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Environmental Factors and Their Impact on the Intestinal Microbiota: A Role for Human Disease?

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Key Words
Microbiota · Inflammatory bowel disease · Ulcerative colitis · Smoking cessation

Abstract
The intestinal microbiota and its potential role in human health and disease have come into the focus of interest in recent years. An important prerequisite for the achieved advances with regard to a better characterization of its complex composition and influencing factors is the increasing availability and affordability of culture-independent methods, such as high-throughput sequencing technologies. We discuss some general aspects of the intestinal microbiota. Recent insights into its potential pathogenetic role in the metabolic syndrome and inflammatory bowel disease will also be discussed that imply an impact of smoking status and smoking cessation on intestinal microbial composition.

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General Aspects

An incredible number of microorganisms colonizes the human gut: around 10–100 trillion [1] in an impressively high density of 10^{11}–10^{12} organisms per milliliter of luminal content [2], with Bacteroidetes and Firmicutes as the most abundant (more than 90%) phyla in humans and mammalians [2–4]. In recent years, the human intestinal microbiota has moved increasingly into focus, via research in a variety of medical and biological subdisciplines. Its important influence in the development of innate immunity [5, 6] and the regulation of epithelial development or nutrition [7, 8] is now well established.

The gut microbiota seems to play an important role in the initiation and perpetuation of intestinal inflammation as seen in animal studies with germ-free maintained mouse models, where intestinal inflammation did not develop [9, 10]. Direct evidence for the thesis that intestinal microbes are necessary to initiate and maintain intestinal inflammation in humans was derived from the observation that, during diversion of the fecal stream, no signs of inflammation were apparent in patients with CD – with prompt recurrence, however, after the restoration of intestinal continuity and the fecal stream [11].

Besides the association of the gut microbiota with inflammatory bowel disease (IBD) already having been known for some time, there is accumulating evidence of a role in the pathogenesis of various other disease states, such as irritable bowel syndrome [12, 13], obesity and the metabolic syndrome [14, 15]. Furthermore, a pathogenic role is also suggested in diseases where a link to intestinal

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microbes appears less obvious, such as rheumatoid arthritis [16], multiple sclerosis [17] and even psychiatric or behavior disorders including autism [18] or alcoholism [19].

Although our knowledge about alterations in microbial composition in various disease states has increased in recent years, achievement of several important research goals in the field of the intestinal microbiota still awaits completion:

What is it exactly, that represents a normal gut microbial composition? What are the main factors influencing the composition – predominantly the environment or genetic factors? What is the impact of global variations in microbial composition – are the findings and implications of different studies investigating the gut microbiota in health and disease from different countries comparable? Is there a core gut microbiota?

An essential prerequisite to being able to identify alterations in microbial composition in various disease states is to characterize a 'normal' microbiota as precisely as possible. However, the impressive interindividual [20, 21] (and to a lesser degree intraindividual) differences with regard to species composition, but also the relative abundance of phyla, constitutes a major obstacle. The debate is ongoing as to whether there is a core gut microbiome, i.e. a certain set of universal microbial organismal lineages that all human individuals share. For instance, although no single bacterial phylotype could be detected in abundant frequency in all examined monozygotic and dizygotic twin groups, the existence of a core gut microbiome at the level of shared genes was postulated [22]. However, as the degree of similarity (revealed to be higher in families when compared to unrelated individuals) did not differ between monozygotic and dizygotic twins in that study, the genetic influence on microbiota composition appears to be weaker than anticipated. Instead, environmental factors, such as nutrition, may exert a strong influence from early on.

Based on mathematical models, it can be concluded that there is no shared universal species in the gut at the 1%-level of abundance among all humans. The same holds true for the microbiota on human hands at a level of 2% [23]. In this calculation and for several other quantitative and qualitative analyses, species was approximated with a 97% OTU concordance. This means that the possibility that we all have bacterial species in common in our guts at a high level of abundance can be ruled out. And although humans share a group of some ubiquitous phyla, the level of abundance is highly variable between individuals.

If there is not a core set of species – what about a core set of genes? And what is it that finally counts with regard to intestinal human physiology and immunology – the cocktail of various bacterial species or the function of the multitude of genes provided to the human organism?

Indeed, there is a high level of variability in the abundance of species, but due to a high redundancy the functional state of the whole genome pool may be very similar in very different microbial communities [24].

Microbiota and Nutrition/Obesity

The intestinal microbiota is not just the umbrella term of the sum of microorganisms harbored in the intestine, rather, it is a highly complex group of organisms that interact with its host and actively influence its metabolism, immunological properties and whole body physiology. Well-known examples of these bacterial-derived properties are the conversion of complex nutrients as well as short-chain fatty acids as a nutrient to intestinal epithelial cells, formations of a dense and highly effective defense barrier, the production of essential vitamins e.g. vitamin K, the metabolism of drugs and of bile salt and the recirculation or the modification of nutritional compounds that results in transformed products with potential beneficial (sometimes also hazardous) properties for the host, e.g. in nutritional flavonoids.

Several years ago, an alteration in the relative abundance of the two predominant bacterial phyla – Firmicutes and Bacteroidetes – was shown in obese compared to lean mice with a marked increase of the Firmicutes to Bacteroidetes ratio of around 50% [4]. A very similar result occurred in human studies [25, 26].

Furthermore, the obese phenotype appeared to be transmissible, in that the introduction of an obesity-associated microbiome into the intestine of germ-free mice induced an obese phenotype [25].

A common postulation to explain these findings is an increase in the energy extraction in the metabolism of enteral food components in a Firmicutes-rich gut microbiota. In this respect, a significantly smaller energy content was found in the feces of obese mice compared to their lean counterparts [25].

Interestingly, the opposite procedure, i.e. transplantation of a 'lean microbiota' into germ-free obese mice to induce a lean phenotype has not been performed so far.

However, the question of weight gain in relation to intestinal microbial composition is much more complex

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than just a simple correlation to the ratio of the two most abundant phyla. For instance, harvesting of energy could not be correlated to the abundance and ratio of the most common phyla in a study of adolescents in a structured weight-loss program [27]. Nevertheless, as mentioned below, correlations with successful weight loss were seen going further down in taxonomy beyond the phylum level. Moreover, a study analyzing weight loss after metabolic surgery (gastric bypass) found a significant increase in the amount of lost kilograms in the group receiving a lactobacillus probiotic for 6 months compared to the placebo group [28]. A direct effect of the lactobacillus strains seems rather doubtful, as bacterial overgrowth was simultaneously revealed to have decreased in the probiotic group, pointing rather towards a contribution of the microbial composition on the whole.

So far, it is not possible to relate or even reduce vulnerability to obesity to the abundance of a certain phyla or set of bacterial species. An interesting approach in increasing our understanding of the key elements that make the difference in the microbiota of obese individuals would be a comparison of their gut microbial composition with that of their lean relatives, aiming at the identification of a core intestinal microbiota for each family which harbors the predisposition to obesity [29].

Intriguingly, not only a shift in microbial composition was found in a study with obese adolescents on a weight-loss program (including a low-calorie diet and structured physical activity) – the success in losing weight was variable and dependent on the individual's microbial composition. Weight loss was substantially higher in individuals rich in *Bacteroides fragilis*, *Lactobacillus* and *Bifidobacterium* in relation to *Clostridium coccoides*, despite a matched dietary intake and an exercise program [27].

What is the nature of this host-microbial interaction and what are the mechanisms underlying weight gain in altered microbial composition?

So far, knowledge on these mechanisms is sparse. An interesting study, performed more than 10 years ago already, investigated the intestinal transcription of genes with DNA microarrays in germ-free mice that were colonized with *Bacteroides thetaiotaomicron* (a Gram-negative anaerobe) which is a highly abundant member of Bacteroidetes in the normal microbiota in both mice and humans. Several genes that play an important role in the intestinal absorption of lipids were shown to be modulated by the existence of this human commensal archaeon. Furthermore, the transcription of genes involved in other intestinal functions, such as xenobiotic metabolism, maturation of the intestine after birth, angiogenesis or even the formation and consolidation of the mucosal barrier was also modified [30].

In addition, it has been proposed that there is an interaction between the gut microbiota and the host via hormonal mechanisms. For instance, short-chain fatty acids, that are produced in a higher quantity by an 'obese microbiota' may interact with G-protein receptors [31], leading to an increase in the production of proteins with important endocrine properties in mediating satiety and the regulation of energy metabolism, such as leptins or peptide YY.

Another potential link between the intestinal microbiota and obesity may be a chronic inflammatory state, which is known to be an important component of the pathophysiology of the metabolic syndrome [32]. One potential association is via lipopolysaccharide (LPS). LPS constitutes an important part of the cell wall in Gram-negative bacteria and is a well-known very potent stimulator of an inflammatory reaction in the host, e.g. in the systemic inflammatory response syndrome and sepsis. It has a high affinity to chylomicrons, which are formed in the digestive processing of triglycerides, rendering the chylomicrons a potential transporter of LPS from the intestinal epithelial cells in the systemic circulation [33], thereby augmenting systemic blood levels.

The alteration of the intestinal microbiota induced by a high-fat diet, specifically the decrease of *Bifidobacteria*, may modulate the amount of LPS prone to enter the host's blood circulation via a glucaogon-like peptide 2-mediated increase in the intestinal barrier [34].

Microbiota and IBD

The gut microbiota and alterations in its complex composition are increasingly considered as a crucial factor in the pathogenesis of IBD [9, 35–42], not just since the observations of an improvement in disease activity after antibiotic therapy in both CD and UC [43–45] or the absence of spontaneous colitis in germ-free mice [9, 10].

The chicken and egg dilemma evidently applies to the interplay of intestinal dysbiosis and IBD and is currently still not completely solved. However, an important study, investigating microbial composition in washed colonic biopsies from patients with IBD and controls, identified distinctive microbial abnormalities that correlated with disease severity, interestingly in both an inflamed and a noninflamed colon specimen [42]. Further studies followed which support a causal role, rather than just effect or the role of an innocent bystander.

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Antigen-presenting cells, such as dendritic cells, are essential in microbial recognition as they are capable of sensing phylogenetically highly conserved microbial-associated molecular patterns (MAMPs) via Toll-like receptors (TLR) or peptidoglycan molecules from the bacterial cell walls via nucleotide-binding oligomerisation domain (NOD)-like receptors (NLR).

It appears that at least some of the intestinal bacteria influence the establishment of an immune tolerogenic response through an induction of regulatory T cells and IgA secretion via dendritic cells.

Furthermore, it was shown, that not only bacteria but also their metabolic products may modulate the host's immune response. For instance, polysaccharide A, produced by *Bacteroides fragilis*, a human symbiontic bacteria, was shown to induce an immune-modulating immunological response, providing protection from exhaustive inflammatory response induced by *Helicobacter hepaticus*, in that the immune response was attenuated including the production of IL-10 and suppression of proinflammatory cytokines [46].

Moreover, a chronic stimulation of NOD-2 in human macrophages led to a decreased proinflammatory response, evident in a decrease of TNF-α, IL-8 and IL-1β, when these cells were restimulated after pretreatment [47].

Accordingly, the gut microbiota and the induced tolerance of the host seem to simultaneously assure protection from invasive pathogens and also from an overreacting inflammatory response. The disruption of this homeostatic coexistence between gut microbiota and the host, including the latter's physiologic immune tolerance, is assumed to have a central role in IBD pathogenesis [40, 48].

In recent years, a magnitude of studies looked at the composition of the commensal microbiota in IBD patients compared to healthy controls or to patients with other diseases. One characteristic finding is a reduced microbial diversity in patients with IBD [39, 49–51]. Further distinctive characteristics of microbial composition in patients with IBD compared to healthy controls have been described, sometimes with conflicting results, including alterations on the phylum level like a reduction of Firmicutes and Bacteroidetes and concomitant increase in the fraction of Proteobacteria and Actinobacteria [36, 52, 53].

Other studies identified characteristic shifts on the species level [54], such as a combination of *Dialister invisus*, a hitherto uncharacterized member of the Clostridiacae family, *Faecalibacterium prausnitzii*, *Bifidobacterium adolescentis* and *Ruminococcus gnavus* as being indicative of dysbiosis associated with CD [55].

Even within a specific form of IBD, differences of microbiota composition were shown according to the phenotype. For instance, in CD patients with ileal disease, the disappearance of well-known commensals *(Faecalibacterium* and *Roseburia)* and an increase in Enterobacteriaceae and *Ruminococcus gnavus* were found [56].

However, some intestinal microbes seem to have protective effects and are therefore used as therapeutic agents in IBD, especially UC [57, 58]. Potential mechanisms underlying this clinical benefit might be an attenuation of adherence and consecutive invasion of pathogenic intestinal *Escherichia coli* strains [59] as well as the positive effects of probiotic bacteria on mucosal integrity, the secretion of proinflammatory cytokines and the induction of β-defensins [60–63].

Finally, the assumption that IBD may be related to a single microbial cause – hence being an infectious disease with a hitherto unidentified agent – is not a new one, but was most likely postulated as soon as research on IBD pathogenesis began. Since then, a variety of candidate agents have been proposed, including mycobacteria (predominantly *Mycobacterium avium paratuberculosis*), Enterobacteriaceae, such as *E. coli* strains, *Listeria monocytogenes* and also viral agents, such as paramyxovirus or reroviridae. However, so far, no single agent has been isolated in all – or at least the vast majority of – affected patients with high consistency and the results of therapeutic trials of antimicrobial agents directed at candidate pathogens have been at best inconclusive, arguing against a single microbial organism harboring a key role in the pathogenesis of IBD.

Microbiota and Graft versus Host Disease

Another intestinal disease, where a pathogenic role of the gut microbiota is assumed is graft versus host disease (GVHD). Bearing in mind the common occurrence of infectious complications in GVHD, including severe life-threatening septicemia often due to Gram-negative rods, a link between the gut microbiota and GVHD susceptibility was already suggested in the 60s and 70s of the last century [64, 65].

In an animal model of GVHD – syngenic GVHD (SGVHD), where lethally irradiated rodents receive an autologous bone marrow transplantation followed by cyclosporine A – a form of colitis develops that is not distinguishable from other murine colitis models for IBD,

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revealing similar microscopic findings, activation of a similar phenotype of CD4+ effector T cells and also a cytokine profile [66]. Interestingly, the in vitro responsiveness of these CD4+ T cells from these animals suffering from GVHD-related colitis showed an increase activity against bacterial antigens, which were isolated from the fecal supernatant of normal animals [67].

In a recent study, an increased responsiveness of CD4+ T cells against microbial antigens from the cecal content of normal animals was shown in a murine model. If animals were treated with a broad-spectrum antibiotic combination of metronidazole and ciprofloxacin after transplantation, no clinical symptoms or characteristic pathological lesions in the intestine of cyclosporine-A-treated animals were observed. On an immunological level, the previously mentioned enhanced responsiveness of CD4+ T cells was likewise significantly decreasing, including decreased levels of various proinflammatory cytokines, such as IFN-γ, IL-17 and TNF-α. These findings led the authors to suggest an important pathogenic role of the intestinal microbiota in SGVHD [68].

Another study in mice identified the Paneth cells as a potential target in GVHD, hereby inducing a decrease in the expression and secretion of α-defensins, which have been shown to be an important defense strategy against pathogenic bacteria. The subsequent decrease of secretion of this antimicrobial peptide induced a loss of microbial diversity, predominantly with regard to commensals, hence increasing the abundance of Gram-negative bacteria, which are a well-established origin of systemic bloodstream infection.

Interestingly, the degree of GVHD correlated with the degree of microbial alterations. Moreover, the use of polymyxin B, which has the potential to limit the often observed overgrowth of *E. coli* in GVHD, attenuated or even improved the severity of the inflammatory affection of the gut [69]. Likewise, the induced shifts towards a proinflammatory gut microbiota in another murine study of GVHD was shown to be depending on TLR sensing (MyD88/TLR9), with a decreasing extent of intestinal pathology in TLR9-deficient mice or antagonizing therapy with a synthetic oligonucleotide inhibiting TLR9 [70]. In conclusion, these findings suggest that – very similar to the proposed role in the pathogenesis of IBD – an induced dysbiosis represents both a key element in the development of GVHD but also a potential target of therapeutic strategies aiming at manipulating microbial composition.

Investigating Microbial Composition in Humans Undergoing Smoking Cessation

Smoking has emerged as an important environmental factor influencing the course of IBD, with differing effects in UC and CD. Tobacco smoking has a protective effect in UC. The opposite is true for CD, where smoking has a detrimental influence on the future disease course. In addition, the risk of developing UC in former smokers is substantially elevated compared to never-smokers [71–76], and the course of the disease is milder in former smokers who resume smoking [77, 78].

Moreover, it is well known that smokers who successfully quit smoking gain weight – on average about 7–8 kg in around 80% of individuals [79]. Although one would intuitively assume that this weight gain is related to an increase in food intake, data addressing this issue are equivocal. For instance, a small study conducted around 25 years ago did not detect an increase in total calorie intake in the people who quit smoking and gained weight, although an increase in carbohydrate calories compared to protein calories was observed [80]. A very large (361,662 men screened and 12,866 randomized) population-based primary prevention trial, the Multiple Risk Factor Intervention Trial (MRFIT), conducted in North America, which investigated the clinical effect on multiple cardiovascular risk factors, found the following interesting results with regard to smoking cessation and weight gain: intriguingly, successful quitters showed a reduced calorie intake compared to those who continued or resumed smoking, and also a generally healthier diet composition. Nevertheless, a weight gain was exclusively observed in men who ceased smoking, while men who continued or resumed smoking lost body weight [81].

We aimed to investigate whether smoking cessation in healthy individuals leads to a change of the composition of the gut microbial composition. We therefore followed up a group of 10 smokers during smoking cessation (intervention group) over a period of 9 weeks with a prespecified complete cessation of smoking after the first week, and compared these results to 10 control subjects, 5 nonsmokers and 5 continuing smokers. Stool samples were obtained 1 week before as well as 4 and 8 weeks after smoking cessation. The hitherto unpublished results revealed firstly an overall change in the microbial composition in the intervention group as indicated by analyses of terminal restriction fragment length polymorphisms with doubly-centered principal component analyses.

Secondly, the sequences obtained by pyrosequencing showed statistically significant changes in the interven-

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tion group but not in the 2 control groups. Significant differences were observed on the phylum level with an increase in relative abundance of Firmicutes and Actinobacteria and a decrease of Proteobacteria. In addition, significant changes on the genus level were found, again exclusively in the intervention group. Phylogenetic diversity showed an increase in the intervention group after smoking cessation.

Our investigations show that smoking status influences the composition of the gut microbiota and that smoking cessation leads to significant changes in intestinal microbial composition. These results point to a potential pathophysiological link between smoking status and IBD, and furthermore, imply that an alteration in microbial composition and consecutive extraction of energy by the host may play a role in weight gain after smoking cessation.

Disclosure Statement

The authors declare no conflicts of interest.

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