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# The Clinical Potential of Exhaled Breath Analysis For Diabetes Mellitus

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## Summary

Various compounds in present human breath have long been loosely associated with pathological states (including acetone smell in uncontrolled diabetes). Only recently, however, the precise measurement of exhaled volatile organic compounds (VOCs) and aerosolized particles was made possible at extremely low concentrations by advances in several analytical methodologies, described in detail in the international literature and each suitable for specific subsets of exhaled compounds. Exhaled gases may be generated endogenously (in the pulmonary tract, blood, or peripheral tissues), as metabolic byproducts of human cells or colonizing micro-organisms, or may be inhaled as atmospheric pollutants; growing evidence indicates that several of these molecules have distinct cell-to-cell signaling functions. Independent of origin and physiological role, exhaled VOCs are attractive candidates as biomarkers of cellular activity/metabolism, and could be incorporated in future non-invasive clinical testing devices. Indeed, several recent studies reported altered exhaled gas profiles in dysmetabolic conditions and relatively accurate predictions of glucose concentrations, at least in controlled experimental conditions, for healthy and diabetic subjects over a broad range of glycemic values. Optimization of this methodology and validation in large-scale trials under a wider range of conditions is needed to determine its true potential to transition into practical clinical use.

### Keywords

breath tests; diabetes mellitus; diagnostic techniques and procedures; gases; volatile organic compounds



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**Conflict of Interest**
The authors declare that they have no conflict of interest.

# A. Background

## A.1. Introduction to Breath Analysis

Breath analysis has long been recognized as a potentially powerful tool for the diagnosis and study of medical diseases. Even in ancient times, physicians recognized that certain breath odors were associated with specific pathological states. For instance, renal failure became associated with a 'fishy' smell and diabetes with a 'fruity' smell. In the 19$^{th}$ century, Nebelthau found acetone in the breath of diabetics, and Anstie that exhaled ethanol was a byproduct of alcohol metabolism. Yet the complexity of exhaled gas composition was largely undefined until 1971, when Linus Pauling systematically used gas chromatography to reveal ~250 gases in human breath[1]. In more recent times, scientists have been able to detect >3000 different volatile organic compounds (VOCs) and other aerosolized particles in the breath milieu[2, 3]. Further advances have led to the US Food and Drug Administration (FDA) approvals for breath-based diagnosis of alcohol intoxication, asthma, heart transplant rejection, *Helicobacter pylori* infection, carbon monoxide (CO) poisoning, and lactose intolerance[4]. Diabetes and its related dysmetabolic states could clearly greatly benefit from the introduction of similar non-invasive tests for diagnostic, preventive and monitoring purposes too. While such tests unfortunately remain currently unavailable, considerable interest has been geared in this direction recently, raising expectations that a breath test for plasma glucose, and possibly parallel tests for plasma insulin and lipids, may become clinically available within a few years. In this article, we review the basics of breath gas physiology and analysis as applied to diabetes mellitus as well as its possible future clinical applications.

## A.2. Rationale of Breath Analysis for Diabetes Mellitus

Twenty years from now, type 1 (T1DM) and type 2 diabetes mellitus (T2DM) are projected to affect nearly 450 million people worldwide[5]; diagnosis and management of this historically unparalleled global health epidemic hinges on blood tests, which may be expensive (adding to the annual multi-billion dollar financial burden), unpractical (e.g. in community-based large-scale screenings), and even painful (especially in patients with difficult venous access or whose skin has become calloused at common testing sites). Frequent blood testing is especially necessary for patients undergoing insulin treatment, for whom the American Diabetes Association recommends to self-monitor blood glucose concentrations 3+ times daily via finger sticks[6]. Additionally, measuring plasma insulin is clinically useful to assess pre-diabetic states (as an indicator of impaired glucose metabolism, it increases earlier than glycemia itself [7]), to stage the progression of T2DM from initial insulin resistance to eventual pancreatic failure, and to differentiate the increasingly common states in which components of both T1DM and T2DM are simultaneously present. Monitoring insulin can also give insight to other aspects of metabolism; insulin not only regulates glucose disposal but also exerts a strong anti-lipolytic effect, which is markedly reduced in patients with insulin resistance[8]. However, tests for insulin concentrations and sensitivity are unfortunately very laborious. Similarly, testing circulating lipids is important for diabetic patients because hyperlipidemia is an independent risk factor for heart disease. As lipids increase ketone body formation[9], their alterations may also be associated with changes in insulin or glucose metabolism. Knowing the interplay of these metabolic variables may allow clinicians to have a more comprehensive insight into their patients' health, and reliable non-invasive monitoring would certainly improve diagnosis, and treatment of diabetes.

While large resources have been invested worldwide in developing non-invasive devices for diabetes management, progress has been slow. Two early patents for non-invasive glucose testing, based on the changes of light through the eye, were filed in 1974 (U.S. Patents

3,958,560 and 3,963,019). However, no FDA-approved products are commercially available nearly 40 years later[10]; in fact, some devices for non-invasive blood glucose testing were even withdrawn from the market. Real-time insulin or lipid meters (comparable to glucose meters) do not yet exist.

Of many potential modalities of non-invasive testing for diabetes management, breath-based devices have multiple advantages. Notably, breath analysis is readily acceptable by patients, promising a potentially marked increase in testing compliance, which is currently one of the major obstacles to good glycemic control. Further, sample collection is easy and can even be obtained from unconscious patients. Such monitoring could thus facilitate tighter glucose management during surgery, which is currently difficult to achieve but believed to result in better clinical outcomes[11]. There is also virtually no limit in breath collection volume, which is a critical issue in neonates with extremely small circulating volumes. Breath collection can also be useful for wide screening when phlebotomy is problematic, i.e. in obese subjects with difficult vein access or in apprehensive primary school children who may refuse to participate in screening procedures involving phlebotomy. Finally, a number of historically challenging analytical issues, relating to the difficulty of accurately measuring the extremely low concentrations of compounds in breath have been satisfactorily solved, now rendering the rapid development of this methodology technically feasible in a reasonable timeframe.

## B. Gases in the Human Body

### B.1. Sources of Breath Gases

VOCs and aerosolized particles in the human breath arise from many sources including inhaled room air, airways surfaces, blood, and peripheral tissues throughout the body. While much is yet to be learned about the role of these gases, some appear to be by-products of biochemical reactions while others may be produced for specific physiological roles, such as cell-to-cell signaling. Production of other gases may only be present, or greatly amplified, during infections or other pathological conditions. Not surprisingly, therefore, as an increasing number of previously undetected gases is being identified in human exhalates, there is growing interest in the direct identification of the origins of these compounds through the study of cellular and tissue gas emissions under a variety of physiological and experimental conditions.

**B.1.i. Atmospheric Air—**One obvious source of breath VOCs is inhalation from a person’s immediate surroundings (e.g. pollutants like ethane and n-pentane). High atmospheric concentrations of VOCs will directly result in high breath concentrations[12]. Such exposure may also lead to increased uptake of the compounds beyond the airways. Some VOCs (e.g. trichloroethene, toluene, tetrachloroethylene), for instance, are absorbed into the systemic circulation and have been reported in blood samples of non-occupationally-exposed adult US populations[13].

It is important to note that inhaled VOCs, once absorbed into the bloodstream, may undergo partial or total endogenous enzymatic metabolism, altering the ratio between inhaled and exhaled concentrations. For instance, only 1–3% of inhaled tetrachloroethylene is metabolized to trichloroacetic acid while the rest is exhaled unaltered[14]. On the other hand, ~80% of ethylbenzene and xylenes are metabolized by hepatic enzymes, leading to exhaled concentrations that are substantially lower than inhaled air[15]. Importantly, acute metabolic changes can modify this inhaled/exhaled ratio, linking the exhaled levels to specific metabolic events. For instance, hyperglycemia, through an increase in glucose load and blood flow to the liver, suppresses enzymatic catabolism of several VOCs, resulting in greater fraction of these compounds being exhaled; one study reported a baseline exhalation

of aromatic compounds at ~40% of inhaled concentrations and a 2-fold increase during experimental hyperglycemia (ethylbenzene: range 46–434 pptv; x/p-xylene: range 105–1294 pptv; o-xylene: range 44–426 pptv)[16]. Similar changes in the kinetics of VOC metabolism that may be associated with chronic dysmetabolic conditions may therefore conceivably be used as indirect biomarkers of tissue function and/or specific aspects of organ metabolism.

**B.1.ii. Lungs—**The internal surfaces of the lungs may also directly contribute gases to the exhaled mixture (i.e. by either generating lung-specific gases or increasing production of gases also produced elsewhere in the body). For example, 10 VOCs (4 hydrocarbons, 2 esters, 2 ketones, 2-methyl-2-propanol, and ethyl tert-butyl ether) were produced by human bronchial epithelial primary cells[17]. Another study found acetaldehyde and carbon dioxide ($CO_2$) produced from NL20 immortalized, nontumorigenic lung epithelial cells (in addition to other peripheral tissues)[18]. Methacrolein has also been hypothesized to be formed in the respiratory tract as a reaction of ozone and isoprene[19]. Other studies compared VOC production of normal and oncogene-transformed lung cancer cell lines as well; a greater variety of released compounds (including 2-methyl-1-propanol, 2-methyl-2-propanol, and 3-methyl-1-butanol) were found in the former than latter[20]. Furthermore, pulmonary surface liquid can become aerosolized into the breath, resulting in measureable concentrations of arachidonic acid metabolites (eicosanoids), isoprostanes, leukotrienes, proteins, and cytokines in exhaled breath condensate (EBC)[3, 21]. Still other VOCs are generated by the lung tissue only in diseased states. Phillips et al found a unique combination of 22 exhaled VOCs (alkanes, alkane derivatives, and benzene derivatives) from lung cancer patients but not healthy subjects[22]. Chen et al then found 3 VOCs (undecane and 2 unknowns) that were exclusively produced by excised lung cancer cells and detected in the breath of patients but not controls[23]. This finding suggests the direct transmission of these VOCs from the lungs to the exhaled breath.

**B.1.iii. Peripheral Tissues—**A variety of peripheral human cells and tissues, either in healthy or pathological states, are thought to contribute to exhaled VOC composition via release of gases into the bloodstream and their subsequent transfer into the airways. Ethane, isoprene, pentane, and other light hydrocarbons have been proposed as biomarkers of metabolic processes occurring on a systemic scale, such as fat oxidation[24], cholesterol/LDL concentrations[25], and oxidative stress levels[26]. Patients with insulin resistance may have increased lipid production and therefore increased breath ketones (acetone, 2-pentanone and 2-butanone) from subsequent lipolysis[9]. Other VOCs appear more localized to specific anatomical regions or processes. For example, C4–C20 alkanes and monomethylalkanes have been associated with heart transplant rejection[27], and pentane with acute myocardial infarction (2.98–6.83 nmol/l vs. 0.30–3.81 nmol/l in controls)[28]. Elevated breath nitric oxide (NO) concentrations (>16 ppbv) are found in asthmatic patients [29] and increased sulfides are present in those with cystic fibrosis (net uptake of 110 pptv carbonyl sulfide vs 250 pptv for controls)[30]. A separate panel of 5 VOCs (2-propanol, 2,3-dihydro-1-phenyl-4(1H)-quinazolinone, 1-phenyl-ethanone, heptanal, and isopropyl myristate) is associated with breast cancer[31], acetone with non-alcoholic steatohepatitis (919 nmol/l with stage 2–3 steatosis vs 675 nmol/l in stage 0–1)[32], and dimethyl sulfide with cirrhosis and fetor hepaticus (29.02 ppbv vs 13.79 ppbv in controls)[33]. Many more biomarkers are likely to be reported in the near future.

The exact mechanisms of transferring systemically-produced VOCs from tissues into the lungs are still unclear. The simplest possibility is that the VOCs are merely dissolved in the blood and released in the breath via pulmonary gas exchange, in which case the liquid/gas partition coefficients could be estimated by classic chemical equations, taking into account ventilation and specific solubility characteristics of each gas. Often, however, measured gas concentrations, both in breath and in blood, seem to diverge significantly from these

estimates. In isoprene, for instance, a gas long thought to be a biomarker for cholesterol biosynthesis, and one of the more actively studied gases in terms of blood/breath partition, a complex interrelation has been identified between exhaled kinetics and peripheral sources[34], with at least some studies showing exhaled levels disproportionately (40-fold) greater than expected from plasma concentrations[35]. Recent evidence now also suggests that compounds also diffuse through the multiple layers of airway tissue throughout the entire bronchial tree. Breath VOC concentrations are thus not determined solely on alveolar blood (or systemic blood) concentrations, but rather may reflect concentrations in tracheo-bronchial lining fluid, especially for water-soluble compounds. Pulmonary gas exchange, therefore, may best be described with complex mathematical models that also consider other factors such as airway temperature, bronchial blood flow and multiple compartments (e.g. airway lumen, surface mucous, connective tissue, bronchial circulation, adventitia, pulmonary circulation)[36, 37]. Furthermore, another possible explanation for this discrepancy is that these VOCs are not detected in blood because they are bound to carrier proteins en route to delivery to the alveolar space. Among candidates for this transport role of isoprene, hemoglobin has been seriously considered (in addition, of course, to its known ability to transport oxygen, $CO_2$, CO, and NO)[38].

**B.1.iv. Blood—**While blood can act as a gas conduit from peripheral tissue to the lungs, there is evidence that blood components themselves can produce VOCs. Miekisch et al reported acetone, dimethyl sulfide, isoflurane, isoprene, and pentane in the headspace of whole blood samples using gas chromatography[39]. Deng et al also identified 23 compounds (including hexanal and heptanal) produced by whole blood that were present in exhaled breath[40]; other studies have since reconfirmed this finding using alternative liquid-chromatography-based methodologies[41].

Of the various blood cell types, the importance of polymorphonuclear leukocytes (PMNs) in diabetes has been rapidly emerging; PMNs potentially emit gases of their own and have been found to be interrelated with multiple aspects of glucose regulation (Figure 1). Shin et al recently discovered that both promyelocytic cells and isolated neutrophils (but not peripheral blood mononuclear cells) emit acetaldehyde[42, 43], a gas speculated to mediate blood vessel relaxation via calcium channel modulation[44]. As PMNs circulate throughout the body and respond to chemotactic factors, they may produce high local concentrations of gases upon aggregation at specific locations. It is possible that these VOCs may be also signaling through yet unknown pathways including supplementary PMN recruitment. Noteworthy, PMNs are often found sequestered in the lungs at high concentrations, especially in pulmonary disease[45], and therefore may result in VOC transfer into exhaled breath. Changing glucose levels also alters glucose transporter activity and impairs important functional aspects of the neutrophil (response to chemotaxis, elongation)[46, 47]; in fact, blood plasma concentrations of antibacterial, neutrophil-derived defensins and myleoperoxidase (MPO) were observed to be elevated in diabetes[48, 49]. Altered VOC production by PMNs may therefore be present in dysmetabolic states, and provide good candidates for novel biomarkers of altered immune function, onset and progression of disease in diabetic populations. To our knowledge, VOC emission patterns from other blood subtypes have not yet been studied.

**B.1.v. Microorganisms—**Bacteria and other microorganisms can also produce unique gas profiles which, if identified within exhaled gas mixtures, may be used for diagnostic or monitoring purposes. A typical example involves the ingestion of radiolabeled urea to detect *Helicobacter pylori*, a urease-positive bacterium which causes gastroduodenal ulcers; only if the microorganism is present, urea will be hydrolyzed into radiolabeled $CO_2$ and ammonia[50]. Hydrogen cyanide has been shown to be released by cells infected by *Pseudomonas aeruginosa* and investigated as target for noninvasive diagnosis too[51].

Lactose ingestion by patients with hypolactasia is also known to increase their breath hydrogen concentrations (48–168 ppmv vs 0–3 ppmv in controls) due to the increased fermentation of carbohydrates by gut flora[52]. A group of VOCs were found produced in vitro by *Mycobacterium tuberculosis* and also associated with active infection (including derivatives of cyclohexane, benzene, heptane and hexane)[53]. Further, as it will be discussed in greater detail below and more pertinent to the field of diabetes, ethanol and other alcohols can be produced by gut bacteria in response to glucose ingestion or changes in systemic glycemia.

### B.2. Roles of Gases

**B.2.i. Gases of Endogenous Origins—**In addition to being mere by-products of biochemical reactions, the concept that some gases may be released with specific signal transmission roles has gained increased prominence in recent years. Known signaling pathways of NO, for instance, whose discovery led to the 1998 Nobel Prize in Physiology or Medicine, are involved in the vasodilation of endothelial cells, neuronal synaptic plasticity, and antibacterial defense by directly targeting soluble guanylate cyclase[44]. With a mechanism similar to NO, CO affects platelet aggregation and inhibits smooth muscle proliferation[44]. Another signaling gas, hydrogen sulfide, seems to affect $K_{ATP}$ channels and vasodilatation[54].

It is also likely that the biological activity of several additional VOC has simply not yet been identified; indirect inferences, however, may be derived for VOCs present in human systems from established signaling pathways in other organisms. An interesting example is the complex interrelationship among ethylene, defensins, and glucose homeostasis in plants. Human neutrophilic α-defensins, which are elevated in T1DM subjects[48], were recently been shown to suppress hepatic glucose production via a pathway independent of insulin[55]. When this findings is considered in light of earlier reports that their structurally and functionally related plant counterparts[56] are upregulated by the common gaseous plant hormone ethylene in certain plant species (i.e. Arabidopsis)[57, 58], new potential signaling pathways are unveiled. As ethylene was found to antagonize glucose signaling pathways in Arabidopsis plants[59] and the related plant-derived compound 1-(3,5-Dimethoxyphenyl)-2-(4-hydroxyphenyl)-ethylene has been demonstrated to have anti-hyperglycemic effects in rats[60], the possibility arises that glucose regulatory properties of human α-defensins are VOC-linked. While these considerations are certainly just speculative, it is indeed possible that neutrophil-derived ethylene and/or other related gases contribute to a complex signaling network involving multiple aspects of glucose regulation. Alterations in this interactive pattern may have obvious implications in patients with diabetes and impaired glucose metabolism.

**B.2.ii. Gases of Exogenous Origins—**Several VOCs have become widespread throughout the earth's atmosphere, and are present at stable and elevated enough concentrations to induce predictable and repeatable changes in human metabolism when inhaled. For instance trichloroethylene, a common industrial solvent, has been shown to upregulate PPARα, inhibiting NF-κB p50 and p65 signaling and to induce NFκB p52 mRNA synthesis[61]. Inhalation of D-limonene, a major odor constituent in several citrus oils, has antitumorigenic activity via antagonism of adenosine A(2A) receptors[62]; further, it has been found to inhibit LPS-induced nitric oxide production and prostaglandin E2 in murine macrophages[63]. β-Pinene inhibits serotoninergic receptors and has anti-inflammatory and analgesic activity as well as antimicrobial properties[64]. Even $CO_2$ is now thought to regulates gene expression via the NF-κB pathway[65] and may attenuate pro-inflammatory gene expression (TNFα) and enhance anti-inflammatory IL-10 production[66]. While pharmacological effects of some other VOCs have been reported,

their mechanisms of action are less understood. Ethyl nitrate possesses vasodilatory activity and suppresses methemoglobin formation[67]. Methyl iodide can dose-dependently increase serum cholesterol (HDL, LDL) and decrease triglycerides in both rat and rabbit toxicity studies[68, 69]. Inhalation of dichloromethane, ethylbenzene, and trichloroethylene were shown to make 1,217 identifiable changes in rat gene expression[70]. Inhalation of a mixture of 22 VOCs (hydrocarbons) perturbed the immune system of healthy subjects, causing a migration of neutrophils to the upper airways[71]. While not obviously related to carbohydrate metabolism, these and other yet unidentified metabolically active exogenous VOCs may display changing exhaled profiles in the presence of fluctuating levels of energy substrates.

## C. Breath Testing for Diabetes Mellitus

### C.1. Features of Diabetes that Can Potentially Influence Breath Testing

Understanding key physical differences induced by diabetes may be a necessary prerequisite to accurate breath-based measurement of diabetes-related metabolic variables. While a “universal” test applicable to both healthy and diabetic populations would be ideal, changes in the lungs and metabolism occurring in diabetic patients may affect breath composition in characteristic ways. On one hand, these changes may be useful for diagnostic purposes; on the other hand, they may also require specific adjustments to the technique for every-day monitoring purposes.

**C.1.i. Lungs—**Lung function and pulmonary vasculature are both thought to be impaired in diabetes, potentially affecting gas exchange kinetics of multiple VOC, concepts that need to be addressed if utilizing these compounds in the development of future breath tests. As the disease worsens, for instance, increasingly severe pathological changes may render it necessary to perform frequent recalibrations or exclude specific VOCs from use in breath testing.

While the underlying pathophysiological mechanisms are still the object of some controversy, a recent meta-analysis concluded that both T1DM and T2DM is associated with modest restrictive lung disease and reduced diffusing capacities due to micropathology, primarily deriving from nonenzymatic glycation and thereby progressive stiffening of the collagen- and elastin-rich lung tissues[72]. Autopsies on diabetic subjects indeed found thicker epithelial and endothelial capillary basal laminae[73]. Additionally, both plasma MPO and α-defensins have also been found elevated in diabetics[48, 49]; MPO generates damaging oxidative species and α-defensins promote atherosclerosis[74] as well as cause lung injury by disrupting the capillary–epithelial barrier[75]. Furthermore, the binding of advanced glycation end products to their receptors (RAGE), which occurs in the lung in healthy subjects and significantly increases in diabetes, further increases ROS production, elevates pro-sclerotic and pro-fibrotic growth factors, and decreases nitric oxide bioavailability[76]. In streptozotocin-induced diabetic rats, decreased nitric oxide bioavailability also resulted in endothelial dysfunction of pulmonary arteries[77]; this may be the mechanism for the increased risk of pulmonary hypertension in T2DM patients. Another study found these rats had triglyceride deposition on their pulmonary arteries as well as altered prostaglandin and leukotriene production[78].

**C.1.ii. Systemic Metabolism—**Direct metabolic changes alter breath composition in subjects with diabetes. Blood glucose may be degraded directly into VOCs measurable in the breath (e.g. through fermentation to ethanol or methanol)[16, 79, 80]. Glucose may also indirectly affect other VOC levels (e.g. hyperglycemia changes the rate of acetone formation through the suppressive effect of concomitant, physiological compensatory hyperinsulinemia). Even greater hyperinsulinemia is a defining characteristic of early-stage

T2DM, also resulting in suppression of lipolysis[8]; changes in cholesterol synthesis may be reflected in exhaled isoprene[81]. Very small alterations in ketone concentrations and other VOCs may therefore reflect the fluctuations of insulin and glucose metabolism[79]. An complicating factor, not directly caused by the disease itself, is that T1DM patients tend to limit carbohydrate ingestion in favor of proteins and lipids while T2DM patients often ingest a large percentage of high-fat nutrients; this excessive fat ingestion leads not only to acute increases in insulin resistance but also to changes in exhaled methyl nitrate (range: 6–15 pptv) and other ketones [82, 83].

### C.2. Current Breath Analysis Technology

While the potential of breath analysis was recognized for decades, difficulties in measuring very low concentrations of breath compounds had severely limited its clinical applicability until recently. Fortunately, the repertoire of analytical techniques with sufficient detection capabilities has now greatly expanded. Breath analysis techniques available today include classic tracer studies that involve the infusion of molecules tagged with either stable or radioactive isotopes followed by isotope quantification in exhaled breath (which can be used to study β-oxidation and other metabolic pathways[84]). Gas chromatography and mass spectroscopy capabilities have also improved to such an extent that previously undetectable concentrations of VOCs can now be routinely identified and quantified with a high degree of accuracy[85, 86]. Additionally, direct measurements of compounds in EBC is now possible[87]; typically, exhaled breath is trapped in a collection tube, cooled to an aqueous form, and analyzed by various chromatographic techniques[3]. Furthermore, new technologies with more frequent gas sampling or real-time analysis have been developed, which allow repeated testing during and following metabolic perturbations. These approaches include atmospheric pressure ionization mass spectrometry coupled with electrospray charging, which has been used for on-line measurements of volatilized fatty acids[21, 88], as well as proton transfer reaction mass spectrometry (PTR-MS) [89] for measurement of breath VOCs.

Detailed technical descriptions of these various methodologies are beyond the scope of this article, especially in light of the several recent reviews that have thoroughly compared their various advantages and disadvantages. We therefore remand the reader to these publications, such as the work of Miekisch et al and Di Francesco et al, who introduced general instrumental techniques (chromatography and electronic sensors) and some clinical applications for breath testing[90, 91]. Buszewski et al later elaborated on several considerations for breath sampling, preconcentration, and analysis[92]. More recently, Smith et al reviewed selected ion flow tube mass spectrometry and PTR-MS technology for VOC breath analysis in diabetes mellitus[93]. On a related note, other non-invasive glucose monitoring methods are still being actively pursued. Tura et al wrote a detailed summary of 14 such technologies[94], including several spectroscopic techniques, and Turner summarized newer breath and skin technologies[95]. These papers should provide the reader with ample technical background for breath analysis technologies that are currently available.

### C.3. Findings

**C.3.i. Associations of VOCs with Aspects of Diabetes—**Some studies have attempted to use exhaled VOCs for the identification of specific aspects of diabetes and energy substrate metabolism. For example, breath acetone has been linked with diabetic ketoacidosis[79] and isoprene with cholesterol synthesis[81]. In addition to associating specific compounds with physiological processes (Table 1), experiments have also focused on diabetic screening. Using proton transfer reaction-mass spectrometry on 8 mass ranges of exhalates, each representing an unknown group of VOCs, Greiter et al distinguished patients

with T2DM from healthy controls with a 90% sensitivity and 92% specificity[96]. Similarly, Kulikov et al found light hydrocarbons (C2-C3 including ethanol and acetaldehyde) to be elevated in the exhaled breath of women who had risk factors for T2DM (i.e. relatives with diabetes and smoking) as compared to those without[97]. While none of these tests have yet been translated into commercial devices, these studies constitute an important foundation for future research (especially on their underlying biochemical pathways) and product development.

**C.3.ii. VOCs for Quantifying Aspects of Diabetes—**Other experiments have focused on using VOCs for the quantification of altered metabolic conditions associated with diabetes. A common feature of all endeavors aimed in this direction is the realization that a one-to-one correspondence between a single exhaled compound and a given plasma metabolite, or the severity of a given metabolic condition, seems to never exist. Still, as some individual compounds seem to carry greater "historical weight", they have received particularly focused attention; a typical example is breath acetone. Efforts have, in fact, been made to correlate breath levels of this gas with blood concentrations of ketone bodies (e.g. acetone, β-hydroxybutyrate), hemoglobin A1c, and blood glucose[98]. During a study on T1DM subjects, a linear relationship was observed between group mean concentrations of breath acetone (range: 0.74–2.92 ppmv) and blood glucose as well as hemoglobin A1c[98]; however, none was found in T2DM subjects[99, 100]. Taken together, these data suggest that acetone is certainly influenced by changes in carbohydrate metabolism and, in the proper context (i.e. integrated with simultaneous changes of other exhaled compounds), can probably be used to help estimate glycemia or other related plasma variables. However, measurement of its concentration alone appears insufficient for quantification, likely due to the considerable variability of its levels related to the degree of insulin resistance, lipolytic activity, diurnal fluctuations, fasting status, gender, and nutrient composition of diet, etc. [100, 101].

Rather, groups of exhaled gases ("signature" gas profiles) may simultaneously change in response to given metabolic stimuli, albeit with different magnitude and kinetics. Simultaneous analysis of a several compounds and selection of combinations of biomarkers therefore appears necessary in the development of clinical devices for breath-based measurements. For instance, to determine the association between hyperglycemia, diabetes, and oxidative stress, Phillips et al quantified "oxidative age" by integrating the area under a 3-dimensional "breath methylated alkane contour" (derived from concentrations of exhaled C4 – C20 alkanes and monomethylated alkanes) in non-fasting subjects at rest. Both T1DM (n=9) and T2DM (n=53) subjects displayed significantly increased oxidative stress as compared to healthy controls (n=39)[26].

Other studies, using multi-linear regression (MLR) on clusters of exhaled VOCs, focused on developing the conceptual background for a future breath-based, hand-held glucometer. Early experiments by Galassetti et al integrated exhaled ethanol (range: 9.6–45.0 ppbv) and acetone concentrations (range: 280–364 ppbv), obtained during a standard 75g oral glucose tolerance tests, into an model for estimating plasma glucose; the average individual correlation coefficient for the 10 healthy young participants (5M/5F) was 0.70[79]. The group then modified their methodology to use intravenous glucose infusion in 10 healthy subjects (5M/5F) and included 2 additional VOCs (methyl nitrate: range 5–216 pptv; ethylbenzene: range 46–434 pptv) into their MLR model, resulting in higher correlations with glucose (methyl nitrate is believed to be a by-product of the interaction of oxidative radicals and NO while ethylbenzene reflects hepatic enzymatic activity). This improved 4-gas model allowed breath-based glucose prediction with a mean correlation coefficient of 0.91 (range r=0.70–0.98) when compared to standard glucose measurements[16]. In a subsequent study, the same 4-VOC MLR model was applied to both T1DM and healthy

subjects, during more complex glycemic fluctuations (4-h glucose clamp with a 60-min baseline, 90-min hyperglycemia at ~220 mg/dl, 90-min hyperinsulinemia-euglycemia). VOC-derived estimates and direct measurements of glycemia had a very high degree of agreement with an average correlation coefficient of >0.86 over 30 study visits[80]. While these results appear extremely promising, their applicability to clinical practice is still limited by the enormous cost and analytical complexity; it is hoped that miniaturization of the procedures will lead within a reasonable timeframe to the development of clinically testable prototypes of portable devices.

While not tested as frequently as plasma glucose, circulating insulin and lipids also constitute diabetes-related variables whose non-invasive measurement may advance disease prevention and monitoring. Initial reports of breath-based estimates of plasma insulin and triglycerides have, in fact, been published. In a cohort of 13 young healthy subjects, undergoing 4-h clamp experiments in which insulin values ranges from basal levels to ~15-fold basal, breath-based plasma insulin predictions correlated with ELISA measurements with a mean correlation coefficients of 0.94. Importantly, in this study, which utilized MLR analysis of 5 exhaled VOCs[102], a common predictive equation was used for all subjects (unlike the glucose prediction studies mentioned above, in which, while the same gases were used in all subjects, individual predictive equations were necessary, implying an individual calibration test). Breath-based insulin testing may be an important screening tool early screening of diabetes and metabolic syndrome, conditions in which hyperinsulinemia by far precedes elevations of glycemia[7]. Exhaled VOC-based predictive models for plasma triglycerides (TG) and free fatty acids (FFA) were also obtained during similar 4-hour in vivo experiments (insulin-induced lipid suppression or i.v. infusion of a lipid emulsion) in 23 healthy volunteers (12m/11f, 28.0±0.3 years)[103]. Strong correlations between measured and breath-based predictions were observed (r=0.86 for TG, r=0.81 for FFA). If developed, a breath-based lipid test could contribute to the overall management of diabetic patients, whose systemic lipid levels represent a critical risk factor for cardiovascular events; of course, this test could be extended to many non-diabetic populations too.

## D. Conclusions

In summary, breath analysis methods appears on the verge of major breakthroughs that will hopefully exponentially accelerate the transition of past theoretical concepts into practical clinical devices relevant to diabetic populations. However, much still remains to be discovered regarding the origins, pathways, and pathophysiological roles of breath components; fortunately, many new analytical tools that can isolate VOC production from specific cells and tissues are available to now help answer these questions. Furthermore, many of the currently available findings were obtained in highly controlled experimental conditions, and their applicability to real life will need to be confirmed in larger scale clinical trials taking into account several confounding variables. These include, but are not limited to, the effects of prior food ingestion, prior exposure to different air mixtures, prior glycemic control, stability over time of predictive algorithms, duration of diabetes, concomitant presence of tissue complications, and efficacy of the methodology in different glycemic ranges (hypo- versus hyperglycemia). Still, given the rapidly increasing interest in the field, the recent promising findings, and wide range of potential applications (devices may be geared towards glycemic monitoring, initial diabetes screening, hyperinsulinemia, hyperlipidemia, inflammatory markers), we believe breath-based testing will become clinically available in the near future, at least partly replacing current blood-based bioassays.

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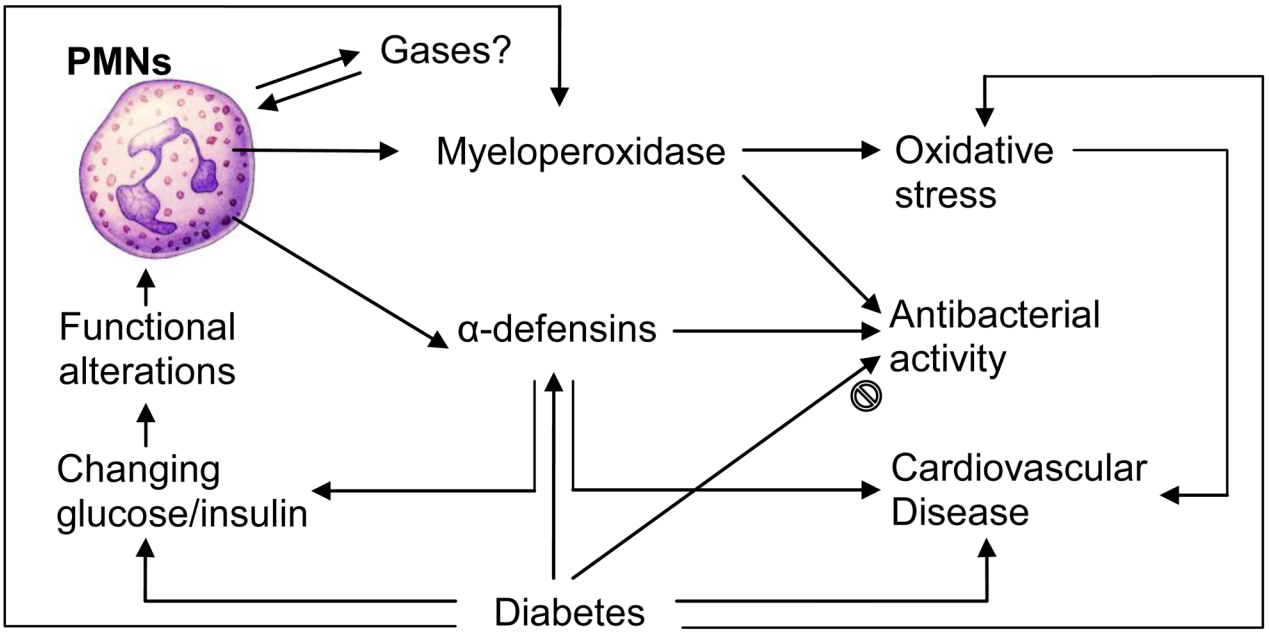


**Figure 1.**
Relationships between Neutrophils (PMN) and Diabetes

## Table 1

Selected Breath Compounds Relevant for Diabetes Mellitus

| Compound | Possible Source/Relevance for Diabetes and Metabolism |
|---|---|
| Aerosolized glucose | Elevated in EBC during experimental hyperglycemia[104] |
| Aromatic compounds (ethylbenzene, o/m/p- xylene, toluene) | Partly metabolized by hepatic enzymes (cytochrome P450 system); rapid-onset hyperglycemia likely suppresses hepatic metabolism of the gases[16, 80] |
| Alkyl nitrates (2-, 3-pentyl nitrate, methyl nitrate) | By-product of the interaction of oxidative radicals and NO[105]<br>Methyl nitrate correlated with plasma glucose in diabetic patients[106] |
| Carbon dioxide | Direct oxidative by-product of energy substrates |
| Carbon monoxide | Marker of oxidative stress (that can be caused by hyperglycemia)[107]<br>Difference in the $^{13}CO$ excretion were found between diabetic and healthy subjects following a labeled oral glucose tolerance test[108] |
| Ethane and pentane | Oxidation of ω3 and ω6 fatty acids[91] |
| Ethanol and methanol | Bacterial fermentation of glucose in intestines and subsequent movement into the portal circulation[79, 109] |
| Ketones (acetone, 2-pentanone) | Increases in diabetic ketoacidosis, fasting, and high-fat/ketogenic diets[9, 79, 82, 101]<br>Suppressed with insulin-mediated suppression of lipolysis[83, 110]<br>Acetone correlates with blood glucose and hemoglobin A1c in T1DM [98] but not in T2DM patients[99, 100]; exhaled at ~1/330 of plasma acetone concentrations [98]; follows a diurnal pattern [98]; elevated in men [100] |
| Isoprene | Cholesterol synthesis (from acetyl-CoA via the melavonic acid pathway) [81] |
| Propane | generated by n-4 fatty acid (18:3) peroxidation[111] or protein oxidation of branched-chain amino acids or production by colonic bacteria[24] |
| Propionic and butanoic acids | Elevated in EBC following sucrose ingestion[88] |