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REVIEW

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Jessica Carrière, Arlette Darfeuille-Michaud, Hang Thi Thu Nguyen

Jessica Carrière, Arlette Darfeuille-Michaud, Hang Thi Thu Nguyen, Clermont Université, M2iSH, UMR 1071 Inserm/University of Auvergne, 63001 Clermont-Ferrand, France
Jessica Carrière, Arlette Darfeuille-Michaud, Hang Thi Thu Nguyen, INRA USC 2018, 63000 Clermont-Ferrand, France
Arlette Darfeuille-Michaud, Centre Hospitalier Universitaire, 63000 Clermont-Ferrand, France
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Correspondence to: Hang Thi Thu Nguyen, PhD, Clermont Université, M2iSH, UMR 1071 Inserm/University of Auvergne, 28 Place Henri Dunant, 63001 Clermont-Ferrand, France. hang.nguyen@udamail.fr
Telephone: +33-4-73178372 Fax: +33-4-73178371
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## Abstract

Important advances during the last decade have been made in understanding the complex etiopathogenesis of Crohn's disease (CD). While many gaps in our knowledge still exist, it has been suggested that the etiology of CD is multifactorial including genetic, environmental and infectious factors. The most widely accepted theory states that CD is caused by an aggressive immune response to infectious agents in genetically predisposed individuals. The rise of genome-wide association studies allowed the identification of loci and genetic variants in several components of host innate and adaptive immune responses to microorganisms in the gut, highlighting an implication of intestinal microbiota in CD etiology. Moreover, numerous independent studies reported a dysbiosis, *i.e.*, a modification of intestinal microbiota composition, with an imbalance between the abundance of beneficial and harmful bacteria. Although microorganisms including viruses, yeasts, fungi and bacteria have been postulated as potential CD pathogens, based on epidemiological, clinicopathological, genetic and experimental evidence, their precise role in this disease is not clearly defined. This review summarizes the current knowledge of the infectious agents associated with an increased risk of developing CD. Therapeutic approaches to modulate the intestinal dysbiosis and to target the putative CD-associated pathogens, as well as their potential mechanisms of action are also discussed.



**Key words:** Crohn's disease; Intestinal microbiota; Dysbiosis; Adherent-invasive *Escherichia coli*; Probiotics; Antibiotics; Fecal microbiota transplantation

**Core tip:** Crohn's disease (CD) is a chronic inflammatory disorder of the gastrointestinal tract of which the etiopathogenesis is not fully understood. Increasing evidence has shown that the etiology of CD is multifactorial involving genetic, environmental and infectious factors. A dysbiosis with an increase in the abundance of putative pathogenic bacteria and a decrease in that of potentially beneficial bacteria has been observed in CD patients, revealing the involvement of intestinal microbiota in such disease. This review aims to summarize the current knowledge of the infectious etiology of CD and to discuss therapeutic approaches to modulate intestinal dysbiosis and to target CD-associated pathogens.

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## INTRODUCTION

The etiopathogenesis of Crohn's disease (CD), a type of inflammatory bowel diseases (IBD), is complex and con-

sists, according to clinical and epidemiological studies, of three interacting elements: environmental factors, genetic susceptibility and infectious agents. While many gaps in our knowledge still exist, the most widely accepted theory holds that the disorder is caused by an aggressive immune response to microorganisms of the intestinal microbiota in genetically predisposed individuals.

The early identification of nucleotide-binding oligomerization domain-containing protein 2 (NOD2), an intracellular sensor of pathogen/microbe-associated molecular patterns, as a susceptibility gene for CD[1,2] has highlighted the role of innate immunity in the disease. This has been substantiated by genome-wide association studies (GWAS) with the identification of genetic association between CD susceptibility and variants in genes involved in autophagy, ATG16L1 (autophagy-related 16-like 1) and IRGM (Immune-Related GTPase M)[3-6]. To date, 70 independent loci or genetic variants linked to various components of innate and adaptive immunity have been identified by GWAS as CD susceptibility factors[4-12]. Arguments in favor of the involvement of environmental factors in CD etiology, which are based on the observation of the irregular distribution of CD cases worldwide, have been raised. Since the appearance of IBD in the middle of the 20$^{th}$ century, the CD incidence and prevalence have shown a continually growing profile in industrialized countries or "Western" countries, such as North Europe and North America, suggesting an involvement of lifestyle[13]. Another epidemiological study also showed that "Western" diet, rich in fat and sugar and poor in fibers, is associated with an increased risk of developing CD[14]. This is recently reported that active smoking is associated with an increased risk of developing CD and that smoking cessation leads to a reduced progression of the disease comparatively to patients who still smoke[15]. Other environmental factors, such as antibiotic use, social status, microbial exposure early in life and during life have been also associated with CD[16]. Whether these factors, along with genetic susceptibility, lead directly to CD, or whether they allow the conditions needed for infectious agents to thrive, is not clear.

Numerous epidemiological studies, clinicopathological data, genetic and experimental evidence increasingly support an implication of microrganisms in CD pathogenesis. Three non-mutually exclusive theories are currently explored to explain the infectious etiology of CD: (1) a "dysbiosis", *i.e.* a modification of intestinal microbiota composition with an imbalance between beneficial and harmful bacteria; (2) an excessive bacterial translocation caused by a disrupted intestinal barrier function and defective immune responses; and (3) persistence of a pathogen. This review summarizes our current knowledge of various organisms that have been postulated as infectious agents in CD, and discusses how this may be relevant to the pathogenesis of CD and the new therapeutic approaches.

## INTESTINAL MICROBIOTA AND CD

### Human intestinal microbiota

The human gastrointestinal (GI) tract contains $10^{14}$ microorganisms of more than 500-1000 different species, forming intestinal microbiota[17]. The density of intestinal microbiota varies along the GI tract, going from $10^2$ colony forming units (CFU) per gram in stomach to $10^{12}$ CFU per gram in colon[18].

The intestinal microbial composition can vary greatly between individuals, and an epidemiological study comparing the fecal microbiota between African and European children showed that its composition is determined in part by hygiene, geography and diet[19]. Higher similarity in fecal bacterial species was reported within twins than in genetically unrelated couples sharing environment and dietary habits[20]. The gut microbiota composition of siblings also showed increased similarity compared to that of spouses, who were living in the same environment and had similar eating habits[21].

Given the complexity of the human intestinal microbiota, the characterization of its composition using conventional culture methods and morphological and biochemical-based traditional techniques is limited. Development of new biomolecular techniques, using high-throughput sequencing, allows circumventing these difficulties. Two approaches are currently available. The first is based on sequencing of the 16S ribosomal RNA coding gene (16S rDNA), which is conserved between all phylogenetic bacterial groups[22]. The second one, namely the metagenomic approach, is based on a complete sequencing of bacterial genome. The evolution of high-throughput technologies with next-generation sequencing allows producing thousands or millions of sequences at once, reducing drastically the costs and facilitating access to full metagenomic sequencing. Dominant bacterial populations in the human intestinal microbiota (> 90%) belong to two phyla: the Firmicutes and the Bacteroidetes; the remainders belong to rarer phyla such as Proteobacteria (containing genuses such as *Escherichia* and *Helicobacter*) and Actinobacteria as well as viruses, protists, and fungi[23-26]. Interestingly, mucosa-associated microbiota is different from the fecal microbiota[27]. The composition of the fecal microbiota may temporally vary following exposure to different types of foods, medications, or physical environments, and also from changes in transit time, as microbial composition in the lumen varies from caecum to rectum[24].

### Intestinal dysbiosis and CD

An imbalance of the intestinal microbiota, *i.e.* a modification of its composition, with decreased complexity of commensal bacterial profiles and higher numbers of mucosa-associated bacteria, has been reported in CD patients.

Using a 16S rDNA-based profiling technique, Ott

and colleagues showed that the diversity of mucosa-associated microbiota in specimens from patients with active CD undergoing surgery was markedly reduced compared with mucosal specimens from control individuals without inflammation[28]. Metagenomic studies have shown a decrease in the abundance of several species of the Firmicutes and the Bacteroidetes phyla in CD patients compared with control subjects[29-32]. The decrease in the abundance of Bacteroidetes could contribute to inflammation since some bacteria belonging to this phyla such as *Bacteroides fragilis* have been shown to exhibit protective effects in a mouse model of colitis induced by *Helicobacter hepaticus*, a murine commensal bacterium with pathogenic properties[33]. Among Firmicutes, a decrease of the amount of *Faecalibacterium prausnitzii* (*F. prausnitzii*) has been observed in CD patients compared with control subjects[34]. In mouse models of intestinal inflammation, administration of *F. prausnitzii* resulted in anti-inflammatory effects[34]. Therefore, the decreased abundance of *F. prausnitzii* could contribute to intestinal inflammation in CD. It has been consistently reported that CD patients have relatively increased amount of Enterobacteriaceae, particularly *Escherichia coli* (*E. coli*) species, compared with control subjects, with a more pronounced difference was observed for mucosa-associated microbiota than fecal samples[35-43]. An increase in the abundance of some mucolytic bacteria, such as *Ruminococcus gnavus* and *Ruminococcus torques*, in CD patients was also observed[44].

## ROLE OF BACTERIA IN THE PATHOGENESIS OF CD

The intestinal mucosal surface is in a continuous contact with the intestinal microbiota. Given the enormous numbers of enteric bacteria and the persistent threat of opportunistic invasion, it is crucial that the host maintains homeostasis at the luminal surface of the intestinal-microbial interface. This is mediated by a perfect integrity of the intestinal barrier and a functional immunotolerance to the intestinal microbiota and luminal antigens.

### *Excessive bacterial translocation caused by intestinal epithelial barrier dysfunction*

The intestinal barrier allows the absorption of water, ions and nutrients without leaving the microorganisms to penetrate across the mucosal surface. The first line of defence between the intestinal lumen and inner milieu, the physical barrier, is made up of a layer of columnar epithelial cells. More than 80% of these cells are enterocytes, and the rest are enteroendocrine, goblet, and Paneth cells[45]. Epithelial cells are connected *via* the intercellular junctional complexes including tight junctions, adherent junctions, desmosomes and gap junctions[46].

Many studies have shown an increased intestinal permeability in CD patients during active phases and a decreased permeability in remission phases[47-51]. Electron microscopy analyses of biopsies from CD patients in active phases revealed a reduced number of tight junctions compared with control subjects[52]. A deregulation of tight junction proteins has been reported in CD patients, with an up-regulation of claudin-2 and a down-regulation of claudin-5 and 8[52]. The alteration of intestinal permeability observed during active phases of CD could explain the chronic inflammation, given the probably resulting transit of bacteria and other luminal antigens through the mucosa, which are able to activate the sub-mucosal innate immune system.

The intestinal epithelial surface is covered by a mucus layer that prevents the contact between the epithelial layer and microorganisms and the diffusion of unwanted substances, as well as protects the physical barrier from sheer stress. The main component of the mucus layer is mucins secreted by goblet cells, which are heavily glycosylated proteins[53]. The outer loose mucus layer contains a limited number of intestinal microbes; whereas the inner adherent mucus layer contains very few microbes, forming a protected zone adjacent to the epithelial surface[54]. It is likely that the antimicrobial proteins, which are secreted by epithelial cells and are retained in the mucus layer, contribute to the maintenance of low bacterial numbers in the inner mucus layer[55]. These "bodyguards" are members of several distinct protein families such as defensins, cathelicidins, and C-type lectins, and they promote bacterial killing by targeting the integrity of bacterial cell walls[56]. Mice lacking the mucin MUC2 are unable to maintain this relative "bacteria-free" zone and suffer from intestinal inflammation[54]. It has been shown that mucin gene expression, mucus composition and secretion are altered by intestinal microbiota and host-derived inflammatory mediators[53].

### *Dysfunction of immunotolerance and innate immune response to bacteria*

Maintenance of immunotolerance and innate immune responses, which allows the control of inflammatory responses in intestinal epithelium, is mediated by several mechanisms: (1) secretion of IgA; (2) bacterial clearance *via* the production of antimicrobial peptides; or (3) a functional autophagic process. Changes in these processes have been observed in CD, which could contribute to abnormal immune responses.

**Defective secretory IgA production in CD:** The IgA immunoglobulins are secreted by B lymphocytes localized in the intestinal lamina propria[57]. The secretory IgA is transcytosed across the epithelium and retained in the mucus layer, where it acts to entrap the luminal antigens and bacteria. Bacteria present in the lumen or penetrating the intestinal epithelium are detected by dendritic cells that will alert B cells in the Peyer's patches, which will, in turn, produce IgA specific for intestinal bacteria[57]. Mice that lack activation-induced cytidine deaminase (AID), which results in defective IgA production in the intestine, exhibit an expansion of mucosa-associated bacteria such as segmented filamentous bacteria (SFB)[58]. This suggests that secreted IgA also regulates the composition and

density of bacterial communities[58]. In IBD patients, a serologic shift from an IgA-dominant to an IgG-dominant response in the intestine, which may act as another local defense line, has been reported[59]. IgG is likely to have an inflammatory effect because in response to flagellin, a common bacterial antigen, the neonatal receptor for IgG FcRn, expressed in hematopoietic cells, promotes inflammation in the presence of anti-flagellin IgG in mice[60].

**Defective bacterial killing through secretion of antimicrobial peptides:** The intestinal epithelia secrete antimicrobial molecules whose function is to kill commensal or pathogenic bacteria. Among these molecules are peptides named defensins. Most defensins function by binding to the microbial cell membrane, and, once embedded, forming pore-like membrane defects that allow efflux of essential ions and nutrients[61,62]. Two classes of defensins have been described in human, α and β-defensins. The α-defensin peptides are mainly secreted by Paneth cells and neutrophils, while β-defensins are more generally secreted by epithelial cells[61]. The biosynthesis of defensins is triggered by the activation of receptors involved in recognition of extracellular and intracellular bacterial components like Toll-like receptors (TLR) and NOD receptors, respectively, leading to a rapid killing of bacteria in contact with the intestinal epithelium[63]. Changes in intestinal microbiota were observed in mice that express the human α-defensin 5 and also in mice that do not produce functional α-defensins[64], suggesting that defensins also regulate the composition and density of bacterial communities. A decrease in α-defensin expression in Paneth cells has been reported in patients with ileal CD, particularly those carrying mutations in *NOD2* gene[65], indicating the link between infectious etiology and host genetic susceptibility. Reduced expression of β-defensins has been observed in patients with colonic CD[65]. Other antimicrobial proteins including lysozyme and RegⅢγ are secreted by Paneth cells upon exposure to bacteria or bacterial antigens[66], thereby contributing to host defense against mucosal penetration of both symbiotic and pathogenic bacteria. Mice with a genetic ablation of Paneth cells exhibit increased translocation of bacteria into the host tissues, indicating that Paneth cells contribute to maintaining luminal compartmentalization of intestinal bacteria[67]. The abnormal synthesis of antimicrobial proteins in CD patients could result in increased intestinal barrier permeability to bacteria that could consequently lead to chronic inflammation.

**Defective bacterial clearance by autophagy:** Autophagy is a homeostatic process that involves degradation of dysfunctional cellular components through the lysosomal machinery. The newly discovered specialized role of autophagy expands autophagic functions as an immune defense mechanism against intracellular pathogens (also referred to as xenophagy)[67,68]. GWAS have revealed CD-associated risk variants in several autophagy genes, such as *ATG16L1*, *IRGM*, *ULK1* (Unc-51 like autophagy activating kinase 1), *PTPN2* (protein tyrosine phosphatase nonreceptor type 2) and *LRRK2* (leucine-rich repeat kinase 2)[68]. This raised autophagy as one of the most attractive molecular pathways in the field of CD. Further efforts have been made to investigate a functional implication of autophagy in CD pathogenesis[68,69]. A link between autophagy and the innate immune receptor NOD2 has been established, the latter recruits and interacts with ATG16L1 at site of bacterial entry in the plasma membrane[70-72]. These studies have also shown that in epithelial cells, macrophages and dendritic cells, one of the *ATG16L1* or *NOD2* risk variants could result in impaired intracellular pathogenic bacterial clearance owing to a defect in xenophagy response. CD patients homozygous for the *ATG16L1* risk allele exhibited structural aberrances in Paneth cells similar to those observed in mice with hypomorphic ATG16L1 expression, *i.e.* decreased granule number and lack of lysosomes in the ileal mucus layer[73]. This indicates that defects in intestinal barrier function in CD could involve dysfunction of Paneth cells related to *ATG16L1* mutation. Interestingly, the CD-associated c.313C>T polymorphism located within the *IRGM* mRNA region results in loss of binding of microRNA-196[74]. This consequently leads to aberrance of regulation of IRGM expression by microRNA-196 and defects in autophagy-mediated control of intracellular replication of the CD-associated adherent-invasive *E. coli*[74]. Together, these studies suggest that a defect in the autophagy machinery in CD patients could lead to an uncontrolled bacterial proliferation inside host cells and consequently cause chronic inflammation.

## INFECTIOUS AGENTS AND CD

Numerous epidemiological, clinicopathological, genetic and experimental evidence has suggested an intervention of infectious agents in CD etiology. Firstly, the preferential location of the lesions in CD are situated in the terminal ileum and colon[75], where the largest population of bacteria is found[18]. Secondly, the use of antibiotics in CD treatment has been proven to be sometimes effective[76]. Thirdly, higher numbers of mucosa-associated and internalized bacteria in biopsies from CD patients compared to control subjects was also reported[37]. These observations, together with the identification of CD-associated polymorphisms in genes encoding innate immune receptors involved in the recognition of bacterial components or proteins participating in the clearance of pathogenic bacteria by autophagy highly support the hypothesis of an involvement of infectious agents in CD etiology. Those who have been suspected to modify the risk of developing CD include viruses, eukaryotes and bacteria.

### *Implication of virus in CD*

Investigations of viral agents in CD patients have been accomplished with the use of PCR and RT-PCR, and allowed to identify the Epstein Barr virus (EBV) in

15% of patients[77]. No enterovirus has been detected in the gut of CD patients[77]. Interestingly, Cadwell *et al*[78] showed that the abnormalities of Paneth cells in hypomorphic ATG16L1$^{HM}$ mice are dependent on a contact with a particular murine norovirus strain CR6, since mice raised in a germ-free condition or mice infected with a non-persistent norovirus strain exhibited normal Paneth cell morphology. In humans, several clinical studies have shown that norovirus infection can aggravate IBD symptoms[79,80]. Although there is no direct evidence showing that viral infection could be a causative factor of CD, the study by Cadwell *et al*[78] suggests that the combination of host genetic susceptibility and the presence of viral factors could lead to CD occurrence.

Bacteriophages are other viral agents that have been suspected to play a role in CD pathogenesis. Indeed, it has been shown that bacteriophages may result in dysbiosis by triggering a destabilization of microbial communities[81]. A study analyzing the bacteriophage population in CD patients reported that each patient is colonized by one dominant phage family[82]. In addition, the amount of bacteriophages is significantly increased in CD patients compared with control subjects, and is decreased in ulcerated areas compared with non-ulcerated areas[82].

### Implication of yeast in CD: Candida albicans

In 2006, the presence of anti-*Saccharomyces cerevisiae* antibodies (ASCA), involved in the recognition of a mannose residue on the surface of the non-pathogenic yeast *Saccharomyces cerevisiae*[83], was shown in the serum of 39%-70% of CD patients *vs* 0%-5% of control subjects[84]. A study proposed that the fungal pathogen *Candida albicans* could act as an intestinal pathogen by triggering the production of ASCA, given that it expresses the ASCA epitope on many surface molecules[85]. The presence of ASCA in CD patients could reflect a decrease of immunotolerance towards specific antigens of this endogenous yeast. It has been observed that CD patients and their unaffected relatives display a greater colonization of the gastrointestinal tract by *Candida albicans* with respective values of 44 and 38% than the general population with 22%[86]. In addition, *Candida albicans* colonizes and aggravates gut inflammation in mice[87]. Although its role in CD etiopathogenesis has not yet been elucidated, the hypothesis of an involvement of *Candida albicans* needs to be taken into consideration.

### Implication of pathogenic bacteria in CD

***Mycobacterium avium* subspecies *paratuberculosis*:** *Mycobacterium avium* subspecies *paratuberculosis* (MAP) is the causative agent of the Johne's disease, a chronic granulomatous ileitis most common in ruminants, but can also affect many other species including primates. Given that this pathology shares some facets with CD, MAP could be an agent implicated in the complex etiology of CD[88,89]. Research groups aiming to identify MAP in CD patients by isolation methods or by amplification of specific DNA sequences have reported contradictory results; while some show the presence of this bacterium in the blood and intestinal biopsies from CD patients[90-94], some do not[95-98]. Furthermore, serologic analyses have highlighted the presence of antibodies against MAP in 90% of CD patients[99]. Administration of antibiotics with strong activity against mycobacteria has resulted in remission in approximately 66%-75% of patients with active CD as reported by three independent studies[100-102]. Although these antibiotics are also active against other bacterial groups and their effect needs to be confirmed, these studies highlight the potential role of MAP in CD etiology.

***Yersinia*:** Yersiniosis, an infectious disease caused by the psychotrophic bacterium *Yersinia*, displays the common facets of CD, including the presence of granulomas and ulcerations along the epithelium[103]. Another study has shown the penetration of *Yersinia enterocolitica* across the epithelium *via* Peyer's patches[104]. A *Yersinia enterocolitica* oral infection induces the secretion of pro-inflammatory cytokines in mice[105]. The presence of *Yersinia enterocolitica* and *Yersinia pseudotuberculosis* strains in the gut of CD patients has been shown[106,107]. It was also reported that two cases of patients displaying terminal ileitis involving *Yersinia paratuberculosis* were diagnosed with CD thereafter[108,109]. These observations support the hypothesis of the involvement of *Yersinia* in CD pathogenesis, but further studies are required to determine their precise role.

***Listeria*:** Numerous studies have been conducted to investigate the role of *Listeria* in CD etiology[110,111]. Immunohistochemical[112] and molecular[111,113] analyses have shown the presence of *Listeria monocytogenes* in CD lesions. *Listeria monocytogenes* has been shown to disrupt and cross the intestinal barrier by entering nonphagocytic cells, escaping from the internalization vacuole, allowing bacteria to move in the cell and to spread from cell to cell[114]. A study reported that NOD2-deficient mice display an increased susceptibility to oral infection by *Listeria monocytogenes*, with a down-regulation of genes coding cryptids, the murine homologs to human α-defensins, in Paneth cells[115]. These elements are in favor of the hypothesis that *Listeria* is involved in CD etiology, but additional studies are required to ascertain its causative role.

***Helicobacter*:** Bacteria belonging to the *Helicobacter* family have been suspected to play a role in CD pathogenesis. An association between the *Helicobacter pylori* strain and the human gastric mucosal system was highlighted since *Helicobacter pylori* provokes mucosal ulcerations[116]. Numerous species of *Helicobacter* have been identified in the human gut[117,118], suggesting that they can cause pathology by colonizing the intestinal mucosa. *In vivo* studies have shown that *Helicobacter hepaticus*, a benign murine commensal bacterium closed to the human *Helicobacter pylori* strain, was able to induce considerable intestinal inflammation in immunocompromised mouse models [mice deficient in T-cell receptor alpha, T-cell receptor

beta or interleukine (IL)-10] by triggering similar immune responses to those observed in CD[119,120]. These experimental data suggest that *Helicobacter* could initiate disease in individuals being genetically susceptible to CD.

## *E. COLI* AND CD

The involvement of *E. coli* in CD etiopathogenesis has been argued for long time. According to serologic studies, the antibodies raised against the outer membrane porin C of *E. coli* (anti-OmpC) have been found in 37%-55% of CD patients[121,122]. Numerous studies have shown the presence of *E. coli*-specific antigens in biopsies from CD patients, particularly in the ulcer areas, along the fissures and within the granulomas and *lamina propria*[37,112,123,124]. These reports are in accordance with numerous independent studies showing increased abundance of *E. coli* in the mucosa-associated microbiota of CD patients with dysbiosis compared with control subjects[37-43]. Specifically, we have shown that *E. coli* abnormally colonize acute and chronic ileal lesions of CD patients comparatively to control subjects[35,36].

### *Pathogenic traits of CD-associated E. coli*

**Adhesion and invasion of epithelial cells:** Phenotypic characterization of the *E. coli* strains isolated from CD patients has evidenced their capacity to adhere to eukaryotic cells *in vitro*. It has been shown that 53%-62% of CD patients carry *E. coli* strains that display adhesion properties to buccal cells *vs* only 5%-6% of control subjects[125,126]. Another study reported that 84.6% of CD patients and 78.9% of patients with disease recurrence carry *E. coli* strains capable of adhering to human intestinal epithelial Caco-2 cells, *vs* only 33.3% of control individuals[35]. Finally, several independent studies have shown the presence of *E. coli* strains internalized in the intestinal mucosa of CD patients and their capacity to invade intestinal epithelial cells (IECs)[36,39,41,42,127]. Our group has more particularly studied the *E. coli* reference strain LF82, isolated from a chronic ileal lesion of a CD patient[35,36], and shown that LF82 is able to adhere to and to invade IECs[128].

**Survival and proliferation in host cells:** Increasing evidence has shown the capability of the CD-associated *E. coli* strains to invade, survive and replicate in IECs and macrophages. The first study showed by electron microscopy that the *E. coli* strain LF82 can trigger, in the same way as other enteropathogens such as *Shigella*, the lysis of endocytic vacuoles to be released in the cytoplasm, where the environment is more favorable for bacterial replication[129]. It has been later reported that CD-associated *E. coli* are able to survive and replicate in macrophages without inducing cell death[130,131]. The mechanism underlying these pathogenic properties of CD-associated *E. coli* has been then investigated. Given the association of polymorphisms in autophagy genes *ATG16L1* and *IRGM* with an increased risk of developing CD, it has been proposed that defects in autophagic process could allow the CD-associated *E. coli* to survive and replicate within host cells. Our group has shown that the *E. coli* strain LF82 replicates more importantly in autophagy-deficient murine fibroblasts than in wild-type fibroblasts, and in human epithelial cells and macrophages with siRNA-mediated ATG16L1 expression silencing[132]. Increased intracellular replication of the LF82 strain was also observed in human cells expressing the *ATG16L1* risk variant[132]. As discussed earlier, the CD-associated C313T mutation in *IRGM* gene results in loss of tight regulation of IRGM protein and therefore autophagy, leading to an increased persistence of the LF82 bacteria in host cells[74]. These studies suggest that impaired capacity of autophagy to handle and clear bacteria could be a mechanism underlying the increased risk of CD patients *via* increased numbers of pro-inflammatory bacteria.

**Disruption of the intestinal barrier function:** Several pathogenic bacteria are capable of disrupting the intestinal barrier to cross the mucosal surface by modulating expression and/or organization of proteins involved in establishment and maintenance of epithelial cell junctions. It has been shown that the CD-associated *E. coli* strains induce disorganization of F-actin and displacement of ZO-1 and E-cadherin from the apical junctional complex in human intestinal Caco-2 cell monolayer, leading to a drop in the trans-epithelial resistance and consequently increased epithelial permeability[127]. Likewise, the LF82 strain induces a redistribution of ZO-1 in Madin-Darby canine kidney-1 cell monolayer, causing a severe disruption of the epithelial barrier[133]. These data suggest that the CD-associated *E. coli* could play a causative role in CD etiopathogenesis by inducing disruption of intestinal barrier function.

**Inducing pro-inflammatory cytokine/chemokine production:** Several *in vitro* and *in vivo* studies have reported that the CD-associated *E. coli* can induce pro-inflammatory responses in host cells. Infection of macrophages with the LF82 strain induces the secretion of high level of TNF-α[127,130], and this is essential for the intramacrophagic replication of the bacteria[130]. This indicates that the CD-associated *E. coli* could induce production of TNF-α to create an amplification loop of replication and of inflammation. Increased production of IL-8 in LF82-infected IECs has been also reported[127,134,135]. In a transgenic CEABAC10 mouse model expressing human Carcinoembryonic antigen-related cell adhesion molecules (CEACAMs), the CD-associated *E. coli* strain LF82 can induce a severe colitis accompanied with an increase in production of the pro-inflammatory cytokines IL-1β, IL-6, and IL-17 and a decrease in that of the anti-inflammatory cytokine IL-10[136]. These *in vitro* and *in vivo* data support the hypothesis of the involvement of CD-associated *E. coli* in the etiopathogenesis of this chronic inflammatory disease.

### *Adherent-invasive E. coli: A new pathovar*

**Pathovar definition:** Analysis of virulence factors and

clinical manifestations engendered by different *E. coli* strains has allowed distinguishing six pathovars: enterotoxinogenic *E. coli*, enterohemorragic *E. coli*, enteroaggregative *E. coli*, diffusely adherent *E. coli*, enteropathogenic *E. coli* and enteroinvasive *E. coli* (EIEC)[137]. CD-associated *E. coli* strains share some virulence features with already established *E. coli* pathovars such as the ability to induce macrophage cell death, but the factors involved in the adhesion and invasion properties of the known pathovars are not present in the CD-associated *E. coli* strains[35,129]. Thus, a new pathovar was defined to classify these strains, and called adherent-invasive *E. coli* (AIEC)[129]. The criteria of this pathovar group include abilities to adhere to and to invade IECs, to survive and replicate in large vacuoles within macrophages without inducing cell death, and to induce secretion of high levels of the pro-inflammatory cytokine TNF-α by infected macrophages. The ability to trigger increased intestinal permeability also constitutes one of the pathogenic characteristics of AIEC[127]. Finally, AIEC have been shown to form biofilm and to induce granulomas formation *in vitro*[138-140]. The *E. coli* LF82 strain displays all of these characteristics, and is therefore considered as the AIEC reference strain.

**AIEC prevalence in CD patients:** Evidence has shown a high prevalence of ileal mucosa-associated AIEC in CD, since AIEC have been identified in the neoterminal ileum of 36.4%-51.9% of CD patients *vs* only 6.2%-16.7% of controls[36,141]. Comparative genomic analyses of AIEC strains isolated from different patients have shown that only one specific strain was not found in all of the patients, nevertheless, some genotypes of particular strains seem to be more frequently associated with ileal lesions of CD[40,142].

**Virulence factors of AIEC:** Genetic determinants of virulence of the AIEC reference strain LF82 are not known and are not similar to those of other invasive *E. coli* strains. Thus, they have been searched by random mutagenesis (insertion of the transposon *Tn5phoA*) and by comparison of the genome of LF82 with that of other pathogens[143,144]. These studies have permitted the identification of the lipoprotein NlpI which appears to be involved in adhesion and invasion capacities of LF82, since the insertion of the *Tn5phoA* transposon in the NlpI-encoding gene leads to a loss of invasion capacity of LF82 and the LF82-Δ*nlpI* isogenic mutant showed a decreased adhesion and invasion capacity in Intestine-407 epithelial cells[145]. Likewise, the analysis of the *Tn5phoA* insertion mutant library and the construction of isogenic mutants led to the identification of flagella and the membrane proteins YfgL, OmpC and OmpA as factors involved in adhesion and invasion properties of the reference strain LF82[146-149]. Another study showed that type 1 pili are a crucial virulence factor that allows AIEC to adhere to IECs *via* the receptor CEACAM6[150]. They are composed of a major subunit with repetition of FimA protein, minor subunits FimG and FimF, and one adhesin called FimH present at the end of the pilus[151]. Our group recently showed that point mutations in FimH confer AIEC bacteria a higher ability to adhere to CEACAM6-expressing human IECs[152]. The replacement of FimH-coding gene having an AIEC-associated mutation in the LF82 strain by a gene coding FimH of the commensal non-pathogenic *E. coli* K12 MG1655 strain decreased the ability of the bacteria to colonize the gut and to induce intestinal inflammation in CEABAC10 transgenic mice[152]. This suggests that selection of amino acid mutations in FimH is a mechanism of AIEC virulence evolution, which could increase the risk of CD development in a genetically susceptible host. Recently, we have identified long polar fimbriae (LPF) as a key factor for AIEC to target microfold cells (or M cells) on the surface of Peyer's patches, and that the prevalence of the AIEC strains harboring the *lpf* operon was markedly higher in CD patients compared with controls[153]. This operon has also been identified in other enteropathogenic bacteria such as *Salmonella* Typhimurium and been shown to be involved in specific adherence of the bacteria to M cells on Peyer's patches[154]. Interestingly, bile salts, of which the composition has been reported to be modified in CD patients[155], induce LPF expression favoring the colonization of the epithelium by AIEC[156]. These data could explain the presence of early lesions in the Peyer's patches of CD patients.

## THERAPEUTIC APPROACHES TARGETING INFECTIOUS AGENTS TO TREAT CD

Current CD treatment strategies aim to control inflammation, relieve symptoms and correct nutritional deficiencies. The treatment depends on the location and severity of disease, complications and response to previous treatment. At this time, treatment can help control the disease, but there is no cure. Established therapies for CD include anti-inflammatory agents [*e.g.*, aminosalicylates (5-ASA), omega 3 fatty acids], immunosuppressive drugs (*e.g.*, corticosteroids, azathioprine and 6-mercaptopurine) and antibiotics. An increasing number of novel and alternative therapeutic approaches are in progress[157]. New biologic therapies include the targeting of pro-inflammatory cytokines, enhancement or infusion of anti-inflammatory cytokines, blocking intravascular adhesion molecules, and modifying T-cell functions[157]. Given the increasing evidence supporting the infectious etiology of CD, therapeutic approaches to manipulate gut microbiota have been attempted by using antibiotics, probiotics, prebiotics and possibly defensins. Although these approaches are widely used, their benefits are variable and certainly not permanent. One important reason for this is the fact that the etiology of CD is complex and multifactorial, and does not include only infectious factors. Therefore, manipulation of the gut microbiota is beneficial, but, on its

own, is insufficient to cure the disease.

### Antibiotics

The beneficial effect of broad-spectrum antibiotics in the treatment of a moderate form of CD has been reported, although it lacked a large-scale clinical trial[158]. A controlled clinical trial conducted in American and Canadian centers reported that metronidazole, an antibiotic active against strictly anaerobic bacteria, is more effective in CD patients than a placebo at both a low dose (10 mg/kg per day) and a high dose (20 mg/kg per day)[159]. A therapy based on ciprofloxacin has been shown to be effective in CD treatment and is also effective in combination with conventional treatments in patients with resistant CD[160,161]. Combination of ciprofloxacin and metronidazole has been tested in treatment of acute phase of the disease and appeared to be effective[162]. Numerous clinical trials have been performed to test the potential benefit of antibiotics during different clinical manifestations of CD. Papi and colleagues showed that administration of antibiotics (metronidazole and ornidazole) is effective in preventing post-operative recurrence of CD, which is inevitable since the surgery is not curative[163]. The efficiency of antibiotics in the treatment of perianal fistulas, a complication of CD, was tested, but did not allow obtaining extended closure of fistulas[164]. The authors of this study suggest the use of antibiotics as a second-line therapy for fistula healing following the use of anti-TNF-α antibodies, which are known to be effective. Preoperative administration of antibiotics seems to reduce the risk of surgery[165]. Although antibiotic treatment is effective in some cases, it has some side effects including non-specific effects against microbiota, the possibility of inducing an antibiotic resistance and the risk of *Clostridium difficile* superinfection. Those antibiotics have been therefore recommended as a second-line treatment for CD.

### Probiotics

Given that intestinal dysbiosis has been postulated to cause CD in genetically predisposed individuals, therapeutic strategies based on the use of probiotics have been developed to modulate the imbalance of intestinal microbiota observed in CD patients.

Potential action mechanisms of probiotics include competitive interactions with enteropathogens, production of antimicrobial metabolites, influences on the epithelium, and immune modulation[166]. The use of the probiotic yeast strain *Saccharomyces boulardii* has been shown to be effective in prevention and treatment of antibiotic-associated and *Clostridium difficile* infection-associated diarrhea, as well as traveler's diarrhea[167]. Several probiotic strains have been tested in CD treatment. Treatment of CD patients with the probiotic *E. coli* strain Nissle 1917 leads to a remission more rapidly than untreated patients, without affecting the number of patients entering remission[168]. One study, although involving only a few subjects, 32 patients, reported the maintenance of remission in CD patients treated with the probiotic strain *Saccharomyces boulardii* comparatively to patients treated with mesalamine[169], of which the effect in maintaining remission has been raised[170,171]. However, a recent randomized, placebo-controlled trial reported no significant effect of the yeast *Saccharomyces boulardii* in preventing relapse following a medically-induced remission[172]. Clinical trials have been carried out to evaluate the potential efficacy of the probiotic strain *Lactobacillus GG* in the prevention of post-operative recurrence in CD patients[173] and on the average time of relapse after a medically induced remission period[174]. The first reported contradictory effects with rates of clinical and endoscopic recurrence of 16.6% and 60%, respectively, in the *Lactobacillus*-treated group *vs* 10.5% and 35.3% in the placebo group. The second showed a shorter average time of relapse of 9.8 mo in patients treated with the probiotic *vs* 11 mo in the placebo group. Another strain of *Lactobacillus*, *Lactobacillus johnsonii*, was tested in CD patients during two double-blind trials, and both reported no significant effect of this strain in preventing clinical recurrence of the disease following a surgically-induced remission in probiotic-treated patients comparatively to the placebo group[175,176]. Although probiotics may be the most physiologic and non-toxic way to prevent and treat CD, it may be transient and has a limited and debatable usefulness at present.

### Fecal microbiota transplantation

Given the potential role played by intestinal microbiota in CD pathogenesis, another therapeutic approach has been considered for CD treatment: fecal microbiota transplantation. The transfer of fecal microbiota from a healthy individual to the gut of a patient, enabling the re-establishment of a normal microbial community, has been shown to be effective in the treatment of ulcerative colitis[177], another form of IBD, or infection with *Clostridium difficile*[178]. *Clostridium difficile* infection has become a major public health problem, occurring after antibiotic treatment or ingestion of spores in the environment. In patients with a recalcitrant infection, fecal microbiota transplantation has been shown to be effective, with an efficiency rate of 90%[179,180]. Only few case reports and case series of fecal microbiota transplantation for the management of CD have been published. The first case was a 31-year-old man diagnosed with terminal ileal CD who remained symptom-free for 4 mo after the transplantation[181]. Among the other cases reported, the use of fecal microbiota transplants leads to CD resolution, *i.e.* to a complete cessation of symptoms or to the absence of active disease confirmed by endoscopic and histologic analyses, but in most cases, it does not[182]. A recent study reported the effectiveness of fecal microbiota transplantation in a case of severe fistulizing CD with a sustained clinical remission for more than 9 mo after the treatment[183]. Fecal microbiota transplants have also been used to manage *Clostridium difficile* infections in CD patients, and it appears to be effective in most of patients with a reduction or a complete resolution of the

infection-associated diarrhea[182]. More clinical trials with better standardized protocols are required to confirm the beneficial effect of fecal microbiota transplantation in treatment of this complex disease.

## CONCLUSION

Since the first description of CD in 1932, numerous research groups worldwide have attempted to unravel the complex and multifactorial etiology of the disease to develop a curative therapy. In addition to the identification of genetic and environmental risk factors in CD, increasing lines of evidence have supported a role for infectious agents in CD etiopathogenesis. These include the disruption of the intestinal barrier function associated with excessive bacterial translocation, an intestinal dysbiosis, defects in the secretion of IgA entrapping antigens and bacteria in the intestinal lumen and inefficacity of autophagy-mediated clearance of intracellular bacteria. These defects, which have been reported in CD patients, can lead to the emergence of infectious agents (viruses, eukaryotes or bacteria) that could induce chronic inflammatory characteristic of CD. Advances in the knowledge of infectious etiology of CD enable to develop different therapies based on the clearance of CD-associated pathogens, the modification of the imbalanced intestinal microbiota and re-establishment of a "healthy" microbiota with the use of antibiotics, probiotics and fecal microbiota transplantation. However, these therapies on their own are insufficient to provide a cure for CD. Therefore, successful CD therapies are likely to require multiple pathway-integrated treatments depending on the stage of the disease and each patient subset.

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