Microbiomarkers in inflammatory bowel diseases: caveats come with caviar

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The largest numbers of commensal bacteria reside within our intestinal tract, with an increasing density from mouth to anus. Recently, a new estimate for the total number of bacteria (3.8×10^{13}) in the 70 kg 'reference man' was reported.[1] For human cells, the same authors revised past estimates to 3.0×10^{13} cells, out of which approximately 90% belong to the haematopoietic lineage. Hence, the widely cited 10:1 ratio of bacteria versus human cells received an update, showing that the number of bacteria in the body is actually of the same order as the number of human cells, and that the cumulative bacterial mass is about 200 g. Still, this large number of bacteria highlights their importance in maintaining health and metabolism. Different parts of the intestinal tract have different functions, tissue structure varies accordingly and gradients exist for several physicochemical parameters such as nutrients, pH or oxygen levels.[2] Consequently, microbiota composition varies along the gut, but also between luminal and mucosa-attached communities of the same intestinal segment, and even along the crypt-villus axis in the epithelium. Thus, host–microbiota interactions are likely regionally specific and the local crosstalk determines intestinal function and physiology. Probably each human individual carries its own 'microbial fingerprint' (especially when considering genomic variation within the bacterial species' populations), which is why forensic scientists started to exploit the use of this non-human organ.[3]

Recent large-scale analyses of population-based cohorts with >1000 samples validated that body mass index, age at sampling and gender are important covariates that need to be included in microbiome association analyses.[4] In sum, Falony *et al* identified 18 covariates, including stool consistency, dietary factors and blood traits, cumulatively explaining 7.7% of the total variation in the gut microbiota, leaving 92.3% of the interindividual microbial variation unexplained. In a recent host-microbiome genome-wide association study, 42 genetic loci were identified that explain another 10% of microbiome variability.[5] Although these percentages need to be confirmed in other cohorts and for other ethnicities, both studies show that (A) a large portion of microbiome variability remains to be explained and (B) any microbiome study needs to account for covariates to prevent false-positive and false-negative results. This means for the field that the meta-data for a particular sample *is at least as important* for the data analysis as the actual microbiome data itself.

The main subtypes of IBD are Crohn's disease (CD) and UC, affecting together more than 2.5 million European individuals. More than 200 genetic susceptibility loci have been identified for IBD, a contribution that significantly improved our disease understanding. In contrast to the static human genome, microbiome composition is more dynamic and can thus be more easily modified by, for example, probiotics, prebiotics, antibiotics or faecal microbiota transfer (FMT) from healthy donors to patients by enema or via the nasoduodenal route. The latter was demonstrated to be perhaps the best method to treat life-threatening recurrent *Clostridium difficile* infections,[6–8] but is a controversial method for IBD and is therefore still regarded as an experimental therapy. Nevertheless, a recent multicentre placebo-controlled trial in Australia (the Faecal Microbiota Transplantation in Ulcerative Colitis (FOCUS) study) demonstrated efficacy of FMT for treating UC (p=0.021; enemas given at 5 days a week for overall 8 weeks).[9] Based on this finding, it was suggested that future work should focus on defining the optimum treatment intensity and the role of donor–recipient matching based on microbial profiles. The growing body of studies using FMT and gnotobiotics started to move the field of microbiota research away from correlation to causality by proving a functional involvement of the microbiota for health and disease.[10–12]

Microbiome studies in IBD have been consistent in finding reduced diversity in patients compared with healthy controls (HC).[13 14] A lack of microbial diversity seems to be a general theme in several diseases (obesity, diabetes, asthma, atopic dermatitis, and so on), suggesting that a species-rich ecosystem is more robust against environmental influences, as functionally related microbes exist in an intact ecosystem that can compensate for the function of another species that has disappeared. Consequently, diversity seems to be a good indicator of a 'healthy gut.'[15] A large study that analysed the microbiota in 447 treatment-naïve patients with new-onset CD (treatment effects on the microbiota can likely be excluded in this unique cohort) also identified different bacteria, which were more abundant in patients compared with controls and vice versa.[16] Gevers *et al* also developed a microbial dysbiosis index that was correlated with the paediatric CD activity index. The dysbiotic signal in their study was stronger in the mucosa-attached microbial communities compared with faecal bacteria, suggesting that disease-relevant and more adherent bacteria are probably 'diluted' in the stool. The recently published longitudinal IBD microbiome study of Halfvarson and colleagues revealed that the microbiome of patients with IBD varies more over time

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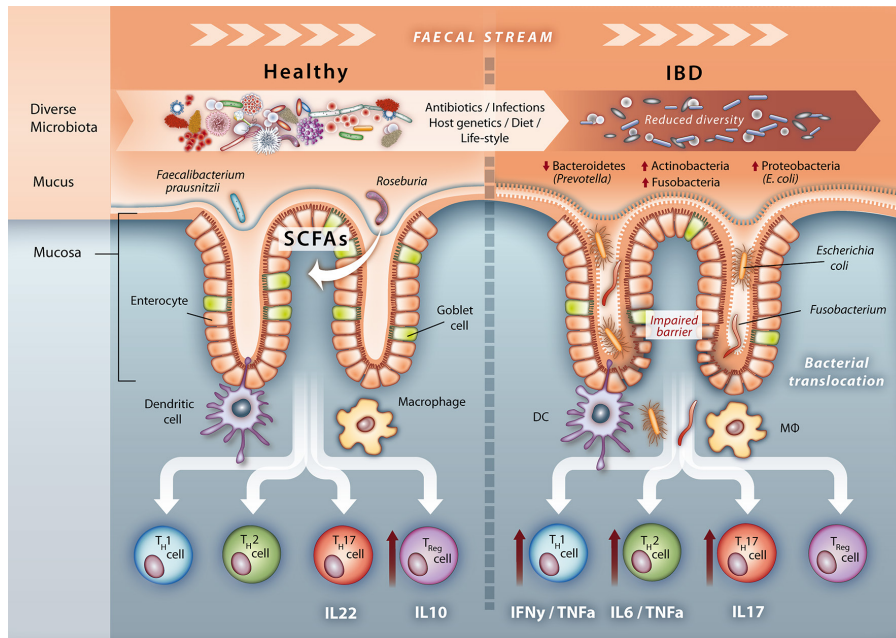
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Figure 1 Microbial signatures of a healthy gut and IBD. Under healthy homeostasis the microbiota is diverse. Goblet cells produce a thick colonic mucus layer, which creates a physical barrier against the microbiota, but also harbours a specific mucus-resident microbiota enriched in, for example, short-chain fatty acid producing bacteria Roseburia and Faecalibacterium prausnitzii. Immune sensory cells such as DC and MΦ sample microbial patterns and induce a T cell profile dominated by IL-10 producing Treg lymphocytes leading to homeostasis. However, the composition of the microbiota is less diverse in patients with IBD with fewer Bacteroidetes mainly attributed to loss of Prevotella species and expansion of Actinobacteria, Proteobacteria such as adherent invasive Escherichia coli and Fusobacteria. Mucosal function is also altered, for example, lipid metabolism, illustrating a cometabolism of the metaorganism. A reduction in Paneth and goblet cell number along with their impaired functions—secretion of antimicrobial substances and mucus—leads to decreased mucus thickness, reduced mucosal integrity and finally to an impaired barrier function. This increases bacterial translocation and stimulates activation of DCs and MΦs, which then induce an altered T cell profile with increased IFNγ/TNFα-producing Th1, IL-6/TNFα-producing Th2 and IL-17-producing Th17 lymphocytes resulting in a proinflammatory response and tissue damage, which in turn stabilises the dysbiotic microbiota and a chronic inflammatory tone. DC, dendritic cells; IFNγ, interferon gamma; IL, interleukin; MΦ, macrophages; TNFα, tumour necrosis factor alpha.

compared with controls, corroborating that patients with IBD harbour a disturbed microbial ecosystem.[17] The highest volatility of the microbiota, that is, dynamic deviation from a 'healthy plane,' was observed for patients with CD with ileal inflammation (lower volatility in colonic CD and UC). In an elegant visualisation study, Swidsinski *et al* demonstrated that the number of mucosa-associated bacteria increases with disease severity[18] and together with reduced diversity, an increase in bacterial load seems to be a key feature of several diseases.[19] Notably, diet can rapidly alter the gut microbiome with effects on disease susceptibility such as pathogenic infection or chronic inflammation.[20–22] For example, removal of carbohydrates from the diet promotes the growth of mucus-utilising bacteria and alters microbial biogeography by attracting luminal bacteria to the mucus

layer, which increases competition for beneficial mucus-resident bacteria such as *Akkermansia muciniphila* or *Faecalibacterium prausnitzii*, which in turn shifts epithelial responses, in particular goblet cell function.[23 24] Oral but even intravenous iron replacement therapy distinctly alters the gut microbiota and metabolome in patients with IBD.[25] Recent studies even suggest that changes in nutrition during the course of human history may have led to decreased microbiota diversity and the increased incidence of chronic inflammatory diseases and obesity.[24 26 27] Another recent study has highlighted the potential effect of a disturbed host–microbiota crosstalk in IBD. This is evidenced by a striking uncoupling of host transcriptional patterns and microbiota signatures in IBD, with the affected pathways indicating a loss of cometabolic functions.[28] It therefore seems imperative to correct for

dietary variance in microbiota-related data and to explore dietary modulation of the metabolic properties of the gut ecosystem (eg, enrichment of dietary fibre) as a possible therapeutic approach for IBD. Figure 1 provides a schematic overview on known gut microbiome changes in IBD.

In the highlighted study, Pascal and colleagues[29] report on an analysis of the faecal microbiome for a large IBD panel from four different countries (40 HC (unrelated HCs)/71 HR (healthy relatives from patients)/34 CD/74 UC from Spain, 54 CD from Belgium, 977 healthy twins from the UK and 158 patients with anorexia from Germany). In sum, they included about 1246 non-IBD and 162 IBD samples. The hypervariable region V4 of the 16S rRNA gene was targeted for microbiome profiling. Pascal *et al* replicate the findings of Gevers *et al*[16] that *F. prausnitzii* is reduced/absent in patients with CD and that patients have a higher abundance of *Escherichia* and *Fusobacterium*.

The key finding of their study is a 'microbial signature' that may be used to discriminate between CD and non-CD by eight prokaryotic groups: *Faecalibacterium* (genus from family Clostridiaceae), Peptostreptococcaceae (family from order Clostridiales), *Anaerostipes* (genus from family of Lachnospiraceae), *Methanobrevibacter* (genus from family Methanobacteriaceae), Christensenellaceae (family from order Clostridiales) and *Collinsella* (genus from family Coriobacteriaceae) being decreased in patients with CD; *Fusobacterium* (genus from family Fusobacteriaceae) and *Escherichia* (genus from family Enterobacteriaceae) having a higher relative abundance in patients with CD. The overall sensitivity of this diagnostic signature was 80%, with specificities of 89%–94% depending on the comparison. In comparison, assessing faecal calprotectin (a heterodimer of S100A8 (calgranulin A) and S100A9 (calgranulin B)), an established, non-invasive test that measures intestinal inflammation, has a sensitivity of 83% and specificity of 84% for distinguishing organic from functional intestinal diseases in symptomatic patients.[30] However, diagnosis of IBD, and especially distinguishing between the different subtypes of IBD, still relies on a combination of diagnostic tests, including endoscopic and histological analyses, which showed high accuracy in a 12-month long follow-up, with only 1%–2% of patients with CD and UC being identified as false positives, but 9%–12% of patients with CD and UC

Table 1 Overview of sequencing-based methods to characterise microbiota composition and function

	Full-length 16S rRNA gene sequencing	Targeted 16S rRNA gene amplicon sequencing	Metagenome sequencing	Metatranscriptomics	Single-cell analysis
Technique	Clade-specific amplification of 16S rRNA gene, vector-based cloning and sequencing	Long-read-based amplification and sequencing of large 16S rRNA gene fragments	PCR-based amplification of target followed by sequencing	Sequencing of entire DNA extracted from samples	Sequencing of entire RNA extracted from samples	Cultivation-based isolation of single bacterial clones	Emulsion or droplet-based single-cell amplification
Target	Entire 16S rRNA gene	Entire 16S rRNA gene	Variable regions, eg, V1-V2; V3-V4; V4; V6	All DNA molecules	All transcribed RNA molecules	Multiple complete genomes	Single bacterial cells
Potential bias	Gene copy-number bias	Amplification bias	Primer/region-specific amplification bias; gene copy-number bias		Transcriptionally more active bacteria are over-represented	Anaerobic and hard-to-culture bacteria are under-represented	More abundant taxa over-represented
Sequencing technology	Sanger	PacBio or Oxford Nanopore	MiSeq	HiSeq/NextSeq	HiSeq/NextSeq	Any NGS technology	HiSeq/NextSeq
Advantages	Species-level resolution	High throughput; species-level resolution	Low cost; high throughput	Information on encoded functional repertoire; high-resolution taxonomic assignment; captures also viruses, fungi and archaea	Information on actively expressed functional content	Reconstruction of multiple complete genomes from complex communities	Reconstruction of multiple complete genomes from complex communities
Disadvantages	No information on functional repertoire; low throughput; much hands-on time	No information on functional repertoire; high error rates; higher cost per base than MiSeq	No information on functional repertoire; low resolution on species level	High cost; high computational burden; large amounts of unannotated data	High cost; high computational burden; large amounts of unannotated data Removal of 16S rRNA required	Tedious culturing and selection approaches (eg, media, oxygen); high cost	High cost

NGS, next generation sequencing.

being reclassified as another subform of IBD.[31]

While the study of Pascal *et al* employed a large sample panel in order to identify potential microbiomarkers of interest, a few words of caution are necessary. First of all, the findings still need to be validated by other centres using well-powered sample collections that include HC, CD, UC and IBD-related diseases, that is, differential diagnoses such as IBS. In a first attempt, Pascal *et al* applied their test to another cohort sequenced in another centre in France with another method (V3-V5 instead of V4). This led to a significantly lower sensitivity and specificity for the prediction of CD versus UC (60% and 68% respectively) and a lower sensitivity (60%) for the prediction of CD versus HC. While these results still suggest a non-random signal in the data, they also imply that larger and multicentre trials are clearly needed to produce a more robust and clinically useful diagnostic signature. It is noteworthy that Pascal *et al* included a very large UK control panel, making up almost half of their sample size, while they did not include a matched patient panel from the same geographical region. Anthropometric features as well as nutritional patterns can have a large impact on the gut's microbial community and are a valuable resource in the investigation of connections between disease states and the microbiota. While these data will certainly lead to more complex models for the determination and distinguishing of dysbiotic states, they will most certainly lead to higher sensitivity and specificity of microbiome-based classifiers and should be considered for future approaches.

The regional specificity of the intestinal microbiota to different anatomical sites might partially explain why the authors failed for CD but were able to describe UC-specific microbiomarkers.[2 16] Faeces may be a suitable surrogate for the colonic microbiota but not for that of the small intestine. Thus, the diagnosis of CD using microbiomarkers seems to require sampling of the local microbiota using biopsies.

To date, endoscopy is the gold standard to diagnose IBD and assess mucosal inflammation. Since endoscopy is an expensive and invasive procedure with the risk for complications, alternative scores, for example, the Harvey-Bradshaw index for CD[32] or the simple clinical colitis activity index for UC,[33] which include non-invasive measures such as general state of health, faecal

properties and frequency or abdominal pain have been developed. These scores, however, do not correlate well with mucosal inflammation[34] and therefore inflammatory markers such as C-reactive protein or faecal calprotectin are additionally used to evaluate disease activity. Still, levels of C-reactive protein are not specific for the intestine, but rather a general marker of systemic inflammation and faecal calprotectin correlates with UC but not CD and thus seems to be a marker of inflammation only for the colon.[35 36] Thus, other non-invasive biomarkers are needed as surrogates for a healthy or inflamed gut ecosystem. Explorative metabolomics of faecal water showed already good differentiation of colonic versus ileal CD with a clear association of metabolites to the core metabolome or to specific microbiota,[37] motivating thus also the use of high-resolution analytical technologies for the description of novel molecule biomarkers. The concept of determining non-invasive microbiomarkers in stool, which can aid gastroenterologists in the diagnosis of IBD and in distinguishing between different intestinal diseases, is a very attractive one and warrants future investigations.[29 38] However, specific bacterial species that are clearly associated with disease activity are not yet identified. With IBD on the rise worldwide and a strong geographical influence on intestinal microbiota signatures,[39] analysis of a core taxa signature (combination of bacterial taxa) in large transethnic cohorts or determining functional deficits of the microbial community seems to be required.[16 29 40] More sophisticated sequencing-based approaches (see table 1) such as metagenomic/metatranscriptomic shotgun sequencing or metabolic modelling,[41] which capture the functional capacity of the microbiota, overcome many limitations of 16S sequencing, yet should be complemented with phenotypic characterisation of individual bacteria using culture-based techniques, proteomics and metabolomics. With more complete microbiome reference data sets becoming available, and with larger international sample panels being collated— similar to the genetics community where several 10 000 DNAs and genome-wide genetic data sets were analysed in a joint effort (see www.ibdgenetics.org)—we are certain that microbial signatures of 'caviar value' may be derived in the future. Until then, we alert the scientists to the 'caveats' that come along with the field of biomarker research and call for

more large-scale efforts by the community.

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