

WJG 20th Anniversary Special Issues (12): Nonalcoholic fatty liver disease**Breath volatile organic compounds for the gut-fatty liver axis: Promise, peril, and path forward**

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Abstract

The worldwide interest in the gut microbiome and its impact on the upstream liver highlight a critical upside to breath research: it can uniquely measure otherwise unmeasurable biology. Bacteria make gases [volatile organic compounds (VOCs)] that are directly relevant to pathophysiology of the fatty liver and associated conditions, including obesity. Measurement of these VOCs and their metabolites in the exhaled breath, therefore, present an opportunity to safely and easily evaluate, on both a personal and a population level, some of our most pressing public health threats. This is an opportunity that must be pursued. To date, however, breath analysis remains a slowly evolving field which only occasionally impacts clinical research or patient care. One major obstacle to progress is that breath analysis is inherently and emphatically multi-disciplinary: it connects engineering, chemistry, breath mechanics, biology and medicine. Unbalanced or incomplete teams may produce inconsistent and often unsatisfactory results. A second impediment is the lack of a well-known stepwise structure for the development of non-invasive diagnostics. As a result, the breath research landscape is replete with orphaned single-center pilot studies. Often, important hypotheses and key observations have not been pursued to maturation. This paper reviews the rationale and requirements for breath VOC research applied to the gut-fatty liver axis and offers some sug-

gestions for future development.

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Key words: Breath; Volatile organic compounds; Fatty liver; Gut flora; Breath analysis

Core tip: The biology of the gut-liver axis has always been fascinating and exceedingly difficult to study. With the rapidly expanding interest in the gut microbiome, however, finding better measurement techniques to evaluate this biology has never been more relevant. Breath volatile organic compounds (VOCs) measurement presents the unmatched potential to address this critical unmet need. Breath measurement can be challenging, however, and requires coherent teams including engineers, breath chemists, and clinical researchers. It also requires long term vision and strategy. This paper describes the rationale for breath VOCs, critically reviews the history of breath VOC development, and offers suggestions for progress.

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INTRODUCTION

The gut flora microbiome and the gut-liver axis are exceptionally difficult to evaluate. However, the now universal appreciation of the microbiome's impact on upstream fatty liver and associated disorders such as obesity compels an even greater interest in improved measurement techniques.

Breath researchers have measured gut flora activity in exhaled breath for decades. However, there has been little

Table 1 "Fatty liver" volatile organic compounds candidates

Property	Examples
Fermentation activity ^[29-31]	Alcohols and their aldehydes
Metabolism ^[32,33]	Acetone and isoprene
Inflammation ^[34-36]	Dimethylamine, trimethylamine, hydrogen sulfide, ethane, methylsulfide, methylmercaptan

sustained success. This paper critically reviews the experience to date and offers suggestions for future progress.

Breath analysis still holds the unique and possibly unmatched potential to better measure this challenging and highly significant physiology.

PROMISE: UNPRECEDENTED OPPORTUNITY FOR BREATH VOLATILE ORGANIC COMPOUNDS

The role of gut flora in fatty liver pathogenesis has been studied for decades. Alcohol fatty liver research, for example, demonstrated that gut flora were necessary but not sufficient for liver disease, and explored the use of gut flora therapy (poorly absorbed antibiotics) using animal models^[1,2]. Various lines of evidence also pointed to a key role in the pathogenesis of non-alcoholic fatty liver disease (NAFLD)^[3-5]. Gut flora, *via* various mechanisms such as altered small bowel motility and impaired mucosal barrier function, have also been long appreciated to affect the clinical course of cirrhosis, regardless of liver disease etiology^[6].

Research connecting the gut flora to the liver has been particularly challenging and fascinating because gut bacterial biology and liver disease are distinct disciplines connected anatomically *via* a nearly inaccessible portal venous system. And although there is a history of gut flora therapies (*i.e.*, prebiotics, probiotics, dietary interventions) for liver disease^[7,8], progress has been slow because the science, especially the details of the gut flora, is underdeveloped. Nevertheless, the potential impact was evident: non-alcohol fatty liver, alcohol fatty liver, and cirrhosis affect many people.

However, with the now-familiar association of gut flora dysbiosis to obesity^[9,10] and insulin resistance^[11-13] interest in gut flora biology and, along with it, the gut-liver axis, has grown and today would be difficult to overstate. The gut flora is now regarded as a newly discovered metabolic organ. Many essential questions persist and have triggered a worldwide effort to better understand this new organ^[14-16]. Multiple comprehensive reviews have addressed the impact of gut flora on fatty liver and/or obesity^[17-20].

The major studies which have propelled these advances have generally used detailed fecal analysis. These analyses can include a variety of techniques including DNA sequencing, culture, and metabolic profiling^[21]. The emerging data indicate several possible mechanisms of

gut flora influence: fermentation, effects on metabolism, inflammatory signaling, or a combination. Notably, it is understood the gut microbiome is personal; one's gut flora, as well as their metabolic response to diet, and upstream liver effects cannot be predicted *a priori*^[22]. Thus, since exogenous ethanol is metabolized to acetaldehyde at a variable and unpredictable rate^[23], the same should follow for endogenous ethanol produced from gut flora. Furthermore, it is acknowledged that there remain many additional unknowns that exist about the gut-liver axis (*i.e.*, motility, mucosal barrier, immune system interactions, molecular mechanisms within the hepatocyte). However, despite both these known differences and true unknowns, there is a rapidly growing interest in the gut flora therapies and dietary interventions premised on these mechanisms^[24].

Therefore, notwithstanding the usefulness of fecal analysis to date, is not clear that it will prove as successful for wide scale clinical research^[25]. Fecal analysis, by virtually any method, has a number of drawbacks: samples are collected infrequently and episodically, are expensive to run, and result in large amount data that nevertheless remains challenging to interpret in the setting of multiple, interrelated physiologic variables: *i.e.*, gut flora modulate mucosal integrity and immune function with differential impact on the liver, and vice-versa^[26,27]. Fecal analysis cannot readily account for a number of factors in the gastrointestinal tract, including transit time, presence or absence of mucosal disease, and the possible differential impact bacterial subpopulations (*e.g.*, distal small bowel *vs* colonic, and so on).

Breath volatile organic compound (VOC) measurement, therefore, may serve to complement fecal analysis^[28]. Individual VOCs can be measured for specific hypothesis driven goals tailored to match the present understanding of the role of gut flora in the gut-liver axis.

Since the pathogenesis of fatty liver (Table 1) is multifactorial and there are many variables which impact the gut-liver axis, the most successful research will likely simultaneously measure multiple VOCs.

It is presumed that some of these metabolites (*e.g.*, ethanol) are produced only by gut flora, whereas others (*e.g.*, acetaldehyde) are produced by both gut flora and human metabolism. Notably, some of these VOCs may potentiate others. For example, ethanol and acetaldehyde can increase the growth of gram negative bacteria and intestinal permeability, respectively, and thereby may promote uptake of inflammatory mediators^[37]. Hydrogen sulfide may reduce gastrointestinal motility and thereby lead to bacterial stasis and overgrowth^[38]. Other VOCs have multiple affects that overlap multiple categories. For example, some gut flora metabolize choline efficiently and their over-abundance can lead both to choline deficiency and an overproduction of the toxic metabolites dimethylamine and trimethylamine^[39]. Both mechanisms have been implicated in the pathogenesis of fatty liver and non-alcoholic steatohepatitis^[40,41]. Each of these VOCs have been measured in exhaled breath, though

Table 2 Exhaled breath uniquely captures the entire output of the gut liver axis in the context of a person

Gut flora	Lumen factors	Hepatic factors	Host
Bacterial diversity and function	Barrier integrity	Enzyme heterogeneity (<i>e.g.</i> , alcohol dehydrogenase)	Diet
Mucosal or lumen associated	Immune defense	Liver disease	Medications
Location (<i>e.g.</i> , small bowel, right colon)	Mucosal disease (<i>e.g.</i> , celiac, crohns) Transit time	Cirrhosis and porto-systemic shunting	Co-morbid conditions (<i>e.g.</i> , diabetes) Age, gender, body mass index

usually separately. However, much like the standard twelve lead electrocardiogram or lipid panels, it is likely that the most meaningful VOC breath data would come from the simultaneous measurement and interpretation of multiple VOCs and/or profiles.

In contrast to fecal analysis, exhaled breath VOC analysis can measure the global activity of the entire gut-liver axis. Because breath measurement is non-invasive, safe, and potentially inexpensive, it easily enables studies with repeated measures. For example, it is simple and highly relevant to envision evaluating the immediate differential effect of various oral challenges (*e.g.*, high/low fiber, high/low fructose) in various subjects (*e.g.*, lean/obesity, fatty liver/cirrhosis) using timed VOC measurements over several hours, days, or longer.

The gut liver axis (Table 2) includes many important, highly variable factors that are difficult to measure physiologically. While fecal analysis is inherently limited, breath VOC measurement may evaluate the global activity of the entire system.

In summary, the microbiome and gut-liver axis are a major research emphasis world-wide, and studies employing fecal analysis are appropriately credited with many advances. However, even if fecal analysis was fully validated, free, easy to perform, and always yielded interpretable results, it still cannot measure many “upstream” factors germane to both fatty liver and the metabolic syndrome and the marked heterogeneity between subjects. Studies using breath VOC analysis, in contrast, can uniquely evaluate the entire organism in real time. The simple capability of repeated measures greatly expands options in clinical research.

PERIL: A HISTORY OF UNMET EXPECTATIONS IN BREATH ANALYSIS

Breath analysis is appealing because it enables the potential for non-invasive, real time, easy to use, point of care measurement of metabolites that are, in some cases, difficult or impossible to measure by blood assays or other means. Previous attempts to apply breath analysis to gut physiology, however, have not been met with great success. Two examples, hydrogen and ammonia, are illustrative.

Hydrogen

Breath hydrogen testing has been available for decades^[42]. The monitors are relatively inexpensive, portable, and simple to operate. Aside from the addition of methane (to

capture preferential methane producers) and carbon dioxide (for quality control), the instrumentation and breath collection process have not significantly changed in many years. Hydrogen measurement is technically easy: it is relatively inert; its measurement is not affected by background ambient air; and it is present at high concentrations (parts per million)^[43]. Breath hydrogen testing has been incorporated into hundreds of published research studies.

The most widely accepted clinical use is in the evaluation of small intestine bacterial overgrowth (SIBO) and carbohydrate mal-absorption. Regarding the former, SIBO has emerged as a possible important and modifiable factor in the pathogenesis of irritable bowel syndrome (IBS) for some patients^[44]. As a result, the use of hydrogen breath testing has surged over the last decade to evaluate SIBO in IBS, including responsiveness to putative gut flora therapy (*i.e.*, rifaximin, a poorly absorbed antibiotic)^[45,46]. Because SIBO or “gut dysbiosis” is challenging to measure by other means, breath hydrogen testing had the potential to fulfill an important unmet need.

However, there remain serious concerns about its validity. An excellent recent review noted many problems, including lack of standardized instructions regarding testing substrates, doses and time intervals, as well as varying definitions of positive *vs* negative tests persist^[47]. Thus, notwithstanding a surging scientific and public interest in the possible role of gut flora in IBS, the American College of Gastroenterology does not endorse routine breath testing^[48].

The results of a recent meeting of the United States Food and Drug Administration (FDA) Gastrointestinal Drugs Advisory Committee (GIDAC) provide additional insight^[49]. The meeting’s purpose was the design of clinical trials to evaluate the safety, efficacy, and durability of response of repeat cycles of Xifaxan (rifaximin). To the author’s knowledge, this was the first time a breath test was seriously considered in the drug evaluation and approval process for a gut disease. But despite its long history, lack of technical issues, and the unmet need, GIDAC and the sponsor (Salix) easily agreed that breath hydrogen testing fails to meet criteria as a valid biomarker for any purpose and should not be utilized^[50]. Future developments seem unlikely.

Ammonia

In contrast to hydrogen, ammonia is highly volatile and difficult to measure by any method^[51,52]. Due to its relevance to gut flora and various disease states^[53], breath

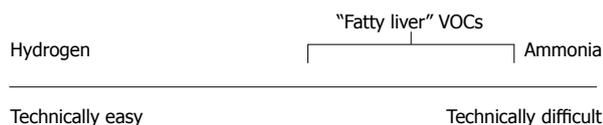


Figure 1 Most volatile organic compounds are challenging to measure. VOCs: Volatile organic compounds.

researchers have aspired to measure it for greater than thirty years^[54]. A progression of highly sophisticated measurement platforms (*e.g.*, GC/MS, quantum cascade lasers^[55]) have been used in the hopes that ever faster and more precise equipment modifications will finally yield accurate and reproducible results usable for clinical research and patient care. Many technical factors must be considered (*e.g.*, temperature, humidity, flow, and mode of breathing) alongside complex biologic concerns (*e.g.*, contamination from oral bacteria)^[56,57]. Despite these major challenges, many small studies were published purporting to demonstrate the utility of breath ammonia measurement for a specific disease or condition (*e.g.*, hepatic encephalopathy, renal dialysis, exercise^[58-60]). However, it now appears from work published by highly experienced groups, that exhaled breath may not reflect systemic levels, at least not be by the methods described to date. Aspirations repeatedly exceeded reality. Not surprisingly, therefore, the current ammonia literature has nearly completely ignored breath research^[53,61,62].

In summary, breath hydrogen is easy to measure and has an established role in clinical research and patient care. However, it is not a valid biomarker and its impact has not grown with in parallel with the rise in interest in gut flora. Breath ammonia is difficult to measure and, notwithstanding intense efforts by multiple breath research groups, has had little influence on clinical ammonia research. Thus, both the easy and difficult extremes of the breath metabolite spectrum reveal that, at times, the breath enterprise exists as only a tangential contributor to overall human research. The literature is replete with orphaned pilot studies. While hydrogen and ammonia serve as prototypical examples, this pattern has been duplicated with many metabolites (Table 3).

Notably, most of the candidate “fatty liver” VOCs are also quite difficult to measure (Figure 1).

PATH FORWARD

Volatility mandates reproducibility. First, test the test

By definition, VOCs are dynamic and changeable. Furthermore, they are present only in trace quantities and are subject to multiple confounders, including environmental factors. Therefore, studies of VOCs carry an exceptional burden of validation that requires the demonstration of reproducibility. Ideally, this includes at least three kinds of reproducibility: immediate (paired samples back to back), day to day, and location to location. The latter is needed because of ambient air influences, especially if human breath is collected in proximity to medical or

Table 3 Breath research often failed to meet its potential for multiple reasons

Technical/scientific factors	
Monitor/interface/biology	Too many interrelated unknowns
Unique data: uncertain utility	Relevance difficult to establish
	Relevance may not exist
Non-technical factors	
Inadequate teams	Engineers, chemists, doctors, statisticians
Inadequate synergy	Single center efforts
Lack of focus	Too many diseases, too little strategy
Lack of common languages	Device development is not drug development
Few models of commercial success	Difficult to envision endgame

research offices and facilities. For example, it must be proven that a subject’s breath ethanol at 200 ppb would be measured the same in low (*e.g.*, 50 ppb) and high (*e.g.*, 5000 ppb) ambient air environments. Once established, then other important influences should be evaluated, including time of day, mode of breathing, mouth rinses, food intake including composition and timing, and so on.

It must be acknowledged that such studies are often tedious, have poor publication value and short term return on investment. However, they are essential. Historians note that when the United States Food and Drug Administration (FDA) first promoted the basic drug safety expectations that evolved into present day preclinical and phase I studies (*i.e.*, the Food, Drug, and Cosmetic Act of 1938), most pharmaceutical companies simply folded^[63]. The survivors, *e.g.*, Merck, not only responded by drastically increasing their research enterprise, their leadership specifically assigned only their best scientists to these early stage efforts in acknowledgement of both their critical importance and tedium.

Breath research has to date failed to uniformly meet these requirements. Breath research papers often detail monitor mechanics and the ability of the monitor to reproducibly measure a targeted VOC against a known laboratory reference gas standard. Without further evaluation, small cross-sectional human studies are then performed purportedly to evaluate a disease state. Unfortunately, this pattern ultimately results in an unconvincing and inherently limited literature, as illustrated above for both breath hydrogen and ammonia. Breath VOC researchers have, therefore, earned skepticism from the broader research community.

Fecal VOC analysis should also meet these standards. For example, a recently published study evaluated fecal VOCs in NAFLD^[64]. Using home stool kits, subjects produced samples once, froze them, and later transported them to the lab. Fecal VOCs were then measured and compared to DNA analysis. Given the large number of VOCs measured (two hundred twenty), small sample size (thirty cases and controls) and observational case-control study design, the strength of the study’s conclusions is largely determined by the confidence in the measurement

process. However, while the authors and accompanying editorial carefully and appropriately discuss multiple other important influences and limitations of the study, neither substantively addresses this more basic issue^[65]. Even for analyses that may be exploratory and descriptive, more complete methods discussion is imperative to build a confidence foundation for additional studies.

Finally, it is noteworthy that while blood VOC analysis may also have important potential, it has similar downsides. For example, Zhu *et al*^[66], recently reported that specific gut flora compositions may drive an elevated endogenous ethanol production in a pediatric population with non-alcoholic steatohepatitis. However, blood assays for VOCs can also be challenging^[67]; for example, despite the fact that ammonia has been measured in the blood for over one hundred years, the proper blood source (venous *vs* arterial)^[68] and state (partial pressure NH_3 *vs* NH_4^+)^[69,70] remain debated. Furthermore, phlebotomy makes studies requiring multiple repeated measures difficult.

BIOMARKER DEVELOPMENT: BREATH SUCCESS REQUIRES EXCEPTIONAL TEAMS AND STRATEGY

In the 1950's and 1960's, the United States FDA promulgated a three phase strategy to evaluate the safety and efficacy of new drugs^[63]. The phases became familiar worldwide and created a uniform path for drug development. It is relatively easy, therefore, to interpret and compare clinical trials as they evolve through the phases. This is helpful not only for medical researchers, scientists, and regulators, but also for other stakeholders including investors and the broader public. Furthermore, drugs are developed and approved for a specific disease indication. Because this process is slow and highly resource intensive, progression through the phases occurs only after careful and continuous consideration of an unmet need and competing alternatives^[71]. As a result of this step-wise structure, regulatory approval, at least in the United States, is a milestone that is almost always associated with at least some commercial potential.

Unfortunately for breath research, an analogous path does not exist for non-invasive diagnostics or biomarker development^[72]. While the FDA indeed regulates non-invasive medical devices, the requirements for approval are much different, generally lower, and not as well known. Furthermore, they are not nearly as meaningful. Therefore, while biomarkers researchers may have lower apparent initial development costs and greater latitude than drug researchers, they risk misunderstanding and misdirection amongst members of the development team.

It is essential, however, that an overall strategy exists. This begins with an extensive and thorough validation of a putative biomarker applied to a particular application, *e.g.*, risk estimation, screening, diagnosis, monitoring, and so on. Moreover, biomarkers should also be characterized

by purpose, *e.g.*, predictive, prognostic, and so on^[73-75]. This compass must guide testing. Poorly designed studies in the wrong population are destined to yield uninterpretable results; this is especially true in breath analysis, where experimental monitors are often touted to measure experimental metabolites *via* experimental interface samplers to describe unknown biology.

Successful breath VOC research requires (1) multiple disciplinary teams; (2) extensive early stage validation studies; and (3) a clear clinical research strategy.

Coherent teams require, at a minimum, the ongoing participation of engineers, breath measurement experts, clinical researchers with experience in gut biology, gastroenterology, hepatology, and statistics. The process should begin with a foundation of knowledge and experience with breath VOCs resulting in focused testable hypotheses that can be transformed into monitors with specific performance specifications and operational capacities. Ideally, multiple monitors are built and are tested clinically side by side first at a single site and then at multiple sites for accuracy and reproducibility. After these are clearly established and normative data are generated, disease specific hypothesis can be pursued. Finally, a clear long term clinical research strategy grounded in the requirements for biomarker development should be articulated. Outside of a few centers of excellence, (*e.g.*, the Austrian Breath Research Institute, ISTM Keele University) such a comprehensive approach would be novel for breath research. The recent publication of comprehensive breath research books^[76], growing interest in breath research conferences, and the development and greater use a specially designed interface sampler^[77] are positive steps.

BREATH VOC METABOLITES FOR THE GUT-LIVER AXIS: CURRENT STATUS

The breath VOC metabolites of interest shown in Figure 1 are nearly as technically challenging as ammonia. Each of them, however, have been measured in breath with the generation of some normative data^[33,78]. Many innovative and useful small, single center studies have been published, as has been recently reviewed^[28].

A few studies have specifically focused on the gut liver axis and demonstrated some physiologic insights. For example, Cope *et al*^[79], evaluated the effect of an intervention (neomycin, a poorly absorbed antibiotic) on exhaled breath ethanol in an obese murine model of fatty liver compared to lean littermates. In addition to utilizing an intervention, this convincing study also reported repeated measures and thereby accounted for diurnal ethanol variations. The follow up human studies did not have these strengths and were therefore less persuasive^[80,81]. At present, though, breath VOCs are most developed not for fatty liver but for use in diabetes monitoring, where multiple groups have many significant recent advances^[82,83].

Finally, it must be acknowledged that liver disease, especially fatty liver, is difficult to accurately measure

by any means, including blood assays, imaging, or biopsy^[84,85]. Moreover, the pathophysiology of fatty liver, its relationship to steatohepatitis, cirrhosis, and associated conditions like obesity is complex, and there are many important mechanisms that do not involve VOCs. Thus, even if a well validated breath VOC panel existed now, it would be difficult to definitely tie such a profile to a clinical outcome of interest, and multiple measurement modalities are likely needed. As a result, breath research groups might aspire to participate in established long term fatty liver research programs (e.g., the United States based Non-Alcoholic Steatohepatitis Clinical Research Network^[86]) as ancillary studies.

ENGINEERS REQUIRED

Many of the pioneers of breath research have creatively adapted existing measurement platforms to breath measurement^[87]. Even now, specifically designed breath monitors are usually built as one-of-a-kind prototypes. The obvious legacy has been the small, single center fundamentally limited studies described above. For the same straightforward reasons, clinical researchers need multiple identical monitors that are portable and measure multiple VOCs simultaneously and accurately. Because clinical research is most convincing when multi-center studies involve large numbers of subjects, the need for multiple identical monitors is imperative. This is especially true due to extra reproducibility requirements of breath VOC research. While engineers may be understandably reluctant to commit the resources to build five monitors (especially after the results of a single prototype may have been equivocal), that is the prescription.

Given engineering advances and the right vision, this is achievable. Breath measurement experts, “breathologists,” should be involved at every stage, from design through maturation. Like drug development, this process will likely require a collaborative effort between academia and industry, but could occur at a fraction of the cost.

CONCLUSION

Breath analysis continues its infancy, and is almost always discussed in terms of its potential. But the rationale for breath has never been greater: breath affords the almost unique opportunity to quickly, cheaply, and non-invasively measure important markers that reflect the global gut-liver axis biology not measurable in other ways. Engineers, if they are willing, are ever more capable of making fast, portable, ultra-sensitive monitors. Comprehensive breath research teams should thoroughly address reproducibility to build a foundation for specific hypothesis driven goals. Those willing to invest in a long term strategy for breath VOC development may yet transform and revolutionize gut-liver axis research and patient care, with major pay-offs in diseases such as fatty liver, obesity, and the metabolic syndrome.

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