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

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English summary

Parts of this summary has been published in:


Application of Fecal Volatile Organic Compound Analysis in Clinical Practice: Current State and Future Perspectives. el Manouni el Hassani S, Berkhout DJC, Bosch S, Benninga MA, de Boer NKH, de Meij TGJ. Chemosensors 2018, 6, 29.
PART I: INTESTINAL MICROBIOTA

This thesis focusses on novel insights concerning the underlying pathophysiology in a wide variety of clinical conditions in the extremely preterm born population. Secondly, this thesis also comprises studies investigating novel diagnostic biomarkers to detect these conditions in a preclinical phase. Extremely preterm born infants harbor an increased risk of developing several inflammatory and infectious diseases, in which the intestinal microbiota is considered to fulfill an essential etiological role. During delivery, colonization of the neonatal gut is realized by microbes deriving from both the mother and the direct environment. However, this initial microbial composition is extremely precarious and highly subjective to change. Environmental factors considered to influence this composition include mode of delivery (vaginal birth versus cesarean section), feeding practices and medication use. Part 1 of this thesis focusses on the intestinal microbiota of the neonate.

In chapter 1 we studied the influence of maternally administered intrapartum antibiotic prophylaxis (IAP) during a cesarean section (CS) on the intestinal microbiota composition of the neonate. This IAP is administered in order to reduce the risk of maternal infectious morbidity. Although this IAP was previously administered after clamping the umbilical cord, novel insights resulted in the development of obstetric guidelines advocating IAP administration prior to skin incision. Although this alteration in timing of antibiotic administration decreases the observed maternal infectious morbidity even further, potential effects on the composition of the neonatal microbiota have never been adequately addressed. Notably, gut microbes are essential in priming of the relatively naïve immune system and early in life aberrations in the intestinal composition are associated with a broad variety of clinical conditions, including asthma, allergies, necrotizing enterocolitis (NEC), obesity, inflammatory bowel disease, and sepsis. To explore the influence of this alteration in timing of IAP administration we conducted a randomized controlled trial, comparing the gut microbiota composition of 20 full-term infants, born by primary CS and exposed to cefuroxime, to the microbial composition of 20 full-term, cesarean delivered infants not exposed to antibiotics. In this study we demonstrated infants allocated to the study group receiving IAP prior to the skin incision to depict increased umbilical cord blood levels of cefuroxime. Moreover, cefuroxime exposed infants demonstrated a proportional increase in Proteobacteria representatives at the postnatal age of 28 days, whereas members of the phyla Firmicutes, Actinobacter, Fusobacteria and Verrucomicrobia (FAFV) demonstrated a deceased relative abundance. Future studies should evaluate the persistence of the observed differences after the postnatal age of 28 days. In addition, current study results advocate the urgent need the evaluate any potential neonatal health implications later in life resulting from implementation of this new obstetric guideline.

Aim of chapter 2 was to describe the intestinal microbiota composition and dynamics of infants developing NEC. Being inversely related to gestational age, NEC is the most
common severe gastrointestinal disease in the preterm born population and despite major improvements in neonatal intensive care, its incidence rate increased over the past decade\textsuperscript{11}. Although the exact underlying pathophysiology remains to be elucidated, gut microbes are considered to fulfill an essential etiological role in this multifactorial disease\textsuperscript{12}. In this study, the intestinal microbiota composition of 53 preterm born infants (gestational age ≤30 weeks) developing NEC were compared to the compositions of 53 matched infants not developing NEC. Fecal samples obtained up to three days prior to clinical disease onset were analyzed in this study. No compositional differences were observed if all infants were included in the analysis. However, after focusing on NEC cases with the severest clinical outcomes, defined as infants staged IIIB (n=11) according to the modified Bell’s staging criteria\textsuperscript{13} or infants not surviving the NEC episode (n=17), an altered microbial composition was demonstrated, suggesting the ability to preclinically identify these most severe phenotypes of NEC. In future this may provide the clinician opportunities for timely detection and initiation of targeted microbiota-based interventions, aimed at preventing NEC. Interestingly, in 68% of the NEC cases enduring a septic episode within 72 hours of disease onset, the sepsis-causing pathogen was already observed to be present in preclinical stools. Presumably, the intestines form a reservoir of potential pathogens, translocating through the mucosal wall into the bloodstream after the gut becomes necrotic, ultimately resulting in sepsis development during the NEC episode\textsuperscript{12}.

To conclude, in the first part we described the intestinal microbiota composition of both diseased and healthy neonates. We first demonstrated that the intestinal microbial composition of infants exposed to cefuroxime during a CS differed from the composition of non-exposed infants at the age of 28 days. Future studies investigating the long-term effects of this antibiotic exposure on neonatal gut microbiota composition and its clinical implication later in life are urgently warranted. Secondly, gut microbiota composition of infants developing a severe phenotype of NEC differed from control infants not developing NEC. Future studies should aim at exploring any causality between these compositional alterations in gut microbiota and the development of NEC. In case of causality, studies investigating the potential of microbiota-based interventions allowing for the early treatment of, or even prevention of NEC development, should be performed.

**PART II: VOLATILE ORGANIC COMPOUNDS**

In part 2 of this thesis we investigated the potential of fecal volatile organic compounds (VOCs) as diagnostic biomarker in several neonatal diseases. These VOCs are carbon-based chemicals, volatile at ambient temperatures and are the cause of most surrounding odors\textsuperscript{12}. VOCs deriving from humans may either have an external origin (e.g. microbes and medication) or are produced during local and systemic (patho)physiological metabolic processes before
being excreted through all conceivable bodily excrements\textsuperscript{12,14}. Currently available detection techniques can roughly be divided in two different categories\textsuperscript{15}. Chemical analytical techniques, including gas chromatography- mass spectrometry (GC-MS), residing at one end of the spectrum. These devices provide valuable information about the presence and concentration of individual VOCs, however are limited by their time-consuminess, high costs and complex (statistical) analyses, hampering implementation of such devices in daily clinical practice\textsuperscript{15}. At the other end of the spectrum are the electronic nose (eNose) devices, allowing for bed-side analysis of complex gaseous mixtures based on pattern recognition algorithms. Although these user-friendly devices are relatively inexpensive and allow for fast obtainment of results, their inability to discriminate between individual VOCs is one of their major limitations. Hypothetically, information about discriminating VOCs identified by using chemical analytical techniques may allow for the development of a tailored eNose, applicable in daily clinical practice and specifically designed to accurately detect specific clinical conditions\textsuperscript{15}. In this thesis we predominantly focused on VOCs deriving from feces. Fecal VOCs are considered to reflect gut microbiota composition, concurrent metabolic activity, and provide information about the interaction between microbiota and the host\textsuperscript{12}. Consequently, fecal VOCs may hypothetically serve as an early diagnostic biomarker in clinical conditions associated with preclinical alterations in gut microbiota composition.

Chapter 3 provides an overview of the available literature regarding the potential of VOCs as diagnostic biomarker in a wide variety of pediatric clinical conditions. In this chapter we mainly focused on gastrointestinal and liver disorders. Despite the exponential increase in proof-of-principle studies over the past decade, inter-study variation in applied methodology and variation in analyzed substrate (e.g. feces, exhaled air, urine etcetera) in combination with the relatively small study populations hampers the ability to compare outcomes between studies. In this chapter we conclude that VOCs harbor great potential in discriminating disease from health. However, the development of standardized study protocols in combination with the validation of study results in large cohorts remain urgently warranted before VOCs could live up to its potential as diagnostic biomarker.

Chapter 4 comprises a validation study investigating the influence of different sampling and environmental factors on fecal VOC profiles as obtained by an eNose. All investigated environmental and sampling factors had a significant influence on detected VOC profiles, emphasizing the need for the universal standardization of sampling techniques. Notably, intra-study variation in sampling conditions prevents the ability to draw unbiased conclusions, whereas inter-study variation in these conditions limits the capacity to adequately compare study outcomes.

In the following chapters we investigated the potential of fecal VOCs to differentiate between diseased and healthy individuals. Based on the hypothesis that late-onset sepsis
(LOS) in the extremely preterm born population is preceded by preclinical alterations in gut microbiota composition, leading to bacterial overgrowth of potential pathogenic species, followed by transmucosal translocation into the bloodstream and ultimately allowing for sepsis development\textsuperscript{16}, we investigated the potential of fecal VOC profiles to detect LOS in a preclinical phase by using two different eNose technologies. In chapter 5 we demonstrated in a proof-of-principle study, performed at three neonatal intensive care units (NICUs), that fecal VOC profiles allow for the preclinical discrimination between LOS cases and matched control infants not developing LOS. More specifically, up to three days prior to clinical disease onset, fecal samples from 36 LOS cases could be discriminated from the fecal samples from 40 controls. However, corresponding discriminative accuracies at each individual time-point were relatively moderate. Since each individual bacterial species produces a species-specific VOC profile\textsuperscript{17}, we hypothesized that the relatively small sample size in combination with the heterogeneity in isolated sepsis-causing pathogens resulted in this suboptimal discriminative accuracy. Another potential cause is the inclusion of LOS cases with a central venous catheter during sepsis onset. Hypothetically, in these particular cases, sepsis onset was not preceded by bacterial transmucosal translocation but originated from the skin. We aimed to investigate these two hypotheses in the following two chapters. In chapter 6, fecal VOC profiles of 127 neonates developing LOS were compared with the profiles from 127 control infants not developing LOS. All neonates were born at a gestation of \( \leq 30 \) weeks and admitted to one of the nine participating NICUs. In contrast to chapter 5, fecal VOC profiles only allowed for discrimination between LOS cases and controls at one day prior to clinical sepsis onset. Presumably, this difference in study outcomes could be explained by the vast heterogeneity in sepsis causing agents in the study of chapter 6. Notably, after focusing on individual bacterial species, more specifically \textit{Escherichia coli}, \textit{Staphylococcus aureus} and \textit{Staphylococcus epidermidis}, discriminative accuracy increased, resulting in a high predictive value up to three days prior to clinical onset. In chapter 7 we compared the fecal VOC profiles from 24 LOS cases not meeting the criteria for central line-associated bloodstream infection, defined as the presence of a central line within 48 hours prior to LOS onset, with the fecal VOC profiles from 24 infants without LOS. Historically, central lines are considered to be the most important source of LOS\textsuperscript{18}. By excluding infants with a central line during sepsis development we aimed at reducing the number of LOS cases originating from skin invasive procedures while concurrently increasing the plausibility of an intestinal origin of the sepsis causing pathogen. Conform the previous two studies we demonstrated that fecal VOC profiles allow for the discrimination between LOS cases and control infants up to three days prior LOS onset. In addition, discriminative accuracy was most optimal in the analysis including only cases with identical sepsis-causing pathogens, more specifically \textit{Staphylococcus epidermidis}. Based on the results of these three studies we increased the plausibility that a LOS episode in preterm born infants is often preceded by
intestinal overgrowth. Presumably, this bacterial overgrowth results in a species-specific fecal VOC profile, allowing the eNose to differentiate disease from health in a preclinical phase.

Aim of chapter 8 was to evaluate if fecal VOC profiles allowed for the differentiation between infants developing severe bronchopulmonary dysplasia (BPD) and infants without BPD. BPD is an inflammatory pulmonary disease characterized by an interruption in its development resulting in fewer and larger alveoli in combination with a dysmorphic pulmonary microvasculature. This clinical condition predominantly affects preterm born infants and results in oxygen dependency for a prolonged period of time. In this study we demonstrated that fecal VOC profiles allowed for the differentiation between infants developing severe BPD and control infants at the postnatal age of 14, 21 and 28 days, but not at 7 days. Potentially, sampling at the postnatal age of 7 days may be too early in the developmental process of BPD to detect any alteration in fecal VOCs allowing for discrimination between BPD and non-BPD. Since microbiota analysis on the samples obtained at the postnatal age of 28 days did not demonstrate any compositional differences between cases and controls, we hypothesize that the observed differences in VOC profiles did not derive from alterations in gut microbiota but resulted from the local and systemic production of inflammatory biomarkers influencing the fecal VOC profile. In addition to BPD, other pulmonary diseases may also be detected using VOC profiles.

Chapter 9 comprises a study in which we compared fecal VOC profiles obtained from 44 NEC cases with the fecal VOC profiles from 44 control infants without NEC. Fecal samples obtained up to three days prior to clinical disease onset were analyzed by means of GC-MS. The number of individual VOCs per analyzed sample ranged between 101-383. Compared to NEC cases, infants not developing NEC demonstrated a relatively stable VOC-pattern over time. However, using different supervised classification methods, both groups could only be discriminated with a relatively modest accuracy ranging between 60 and 70%. The reason for this suboptimal discriminative accuracy could only be speculated upon. Although gut microbes are considered to fulfill an essential role in NEC etiology, we already demonstrated in chapter 2 that a uniform and NEC-specific microbial composition could not be identified. Therefore, we hypothesize that NEC is a clinical condition with a wide variety of different underlying mechanisms all resulting in clinical symptoms typical for NEC, such as pneumatosis intestinalis and intestinal necrosis. This non-uniformity in underlying pathophysiology may potentially be the explanation of the absence of a NEC-specific VOC profile in the present study. In the future, we aim to perform two additional analysis based on the results discussed in chapter 2. In the first analysis, only NEC cases with the most severe clinical outcomes be will included, investigating the discriminative potential of fecal VOCs in these most severe phenotypes of NEC. In the second analysis, focusing on infants developing sepsis during their NEC episode, the potential of fecal VOCs to preclinically identify the sepsis-causing pathogen will be investigated.
In majority of the chapters in this thesis, fecal samples collected from preterm born infants admitted to one of the nine participating NICUs were subjected to analysis. In addition to fecal samples, a wide variety of different demographic and clinical variables were collected from all included infants. **Chapter 10** comprises an epidemiological study aimed at identifying independent risk factors associated with the development of NEC. In this prospective case-control study, a total of 843 preterm born infants (gestational age ≤ 30 weeks) were included, of which 56 infants endured a NEC episode during the first 28 postnatal days. Conform literature, NEC incidence was inversely related to gestational age. In the multivariable logistic regression model, number of parenterally fed days and formula feeding prior NEC onset were both associated with an increased risk of NEC development. In contrast, administration of any antibiotic within 24 hours after birth was associated with a reduced risk in NEC development, underlining the increasing notion that early intestinal colonization may play a pivotal role in NEC pathogenesis.
REFERENCES


