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Volatile organic compounds analysis in the context of gastrointestinal health and disease

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Summary and general discussion

This thesis is focused on the untargeted analysis of volatile organic compounds (VOCs) in the context of gastrointestinal health and disease. Breath analysis, with its non-invasive nature, is an attractive prospect for diseases diagnosis and monitoring. It is widely assumed that VOCs in breath provide a reflection of a metabolic state of the organism but understanding and capturing that reflection with pinpointing its source is still an incredibly challenging task. In addition, results published in the literature are based on small patient numbers that impacts statistical validity of the results and where it is virtually impossible to study confounding factors [1]. **Chapter 2** of this thesis is looking at the general influence of endogenous and exogenous factors on exhaled breath composition to gain better understanding which potential confounding factors must be accounted for when studying exhaled VOCs. Thanks to support of Top Institute Food and Nutrition, we had an opportunity to study confounding factors in breath samples from the 1417 well-characterised participants from the LifeLine (LL) population cohort in the Netherlands: *i.e.,* age, BMI, smoking, blood cell count, several metabolic parameters, and a group of 16 recorded medications. The results showed that smoking impacted the VOCs profile most significantly.

Age, gender and BMI influence an individual's metabolism. Although they impact breath composition to lesser extent than smoking, they should also be considered in study designs. In our study, no evidence was found that cholesterol or triglyceride levels influenced overall VOCs profile. The immune response is expected to influence VOCs, but surprisingly no significant differences were detected related to the number of various types of white blood cells. This latter may be explained by the lack of statistical power, as in this general population cohort the number of people with increased neutrophil counts was small (n=11). Medications can affect both host and/or microbial metabolism and as such were expected to influence VOCs profiles [2]. Within the available cohort set up we were only able to compare VOCs profiles of women taking chemical contraceptives vs. those not taking them. However, no significant impact on VOCs was noted. For all other medications, because we were

not able to compare individuals with a certain disease taking medication versus individuals with the same disease not taking the medications, it was impossible to distinguish disease effect from treatment effect on VOCs. It is important to note that medication may affect the breath profile and this information should therefore be considered in breath analysis studies [1]. The effect of external factors, *i.e.,* age, gender, smoking, presence of anxiety and depression was also included in **Chapter 5**, where the use of VOC as a potential diagnostic biomarker for IBS was investigated. No statistically significant correlations were observed, indicating they were not confounding the separating ability of the VOCs profile for diagnosis of IBS.

Chapter 3 and **Chapter 4** focus on exploration if and how exhaled breath was affected by dietary changes in healthy individuals. First, a set of 12 volatile compounds distinguished samples obtained during a gluten-free diet from those obtained during a normal diet (**Chapter 3**). Our findings indicate that a gluten-free diet had a reversible impact on participants' excreted metabolites visible in breath. Nine weeks after ending the gluten-free diet, the VOC profile returned to the one measured prior to intervention. In the research of Palma *et al.,* gluten-free diet appeared to be associated with reduction of polysaccharide intake and since undigested carbohydrates are considered as a main source of energy for commensal microbiota, it might explain the drift of the gut microbial composition [3]. This in turn is expected to be associated with a change of volatile metabolites produced by bacterial communities. Those metabolites when absorbed into the bloodstream, can be further metabolized, and finally be excreted in urine or breath [4, 5]. An interesting observation was that even though the change in VOCs caused by diet followed the same pattern, the dynamic of this change differed between individuals. Heinzmann *et.al.,* in research on stability and robustness of human metabolic phenotypes in response to food challenges, highlights the importance of understanding impact of individual differences in metabolic baseline and in response to dietary interventions. In their study, some individuals displayed greater stability of metabolic profile than others [6]. Therefore, it is possible that differences in dynamic of changes visible in our study could relate to diverse individual metabolic phenotypes. Secondly, in **Chapter 4**, we investigated whether breath analysis can be indicative for altered

metabolism of two studied infant formulas, where the only difference was a droplet size. In a double-blind, cross-over design study, 29 healthy, non-smoking adult males, consumed two different milk formulas and delivered exhaled breath samples at various time points. Results showed significant differences in exhaled breath composition between the two formulas 240 minutes after ingestion, which corresponds to the moment of food entering the small intestine. We speculated that this (in part) may be related to differences in the digestion, absorption, or metabolism of nutrients and/or differences in GI motility [7, 8]. Here also a role of microbial activity could be considered, but this was not investigated in that study. No significant changes were observed at earlier time points. Although the exact origin of the discriminating compounds was not confirmed and pathophysiological consequences are not clear, the findings do show the potential of VOC analyses in fields of nutrition and metabolism.

Furthermore, monitoring the effect of the diet by exhaled breath composition has potential for example to aid checking dietary compliance or to discover different digestion or absorption patterns. Nowadays, there are several simple commercial breath tests available that aid diagnosis of metabolic disturbances. These include for example measurements of carbohydrate malabsorption or detection of small intestine bacterial overgrowth (SIBO). Enzyme activities and organ functions can also be assessed by using stable isotope-labelled probes, such as ^{13}C-labelled urea for the diagnosis of the gastric bacterium *Helicobacter pylori.* An approach based on the use of exogenous VOCs probes (EVOC) as a potential strategy to measure activity of metabolic enzymes *in vivo* and by that aid development of breath based diagnostic and prognostic tests, was recently proposed by Owlstone Medical. Exogenous volatiles used as a probe allow direct monitoring of the substrate (probe itself) and products of its metabolism. The recent study by Ferrandino *et al.* has successfully demonstrated the use of exhaled breath limonene as a biomarker for liver cirrhosis [9]. Identifying specific conditions and diseases that can be targeted via this strategy opens a door for more targeted breath applications.

It was suggested that any exogenous VOC that is metabolised by the human body can offer opportunity to assess metabolic enzyme or organ function [10]. Identifying

specific conditions and diseases that can be targeted via this strategy opens a door for more targeted breath application.

The diagnostic and monitoring potential of VOCs analysis has been described in **Chapter 5** and **6**. First, we determined a discriminating VOCs profile between clinically confirmed IBS patients, obtained from the clinical Maastricht IBS (MIBS) Cohort, and GI healthy controls. Next, we determined how this specific VOC pattern correlated with GI symptoms in the MIBS and in LL DEEP general population cohort (**Chapter 5**). A set of 16 VOCs correctly predicted 89.4% of the IBS patients and 73.3% of the healthy controls (AUC=0.83). To our knowledge, this was the first time that a set of VOCs in exhaled air was able to predict the presence of a prevalent functional GI disorder, which could be considered an important first step forward in the design and development of reliable non-invasive biomarkers for IBS. Random Forest analysis was validated twice to ensure the reliability of the classification and correct selection of the set of discriminatory VOCs. One of the big advantages of the study was the relatively high number of well-characterised people included. As the diagnosis of IBS is based on symptoms (as defined by the Rome III criteria), our second aim was to test whether our VOC biomarker set would also correlate with the severity of GI symptoms. Here results showed that the VOC-biomarker set correlated moderately with a set of GI symptoms in the MIBS (r=0.55, p=0.0003). Since functional GI symptoms observed in IBS patients are also rather common in the general population, we then confirmed that the VOC biomarker showed a moderate but significant correlation with a set of GI symptoms in the general population (r=0.54, p=0.0004) [11]. Correlation with symptoms shows promising potential of VOCs analysis in evaluation of treatments effect in IBS. As lifestyle factors, co-morbid diseases and mediation used are associated with IBS, potential confounding factors should be included in the analysis as also found in **Chapter 2**. Kruskal-Wallis test showed however no influence from possible confounding factors in distinguishing IBS patients from healthy controls. We have speculated that both increase in inflammation and oxidative stress as well as microbiota changes could be highly related to the discriminatory power of the set. Further in-depth research is however still needed to investigate the origin of the discriminatory VOCs, to confirm

their identity, and to investigate the potential link to the underlying causes of IBS. Furthermore, to move from the reported potential closer to clinical applications, the results should be validated in an external cohort.

In **Chapter 6**, the diagnostic value of breath VOCs to monitor mucosal inflammation in inflammatory bowel disease (IBD) was investigated. In 2005, Lechner *et al.,*. [12] already demonstrated the feasibility of exhaled air as a novel diagnostic tool in the differential diagnosis of GI diseases but used a limited number of IBD patients (n=10) and did not identify the significant compounds. At that time, to our knowledge, it was the first large prospective study evaluating the role of VOCs profiles in exhaled air in relation to disease activity of Crohn's Disease (CD) patients [13]. 140 samples originated from an active disease stage, based on fecal calprotectin (FC >250 µg/g) and 135 samples from inactive disease stage (with a clinical HBI score <4 and serum CRP<5mg/l and FC<100µg/g). A third group consisted of samples from 110 healthy controls. A set of 10 discriminatory VOCs correctly predicted active CD in 81.5% and remission in 86.4% (sensitivity 0.81, specificity 0.80, AUC 0.80). Among tentatively identified discriminatory compounds, enhanced levels of alkanes, methylated alkanes, aldehydes were found, which may be connected to increased inflammation. Several studies show that oxygen mediated injury through increased free radical production and impaired antioxidant defense systems plays an important role in the pathophysiology of IBD [14, 15]. Hydrocarbons as the end products of lipid peroxidation show low solubility in blood and are quickly excreted into breath after formation and can be used to monitor the degree of oxidative damage [16]. Hydrocarbons and aldehydes were previously reported to be produced also by intestinal microbiota [17]. In recent work by Smolinska *et al.,* a strong correlation between volatiles in breath and the fecal microbiome was shown in CD patients [18]. It was speculated that this may be due to anabolism/catabolism of volatile metabolites by microbes and/or stimulation/inhibition of microbial growth by metabolites. Volatile metabolites such as short chain fatty acids (SCFAs; butyrate, propionate, acetate) and alcohols (ethanol and propanol) are products of bacterial fermentation mainly from non-digestible oligo- and polysaccharide [19, 20]. SFCAs have been shown to have anti-inflammatory and anti-carcinogenic effects [21].

Additionally, acetone and isoprene came up as discriminatory compounds when comparing healthy controls versus active CD or CD in remission (**Chapter 6)**. Both isoprene and acetone were present in high abundances in every breath sample, but they were both found to be part of discriminating sets of compounds in multitude of conditions [22-29]. As they are the result of 'common' biochemical processes in the body, it remains the question whether they ever will be specific enough to distinguish one condition from another. Furthermore, these compounds change considerably unrelated to the diseased conditions and are quite variably exhaled: their breath levels were reported to vary due to the inhalation from ambient air, diet, fasting, resting and exercise and even circadian rhythms [23, 30-34]. These uncertainties, among others are reasons why mentioned VOCs despite reported discriminatory powers have not yet found the way to clinical applications.

Inflammation, oxidative stress, metabolic changes caused by normal and pathological processes as well as microbiome perturbations, are all factors simultaneously reported to be involved in a multitude of conditions. What one can observe in breath profiles may collectively be impacted by these factors. However, it is not yet fully understood how specific VOCs or combinations thereof can be related to shared underlying pathophysiological mechanisms rather than the specific disease condition per se. Furthermore, more validation is needed, not only to study the prospective biomarker sets in new external cohorts, but also to study them against other diseases.

While the statistical significance or discriminatory power of exhaled breath is crucial for development of a test, it is also crucial to understand the biological origin of VOCs to study specificity of its production and to aid interpretation of results. In **Chapter 7**, we showed the validation of a system that would allow measuring VOCs in headspace of an *in vitro* cultured cell line. Considering interest in GI disorders, we have chosen the Caco-2 human, epithelial cell line that has been wildly used as a model for among others gut barrier function. The *in vitro* system allows for the application of different cell lines, as well as different experimental setups including varying exposure times and treatment options while preserving cell viability. High reproducibility of the collection system was confirmed by checking correlations

($p \leq 0.0001$) between replicate samples. When studying the influence of oxidative stress on the VOCs composition, a total of 10 VOC's showed either increased or decreased abundance in the headspace of the cell cultures due to the presence of the H_2O_2 stressor. An advantage of the developed system is that the released compounds accumulate and can be detected without affecting cell viability. Further, our study design ensured relatively high number of replicate experiments ($n \geq 20$) and inclusion of appropriate controls. Studying the relation between certain biochemical pathways and the excretion of VOCs *in vitro*, has the benefit of an enhanced level of control, which is not as simple in *in vivo* studies. *In vitro,* we can for example isolate much easier the matrix effect by looking at the volatilome produced by cells with medium, by medium itself, by medium exposed to a trigger (***e.g.,*** H_2O_2) and by medium exposed with cells. Thereby, we can exactly pinpoint where discriminatory compounds are coming from and avoid drawing wrongful conclusions. In **Chapter 7**, we investigated cells exposure to hydrogen peroxide but in similar manner we could study genetically altered metabolic pathways, cells co-cultures (***e.g.,*** with macrophages), exposures to other agents – either oxidative stress inducing and/or suppressing anti-inflammatory properties, or VOCs produces/consumed by pathogens. We presented a potential and a concept but to fully benefit from *in vitro* experiments it is important to implement standards to confirm the identified compounds and to further study potential biological origin of the changes observed.