CHAPTER 11

Early detection of necrotizing enterocolitis by fecal volatile organic compounds analysis

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ABSTRACT

Objectives

To test the hypothesis that fecal volatile organic compounds (VOCs) analysis by electronic nose (eNose) allows for early detection of necrotizing enterocolitis (NEC).

Study design

In three neonatal intensive care units, fecal samples of infants born at gestational age ≤ 30 weeks were collected daily, up to the 28th day of life. Included infants were allocated in three subgroups: NEC, sepsis and matched controls. Three time-intervals were defined; (a) $T_{-5,-4}$ (five and four days before diagnosis); (b) $T_{-3,-2}$ (three and two days before diagnosis) and (c) $T_{-1,0}$ (day before and day of diagnosis). Three subgroups were analyzed by eNose.

Results

Fecal VOC profiles of infants with NEC (n = 13) could significantly be discriminated from matched controls (n = 14) at $T_{-3,-2}$ (AUC ± 95% CI, p-value, sensitivity, specificity: 0.77 ± 0.21, p = 0.02, 83%, 75%), the accuracy increased at $T_{-1,0}$ (0.99 ± 0.04, p < 0.001, 89%, 89%). VOC profiles of infants with NEC were also significantly different from those with sepsis (n = 31) at $T_{-3,-2}$ (0.80 ± 0.17, p = 0.004, 83%, 75%), but not at $T_{-1,0}$ (0.64 ± 0.18, p = 0.216, 89%, 57%).

Conclusion

In this proof of principle study we observed that fecal VOC profiles of infants with NEC could be discriminated from controls, from two to three days predating onset of clinical symptoms. Our observations suggest that VOC-profiling by eNose has potential as non-invasive tool for the early prediction of NEC.

INTRODUCTION

Necrotizing enterocolitis (NEC) is the most common severe gastro-intestinal disease in very low birth weight infants, with reported incidence rates varying between 3-15%.[1,2] Treatment consists of prompt cessation of enteral feeding, administration of broad spectrum antibiotics alongside supportive care. Of affected infants, 30 to 40% will need surgery at some point for gut necrosis or bowel perforation.[3] Mortality due to NEC remained disturbingly high over the past years, with rates varying between 15 and 30%.[2] In survivors of NEC, neurocognitive and gastro-intestinal impairments, like short bowel syndrome, are common complications.[4,5]

The pathophysiology of NEC is to be considered multifactorial. Immaturity of the gut, enteral (formula) feeding and altered intestinal microbiota composition are the principal inducers of an excessive inflammatory response which leads to intestinal injury.[6] This inflammatory cascade causes non-specific clinical symptoms which may resemble sepsis, commonly leading to delayed diagnosis.[6]

Early diagnosis and initiation of therapy are of pivotal prognostic importance and invasive diagnostic procedures may contribute to adverse neurocognitive outcome.[7] Different biomarkers for NEC have been studied so far, mostly at the time when NEC was already suspected clinically. Unfortunately, the majority of these biomarkers lack accuracy to detect NEC in pre-clinical stage and do not allow proper discrimination from sepsis.[6,8] Therefore, the search for disease-specific, early and non-invasive diagnostic biomarkers for NEC remains warranted.

Since the pre-clinical stage of NEC is associated with alterations in gut microbiota composition, fecal volatile organic compounds (VOCs) could hypothetically serve as non-invasive biomarkers for the early detection of NEC.[9,10] Fecal VOCs are carbon-based gaseous chemicals, originating from fermentation processes of colonic microbes and from host metabolism.

Fecal VOC profile analysis can be performed by electronic nose (nose) technology. This odour sensing method is based on pattern recognition algorithms, mimicking the mammalian sensory system. Fecal gas analysis by eNose has previously been shown potential in the (early) detection and assessment of disease activity in colorectal cancer and in pediatric inflammatory bowel disease respectively, disorders characterized by alterations in microbiota composition.[11,12]

We, therefore, hypothesized that analysis of fecal VOCs by eNose allows for detection of NEC before the onset of clinical disease. We aimed to study this in a prospective, multi-

center proof of principle study in preterm infants, by comparing fecal VOC profiles of infants with NEC with matched controls and infants with sepsis.

MATERIALS AND METHODS

Subjects

This prospective study was performed between September 2013 and March 2014 at the Neonatal Intensive Care Unit (NICU) of the VU medical center in Amsterdam, the Emma Children's Hospital/Academic Medical Centre in Amsterdam, and the Máxima Medical Centre in Veldhoven, The Netherlands. None of the centers used probiotics as prevention for NEC. The study was approved by the local Institutional Review Boards.

Preterm infants were eligible for the study if they were born at a gestational age ≤ 30 weeks and written informed consent was obtained from both parents. Exclusion criteria were congenital intestinal anomalies (anal atresia, Hirschsprung's disease, short bowel syndrome) and intestinal surgery (bowel resection or stomata) during the period of stool collection, since this might influence fecal VOC profiles.

In addition to standard demographic parameters, the following clinical data were prospectively collected: mode of delivery, enteral and parenteral feeding pattern, medication (including antibiotics), erythrocyte transfusions, respiratory support, sepsis and development of NEC.

All cases were reviewed independently by two experts (TdM and HN) and allocated to one of the following groups: (i) infants with NEC stage IIA and higher, according to the international classification of Walsh and Kliegman,[13] (ii) infants with (late-onset) sepsis and (iii) matched controls. Full agreement was reached in all cases. Infants with sepsis were defined as subjects with clinical signs or symptoms of infection, combined with positive blood culture.[14] Controls were defined as infants without clinical evidence of sepsis or NEC.

Sample size calculation

Based on results from our previous studies on fecal gas analysis (effect size 1.340), we concluded that a sample size of ten subjects per subgroup would be sufficient to obtain a power of 0.80 to reject the null hypothesis that no differences exist between fecal VOC profiles of patients with NEC and controls at $p < 0.05$ (Nquery advisor 7.0).[11,12]

Fecal sample collection

Fecal samples of included preterm neonates were collected daily by the nurse from the diaper, during the first twenty-eight days of life. Approximately 0.5 gram of feces was stored in a sterile plastic container and stored at -20°C in a freezer immediately following collection. In case the infant was discharged from the NICU, or transferred to another hospital, before age of twenty-eight days, stool sampling was terminated. If an infant passed more than one stool per day, only the first produced sample was stored. In case of absence of daily bowel movements, the subsequently produced fecal sample was collected.

Sample selection

The stored stool samples produced up to five days before the diagnosis of NEC and sepsis were used for fecal VOC analysis. Based on sample size calculation, the necessary number of fecal samples per time interval was obtained by clustering samples into three time windows: (a) $T_{-5,-4}$ (five and four days before diagnosis; (b) $T_{-3,-2}$ (three and two days before diagnosis) and (c) $T_{-1,0}$ (day before and day of diagnosis). To investigate whether fecal VOC profiles of preterm infants with NEC differed from controls and from sepsis per defined time window, each fecal NEC sample was matched with one control sample by center of birth, gestational age, postnatal age, number of days exposed to antibiotics and birth weight. Furthermore, in order to investigate whether fecal VOC profiles of infants with NEC differed from infants with sepsis, VOC-profiles of both subgroups were compared.

VOC analysis by eNose

VOC analysis of the selected fecal samples was performed with use of a Cyranose 320 eNose® (Smiths Detections, Pasadena, CA, USA). The preparation of samples and technique of VOC-measurements in fecal gas were in accordance with methods described in detail in previous studies.[11,12] In short, approximately 1 gram of frozen feces was transferred from the stored containers into a sealed vacutainer (BD vacutainer, Belliver Industrial Estate, Plymouth, UK). This vacutainers were resealed and gradually heated to 37°C for 1 hour in an incubator, to enhance vapor release from the stools. The heated vacutainers were subsequently connected to the e-nose and analysed in an air-tight closed loop system in order to prevent headspace dilution. Headspace sampling was performed using the Cyranose 320 eNose®, a handheld chemical vapor analyzer, containing a fully-integrated nanocomposite array comprising 32 polymer sensors. Upon exposure to a gaseous mixture,

these polymer sensors interact competitively with VOCs, causing the sensor material to swell, thereby increasing the electrical resistance of the sensor matrix. Multiple biomarkers interact with each individual sensor and individual biomarkers may interact with multiple sensors. The resulting alterations in resistance depend on the chemical characteristics of both the sensor material and interacting VOCs and are. combined into a so-called smellprint. This smellprint can subsequently be used to differentiate clinical groups by pattern recognition analysis.[15] Samples were analyzed in random order, using www.randomizer.org. The investigators performing the eNose analysis were blinded to clinical data.

Data analysis

Basic patient demographic data were tabulated and compared by independent t-test, chi-square test or non-parametric tests where appropriate.

The eNose provides a raw data output consisting of resistance changes of the sensors upon exposure to the VOC-mixture. Principle Component Analysis was performed on the raw data to recombine the variance of the original dataset into a set of orthogonal principle components or factors. This unsupervised method captures the highest amount of variability of the original dataset in the lowest number of variables, thereby reducing the risk of overfitting our diagnostic algorithm.

As previously described samples were pooled into 3 time periods. Principle components differentiating between cases and controls at this time point (e.g. NEC versus Controls) were selected by means of student t-test ($p < 0.05$). These principle components were used in a supervised Canonical Discriminant Analysis (CDA) internally validated by a leave one-out method in view of the relatively low sample size. This method builds a diagnostic algorithm using data from all but one of the subjects. The remaining subject is subsequently introduced to the algorithm and classified providing a probability of disease for that case. This iterative process is repeated until each subject has been excluded from the primary algorithm once. The combined disease probabilities for all cases are used to construct a Receiver Operator Characteristic (ROC)-curve and compute single point sensitivity, specificity, negative and positive predictive values. The overall accuracy of the algorithm was assessed by the Area Under The Curve (AUC) and associated 95% confidence interval.

RESULTS

Study population and demographics

In the study period 128 infants (13 NEC, 31 sepsis, 84 controls) were included, accounting for a total of 2110 stored stool samples. Fecal samples of all NEC and sepsis cases and of 14 controls were used for fecal gas analysis in order to strictly match each fecal NEC-sample per defined time window with one control sample. An overview of the number of fecal samples per subgroup and per time window used for VOC analysis is given in Table 11.1. Patient characteristics of the three clinical subgroups are depicted in Table 11.2. Individual

Table 11.1 Number of fecal samples (n) in each group per selected time-interval used for VOC analysis

Interval	(n)		
	NEC	Control	Sepsis
$T_{-5,-4}$	10	10	18
$T_{-3,-2}$	12	12	20
$T_{,-1,0}$	9	9	23

Table 11.2 Subject characteristics of the three subgroups NEC, sepsis and controls

Number (n)	NEC 13	Sepsis 31	Control 14
Sex			
Male (n [%])	4* [31]	18 [58]	11* [79]
Birth weight (median [IQR]), g	740 [155]	880 [300]	937 [346]
Gestational age (median[IQR]), weeks + days [days]	26 + 6 [6]	26 + 3 [17.5]	26 + 5 [15.3]
Feeding pattern (n [%])			
Breast milk ± formula	12 [92]	29 [94]	13 [93]
Exclusive formula	1 [8]	1 [3]	0 [0]
Missing value	0 [0]	1 [3]	1 [7]
Postnatal age at T_0 (median, [IQR] (days)	18.5† [8]	10†‡ [7]	17‡ [0]
AB use before T_0 (%)	n = 13 (100)	n = 29 (94)	n = 13 (93)
Days AB use before T_0 median [IQR] (days)	7 [5]	5 [4]	7 [4]
Deceased (n [%])	7 [54]	3 [10]	0 [0]
Age deceased median (days)	20	18	NA

AB = antibiotics; NA = not applicable.
* p = 0.013.
† p = 0.005.
‡ p = 0.008.

characteristics of neonates who developed NEC are depicted in Table 11.3. Seven (54%) of thirteen infants with NEC died (five within one day following diagnosis, one after three days and one after thirteen days). An overview of the isolated pathogens from blood cultures in the sepsis group is given in Table 11.4.

Fecal gas analysis

NEC versus control

Patients with NEC could not be discriminated from matched controls at time window $T_{-5,-4}$ (AUC ± 95% CI, p-value, sensitivity, specificity; 0.65 ± 0.25, 0.257, 60.0%, 60.0%). At $T_{-3,-2}$, VOC-analysis discriminated infants with NEC from controls with an accuracy of 0.77 ± 0.21, 0.024, 83.3%, 75.0%. This further increased to a high accuracy at $T_{-1,0}$: 0.99 ± 0.04, 0.001, 88.9%, 88.9%. A scatter plot for the discrimination of infants with NEC and controls is shown in Figure 11.1.

Table 11.3 Patient characteristics of infants with NEC

Subject	Sex	BW (g)	GA (wk)	Feeding pattern	Diagnosis NEC (day)	NEC stadium[a]	AB prior to T_0 (days)	Deceased[d]
1	M	545	26 + 3	BM[c]	23	IIIB	15	Yes (24)
2	F	685	27 + 0	BM[c]	15	IIIB	10	No
3	F	725	26 + 1	BM[c]	20	IIIA	9	Yes (28)
4	F	840	26 + 1	BM[c]	19	IIIA	9	Yes (20)
5	F	1185	27 + 0	Fo	8	IIB	4	No
6	M	810	29 + 2	BM[c]	20	IIB	8	No
7	F	1020	25 + 6	BM[c]	10	IIB	2	Yes (11)
8	F	730	27 + 4	BM[c]	21	IIB	3	No
9	F	640	24 + 0	BM[c]	15	IIIB	13	No
10	M	740	24 + 5	BM[c]	18	IIA	7	Yes (19)
11	F	610	27 + 0	BM[c]	27	IIIA	7	Yes (28)
12	F	850	27 + 0	BM[c]	12	IIB	2	No
13	M	770	26 + 6	BM[c]	11	IIB	5	Yes (14)

BW = birth weight; MV = missing value; BM = breast milk; F = female; Fo = formula; GA = gestational age; M=male; NEC = necrotizing enterocolitis; AB = antibiotic.
[a] Classified with modified Bell staging criteria for NEC; [b] t0 = day of diagnosis; [c] exclusive breastmilk or combination breastmilk/formula; [d] Deceased during inclusion period of 28 days postnatal, displayed in postnatal age (days).

Table 11.4 Isolated pathogens n (%) from blood cultures in 31 sepsis patients

Staphylococcus Aureus	2 [6]
Coagulase Negative Staphylococcus (CNS)	21 [68]
Staphylococcus Epidermidis	10 [32]
Staphylococcus Capitis	5 [16]
Staphylococcus Haemolyticus	1 [3]
Staphylococcus Warneri	2 [6]
Combination of 2 different CNS	3 [10]*1
Escherichia Coli	3 [10]
Enterococcus Faecalis	1 [3]
Serratia Marcescens	1 [3]
Candida Albicans	1 [3]
More than 1 pathogen cultured	2 [6]*2

*1 - 2x Staphylococcus Capitis & Staphylococcus Epidermidis
- 1x Staphylococcus Capitis & Staphylococcus Haemolyticus
*2 - 1x Enterobacter Cloacae & Staphylococcus Warneri
- 1x Acinetobacter Baumanii & Staphylococcus Capitis

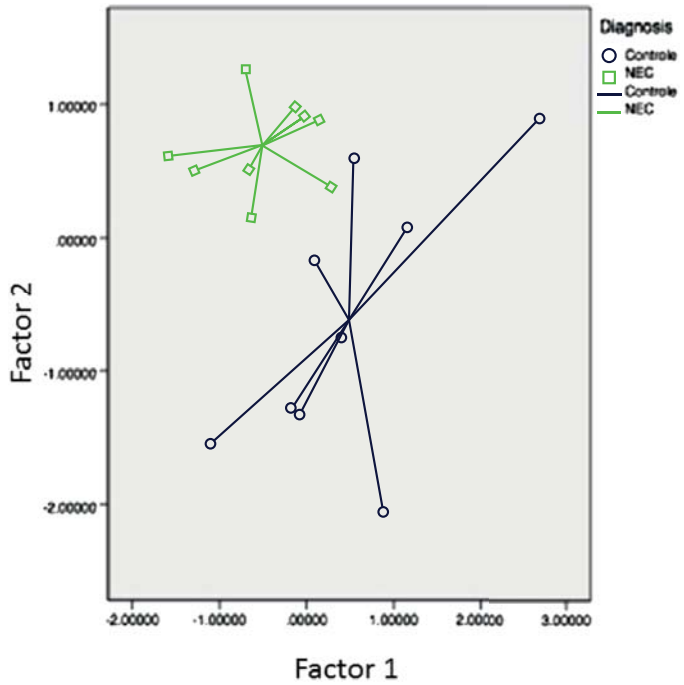

Figure 11.1 Scatter plot for the discrimination of infants with NEC (green circles) and controls (blue circles) at time window $T_{0,-1}$ by electronic nose.
Axes depict two orthogonal linear recombinations of the original 32 sensor data, designed to capture the highest amount of original data variance, by means of principal component analysis. These variables are called factors.

NEC versus sepsis

Patients with sepsis could be differentiated from patients with NEC at 2 to 3 days prior to clinical onset: 0.80 ± 0.17, 0.004, 83.3%, 75.0%. This discrimination was not possible at $T_{-1,0}$ (0.64 ± 0.18, 0.216, 88.9%, 56.5%) and $T_{-5,-4}$ (0.52 ± 0.23, 0.886, 50.0%, 40.0%).

Detailed test-characteristics for all classification algorithms are depicted in Table 11.5. Corresponding receiver operating characteristic (ROC) curves for diagnosis of NEC compared with controls and NEC versus sepsis are displayed in Figure 11.2.

DISCUSSION

In this prospective multicentre study, we hypothesized that fecal VOC profiles measured by eNose could serve as an early diagnostic biomarker for NEC. We observed that two to three days before the clinical onset of NEC, fecal VOC profiles of affected infants could be discriminated significantly from controls, while the difference in the VOC profiles of the two groups increased even further towards onset of NEC. Besides, infants with NEC could be discriminated from subjects with sepsis, two to three days before diagnosis, but not at time-interval $T_{-1,0}$.

The limited available information in the literature on fecal gas analysis in the early detection of NEC is derived from a pilot study using gas chromatography mass spectroscopy (GC-MS). Garner and colleagues described in this relatively small scaled study (six NEC cases versus

Table 11.5 Performance characteristics of fecal VOC-analysis for the discrimination of NEC, sepsis and controls. Sensitivities, specificities, positive and negative likelihood ratios are reported for the optimum cut-points.

Time window	AUC ± 95% CI	p-value	Sensitivity	Specificity	+ LR	- LR
NEC versus control						
$T_{0,-1}$	0.99 ± 0.04	> 0.001	88.9	88.9	8.1	0.1
$T_{-2,-3}$	0.77 ± 0.21	0.024	83.3	75.0	3.3	0.2
$T_{-4,-5}$	0.65 ± 0.25	0.257	60.0	60.0	1.5	0.7
NEC versus sepsis						
$T_{0,-1}$	0.64 ± 0.18	0.216	88.9	56.5	2.1	0.2
$T_{-2,-3}$	0.80 ± 0.17	0.004	83.3	75.0	3.3	0.2
$T_{-4,-5}$	0.52 ± 0.23	0.886	50.0	40.0	0.8	1.3

AUC ± 95% CI = area under the curve with 95% confidence interval; + LR = positive likelihood ratio; - LR = negative likelihood ratio.

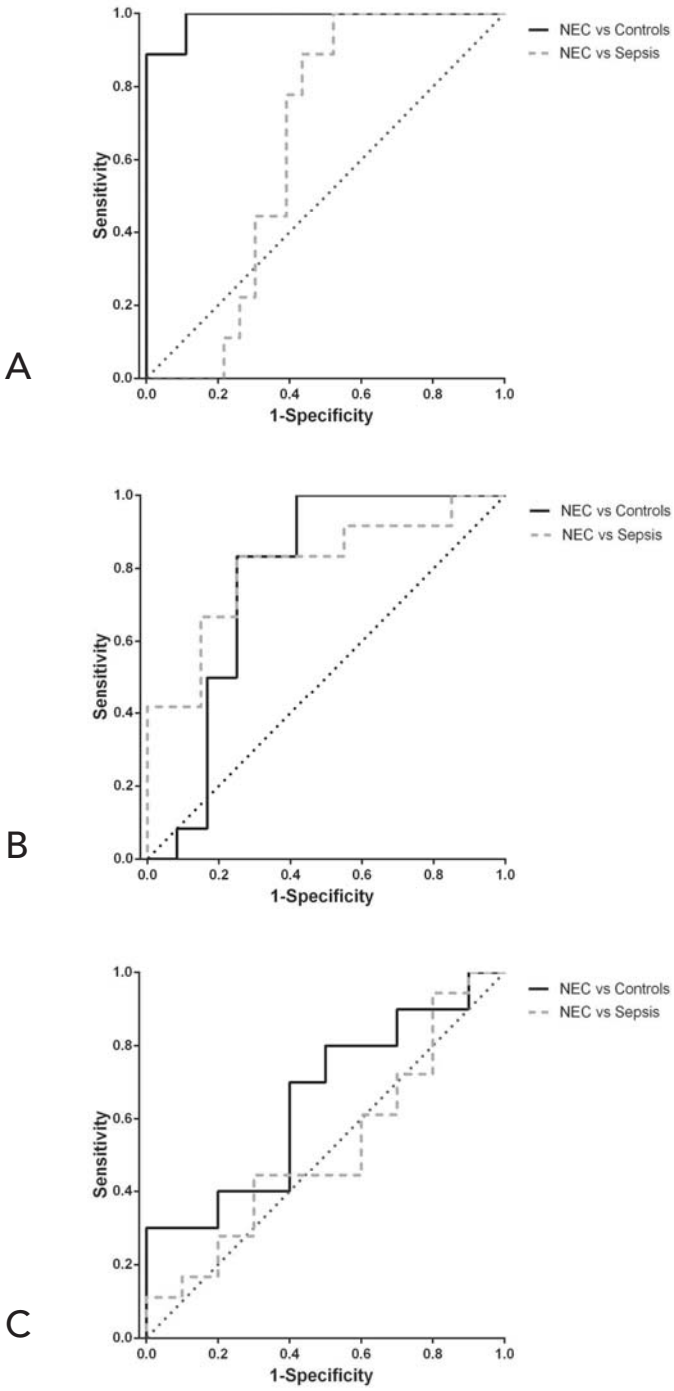

Figure 11.2 ROC-curve with 95% confidence interval for diagnosis of NEC compared with controls and NEC versus sepsis, at time-intervals $T_{-1,0}$ (A), $T_{-3,-2}$ (B) and $T_{-5,-4}$ (C).

seven controls) specific differences in VOC-composition next to a decreased total number of present VOCs in NEC subjects in the four days before clinical onset of disease, compared to controls.[16] Our present study was the first to describe eNose technology in the search for novel diagnostic biomarkers for NEC. Our findings underline that VOC analysis might be an effective tool to detect NEC in the pre-clinical stage. Benefits of eNose devices over GC-MS include lower costs, high-throughput capacities, user-friendly hard- and software, all allowing for the future applicability in clinical practice. We performed the fecal gas analysis following gradually heating of the faecal samples to 37°C, to enhance vapor release from the stools. This particular temperature was chosen to evaluate whether eNose devices have potential as bedside screening tool, when using freshly produced fecal samples. In a previous study we have performed validation sessions on fecal gas analysis by eNose, in which we observed no statistically significant effects of thawing and refreezing and of different temperatures of fecal samples (varying from 15°C to 35°C) on measured VOC profiles.[11]

The major reservoir of fecal VOC source is considered to be microbes residing in the intestinal tract. The microbiome plays an essential factor in the development of NEC, and has been subject to numerous microbial studies analyzing its composition in NEC.[9,10,17-21] Recent reports on microbiota composition in NEC described significant differences in species mainly belonging to the phylum *Proteobacteria*, detectable up to two weeks prior to onset of NEC, next to an increase in Citrobacter and a reduced diversity and depletion of enterococcus.[9,10,17-19] Current opinion is that these microbiota alterations in infants with NEC subjects result from manipulation by environmental factors linked to the development of NEC, such as the antenatal or early postnatal use of antibiotics and feeding type.[22,23] Here, we observed that differences in VOC-profiles between NEC and controls gradually increased from three days before, towards the onset of clinical diagnosis of NEC. The observed VOC-shifts can however not solely be assigned to shifts in microbial presence. Firstly, VOCs with a microbial origin reflect, at least in part, functional metabolic aspects of these bacteria. Many of these VOCs are therefore non species specific. Fecal gas analysis by eNose may be able to detect sudden shifts in microbial activity not observed by microbiome analysis such as the sudden increase in the production of hydrogen, associated with pneumatosis intestinalis, a pathognomonic radiologic sign of NEC.[24,25] Secondly, VOCs are not merely be produced by the gut microbiota, but at least partly result from the intestinal mucosal inflammatory process preceding the clinical onset of NEC.

Although fecal VOCs measured in the days preceding clinical NEC were highly discriminative from controls, this does not automatically implicate that observed smellprints are disease-

specific. This is illustrated by the finding that VOC profiles of infants with NEC and sepsis were distinguishable at $T_{-3,-2}$, but not at $T_{-1,0}$. Possible explanation for this apparent discrepancy might be that both disorders have their unique microbial and metabolic shifts predating clinical diagnosis, reflected by the early observed differences in VOC profiles. Subsequent merging of VOC profiles might result from increased intestinal permeability by gut barrier failure in NEC cases, leading to bacterial translocation (with or without sepsis) and by production of non-specific biomarkers of inflammation.[26,27] Further analysis by GC-MS might shed light on this subject by identification of those molecular compounds that are present at different stages and different underlying causes of inflammation. In addition, infants of the sepsis group had significantly lower postnatal age at time of sepsis onset compared to onset of NEC, which hypothetically could have influenced our observations. To obtain detailed insight in the potential of fecal VOC analysis in (early) discrimination between sepsis and NEC, understanding of the course of VOC profiles in sepsis compared to controls is indispensable. We performed a post hoc analysis to compare VOC profiles of infants with sepsis and controls. Here, fecal VOC profiles of controls were used from similar time-intervals as in the NEC versus controls analysis (Table 11.1). No significant differences were observed between theses subgroups, at all defined time windows (range AUC 0.64-0.67; range p-value 0.08-0.13). However, our study design do not allow reliable comparison between these two subgroups, since infants with sepsis were not strictly matched with controls.

A strength of this study is the multi-centre, prospective design including infants with sepsis as a separate subgroup, allowing to compare (early) VOC profiles of sepsis and NEC, diseases which are typically difficult to distinguish in clinical practice, especially at an early or pre-clinical stage. Furthermore, the control group was strictly matched and consisted of infants belonging to the intention-to-diagnose group.

This study has several limitations. Since the number of NEC cases was limited, samples were clustered in two-day intervals, preventing detailed day-to-day description of VOC course. At interval $T_{-1,0}$ a total of nine samples of NEC subjects were available for VOC analysis, two of them collected at T_0, before diagnosis of NEC was established. VOC profiles of those two infants closely resembled profiles of seven samples at T_{-1}. Obviously, results of this study have to be externally validated in a study comprising larger number of subjects with NEC. Since different gut pathogens have been reported to different institutional outbreaks of NEC, next to the presence of seasonal variation, validation studies should preferably be performed in a multi-center setting, in different countries, and compromising all seasons. Furthermore, findings per race, ethnicity, and gender may not make the findings applicable

to all populations, especially to populations in developing countries. Future studies are needed to ascertain detailed insight in how metabolic, immunologic and inflammatory processes in NEC shape fecal VOC composition, and whether found differences between NEC and controls are NEC-specific or not. Since the eNose method is a non-invasive and cost-effective technique allowing for real-time and bedside analysis of fecal VOC profiles, it is an interesting candidate for future application in daily clinical practice, especially in countries with fewer resources.

Because fecal VOC-profiles could be identified already several days before clinical onset of NEC, this technique may offer opportunities for early intervention strategies.

In conclusion, fecal VOC profiles of infants with NEC could strongly be discriminated from controls, from two to three days predating the onset of clinical symptoms. Our observations imply that VOC-profiling has large potential as a non-invasive bedside tool for the early prediction of NEC.

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