Fecal volatile organic compound analysis and intestinal microbiota profiling in healthy and diseased infants

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General introduction and outline of the thesis

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PART 1: INTESTINAL MICROBIOTA

Approximately 38 trillion (10^{14}) bacteria reside in and on our body, whereas the human body itself consists of 30 trillion (10^{14}) individual cells. The gastrointestinal tract (GIT), especially the colon, harbors the vast majority of these microbes. Studies investigating the function of these bacteria and their role in maintaining health has gained momentum over the past decades.

Function of the intestinal microbiota

The gut microbiota is considered to play a substantial role in maintaining immune and metabolic homeostasis. Besides, these intestinal microbes are crucial for the uptake of energy and nutrients from the fecal stream. Fermentation of non-digestible carbohydrates, resulting in the production of several short chain fatty acids (SCFAs), is another important function of the gut microbiota. These SCFAs are an important source of energy for colonic epithelium cells and are essential in maintenance of mucosal barrier integrity and play a crucial role in several other physiological processes including glucose homeostasis, lipid metabolism, and immune system regulation. Directly after birth, the gut is exposed to a wide variety of different microbes. These microbes are required for the development and maturation of the intestinal mucosa and priming of the relatively naïve immune system. Mucosal barrier integrity and consequently intestinal permeability are regulated by tight intercellular junctions. Several commensals demonstrated beneficial effects on intestinal barrier integrity, whereas others prevent -or even reverse- adverse effects of pathogens on this barrier function. Moreover, for prevention of epithelial injury, interaction between host and commensals through Toll-like receptors (TLR) is crucial. Signaling through TLR leads to an increased expression of protective factors while simultaneously interferes with mechanisms resulting in epithelial cell turnover. Commensals are also considered to be involved in modulating immune cell differentiation, for example the differentiation of B cells into IgA-producing plasma cells. Overall, it has become increasingly clear that initial intestinal colonization plays an essential role in maintaining a healthy state, both early and later in life. In line with this, bacterial dysbiosis early in life, defined as any compositional alteration of resident commensal communities relative to the community found in healthy individuals, is associated with the development of numerous immunological, inflammatory and infectious diseases. To increase understanding of the etiological role of gut microbiota in these diseases, it is first key to discuss the initial intestinal colonization process and factors influencing gut microbiota composition in the neonatal period.

Intestinal colonization and influencing factors

Although the fetal environment was long considered to be sterile, recent studies suggest that colonization of the GIT may already be initiated in utero. It is assumed that micro-organisms reach the amniotic fluid through the maternal genitourinary tract or may translocate from...
maternal oral and gut niches into the bloodstream, eventually reaching the placenta by hematogenous spreading. Subsequently, microbes translocating into the feto-placental interface may eventually colonize the GIT after the fetus swallows amniotic fluid. However, studies investigating this phenomenon are scarce and therefore, much remains to be elucidated about antenatal colonization and environmental and genetic factors influencing this process.

Mode of delivery is the first perinatal factor influencing gut microbiota composition. Vaginally delivered infants harbor a microbiota comparable to the maternal vaginal microbial composition, whereas infants born by cesarean section depict a more skin resembling microbiota. Differences in microbiota composition between infants born vaginally or by cesarean section may persist up to the age of seven years. In addition, neonates exposed to intrapartum antibiotic prophylaxis (IAP), directed against group B Streptococcus (GBS), depict an altered microbiota compared to non-exposed infants. Overall, exposed infants depict a delayed progression towards a more mature microbial composition with a decrease in α-diversity and Bifidobacterium counts, whereas members of the Enterobacteriaceae family are more abundantly present. In chapter 1 we investigated the influence of timing of maternally administered IAP during a cesarean section on the intestinal microbiota of the neonate. In order to reduce maternal complications, obstetric guidelines regarding timing of antibiotic administration now advocate antibiotic administration prior to skin incision. A consequence of this revised guideline is that all infants born by cesarean section are exposed to broad-spectrum antibiotics by transplacental transfer. Although this has resulted in an overall decrease in maternal infectious morbidity, potential effects on neonatal gut microbiota composition, immunological priming and potential health implications later in life have not been adequately addressed so far.

Gut microbiota development of the preterm infant is also highly influenced by feeding practices, which can be divided in parenteral and enteral feeding. In preterm infants, microbial diversity was demonstrated to be inversely related to duration of parenterally fed days. Focusing on enteral feeding types, infants receiving mothers’ own milk (MOM) depict a diverse gut microbial community. Compared to formula fed infants, less inter-individual variance in gut microbiota composition was observed between infants receiving MOM, indicating that the gut microbiota of formula fed infants is more susceptible to influences by other factors. Moreover, immunoglobulins excreted in MOM forms the first line of defense in the prevention of enteric infections by coating the mucosal surfaces of the infant GIT.

Another factor considered to have a significant impact on microbiota composition is the degree of prematurity. Although gestational age-based differences are most prominent directly following birth, alterations are still detectable after several months. Gut microbiota in preterm infants show a delayed progression towards a composition resembling full-term
infants, characterized by a *Bifidobacterium*-dominated community\(^{20,21}\). Since preterm births are often suspected of intra-uterine infections, antibiotics are almost universally administered to (extremely) preterm born infants early in life\(^{22}\). In addition to the bactericide and bacteriostatic properties of antibiotics, leading to a decreased bacterial diversity\(^{23}\), recent studies suggest that antibiotics prevent granulocytosis and thereby disturb the innate immunity\(^{24}\). After birth, the ongoing interaction between the neonate and environmental microbes significantly influences the shaping of the gut microbiota composition\(^{25}\). Although preterm infants depict a unique microbiota composition directly postnatally, these inter-individual compositional differences become less prominent with increasing duration of neonatal intensive care unit (NICU) admission\(^{26}\). This observation underlines the hypothesis of the existence of a vicious circle between hospital room and infant colonization. In fact, by the age of 18-24 months, the infant gut microbiota composition has shifted towards a more adult-like, diverse microbial composition\(^{27}\). Because of the lower diversity, the infant microbial composition is less stable over time compared to adults, and is highly mediated by external factors.

**Intestinal microbiota in necrotizing enterocolitis (NEC)**

Despite advances in neonatal intensive care, incidence and mortality rates of NEC remain disturbingly high in the preterm population\(^{28-30}\). Characterized by necrosis of the intestinal mucosa, initial clinical symptoms include abdominal tenderness, feeding intolerance, bloody stools and abdominal distention\(^{31}\). In a subset of the affected infants, intestinal necrosis may result in an intestinal perforation. Mortality rates range between 20% and 50%, depending on the need for surgical intervention\(^{32}\). Survivors of NEC often cope with adverse long-term outcomes such as growth delay, neurodevelopmental disabilities and GIT complications including short bowel syndrome\(^{33-35}\). To date, the exact pathophysiology of NEC is still largely unknown. However, preterm birth is considered to be an important risk factor since NEC development is reversely related to gestational age. In chapter 10 we aimed to identify other demographic and clinical variables associated with the development of NEC in a prospective, multicenter cohort study. Although NEC is considered a multifactorial disease, evidence is emerging that the intestinal microbiota plays a pivotal etiological role. Several studies observed microbial compositional differences between NEC cases and controls to be already present in meconium\(^{36-38}\). Other studies demonstrated a signature NEC microbiota profile to be dependent on age of onset. Infants developing an early-onset NEC, defined as NEC development before the age of 20 days, demonstrate a *Firmicute* dominance with a concurrent decrease in *Proteobacteria* preceding onset, whereas in late-onset NEC, gut microbiota composition demonstrates an opposite trend. However, not all available studies observed a statistical significant alteration in gut microbiota between NEC cases and controls\(^{39-41}\). In 2016, a meta-analysis including eight studies comparing gut microbiota of infants developing NEC (n=106) and controls (n=278) demonstrated a proportional increase in *Proteobacteria*.
with concurrent decrease in *Firmicutes* and *Bacteroidetes*². Yet, considerable heterogeneity in methodology among included studies hampers the ability to draw reliable conclusions based on outcomes.

In summary, numerous studies have attempted to identify a ‘NEC-specific’ microbial composition resulting in NEC onset. So far, no single or set of microbes have consistently been associated with the development of NEC. This may have resulted from substantial inter-study variation in applied analytical techniques, the relative small study populations, the limited number of participating centers, and differences in sampling time-points, inhibiting the identification of a universal ‘NEC-specific’ microbial composition. Therefore, the aim of chapter 2 was to describe the intestinal microbiota composition of the largest cohort NEC cases so far by performing microbiota analysis on fecal samples obtained within three days prior to NEC onset and compare them with samples from matched control infants.

**PART II: VOLATILE ORGANIC COMPOUNDS**

While molecular microbiota detection techniques can provide insight in composition of microbial communities present, no information can be obtained on their functional characteristics and concurrent interaction with the host. Consequently, revealing and understanding of underlying pathophysiological mechanisms resulting in clinical conditions associated with microbial aberrations early in life is currently hampered. Therefore, novel biomarkers providing additional information on bacterial activity and concurrent host-microbiota interaction are awaited. Over the past years, metabolomics, implying the analysis of the whole spectrum of metabolites⁴³, has gained momentum as potential early biomarker. In particular volatile organic compounds (VOCs), a particular field within metabolomics, has gained considerable clinical interest. VOCs deriving from humans may originate from exogenous sources (e.g. medication or microbiota) or are produced during local and systemic (patho)physiological metabolic processes before being excreted through all conceivable bodily excrements (e.g. sweat, exhaled breath, urine, feces etc.)⁴⁴. Consequently, VOCs may serve as an ideal candidate for diagnostic, screening and monitoring purposes⁴⁴. Chapter 3 provides an overview of the available literature regarding the potential of VOCs as biomarker in gastrointestinal diseases in the pediatric population.

**VOC detection techniques**

VOCs are carbon-based chemicals, volatile under normal atmospheric conditions and temperatures, and can be perceived by mammalian olfactory sense. Besides the conventional olfactory sense in mammals, available VOC detection techniques can roughly be divided into two different categories. Chemical analytical techniques residing at one end of the spectrum allow for the quantitative and qualitative detection of individual VOCs, however, are limited
by their time-consuminnes, expensiveness and complex analyses. Gas-chromatography mass spectrometry (GC-MS) is currently considered the gold standard in VOC detection and can be placed under the chemical analytical techniques. Electronic nose devices, residing the other end of the spectrum, come in many different sort of shapes and sizes. They enable real-time and high-throughput analysis, entail inexpensive purchase and measurement costs and produce relatively easy to interpret outcomes. A complex gaseous VOC mixture is presented to an array of different sensors, influencing the measurable attribute (for example electrical resistance or oscillation frequency) of each individual sensor. Based on pattern recognition algorithms, obtained VOC-profiles could be compared and potentially discriminated from each other. However, main limitation of such eNose devices is their inability to identify individual VOCs, consequently hampering inter-device outcome comparison.

**Fecal volatile organic compounds**

Recently, a total of 1840 VOCs emanating from the human body have been identified, of which 381 volatiles are obtainable from the feces of healthy individuals. VOCs emanating from feces are considered to provide insight about gut microbiota composition, microbial metabolic activity and concurrent host-microbiota interaction. Therefore, fecal VOCs may serve as an interesting biomarker in diseases characterized by preclinical aberrations in gut microbiota composition and activity. Before fecal VOC analysis could be implemented in daily clinical practice, it is first key to investigate the influence of non-relevant or non-avoidable environmental and sampling-specific sources on detected fecal VOC composition. Therefore, in chapter 4, the influence of several environmental factors, sampling conditions and different sampling methods on fecal VOC composition was assessed using an eNose.

**Necrotizing enterocolitis**

To date, the potential of fecal VOCs for the detection of NEC in a preclinical phase has been explored in two studies. Gas chromatography-mass spectrometry (GC-MS) demonstrated the number of fecal VOCs to significantly increase with age in non-NEC infants (n=7), whereas a reduction was observed in NEC infants (n=6) days before diagnosis. In the second study, fecal VOCs were analyzed by means of an electronic nose (eNose) device. Up to 3 days prior to clinical NEC onset, fecal VOC profiles allowed for the discrimination between NEC cases (n=13) and controls (n=14) with increasing discriminative accuracy towards onset of NEC. In chapter 9 we compared the fecal VOCs of infants developing NEC with the profiles from matched control infants using GC-MS, attempting to identify discriminating VOCs allowing for the preclinical detection of NEC.
Late-onset sepsis

Historically, the origin of late-onset sepsis (LOS) has been linked to colonization of intravascular catheters. However, recently, a genetic incongruity between the microbes obtained from the blood culture and the organisms cultured from the intravascular catheter tip was demonstrated, suggesting another origin\textsuperscript{46-48}. Genetic similarity between intestinal isolates and the LOS causing pathogen obtained from the blood culture led to the hypothesis that gut microbes may be involved in LOS etiology. Overall, LOS development has been associated with a slow transition towards a predominantly anaerobe gut microbiota composition, considered to reflect a healthy gut state. Consequently, potential pathogenic aerotolerant microbes remain present in the intestines for a prolonged period, promoting development of a reservoir of pathogens, and consequently increasing the risk for developing gut-derived sepsis\textsuperscript{49}. In line with this observation, the LOS causing agent was demonstrated to be abundantly present in the gut in 57%-86\% of the cases, even preceding clinical disease onset \textsuperscript{49-52}. Therefore, since LOS is associated with preclinical alterations in gut microbiota composition, we investigated the potential of fecal VOCs as early biomarker in LOS in chapter 5, 6 and 7.

Bronchopulmonary dysplasia

With a current incidence of 30\%, bronchopulmonary dysplasia (BPD) is the most common adverse outcome in preterm infants born with a very low birth weight (<1500g)\textsuperscript{53}. This chronic pulmonary disease is characterized by an impaired lung growth, reflected by a disruption in alveolar growth and pulmonary vasculogenesis\textsuperscript{54}. BPD etiology is considered to be multifactorial with a wide variety of different antenatal and postnatal factors contributing to its development\textsuperscript{55}. Over the past years, an increasing number of animal and adult studies have demonstrated the existence of vital cross-talks between the gut and lungs\textsuperscript{56,57}. This gut-lung axis has been considered to be involved in the pathogenesis of several chronic respiratory diseases, including asthma and chronic obstructive pulmonary disease (COPD)\textsuperscript{56}. Whether the gut-lung axis is involved in the development of respiratory microbiota alteration and possibly in the pathophysiology of BPD, has not been investigated so far. Therefore, the aim of chapter 8 was to explore whether development of severe BPD was associated with early shifts in intestinal microbiota and fecal volatile metabolites.
REFERENCES


