genes are categorized into four groups to (1) acquired immunity (IL23R, IL12B, JAK2, STAT3), (2) bacterial recognition and processing (NOD2/CARD15), (3) autophagy (ATG16L, IRGM, ATG5), and (4) mucosal barrier (ECM1, CDH1, LAMB1) [81]. These CD susceptibility genes are associated with bacterial recognition and processing, and are related to mucosal barrier function, suggesting that impaired handling of bacteria or disruption of the mucosal barrier function leads to breakdown of tolerance against the commensal bacterial in the gut in CD.

NOD2 / CARD15 were the first CD susceptibility gene described,supporting the link between gut microbiome and IBD etiopathogenesis [82]. This gene is located on chromosome 16 and is expressed in monocytes/Paneth cells in the terminal ileum, encoding an intracellular receptor for the bacterial peptidoglycan muramyl dipeptide (MDP), a component of the cell wall of gram-positive bacteria. NOD2 recognizes components in the bacterial cell membrane and, together with other genes associated with IBD susceptibility (e.g., ATG16L1, CARD 9, IL23R) determines how patients will respond to microbial stimuli. NOD2 mutations showed a decreased production of antimicrobial peptides from Paneth cells [82] and reduction of production of anti-inflammatory cytokine IL-10 from peripheral mononuclear cells in CD patients [83].

Autophagy in Paneth cells is essential to maintain intestinal homeostasis, probably through the regulation of intestinal microbiota by AMP production. Abnormalities in the size, number, and distribution of granules in Paneth cells, which contain AMPs, have been observed in CD. These morphological abnormalities were reported to be more frequent in CD patients with NOD2 or ATG16L mutations [84]. Impairment of the function of Paneth cells may be an important element in the development and perpetuation of intestinal inflammation in CD. Genetic alterations support the notion that IBD is due to the inability of the host to protect against microbial invasion together with uncontrolled immune activation

MICROBIOTA AS THERAPHY

The role of dysbiosis has emerged as an important alternative for control to gut immunity in producing intestinal inflammation, modulating or renewing gut microbial populations [85].

Strategies for treating IBDs including probiotics, prebiotics and antibiotics [86]. Lactobacillus, Bifidobacterium, and Streptococcus species are clinically beneficial for the downregulation of intestinal inflammation. So, Lactobacillus strains reduces intestinal inflammation by inducing regulatory dendritic cells, which produce IL-10, TGF-β, and indoleamine 2,3-dioxygenase that promote the generation of Treg cells [87]. The efficacy of probiotics for the treatment of IBD is controversial and difficult to assess due to differences in preparations (types of microbes and quality control), administration, patient groups, and endpoints. In general, probiotics in CD patients have not shown any additional benefit in inducing clinical remission or preventing clinical relapse [88].

Prebiotics provide metabolic fuel (e.g. oligosaccharides) for the support and proliferation of beneficial commensal bacteria [89]. Other important microbial metabolite include short-chain fatty acids that mediate the differentiation of naive T cells into Treg cells [90].

Fecal Microbiota Transplantation **(FMT)** offers a possibility for successfully decreasing intestinal inflammation [86]. In FMT, faeces from a healthy donor are transferred into the intestinal tract of a patient with intestinal inflammation. In doing so, a healthy intestinal microbial community is restored in the patient with inflammation. FMT is an effective form of therapy in recurrent *Clostridium difficile* infection where the experience is much greater. In IBD, the data is more controversial, where there are just a handful of case reports and case series. In a systematic review, improvement of IBD symptoms and disease activity index was observed in 76 and 63 % of patients with FMT treatment [91]. Other groups showed transient improvement of Crohn's colitis after FMT, but the disease recurred after 6 months of treatment [92]. Despite insufficient data on FMT in IBD it may be suggested as potentially effective and safe treatment for subjects who failed conventional treatments. It is necessary to perform new well-designed and randomized trials to obtain more data about clinical therapy in IBD to evaluate safety and success rate and standardize protocols.

The manipulation of the intestinal microbiota may represent an alternative strategy not only for intestinal disorders but also extraintestinal diseases. This therapeutic approach will likely need a personalized understanding of individual (donor and recipient) genetics, diet, gut microbiota and other environmental factors to be effective.

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