Microbiota and Flatography in Pediatric Gastrointestinal Disease

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CHAPTER 1

Introduction

PART I: PAEDIATRIC INTESTINAL MICROBIOTA

In medicine, bacteria have for centuries almost exclusively been regarded in the context of disease, but it has now become evident that human life without bacteria is neither possible nor should be pursued. Since the nineteenth century conventional *in vitro* culture techniques have been the cornerstone into investigating the microbiota composition. However, the majority of microbial species inhabiting the intestinal tract was and is not cultivable. Over the past decades, knowledge on the microbiota has profoundly expanded with the emergence of DNA-based detection techniques to describe microbial communities.[1] The human microbiota consists of approximately 30 trillion (10^{14}) bacteria, which is approximately equal to the total number of human body cells.[2] The primary sites of microbiota colonisation include skin, oral cavity and gut. The commensal intestinal microbiota compromises over 1000 different bacterial species, primarily belonging to the phyla *Bacteroidetes*, *Firmicutes*, *Actinobacteria*, *Fusobacteria*, *Verrucomicrobia* and *Proteobacteria*.[3] The functions of the intestinal microbiota are highly diverse and are considered to play a significant role in a variety of anti-inflammatory, immunological and metabolic pathways, all contributing to maintenance of individual's homeostasis.[4-6] This may indicate the importance of a microbial balance, which is considered unique for each individual. Disturbances of the precarious microbial balance have been associated with an increasingly wide range of gastrointestinal and, interestingly, also many extra-intestinal diseases. A wide spectrum of diseases has been linked to microbial disturbances, which is believed to find its origin in (early) childhood, illustrating the importance of the early development of microbiota from infancy onwards.[7] Therefore, detailed characterisation of physiological and pathophysiological dynamics of the intestinal microbiota, including insight in genetic and environmental factors influencing its configuration, has become pivotal.

Microbiota composition in early childhood is highly dynamic and influenced by numerous environmental factors, including duration of pregnancy, way of delivery, maternal microbial composition, neonatal feeding pattern, introduction of solids, medication and socio-economic status.[8] Current efforts to characterise microbial composition and stability have mainly focused on microbiota acquisition during the first years of human life and on microbial dynamics in adulthood. In adulthood, microbial communities are characterised by a more stable and highly resilient composition.[9-13] So far, insight into the development and dynamics of bacterial communities in the intermediate period, between infancy and adolescence, is very limited. Consequently, the way in which (disturbance of) paediatric intestinal microbiota may influence health and provoke disease remains largely to be explored.

Characterizing paediatric microbiota in health and disease

Currently, most relevant molecular-biological approaches used in microbiota research include targeted detection methods, such as 16S rRNA gene next-generation sequencing (NGS), shot-gun sequencing, and techniques focusing on the bacteria-specific ribosomal 23s subunit or 16S-23S spacer region.[14] Limitations of sequencing techniques include the need for extensive analytic capacities, while outcome is decidedly dependent on the extraction protocol used and the choice of primers to amplify the targeted 16S region, potentially leading to selection bias. Target of the microbiota profiling technique IS-pro is the rRNA operon between the 16S and 23S ribosomal subunits, the intergenic spacer region.[15] This approach allows for high-throughput microbiota profiling on both phylum and species level, by the usage of fluorescent phylum-specific primers. One specific advantage of IS-pro over sequencing techniques is its capacity to generate results within hours following sampling, opening avenues for the development and implementation of bedside, microbiota-based diagnostic, prognostic and predictive algorithms in daily clinical practice, in a standardized matter.

Several international collaboration projects including the Human Microbiome Project (HMP) and the European MetaHit project have applied these novel molecular microbiota detection techniques to characterize 'normalcy and variation of the human microbiome' in adults.[16-18] These initiatives have largely increased our knowledge on the capacity of intestinal bacterial communities to affect physiological state. Furthermore, these studies revealed that the aetiology of numerous diseases may be associated with disturbance of this balanced state. Consequently, the intestinal microbiota has been considered to harbour potential to serve as a diagnostic biomarker of disease and, additionally, to monitor disease activity.[19,20] Furthermore, the natural course of disease may be influenced by therapeutic strategies aimed at manipulation of the microbiota composition, hypothetically by restoration of microbial dysbalances.

Manipulation of the intestinal microbiota in children is currently rather disappointing in terms of treatment outcome; it is hampered by insufficient knowledge about composition and dynamics in health and insight in disease-and site-specific microbial signatures. Expanding the experience regarding microbial dynamics towards the paediatric population may provide opportunities to further explore the options to develop individualised preventative and therapeutic interventions, including the use of pre- and pro-, syn-, and antibiotics and faecal transplantation.[21]

The list of paediatric gastro-intestinal diseases possibly linked to microbial imbalance seems to be infinite. It includes inflammatory bowel disease (IBD), functional constipation, coeliac disease, irritable bowel syndrome (IBS), necrotising enterocolitis (NEC), obesity, allergy and juvenile idiopathic arthritis (JIA).[22-29] Limitations of most paediatric studies aiming at identification of disease-specific microbial signatures include the limited number of subjects and controls included in the studies and the cross-sectional design of most studies, precluding accurate comparison of microbial dynamics in the diseased state versus the physiological course through time. Moreover, robust conclusions on diagnostic accuracy of tests using disease-specific microbial signatures cannot be drawn easily since the influence of environmental factors such as diet and medication is not taken into account in most studies, potentially leading to type I errors. Furthermore, reported patterns of microbial dysbiosis in affected subjects are inconsistent between the various studies, partly, or maybe largely, due to differences in strategies regarding sample harvesting, collection and storage and microbiota detection technique.[14] For example, studies assessing microbial signatures in paediatric IBD comparing ileal, rectal and faecal samples suggest that in particular assessment of the rectal mucosa-associated microbiota offers a unique potential for early diagnosis of CD.[30] These findings above underline the need for methodological standardisation of studies on microbiota characterisation in (paediatric) health and disease.

PART II: VOLATILE METABOLOMICS

While molecular microbiota detection techniques can provide insight in composition of microbial communities present in a particular sample, no information can be obtained on their functional characteristics. This means that they provide insight into 'who is in there?' but not into 'what are they doing?' Consequently, one cannot easily infer an underlying pathophysiological mechanism based on any observed associations between (alterations in) microbiota composition and particular disease states. Furthermore, molecular microbiota techniques usually include the need for intensively trained personnel for sample preparation and to perform the complex (statistical) analyses. Real-time identification of bacteria may have major implications in terms of treatment of microbiota-associated diseases in clinical practice, novel tools are therefore awaited. One of these new approaches to obtain functional biochemical information is metabolomics: the analysis of the whole spectrum of metabolites.[31] Metabolomics provides insight into the interaction between (alterations in) gut microbiota and human metabolic processes, taking into account potential influences of environmental factors

such as diet and medication. Analysis of end products of metabolic pathways is considered to provide the most accurate assessment of human biological state.[32] Furthermore, it enables the development of individualised metabolic biomarkers to be used for diagnostic and prognostic purposes and for prediction of disease course. Over the past years, there has been a rapidly increasing interest in the potential of a particular field within metabolomics, the analysis of volatile organic compounds (VOCs).[33] VOCs are carbon-based chemicals, typically in volatile state at ambient temperature and for instance responsible for the smell deriving from all conceivable bodily excrements, including faeces, breath, urine and sweat.[34] VOCs originate from physiological and pathophysiological metabolic processes within the human body and may therefore be considered metabolic smell-prints. Over the last years, VOCs from a broad range of human samples have been characterised, identifying over 1800 unique compounds.[34] This VOC pool harbours a magnificent source of information with the potential to serve as noninvasive diagnostic biomarkers and as markers of disease activity, including malignancies, infections and metabolic conditions.[35] Diseases linked to changes in the composition of the intestinal microbiome, like IBD, NEC and IBS, are of particular interest, as these changes may be associated with changes in the production of faecal VOCs.[36] Identification of bacterial VOCs may enforce its potential as a diagnostic tool, with VOCs acting as biomarkers for the presence or absence of (a set of) specific bacteria. For example, specific long-chain alcohols have been described to serve as markers for Gram-negative enteric bacteria, production of hydrogen cyanide indicates the presence of *Pseudomonas aeruginosa*, and indole, acetoin, pyrovate and 2,3-butanediol are characteristic metabolic products of coliform bacteria.[37-40] VOCs are considered to reflect microbiota composition but also host-microbiota interaction. Microbiota- and VOC detection techniques can therefore be considered complementary; The questions 'who is in there' and 'what are they doing' could be addressed by combining both techniques. This fascinating field is yet largely unexplored; only very few studies have evaluated the potential of faecal VOC analysis in clinical practice, especially in paediatrics.[36]

VOC detection techniques and clinical applications

VOC detection techniques can be divided into two main categories; chemical analytical techniques, like gas chromatography-mass spectrometry (GC-MS), enable identification of individual molecular compounds, and electronic devices are based on pattern-based recognition algorithms.[33] Electronic nose (eNose) technology allows for real-time, high-throughput analysis of VOCs in gaseous mixtures.[41] The complete spectrum VOCs interact with a matrix of eNose sensors, influencing a measurable feature of each individual sensor,

such as electrical resistance. Since eNoses are relatively cheap, easy in use, and results can be obtained within minutes, they are considered appropriate as point-of-care instrument. In addition, they can be learned to recognize disease-specific VOC patterns.[41] Chemical analytical and eNose technologies are based on basically different approaches and are considered highly complementary. Identification by GC-MS of individual key VOCs may allow for the development of primed, disease-specific eNose sensors, which may be used as bedside diagnostic tool. So far, however, VOC analysis has hardly found its way towards application in clinical practice; it is almost exclusively used in research settings. Moreover, the vast majority of VOC studies have been performed in adults, while exhaled breath until now is the main substrate.[35,42] To a large extent, the relative contributions of certain VOC to the overall VOC profile are determined to a great extent by the substrate used for analysis. Gastrointestinal diseases such as IBD, colorectal cancer and NEC are characterised by specific inflammation of the gut mucosa and are being associated with intestinal microbial shifts, resulting in local production of disease-specific VOCs. It may therefore be hypothesised that in diseases originating from the gastro-intestinal tract, analysis of faecal VOCs may offer a more sensitive, direct and integral view on disease activity if compared with breath (or urine) sampling.

OUTLINE OF THE THESIS

As can be inferred from the discussion above, microbiota profiling and VOC analysis seem to have potential as diagnostic, predictive and prognostic techniques for health and gastrointestinal disease in childhood. In this thesis we have taken advantage of two recent developments. Part I of this thesis focuses on the study of microbiota composition and dynamics in paediatric health and disease using the recently developed and validated IS-pro technique.[15] In part II, application of IS-pro in clinical practice is illustrated by several cases. In part III, adoption of an 'electronic nose' for medical applications will be evaluated/assessed. The studied electronic nose has been developed by US army to detect biochemical weapons several decades ago and is currently, next to army purposes, being used in the food industry in particular. For our study purposes, we had to convert this device into a closed-loop system for transferring the VOC mixture without contamination by exogenous, environmental VOCs. The potential of generated faecal VOC profiles as a noninvasive diagnostic biomarker for disease activity in paediatric inflammatory diseases is assessed in several proof-of-principle studies. Combining techniques to analyse microbiota and volatile metabolomics could provide a more integral view on microbial related processes in the intestinal tract.

The thesis is divided in three parts. The **first part** consists of five chapters describing microbiota composition in healthy state and in a variety of paediatric gastro-intestinal diseases in which aetiology is presumably linked to microbial dysbalance, using the IS-pro technique. In the second chapter reference values of microbial composition, diversity and stability are defined by analysis of a large cohort of healthy Dutch children, aged 4-18 years. In this chapter, we also describe the presence of a core microbiota. In the third chapter, we describe temporal microbial dynamics in a cohort of children with de novo inflammatory bowel disease, and compare these data to the reference values on composition and stability presented in chapter two. In the fourth chapter, microbiota composition in children with functional composition is analysed and compared to healthy controls. In chapter five, we compare duodenal mucosa-associated microbiota in untreated paediatric coeliac disease with mucosal microbial signatures from non-coeliac, matched controls. In chapter six the intestinal microbiota in juvenile idiopathic arthritis patients is explored, prior to initiation of disease-modifying antirheumatic drugs, and compared to matched controls.

The **second part** consists of two chapters in which application of the IS-pro technique in clinical practice is demonstrated. The first chapter describes two children with severe ulcerative gastritis, in whom the presence of *Sarcina ventriculi* was demonstrated by means of IS-pro analysis. The second chapter describes the impact of faecal microbiota transfer on microbiota composition over a period of months in a child with *Clostridium difficile* infection, and linked to clinical outcome.

The **third part** consists of five chapters evaluating the potential of faecal VOCs as biomarker in a variety of paediatric gastro-intestinal diseases. The first chapter provides an overview of the currently available evidence on faecal volatiles as biomarkers for paediatric gastro-intestinal diseases. In the second chapter we perform faecal gas analysis in samples from de novo paediatric IBD patients over time. In the third chapter, we perform faecal VOC analysis on samples collected at three neonatal intensive care units in the Netherlands to evaluate if this technique is a promising early, diagnostic biomarker for NEC. In the fourth chapter we focus on faecal VOC profiles as early, preclinical biomarker for late-onset sepsis in premature infants. The fifth provides an overview of available biomarkers for NEC, with the focus on (early) microbiota alterations. So far, no reliable biomarkers are available to be used in clinical practice, underling the urgent need to develop novel, preferably noninvasive biomarkers allowing for detection of NEC in a preclinical stage.

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