CHAPTER 7

SUMMARIZING DISCUSSION AND FUTURE PERSPECTIVES

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SUMMARIZING DISCUSSION

Crohn’s disease (CD) and ulcerative colitis (UC) are autoimmune chronic inflammatory disorders of the intestine that are collectively known as inflammatory bowel diseases (IBDs). The pathological basis of IBD is incompletely understood, but it is generally assumed that it finds its origin in an exaggerated inflammatory response to gut luminal contents, caused by an inaccurate response to environmental factors due to the specific genetic makeup of the individual. This complex interplay of genetic, microbial and environmental factors concludes in a sustained activation of the mucosal immune system, resulting in chronic inflammation and tissue destruction caused by excessive secretion of pro-inflammatory molecules. Moreover, the inability to accurately suppress the immunological response and repair the epithelium primes the gut for future flare-ups that could be triggered by rather subtle environmental changes, such as a mild infection. A typical factor from the Western lifestyle is cigarette smoking, which is beneficial for UC, but detrimental for CD. Due to these remarkable opposite effects, cigarette smoking is one of the best studied and most replicated environmental factor in IBD. Despite strong epidemiologic data, the mechanism(s) how smoking impacts on the IBDs remains unclear. However, it is very likely that the smoke-induced effect(s) strongly depend(s) on the specific host genome and only manifests in combination with specific gene variants. In this thesis, we undertook in-depth studies to characterize individual and combined effects of cigarette smoke and the CD-associated genetic variant ATG16L1-T300A on various human cell types and the microbiome in order to clarify their role in disease development, in particular the opposite effects of smoking on CD and UC.

The current knowledge on the etiology and the influence of susceptibility factors (e.g. genetic variants, environment, and intestinal flora) is summarized in Chapter 1. Substantial advances in our understanding of the pathogenesis and management of IBD have been made in the last decade. The combined power of genetics and cellular/molecular biology has begun to expose the key mechanism in the etiology of IBD as well as help to predict the response to specific therapies. CD has been a showcase for the discovery of susceptibility genes in complex polygenic disorders. Most prominent CD-associated genes appear to be involved in recognition and processing of microbial antigens at the mucosal surface. In 2010 (Chapter 2), we reviewed the contribution of CD-associated genes NOD2, ATG16L1 and IRGM on the functionality of innate immune responses in controlling the intestinal bacterial load. Since then, even more information has emerged from large meta-analysis
of population-based genome-wide association studies and at present 163 IBD susceptibility loci are identified. The main task we are facing now is to define the causal mutations in the IBD susceptibility loci and to determine the functional effects that underlie the development of chronic intestinal inflammation, in particular in combination with the disease-associated environmental factors.

**GENE-ENVIRONMENT INTERACTIONS ~ CIGARETTE SMOKE-MEDIATED AMELIORATION OF UC BY ANTI-APOPTOTIC SIGNALING**

The relationship between smoking and inflammatory bowel disease (IBD) is well recognized since many years. A large number of epidemiological studies have described the dual effect of smoking. It is associated with a higher risk for and worse outcome in CD, whereas UC is primarily a disease of non-smokers and former smokers. The wealth of descriptive information on smoking and IBD is in sharp contrast with the limited amount of mechanistic studies exploring the effect of cigarette smoke on the gut and on intestinal inflammation in particular. The molecular and cellular mechanisms by which smoking affects the pathogenesis of CD and UC are only marginally understood and no consensus exist about the most important pathways that play a role. Cigarette smoke has been shown to modulate mucosal immune responses, alter intestinal cytokine and eicosanoid levels and modify gut permeability, but none of these hypotheses offer an explanation for its opposite effect in CD and UC. In Chapter 3 we aimed to identify smoke-induced mechanisms that could explain the dichotomy in UC and CD by focusing on the phosphorylation events that are induced by it. Cigarette smoke induced the phosphorylation of several kinases, including STAT5B, ERK1/2, PI3K, Akt and AMPK in both intestinal epithelial cells (DLD-1) and T-lymphocytes (Jurkat). These effects of smoke have also been described in previous studies on COPD and lung cancer, suggesting that this is a conserved effect of cigarette smoke in different tissues of the human body. Several of these kinases are involved in anti-apoptotic signaling, in particular through the anti-apoptotic proteins Bcl-XL and Bcl-2. In accordance, we observed enhanced expression of Bcl-XL/Bcl-2 proteins, as well as the pro-survival proteins GSK3β, cyclin D1 and LC3-II. Moreover, we found that cigarette smoke enhanced the metabolic activity of DLD-1 and Jurkat cells, suggesting that it enhances cell survival at these concentrations. These observations directed us to investigate the protective effects of smoke during inflammatory conditions in more detail. Indeed, cigarette smoke exposure protected DLD-1 and Jurkat cells against anti-Fas-, cytokine- and reactive oxygen stress-induced apoptosis. Since this effect
is observed in both cell types, with DLD-1 cells serving as a model for epithelial cells in UC and Jurkat cells as a model for T-cells in CD, we hypothesized that a general anti-apoptotic effect of smoking could have opposite effects in both IBDs. Apoptosis is a key process in maintaining intestinal homeostasis, as it preserves the epithelial barrier by regulating the balance between cell proliferation and cell death in the surface epithelium. Minor perturbations in the proliferation/apoptosis balance may disturb the epithelial barrier leading to increased permeability. This will allow luminal pathogens to pass the epithelium, triggering the immune system and inflammation of the gut, a typical condition observed for UC\textsuperscript{6-8}. We propose that the beneficial effect of cigarette smoking as observed in UC patients is due to inhibition of inflammation-induced apoptosis of intestinal epithelial cells, thereby improving barrier function. Apoptosis is also necessary for maintaining the T-cell population to appropriate numbers by preventing an uncontrolled inflammatory response. CD is characterized by apoptosis-resistant T-cells, which results in an inappropriate cellular expansion and subsequent inflammation\textsuperscript{9,10}. In this chapter, we show that cigarette smoking protects T-cells against inflammation-induced apoptosis, exerting its detrimental effect by further enhancing the resistance of T-cell to apoptosis. The action of the anti-TNF drug, infliximab, has partly been assigned to the induction of apoptosis in T-cells, thereby restoring gut homeostasis\textsuperscript{11,12}. Exposure to cigarette smoke may therefore antagonize the therapeutic effect of this drug. Indeed, smoking has been reported to be associated with primary failure of anti-TNF therapy, as well as being a risk factor for loss of response\textsuperscript{13-17}. From these data, we propose that a generalized anti-apoptotic effect of cigarette smoke may lead to mucosal healing in UC, but at the same time further impair proper T-cell termination in CD, and thus explain the dichotomy of smoking in the IBDs.

In Chapter 4 we extended our search for genes and/or proteins that are involved in smoke-induced anti-apoptotic signaling by performing microarray analysis on smoke-exposed DLD-1 and Jurkat cells. The main aim of this study was to identify genes and/or proteins that may contribute to the protective effect of smoking in UC. UC predominantly affects non-smokers and former smokers and smoking reduces the severity and extent of colitis\textsuperscript{18-20}. Flare-up episodes, hospitalization rates, need for oral steroids and colectomy rates are reported to be lower in smokers compared to non-smokers\textsuperscript{2,19,21}. However, the reported effect of smoking on disease initiation and progression are sometimes ambiguous and/or inconsistent as the effects of smoking may depend on the smoking dose and manner (e.g. active, passive, filter), the gender, disease location and severity\textsuperscript{21,22}. Regardless of the fact that smoking
ameliors UC, gastroenterologists still advice these patients to quit smoking, although the favorable outcome of cigarette smoking in UC patients makes it probably difficult for patients to do so. In addition, smoking is a known stress-reliever that might act through the gut-brain axis to reduce stress responses in UC patients and thereby suppress flare-ups\(^2\). Nevertheless, patients have to remain aware of the health risks of smoking as it predisposes for all kinds of cancer.

Whole transcriptome analysis described in this chapter, revealed that the most prominently smoke-induced genes and enriched pathways belong to the heat shock protein family. Analysis of mRNA as well as protein levels showed that smoke dose-dependently enhanced the expression of several heat shock proteins, in particular \textit{HSPA6}. Immuneprecipitation experiments revealed that HSPA6 physically interacts with and stabilizes the anti-apoptotic protein Bcl-XL, which is in accordance with the enhanced expression of Bcl-XL/Bcl-2 in smoke-exposed DLD-1 and Jurkat cells in Chapter 3. Together with an earlier study that showed that another 70 kDa HSP (HSP70) has anti-apoptotic properties due to its interaction with Bcl-XL\(^2\), these data support a role for HSPA6, and HSP70s in general, in controlling apoptosis. This shines a new light on the potential mechanisms that are involved in the protective effect of cigarette smoking in UC. Heat shock proteins are stress-responsive proteins serving as molecular chaperones that respond strongly to environmental changes and maintain protein homeostasis (e.g. preventing protein misfolding and aggregation and assisting in folding of proteins into their native state), thereby providing cytoprotection\(^2\). The gastrointestinal tract holds a highly hostile environment, e.g. acidic condition in the stomach and the colon, which continuously faces potentially disease-causing bacteria and luminal antigens. Cell protective mechanisms, in particular in the mucosal epithelium, may therefore be of utmost importance in this area of the human body. Indeed, several HSP70s are constitutively expressed in colonic mucosa and they are important for intestinal homeostasis\(^2\). These studies correspond well to our findings that show that HSPA6 is highly expressed at the surface of epithelial cells in colon biopsies of healthy non-smoking volunteers. Cigarette smoke exposure leads to a rapid, but short-lived induction of HSPA6 in DLD-1 cells. Higher levels of HSPA6 were not observed in the colonic mucosa of healthy volunteers who smoke, though the HSPA6 expression in this group was highly variable. The high basal levels of HSPA6 and the short-lived induction of the protein may explain why we did not observe a strong smoke-dependent response of HSPA6 in colon biopsies of healthy smoking volunteers. The level of various HSP's
is enhanced in the mucosal epithelium of IBD patients and this has led to the view that these proteins may promote intestinal inflammation, rather than supporting cellular intestinal protection. However, animal models of colitis have clearly shown the opposite, e.g. overexpression of HSPs suppresses the levels of pro-inflammatory cytokines and subsequent intestinal inflammation\textsuperscript{32-34}. Thus, enhanced epithelial HSP expression in IBD patients is most likely the result of the inflammatory conditions in the gut. HSPA6 is exclusively present in large mammals\textsuperscript{35}, suggesting that this protein may have a highly specialized function in these animals and humans. Unfortunately, no clear and definitive function of HSPA6 is established yet, but it does not seem to carry the typical protein aggregation-suppressing activity, nor is it able to protect cells from heat-induced cell death\textsuperscript{27}. Since the function of endogenous HSPA6 cannot be studied in rodents, we need human (genetic) studies to identify possible roles for HSPA6. A comparison of smoke-induced genes to genes that reside in UC susceptibility loci revealed that HSPA6 is located in the UC susceptibility locus rs1801274, together with genes encoding immunoglobulin receptor FCGR2A and FCGR2B. FCGR2A in this locus is considered as the potential disease-modifying gene. However, the non-synonymous rs1801274 is associated with decreased HSPA6 mRNA levels in individuals homozygous for this risk variant and may therefore also increase the risk for UC\textsuperscript{1,36}. The presence of more than one disease risk gene in one locus is not uncommon as it has also been described for the UC susceptibility genes IL-2 and IL-2\textsuperscript{37} and the rheumatoid arthritis susceptibility genes\textsuperscript{38}.

The smoke-contained component(s) that is (are) responsible for the induction of HSPA6 and contribute to the anti-apoptotic effect of cigarette smoking and potential protection against colitis need still to be identified. This is complex, because of the large number (~5,000) of components in cigarette smoke. Although nicotine, CO and acrolein showed, to variable degrees, cytoprotection against inflammation-induced apoptosis (Chapter 3), it remains to be determined whether they and/or other compounds selectively induce HSPA6. Interestingly, acrolein and the anti-ulcer drug geranyl-geranyl acetone (GGA) have already been identified as HSP70-inducing compounds\textsuperscript{39-42}, but this is not established yet for HSPA6. Studying the exact (anti-apoptotic) role of HSPA6 will provide more insight in the smoke-dependent effect on UC and may lead to new therapeutic approaches.
GENE-ENVIRONMENT INTERACTIONS ~ CIGARETTE SMOKE AGGRAVATES CD WHEN COMBINED WITH DYSFUNCTIONAL AUTOPHAGY OF PATHOGENIC BACTERIA

Smoking is associated with more flare-up episodes and complications in CD, as well as increased need for steroids and immunosuppressants. As stated before, the effect of smoking depends on the gender and disease location, therefore more severe disease is observed in smoking women with distal ileal disease. In addition to active smoking, CD patients are more often exposed to cigarette smoking in childhood than controls, suggesting that passive smoking is also an important risk factor for the development of CD. Although cigarette smoking is the most prominent environmental risk factor for CD, its potential interaction with genetic susceptibility remains almost completely unexplored. The heritability of CD is well established and could be the main prerequisite for developing disease, however, it likely requires a trigger from the environment. In fact, these triggers are thought to dominate the heritability of IBD and have a decisive role in IBD development. In other words, if IBD is purely genetically determined, the genetic linkage should be 100%. Since this is not reflected in, for example, twin concordance studies and the genetic heterogeneity of the human population, complex gene-environment interactions are key in developing IBD.

The discovery of CD-associated mutations in the NOD2 gene highlighted the role of innate immunity in CD pathogenesis and causes exaggerated intestinal due to an impaired response to bacterial ligands. This primed the search for other genetic factors that are associated with CD, which has been boosted significantly by recent Genome Wide Association Studies (GWAS). These GWAS revealed for the first time the important role of autophagy in CD, with two of the most significant associations in ATG16L1 and IRGM. The majority of functional studies on NOD2, ATG16L1 and IRGM have mainly focused on degradation of internalized bacteria by a specialized form of autophagy, namely xenophagy. Autophagy was originally described as a starvation-induced mechanism in which organelles and cytosolic material are randomly encapsulated, broken down and recycled to provide nutrients to support cell survival. Besides this conventional function, it plays a crucial role in conferring resistance to infection by elimination intracellular pathogens, thereby defining mucosal tolerance. It has recently been shown that a physical interaction between NOD2 and ATG16L1 is required for autophagic clearance of intracellular pathogens. CD associated polymorphisms in NOD2 and ATG16L1 impair the autophage processing of internalized bacteria, which may alter the microbial composition in the gut mucosa leading to chronic...
inflammation. As autophagy is strongly controlled by the cellular environment, it is likely that environmental factors, e.g. smoking, interact at the level of autophagy and support disease initiation and/or maintenance. Studies on chronic obstructive pulmonary disease (COPD) have shown that smoking strongly enhances autophagy levels in alveolar macrophages\textsuperscript{70-74}. The conditions in lung resemble those in the gut, as both alveolar and gut macrophages are essential for clearing bacteria at the epithelial surface. Defects in autophagy have shown to increase the susceptibility to pneumonia\textsuperscript{75,76}, perhaps in a similar way it does for CD. A possible smoke-gene interaction was reported by Fowler et al., showing that the allele frequency of the \textit{ATG16L1-T300A} risk variant is significantly higher in CD patients who smoke compared to non-smokers. The collective risk effect of smoking, SNP status and CD risk was higher than the cumulative effect of the individual factors, suggesting a synergistic action of these factors on CD pathogenesis\textsuperscript{77}. However, so far there is no experimental evidence for the potential interaction of the \textit{ATG16L1} genotype and cigarette smoke.

In Chapter 5 we assessed the interaction between cigarette smoking and innate immune processes associated with CD (e.g. phagocytosis and autophagy). A remarkable first finding was the increased phagocytotic activity of primary human monocytes homozygous for the \textit{ATG16L1-T300A} risk variant compared to those homozygous for the wildtype variant. The enhanced phagocytosis was non-specific, as it was detected for commensal \textit{E. coli}, CD-associated adherent invasive \textit{E. coli} (AIEC) as well as zymosan (yeast) particles. During internalization and degradation of microbes, adaptive immune responses are also activated. When the degradation system becomes inadequate, in this case through the T300A polymorphism, these adaptive responses become overactivated (e.g. enhanced levels of pro-inflammatory cytokines; IL-6, IL-1\(\beta\)) to compensate for this loss\textsuperscript{52,53,58,59,69}. This could result in a higher percentage of activated monocytes. The second remarkable finding was that cigarette smoke impaired the uptake of commensal \textit{E. coli} in \textit{ATG16L1-T300A} homozygous monocytes, while uptake of AIEC was not affected. In contrast, cigarette smoke did not affect the phagocytotic activity of \textit{ATG16L1-T300A} monocytes, neither of commensal \textit{E. coli} nor that of AIEC. In addition, the \textit{ATG16L1-T300A} genotype was associated with enhanced non-selective autophagy, but cigarette smoke increased autophagy in an \textit{ATG16L1} genotype-independent manner. Cigarette smoke exposure led to more efficient killing of AIEC by \textit{ATG16L1-T300} homozygous monocytes, but was completely ineffective in \textit{ATG16L1-T300A} homozygous monocytes. These data may provide an explanation for the abnormal AIEC colonization of ileal lesions in CD.
The ATG16L1 risk variant enhances uptake of AIEC by innate immune cells, but these are unable to properly degrade them due to impaired xenophagy. Cigarette smoke enhances xenophagy/bactericidal activity of the ATG16L1-T300 innate immune cells, but is unable to do so in the ATG16L1-T300A genetic background, thereby promoting inflammation. Consequently, the abundant occurrence of pathogenic bacteria, such as AIEC, might cause relevant changes in gut microbiota, also referred to as dysbiosis, a condition often observed in CD patients. These alterations disturb the balance between the four most dominant phyla: Bacteroidetes, Firmicutes and to a lesser degree Proteobacteria and Actinobacteria. Moreover, it also decreases the biodiversity of bacterial species in the gut. Consequently, CD patients have reduced numbers of bacteria with anti-inflammatory properties and/or more bacteria with pro-inflammatory properties compared to healthy controls. It remains largely unknown whether the level of gut dysbiosis is the cause or consequence of disease activity in the gut.

In conclusion, we show that smoke-induced autophagic processing of pro-inflammatory microbes is ineffective in genetically predisposed monocytes and may be the initiator for gut dysbiosis. This may cause an uncontrolled inflammatory response to intestinal pathogens and/or commensals.

In Chapter 6 we showed that biopsies of inflamed ileal mucosa from CD patients homozygous for the ATG16L1-T300A risk variant contain enhanced (relative) numbers of pathogenic bacteria species from the Enterobacteriacea (E. coli), Bacteroidaceae (B. fragilis) and Fusobacteriaceae phyla, compared to inflamed ileum of ATG16L1-T300 homozygous patients. In contrast, Lachnospiraceae (Firmicutes) were more common in inflamed ileal biopsies of the later patient group and those commensal gut microbes are considered to be beneficial for gut homeostasis. Notably, these differences were only observed in the inflamed tissue. In line, ATG16L1-T300A homozygous monocytes showed impaired killing of AIEC upon stimulation with PMA in an in vitro model for inflammation. Thus, the ATG16L1-T300A phenotype causes bacterial dysbiosis in the ileal mucosa, which is only apparent under inflammatory conditions. These observations support the fact that the microbial composition is modulated by the host genetic background and may impair proper handling and killing of bacteria by the innate immune system thereby potentiating disease. Especially in combination with cigarette smoking that exerts both immune-suppressive (phagocytosis) and immune-stimulating (autophagy) effects in monocytes carrying the ATG16L1 risk variant (Chapter 5), this may further aggravate CD pathology. It is therefore now of importance to determine whether cigarette smoke leads to altered...
immune responses to the gut microbiome in patients that are (genetically) unable to effectively handle and kill intestinal microbes. The integrated analysis of the triangle of genetic susceptibility, gut microbiome and environmental factors holds the key for elucidating the etiopathology of IBD and will provide new crucial leads for disease management and therapy.

FUTURE PERSPECTIVES

Important progress has been made in recent years in our understanding of IBD pathogenesis. The general view that “IBD is caused by an inappropriate immune response to commensal gut bacteria due to a combination of genetic and environmental factors” has not been changed, but we know much more details about the specific factors and cellular pathways involved. This knowledge is obtained from high throughput genomic approaches, investigation of environmental changes, molecular analysis of gut bacteria flora, and a more integrated understanding of the interaction between innate and adaptive immune responses. The growing number and diversity of genetic loci associated with IBD (163 to 177)\textsuperscript{1,92}, provide major challenges to the investigation of how they functionally impact immunity and inflammation in susceptible individuals. The bulk of the research on IBD genetics has focused on the impact of mutations in coding regions of genes to allow hypothesis-driven research in studying the pathogenic mechanisms and exploring potential therapeutic targets. While these studies have been valuable in helping to characterize IBD susceptibility genes, approximately 70% of known IBD susceptibility loci are not linked to any protein coding variant or does not contain protein coding genes at all. The ENCODE (Encyclopedia of DNA elements) project addressed the importance of non-coding DNA elements and revealed that the majority of the disease-causing SNPs reside in region that affect gene expression\textsuperscript{93-96}. They could act at the level of DNA, through modification of transcription factor binding sites or epigenetic modification of regulatory regions that control the expression level of a given gene, as well as at the RNA level, through long intergenic non-coding RNAs or microRNAs (miRNAs)\textsuperscript{97}. For example, a specific group of miRNAs was enhanced in muramyl dipeptide (MDP)-treated cells, which activates the NOD2 pathway, implying that these miRNAs may be involved in fine-tuning the inflammatory response and act synergistically with IBD variants in these inflammatory pathways\textsuperscript{98}. However, the precise role of non-coding RNAs in IBD pathogenesis needs still to be defined. Nevertheless, it remains crucial that we further study the real contribution of the locus and the role of both coding and non-coding variation to disease susceptibility.
and design functional studies to examine the putative role in IBD pathogenesis.

The importance task for us is to analyze in much more detail the role of environmental factors and their putative interaction with IBD susceptibility loci in disease initiation, maintenance and/or progression. We set out to determine the effect of cigarette smoke in IBD, being an intriguing environmental IBD risk factor as it exerts opposite effects on CD and UC; good for UC and bad for CD. While this disease-based discrimination of the smoke effect is most often named, one could also speculate that cigarette smoke has opposite effects on colon versus ileum and as a result affect the localization of disease. So regardless of the IBD subtype (CD or UC), it may be beneficial for colonic inflammation and detrimental for ileal inflammation. This hypothesis is supported by the observation that CD patients who smoke have increased ileal involvement and less colonic involvement in the disease.7,46 In addition, ileal involvement was more pronounced in smokers with IBD, especially in women.99-101

The effects of cigarette smoking on the development and course of IBD have been studied for decades, but it is still unknown which pathways are directly influenced by smoking and which components of cigarette smoke are involved. In this thesis, we report an anti-apoptotic effect that may aid to the smoking-mediated protection in UC. This is most likely driven by heat shock proteins, in particularly HSPA6, a gene unique to large mammals. Other heat shock proteins, such as HO-1, are also under investigation in explaining why smoking is beneficial for UC. HO-1 converts biliverdin to bilirubin and carbon monoxide (CO) and the latter product is able to reduce intestinal injury. Thus, CO is a good candidate to be one of the effector molecules in smoke that suppresses intestinal inflammation in UC. Experiments are underway to test CO-releasing molecules for their therapeutic effect in animal models of colitis. This may eventually lead to new therapeutic approaches in which CO-releasing molecules are used in the treatment of UC. In addition, future studies may also focus on identifying components in cigarette smoke that selectively induce HSPA6 that through an anti-apoptotic action can assist in healing of the mucosal epithelium in UC.

Smoking has a pronounced effect on the first line immune defense, the innate immune response, where it impairs uptake (phagocytosis) and enhances degradation of internalized bacteria. However, in genetically predisposed individuals, e.g. those homozygous for the ATG16L1 risk allele, both processes are unfavorably affected. Innate immune cells of these individual take up more bacteria, but cigarette smoke...
does not enhance their intracellular processing, which triggers sustained release of pro-inflammatory cytokines. Moreover this provokes an overly active T-cell-mediated response, which is further enhanced by smoking by increasing the resistance of T-cell toward apoptosis. It remains important to target these T-cells with a therapy to enhance apoptotic cell death primarily aiming at induction and/or maintaining remission of the disease. However, if the impaired innate immune response is not targeted as well, such therapies may only be affective in subgroups of CD patients. In line with our data, it has been reported that infliximab therapy is less effective in CD patients who smoke.

Since autophagy is an important regulator of both innate and adaptive immune and/or inflammatory processes, stimulation of this signaling pathway might be an appealing approach to suppress and restore the imbalanced inflammatory responses in patients with CD. In contrast to targeting a major inflammatory initiator, such as TNF-\(\alpha\) and subsequent harsh suppression, modulation of autophagy might be a more gentle method to control and normalize the imbalanced immunity. Several autophagy modulators are investigated. For example, sirolimus, an autophagy inducer, is used as maintenance therapy to prevent rejection after organ transplantation. Further, in a case study it was shown that sirolimus markedly improved disease symptoms and induced healing in a patient with severe refractory CD\textsuperscript{102}. Also, the sirolimus-derivate everolimus stimulates autophagy and was shown to have therapeutic effects in rheumatoid arthritis and CD\textsuperscript{103,104}. With the growing interest in modulators of autophagy as potential treatment options, beneficial effects of curcumin, the yellow pigment in the spice turmeric, in IBD have been ascribed in part by its anti-inflammatory and autophagy-inducing effects\textsuperscript{105-109}. Therefore, autophagy-modulating compounds may have great value in the treatment of CD, although the question remains to what extent genetic factors limit or interfere with the therapeutic effect of these modulators.

Similarly, it remains to be determined whether specific IBD-risk loci require specific environmental factors to enhance the chance for getting IBD. More specifically, does ATG16L1-T300A also increase the risk for CD in a cohort of non-smokers? So far, no GWAS has made a subdivision in smokers versus non-smokers and the effect on IBD susceptibility loci. Cigarette smoke prevents correct degradation of engulfed microbes in innate immune cells homozygous for ATG16L1-T300A, which leads to enhanced secretion of pro-inflammatory cytokines. Individuals who smoke are overrepresented in CD cohorts and thus are biased to show the association of ATG16L1 with CD. It remains to be determined whether ATG16L1-T300A is a risk
factor in non-smoking CD patients. The fact that the ATG16L1-T300A variant is the dominant allele in Caucasians implies that at some time in history there was positive selection for this allele. Indeed, several studies have implicated protective roles for this variant in the protection against acute urinary tract infections\textsuperscript{110} and enhanced resistance to \textit{Citrobacter rodentium} infection\textsuperscript{111}.

Accumulating evidence suggest that there is a causal relationship between an abnormal composition of the gut microbiota and IBD. Thus, approaches to change this composition might be considered as therapy. Antibiotics that selectively eliminate pro-inflammatory bacteria or administration of probiotics to promote anti-inflammatory bacteria might be used as primary therapies instead of immunosuppressive drugs that can cause severe side effects. The use of probiotics to increase concentrations of beneficial species, such as \textit{Bifidobacteria}, \textit{Lactobacillus} and \textit{Faecalibacterium prausnitzii} holds promise and could be established with nutritional intervention\textsuperscript{112,113}. The same accounts for fecal microbiota transplantation, a treatment that involves transplanting the intestinal bacteria of a healthy person to a person with IBD. These approaches have been shown to maintain remission of IBD, particularly in UC and to a lesser extend in CD patients\textsuperscript{114-116}. Furthermore, ingestion of \textit{Bifidobacteria}-fermented milk by UC patients increased the levels of short-chain fatty acids, including butyrate, which are important energy sources of \textit{Bacteroides} and \textit{Clostridium} species. Thus, therapeutic effects can be established by probiotic dietary supplementations in IBD patients. On the other hand, aberrant composition of the gut microbiome is also dictated by the host genome and exposure to specific environmental conditions, which are both difficult, if not impossible, to modify. Environmental conditions may trigger the genetically defined host immune response in such a way that some individuals become more susceptible to IBD. Given the heterogeneity of the environmental conditions (e.g. smoking, diet, NSAIDs, appendectomy) and the lack of detailed description of these environmental factors in large cohorts, it is difficult to construct an integrated model of disease development for IBD at this moment. Large population-based studies, like the Lifelines project in the Netherlands, are essential to study the interaction between genetic and environmental factors in complex disease, such as CD and UC. One hundred sixty five thousand (165,000) participants are enrolled in the Lifelines project and will be followed for 30 years in which life style details are documented and biomaterials (blood, urine, stool) are collected. This will provide researchers with a wealth of information on how and why individuals develop chronic diseases.
The results of such well-designed prospective cohort studies are essential to obtain detailed information about the contribution of environmental factors to disease development.

For effective management of IBD, we need this information to identify the driving factors in the host genome, the environment and the gut microbiome, as well as their mutual interactions. This needs to be the primary focus of future research, in order to develop personalized therapies for patients with IBD.

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