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

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Sniffing Out Pediatric Gastrointestinal Diseases: The Potential of Volatile Organic Compounds as Biomarkers for Disease

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ABSTRACT

The diagnostic work-up and follow-up of pediatric functional gastrointestinal disorders and organic conditions usually includes invasive tests, carrying a high burden on patients. There is a place, therefore, for novel, noninvasive disease-specific biomarkers. Volatile organic compounds (VOCs), originating from (patho)physiological metabolic processes in the human body, are excreted as waste products through all conceivable bodily excrements. The spectrum of VOCs harbors a magnificent source of information, with the potential to serve as noninvasive diagnostic biomarkers and to monitor disease activity. VOC analysis has been studied in children and infants with a variety of gastrointestinal diseases, including inflammatory bowel disease, liver diseases, irritable bowel syndrome, necrotizing enterocolitis and infectious diarrhea. Most of these studies, although limited in sample size, show that patients can be discriminated from controls based on their VOC profiles, underscoring the potential of VOC analysis in diagnosis and follow-up. Currently, however, the application of VOC analysis in clinical practice is limited; substantial challenges, including methodological, biological, and analytical problems, still need to be met. In this review we provide an overview of the available literature on the potential of VOCs as biomarkers for pediatric gastrointestinal diseases. We discuss the available techniques to analyses VOCs and provide topics for VOC-related research, which need to be addressed before VOC diagnostics can be implemented in daily clinical practice.
INTRODUCTION

In Western medicine, the earliest description of a diagnostic test based on odor originates from Hippocrates, who noted in his Aphorisms that the sputum of patients with tuberculosis spreads a characteristic, foul smell when poured upon hot coals. Over the past decades, there has been a rapidly increasing interest in the potential of analysis of the responsible molecules (volatile organic compounds [VOC]) as diagnostic biomarkers of diseases, including malignancies, infections, and metabolic conditions. This can largely be attributed to the development of nanotechnology-based sensors enabling fast, point-of-care analysis of the VOCs present in complex gaseous mixtures. VOCs are carbon-based chemicals, typically in volatile state at ambient temperature, originating from physiological and pathophysiological metabolic processes in the human body. Their origin may be local, systemic, and exogenous. Exogenous VOCs originate from nonhuman sources and are absorbed by the body, for example, through skin, lung alveoli, or mucosa. Researchers have characterized the VOCs of a broad range of human samples, identifying more than 1840 different compounds.

As VOCs are excreted as waste products through all conceivable bodily excrements (e.g., breath, sweat, urine, and feces), they can be analyzed by noninvasive techniques. The VOC pool harbors a magnificent source of information with the potential to serve as noninvasive diagnostic biomarkers and as markers of disease activity. In addition, VOC analysis may increase understanding of the pathophysiological metabolic pathways underlying diseases, possibly leading to new therapeutic strategies.

Currently, diagnostic work-up and follow-up of pediatric diseases frequently requires a myriad of invasive, time-consuming, and expensive tests, carrying a high physical burden on the patients and economic consequences for the health system. The need for novel, noninvasive, disease-specific biomarkers particularly exists for the pediatric population; the research into pediatric applications of volatile biomarkers is consequently emerging. In this review we provide an overview of the literature regarding the potential of VOCs as biomarkers in pediatric gastrointestinal diseases. Furthermore, we will discuss the available techniques for the analysis of VOCs and the challenges to be overcome before widespread implementation of VOC-based diagnostics in daily clinical practice is feasible.

ANALYTICAL TECHNIQUES

The available VOC detection techniques can roughly be divided into 2 categories: chemical analytical techniques and electronic devices using pattern-based recognition algorithms. As a detailed evaluation of all available methods within these 2 categories would be beyond the scope of this review, we will focus on the devices applied in the reviewed studies.
Chemical analytical techniques, such as gas chromatography-mass spectrometry (GC-MS), can identify VOCs in a complex mixture based on their physiochemical properties. GC-MS is currently the most commonly used technique for VOC analysis. Limitations of this technique include the need for intensively trained personnel to perform the complex (statistical) analyses and labor intensiveness, because the samples need to undergo condensation and desorption before analysis. In contrast, selected ion flow tube-MS (SIFT-MS) allows for real-time analysis of selected compounds, does not need preanalysis preparations, is easier in use, and can provide results within seconds. SIFT-MS involves ionization of VOCs that are subsequently detected downstream by quadruple MS and an ion-counting system. Analytical devices such as SIFT-MS and GC-MS are relatively expensive, >$200,000 and >$150,000, respectively. GC-MS is, however, available in many general and academic hospital laboratories as the technique is widely used for both clinical and research purposes.

At the other end of the spectrum are electronic devices (“eNose”), with costs <$40,000, containing an array of different gas sensors and deploying pattern recognition algorithms for the discrimination of VOC combinations. Electronic nose technology enables real-time, high-throughput analysis of the complete spectrum of VOCs in complex gaseous mixtures. The VOCs in a given mixture interact with a matrix of eNose sensors, influencing a measurable attribute of each individual sensor, such as electrical resistance or oscillation frequency. Because eNose sensors do not identify individual chemical compounds, the specificity of this approach is generally lower compared with other VOC detection techniques. As they are inexpensive, small, and easy to use, however, they are suited as point-of-care tools. In addition, they can be learned to recognize different pathologic conditions.

So while chemical analytical techniques are expensive, but provide robust and reproducible results, eNose devices carry the promise to perform adequate for clinical purposes at an affordable price. Recent research efforts have focused on the development of eNose sensors with a coating that specifically interacts with target compounds by employing miniaturized classic chemical analytical techniques such as (field asymmetric) ion mobility spectrometry. These developments aim at combining optimal sensor accuracy with user friendliness at minimal cost.

**VOCS IN PAEDIATRIC GASTROINTESTINAL DISEASES**

In the following sections the available evidence will be discussed regarding the potential of VOC analysis in children and infants with inflammatory bowel disease (IBD), liver disease, irritable bowel syndrome (IBS), necrotizing enterocolitis (NEC), and infectious diarrhea. Table 1 provides an overview of available studies.
Inflammatory bowel disease

IBD is a chronic, relapsing inflammatory condition of the intestinal tract, the 2 main phenotypes being ulcerative colitis (UC) and Crohn disease (CD). Over the past years, worldwide incidence rates of pediatric IBD have shown an upward trend, whereas the age at presentation is decreasing\textsuperscript{23,24}. Although there are numerous studies on VOC analysis in adult IBD, so far only 2 studies have focused on the potential of VOCs in diagnosing and monitoring IBD in the pediatric population\textsuperscript{9,13}. Patel and colleagues\textsuperscript{13} aimed to identify a unique breath VOC pattern allowing to differentiate between pediatric patients with IBD and healthy controls by means of SIFT-MS. Exhaled breath samples of 62 pediatric patients with IBD (51 CD, 11 UC) were compared to those of 55 healthy controls. Six VOCs differed significantly between the 2 groups: 1-octene, 1-decene, (E)-2-nonene, 1-nonene, 3-methylhexane, and hydrogen sulphide. Assumedly gut microbiome dysbiosis, which has been found in several studies\textsuperscript{25,26} explains the VOC pattern in the diseased group. For example, it has been shown that hydrogen sulphide is produced by Escherichia coli and Enterococcus faecalis\textsuperscript{27}, species that have been observed in higher concentrations in the mucosa of patients with IBD\textsuperscript{28,29}. Yet, the origin of the majority of VOCs remains unclear. Furthermore, the study could not discriminate between UC and CD based on exhaled VOCs and no correlation was observed between VOC composition and disease activity\textsuperscript{13}. The latter might be due to the small sample size; only 20% of patients had active disease upon inclusion. Another limitation of the study was that none of the patients were treatment-naïve at inclusion, leaving the possibility open that the exhaled VOC profile was biased by the use of medication.

In a recent study using an eNose device for fecal VOC analysis, newly diagnosed, treatment-naïve pediatric patients with IBD (29 CD, 26 UC) could be discriminated from 28 matched healthy controls\textsuperscript{8}. Interestingly, patients with UC could also be discriminated from patients with CD with high accuracy. This was both true for active disease (area under the curve = 0.96) and for clinical remission after 6 weeks follow-up (area under the curve = 0.81). It is tempting to hypothesis that this method can be helpful in cases where diagnosis is unclear, such as IBD-unclassified, leading to improved, personalized care for this subgroup. An interesting observation in the present study was that after 6 weeks, fecal VOC profiles of children with CD had changed from active disease towards clinical remission state, whereas those from children with UC remained unchanged despite the decrease in disease activity and the initiation of medication\textsuperscript{9}. Interestingly, these observations correspond with the microbial changes in IBD subjects with active and inactive disease\textsuperscript{30}. Even in healthy children, however, VOC profiles underwent statistically significant changes over periods as short as 1 week, illustrating the difficulty of linking VOC changes in IBD to changes in disease activity. As nearly 50% of the entire stool VOC composition can be assigned to the diet, both directly by VOCs originating from diet components and indirectly as a result of bacterial metabolism\textsuperscript{2,28,31-34}, VOC changes in healthy children may be attributable to the day-to-day variation in dietary intake. This
emphasizes that information on the diet is pivotal when comparing fecal VOC profiles between cohorts, to minimize the risk of type I errors.

Both studies show that VOC profiling holds promise as a noninvasive tool in diagnosing and monitoring pediatric IBD, with fecal, but not exhaled VOCs, providing information differentiating UC from CD. Supposedly, fecal samples harbor more local and exogenous (derived from the resident microbiome) VOCs than exhaled breath, which predominantly harbors VOCs of systemic origin.

Although at present VOC analysis cannot replace the tools currently used in the diagnostic work-up of IBD, it might improve their performance. The commonly used noninvasive biomarker fecal calprotectin is characterized by high sensitivity for mucosal inflammation (97%), but a relatively low specificity (70%) for pediatric IBD as the cause of this inflammation. Fecal VOC profiling has a reported pooled sensitivity of 86% and specificity of 85% for IBD. Consequently, adding VOC analysis to fecal calprotectin in the diagnostic work-up of IBD might increase specificity, leading to better selection of the children suspected of IBD who need to undergo further diagnostic procedures such as endoscopy.

Liver disease

More than 20% of children and adolescents in developed countries are overweight or obese. Because of this epidemiology of childhood overweight and obesity, an increasing number of children are at early age confronted with liver problems. Nonalcoholic fatty liver disease (NAFLD), affecting approximately 10% of children with obesity and with nonalcoholic steatohepatitis (NASH) as the most aggressive phenotype, is the most frequently encountered liver complication in obesity. The current criterion standard for diagnosis is histological evaluation of a liver biopsy.

Three studies have aimed at linking specific VOCs in exhaled breath to the presence of pediatric liver diseases, including NAFLD, NASH, and chronic liver disease. All children (both cases and controls) participating in the NAFLD and NASH studies were either overweight or obese, whereas the majority of the children in the chronic liver disease study had normal weights.
Sniffing out pediatric gastrointestinal disease

The 3 studies together identified 10 different VOCs with significantly higher concentrations in the cases as compared to controls, all but 2 of them, pentane and 3-methylhexane, identified in only 1 study (Table 2). In addition, only the study in children with chronic liver disease reported significantly lower concentrations of some VOCs —1-nonene, (E)-2-nonene, and dimethyl sulphide—compared to healthy controls. This illustrates the limitation of the VOC studies performed so far, with a lack of methodological standardization and a high rate of type I errors thwarting the possibility to draw firm conclusions.

In summary, VOC analysis in pediatric liver disorders shows promising results, which however are not yet translatable into a practical approach. Standardization of methodology is necessary before the clinical potential of VOCs in pediatric liver diseases can be valued.

Irritable bowel syndrome

Functional abdominal pain disorders are the most frequently encountered gastrointestinal conditions in the pediatric population with a worldwide prevalence of 8.8%, IBS being the most prevalent phenotype. The exact pathogenesis of IBS remains to be elucidated, but it presumably involves a complex interplay between altered gut motility, visceral hyperalgesia, diet-induced microbial dysbiosis, altered immune responses, and psychosocial disturbance. Only a single study has been performed aiming at the identification of IBS-specific VOCs in a pediatric population. VOC analysis of exhaled breath by means of SIFT-MS showed significant higher concentrations of benzene, dimethyl sulphide, 1-octene, and 3-methylhexane in patients with IBS compared to healthy controls. Based on these specific VOCs, patients with IBS could be discriminated from controls with an accuracy of 96%. Limitation of the present study was the presence of a significantly higher mean body mass index percentile in the IBS group. Several studies have demonstrated higher concentrations of 1-octene and 3-methylhexane in overweight and obese children compared to lean controls. Because the discriminate analysis model was not externally validated, therefore, it is impossible to state whether the observed differences were really IBS specific or the consequence of the increased body mass index.

Although the exact pathogenesis of IBS remains unclear, there is no doubt that it involves both somatic as psychological factors. Although this complex interplay may thwart the detection of IBS-specific VOCs, it is tempting to speculate that the identification of an IBS-specific VOC profile might increase our insight into the pathophysiological processes underlying IBS.
<table>
<thead>
<tr>
<th>Disease</th>
<th>Study group</th>
<th>Sample</th>
<th>Method</th>
<th>Medium</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Study (ref.)</td>
<td>cases/controls</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IBD</td>
<td>de Meij et al(^9)</td>
<td>55 (26 UC, 29 CD)/28</td>
<td>eNose</td>
<td>Feces</td>
</tr>
<tr>
<td></td>
<td>Patel et al(^{13})</td>
<td>62 (11 UC, 51 CD)/55</td>
<td>SIFT-MS</td>
<td>Breath</td>
</tr>
<tr>
<td>NEC</td>
<td>Garner et al(^{12})</td>
<td>6/7</td>
<td>GC-MS</td>
<td>Feces</td>
</tr>
<tr>
<td></td>
<td>Mayor(^{46})</td>
<td>34/70</td>
<td>GC-MS</td>
<td>Feces</td>
</tr>
<tr>
<td></td>
<td>de Meij et al(^{10})</td>
<td>13/14</td>
<td>eNose</td>
<td>Feces</td>
</tr>
<tr>
<td>Liver disease</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- NAFLD</td>
<td>Alkhouri et al(^8)</td>
<td>37/23</td>
<td>SIFT-MS</td>
<td>Breath</td>
</tr>
<tr>
<td>- NASH</td>
<td>Okwu et al(^{19})</td>
<td>22/74</td>
<td>SIFT-MS</td>
<td>Breath</td>
</tr>
<tr>
<td>- CLD</td>
<td>Eng et al(^{11})</td>
<td>49/55</td>
<td>SIFT-MS</td>
<td>Breath</td>
</tr>
<tr>
<td>Acute diarrhea</td>
<td>Al-Kateb(^{20})</td>
<td>27/26</td>
<td>GC-MS</td>
<td>Feces</td>
</tr>
<tr>
<td></td>
<td>Poulton(^{21})</td>
<td>10/13</td>
<td>Human smell</td>
<td>Feces</td>
</tr>
<tr>
<td></td>
<td>Probert et al(^{22})</td>
<td>38/6</td>
<td>GC-MS</td>
<td>Feces</td>
</tr>
<tr>
<td>IBS</td>
<td>Patel et al(^{14})</td>
<td>22/55</td>
<td>SIFT-MS</td>
<td>Breath</td>
</tr>
</tbody>
</table>

AUC = area under the curve; CD = Crohn disease; CLD = chronic liver disease; eNose = electronic nose; GC-MS = gas chromatography-mass spectrometry; IBD = inflammatory bowel disease; IBS = irritable bowel syndrome; NAFLD = nonalcoholic fatty liver disease; NASH = nonalcoholic steatohepatitis; NEC = necrotizing enterocolitis; ROC = receiver operating characteristics; SIFT-MS = selected ion flow tube-mass spectrometry; UC = ulcerative colitis; VOCs = volatile organic compounds.
<table>
<thead>
<tr>
<th>Study groups</th>
<th>VOCs used in</th>
<th>P; AUC (±95% CI or range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>UC vs control: - active</td>
<td>Not applicable</td>
<td>P &lt; 0.001; AUC 1.00 (±0.00)</td>
</tr>
<tr>
<td>- Remission</td>
<td></td>
<td>P &lt; 0.001; AUC 0.94 (±0.05)</td>
</tr>
<tr>
<td>CD vs control: - active</td>
<td>Not applicable</td>
<td>P &lt; 0.001; AUC 0.85 (±0.05)</td>
</tr>
<tr>
<td>- Remission</td>
<td></td>
<td>P &lt; 0.001; AUC 0.94 (±0.06)</td>
</tr>
<tr>
<td>UC vs CD: - active</td>
<td>Not applicable</td>
<td>P &lt; 0.001; AUC 0.96 (±0.03)</td>
</tr>
<tr>
<td>- Remission</td>
<td></td>
<td>P = 0.002; AUC 0.81 (±0.08)</td>
</tr>
<tr>
<td>IBD vs control</td>
<td>Three different VOCs</td>
<td>P &lt; 0.001; AUC 0.96 (0.93–0.99)</td>
</tr>
<tr>
<td>CU vs CD</td>
<td></td>
<td>P &gt; 0.05</td>
</tr>
</tbody>
</table>

Number of VOCs increased with age for non-NEC infants, not for NEC infants

Total absence of 4 particular VOCs up to 4 days before NEC

<table>
<thead>
<tr>
<th>NEC vs control</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>- Overall; training set</td>
<td>Six different VOCs</td>
<td>AUC 0.83</td>
</tr>
<tr>
<td>- t(-1) to t(-6); training set</td>
<td></td>
<td>AUC 0.78–0.9</td>
</tr>
<tr>
<td>NEC vs control</td>
<td>Not applicable</td>
<td></td>
</tr>
<tr>
<td>- t(-1), t(0)</td>
<td></td>
<td>P &lt; 0.001; AUC 0.99 (±0.04)</td>
</tr>
<tr>
<td>- t(-3), t(-2)</td>
<td></td>
<td>P = 0.02; AUC 0.77 (±0.21)</td>
</tr>
</tbody>
</table>

| -NAFLD vs control; validation set | Four different VOCs | AUC 0.763 |
| -NASH vs control; training set | Two different VOCs | AUC 0.73 |
| -CLD vs control; training set | Five different VOCs | AUC 0.97 |

Rotavirus infected vs noninfected | Not applicable | “significant” |
Rotavirus infected vs noninfected | Not applicable | P < 0.009 |
Rotavirus infected vs noninfected | Not applicable | P < 0.0001 |
C. difficile infection vs noninfected | | P < 0.0001 |
IBS vs control; training set | Four different VOCs | AUC 0.99 |
Table 2. Overview of significantly increased exhaled volatile organic compound concentrations in pediatric liver disease linked to the corresponding pathophysiological mechanism

<table>
<thead>
<tr>
<th>Increased VOC concentrations</th>
<th>Condition</th>
<th>P vs controls</th>
<th>Pathophysiological mechanism</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unsaturated hydrocarbons</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1-Decene</td>
<td>CLD†</td>
<td>&lt;0.001</td>
<td>Oxidative stress involved in the pathogenesis of liver disease (11)</td>
</tr>
<tr>
<td>1-Octene</td>
<td>CLD†</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>1-Heptene</td>
<td>CLD†</td>
<td>0.035</td>
<td></td>
</tr>
<tr>
<td>3-Methylhexane</td>
<td>CLD†</td>
<td>0.004</td>
<td></td>
</tr>
<tr>
<td>Pentane</td>
<td>NAFLD</td>
<td>0.002</td>
<td>Lipid peroxidation mediated by oxidative stress (8,11,38)</td>
</tr>
<tr>
<td></td>
<td>NASH</td>
<td>0.023</td>
<td></td>
</tr>
<tr>
<td>Saturated hydrocarbons</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Isoprene</td>
<td>NAFLD</td>
<td>0.022</td>
<td>Byproduct of cholesterol biosynthesis (38)</td>
</tr>
<tr>
<td>Oxygen containing compounds</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acetone</td>
<td>NAFLD</td>
<td>0.008</td>
<td>Produced by hepatocytes during lipolysis or lipid peroxidation (8,39)</td>
</tr>
<tr>
<td>Acetaldehyde</td>
<td>NAFLD</td>
<td>0.034</td>
<td>Intermediate breakdown product of ethanol metabolism (8)</td>
</tr>
<tr>
<td>Nitrogen-containing compounds</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trimethylamine</td>
<td>NAFLD</td>
<td>0.003</td>
<td>Produced by intestinal microbiota and subsequently metabolized in liver (8)</td>
</tr>
<tr>
<td>Alcohols</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ethanol</td>
<td>NASH</td>
<td>0.021</td>
<td>Metabolized in liver (39)</td>
</tr>
</tbody>
</table>

CLD = chronic liver disease; NAFLD = nonalcoholic fatty liver disease; NASH = nonalcoholic steatohepatitis; VOC = volatile organic compounds.
†Includes children with autoimmune hepatitis, primary sclerosing cholangitis, biliary atresia, NAFLD, viral hepatitis, alpha-1 antitrypsin deficiency, congenital hepatic fibrosis, progressive familial intrahepatic cholestasis, Alagille syndrome, vascular disorders, idiopathic hepatitis, parenteral nutrition-related cholestasis, and idiopathic cholestatic liver disease.

Necrotizing enterocolitis

NEC is the most common severe gastrointestinal disease in premature infants with very low birthweights. Despite a decline in overall mortality among extremely premature infants, NEC-related mortality in this specific population has risen. At present, 3 studies have looked into the potential of fecal VOCs as (early) biomarkers of NEC.

Garner and colleagues analyzed fecal VOCs in 6 infants with NEC and 7 matched controls by means of GC-MS. Although in the control group the abundance of VOCs increased significantly with age, this was not the case in the NEC group. Moreover, 4 particular esters were consistently absent in all fecal samples up to 4 days before the clinical onset of NEC: 2-ethylhexyl acetic ester and decanoic, dodecanoic, and hexadecanoic acid ethyl ester. All of these esters were found in at least one of the fecal samples of the non-NEC infants; however,
none of the esters was present in every sample of each control. The authors suggest that this finding is a reflection of changing physiology or changing gut microbiome. They speculate that the absent esters might have been oxidized or digested by esterases or are lacking due to downregulation of their synthesis.

In a recently published abstract, the same group confirmed their previous findings in a much larger cohort. Using the same technique, they analyzed fecal samples from up to 6 days before the clinical diagnosis of NEC from 34 premature infants with NEC and 70 matched healthy premature controls. In total, 6 VOCs differed significantly between NEC and control samples, 3 being positively and 3 negatively associated with NEC. Using these 6 particular VOCs, NEC cases could be discriminated from controls with an area under the receiver operating characteristic curve of 0.83. Between 1 and 6 days before the clinical onset of NEC, the area under the receiver operating characteristic curve ranged from 0.78 to 0.9. Information on sensitivity and specificity is lacking.

Recently, the eNose was used to follow the course of VOC profiles of 13 infants developing NEC compared with that of 14 matched preterm controls. The present study also showed that NEC is preceded by changes in VOC production. From 3 days before the clinical onset of NEC onward, VOC profiles differed significantly between the 2 study groups, with increasing accuracy towards the onset of NEC.

In conclusion, VOC analysis appears to be a promising method for the detection of NEC in a preclinical stage. Timely recognition of this stage could steer treatment and prevent the development of (more serious stages of) NEC. Future, prospective multicenter studies including large numbers of subjects are needed to determine which molecular compounds emerge preceding and during NEC, to assess the influence of different variables (e.g., gestational age, feeding pattern, and antibiotic therapy) on VOC composition, and to discriminate NEC from other causes of inflammation, before VOC-based decisions can be made in daily practice. Obviously, assessment of optimal therapeutic interventions upon early detection of NEC, to decrease the related high morbidity and mortality rates, will become the next challenge to be met.

**Infectious diarrhea**

Diarrheal diseases remain a leading cause of death in infants worldwide. In 2010, over 800,000 (10.5% of total) deaths among children younger than 5 years were attributable to diarrhea, rotavirus being the leading infectious cause. Experienced nurses working at pediatric wards often report that stool and flatus of infants with rotavirus gastroenteritis produce a characteristic smell. A study dating from 1987 demonstrated that based on their olfactory skills, nurses were able to differentiate between rotavirus and nonrotavirus diarrhea with an
accuracy of 69%\(^1\). Presently, only 2 studies have been published that tried to objectify this finding.

Al-Kateb and colleagues\(^2\) used GC-MS to compare fecal VOCs from 27 Malawian children with rotavirus gastroenteritis with those from 26 controls with unspecified gastrointestinal problems. They found VOCs, in particular aldehydes and 2,3-butanedione, to be more abundant in the rotavirus group. Probert et al\(^2\) published a study comparing fecal samples from 38 patients, adults, and children with infectious diarrhea, with fecal samples from 6 healthy adults by means of GC-MS. Depending on the VOC pattern they could differentiate rotavirus, Campylobacter jejuni and Clostridium difficile diarrhea (5–6 patients each, adults and children) from each other with positive and negative predictive values of between 40% and 100% and between 77% and 100%, respectively. C. difficile diarrhea was associated with increased levels of 5-methyl-2-furancarboxaldehyde and/or 2-furancarboxaldehyde and total absence of 3-methylindole; rotavirus diarrhea with the absence of ethyl decanoate combined with the presence of ammonia; and C. jejuni diarrhea with the absence of terpenes and hydrocarbons\(^2\).

Given the small groups in both studies, it is impossible to draw firm conclusions. It should be noted that the study by Probert et al was not confined to children, which makes comparison with the Malawi study difficult.

FUTURE PERSPECTIVES AND CONCLUSION

In this review we have focused on the use of VOCs as biomarkers in pediatric gastrointestinal conditions. Although most studies are limited in sample size, present evidence suggests that infants and children with both functional and organic conditions can be discriminated from controls based on their VOC profiles, enforcing the potential of VOC analysis in diagnosis and follow-up. Although this field is gaining momentum, research is in early stages and several barriers are still on the road towards successful application of this novel approach in daily clinical practice. One caveat with every study using VOC analysis, however, is that the number of measured variables by far exceeds the number of analyzed samples. As a result, one can expect an excess in false-positive associations\(^4\). This is underscored by the fact that there is little correlation between the VOC profiles reported for the different conditions. At present, therefore, the clinical implications of the findings remain largely uncertain. In addition, most studies did not control for environmental factors, which makes it difficult to determine if the observed differences were caused by the condition of interest or also, or even largely, by the presence of confounding variables (e.g., diet, age, exogenous VOC exposure). Unfortunately, none of the results so far have been validated in external cohorts.

Another challenging aspect to confront is standardization of sampling methods and assessment. Unpublished results from our laboratory suggest that differences in collecting,
storing, and pre-paring the samples may have major consequences for the test results. Differences in methodology between studies, therefore, may yield incomparable outcomes\textsuperscript{50}. For the further development of VOC analysis it is recommendable to work along guidelines with proven efficacy, such as STARD and TRIPOD\textsuperscript{51,52}. Ideally, future studies should take effort in standardizing and protocolling sampling methods and analyzation techniques. Thus the conditions can be set up to enable multicenter trials and pooling of data, which is necessary for the confirmation of the causal relation between certain conditions and the associated VOC changes.

To implement VOC analysis as a diagnostic tool, therefore, several analytical and clinical aspects need to be attacked. First of all, selection of the optimal analytical technique is needed. Obviously, the choice of technique also depends on the purpose of VOC assessment. Chemical analysis provides detailed (molecular) insight into the VOC composition in a given situation, but at high costs in terms of purchase and maintenance. Pattern recognition (eNose) devices are relatively cheap and highly user friendly, but at present there is uncertainty regarding the robustness and reproducibility of the test results. We strongly believe, therefore, that technical validation is an essential step in the development of any VOC-based application. So chemical analytical and pattern recognition techniques have their own places as complementary approaches. Understanding the compounds relevant to a specific condition can drive the selection and development of optimal sensors for that condition. Alternatively, in research situations, when there is no need for a point-of-care application, chemical analytical techniques may be preferable.

Another issue is the choice of the optimal substrate. Because VOC profiles originate from a combination of exogenous, local, and systemic sources, the choice of substrate will influence VOC outcome\textsuperscript{3}. This choice will also be influenced by the organ or condition under consideration. For instance, feces may be superior to breath in case of gastrointestinal conditions, as is suggested by the IBD studies discussed earlier, in which fecal VOCs, and not breath VOCs, enabled discrimination between CD and UC. For liver disease, in contrast, it could be argued that breath analysis is preferable, although at present there are no comparative studies.

Lastly, the clinical applicability of VOCs as a diagnostic tool needs to be validated. Most studies so far have compared VOC profiles of patient groups with those of healthy controls. In clinical practice, however, tests are performed to confirm or exclude a certain condition in a given patient. This requires biomarkers with optimal accuracy. Any VOC-based test used in clinical practice needs to be tailored to that extent and evaluated under nonstandardized conditions.

In conclusion, a considerable volume of proof-of-principle studies have shown the potential of volatile biomarkers in diagnosing, monitoring, and predicting outcomes in
pediatric gastrointestinal conditions. The study groups are, however, generally small and there is lack of standardized methodology, whereas very few study results are reproduced. We are confident that, when these issues are adequately addressed, there is a high potential in clinical practice for this novel approach. VOC analysis harbors promises as a future clinical screening and monitoring tool in pediatric gastrointestinal condition. Strong points are its noninvasive character and the possibility to deliver fast results. Next to standardization of methodology, priority should be given to the identification of key volatiles, enabling the development of disease-specific eNose sensors.
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