

Breath Metabolites to Diagnose Infection

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BACKGROUND: Starkly highlighted by the current COVID-19 pandemic, infectious diseases continue to have an outsized impact on human health worldwide. Diagnostic testing for infection can be challenging due to resource limitations, time constraints, or shortcomings in the accuracy of existing diagnostics. Rapid, simple diagnostics are highly desirable. There is increasing interest in the development of diagnostics that use exhaled breath analysis as a convenient and safe diagnostic method, as breath sampling is noninvasive, secure, and easy to perform. Volatile organic compounds (VOCs) present in exhaled breath reflect the fingerprint of the underlying metabolic and biophysical processes during disease.

CONTENT: In this review, we overview the major biomarkers present in exhaled breath in infectious diseases. We outline the promising recent advances in breath-based diagnosis of respiratory infections, including those caused by influenza virus, SARS-CoV-2, *Mycobacterium tuberculosis*, *Pseudomonas aeruginosa*, and *Aspergillus fumigatus*. In addition, we review the current landscape of diagnosis of 2 other globally important infections: *Helicobacter pylori* gastrointestinal infection and malaria.

SUMMARY: Characteristic and reproducible breath VOCs are associated with several infectious diseases, suggesting breath analysis as a promising strategy for diagnostic development. Ongoing challenges include poor standardization of breath collection and analysis and lack of validation studies. Further research is required to expand the applicability of breath analysis to clinical settings.

Introduction

Breath analysis may be one of the oldest strategies used for clinical diagnosis. Around 400 BC, Hippocrates recognized the diagnostic usefulness of body odors, reporting on several disease-specific odors in urine and sputum (1). In 1782, Lavoisier identified carbon dioxide

as a combustion product in the breath of guinea pigs, (2) and, by the mid-1800s, Nebelthau had identified exhaled acetone as a characteristic of individuals with diabetes (3). A major breakthrough in the scientific study of breath came in the 1970s, when Nobel Laureate Linus Pauling used mass spectrometry to demonstrate the chemical complexity present in exhaled human breath, which contains over 200 volatile organic compounds (VOCs) (4). Modern instruments now reveal that human breath is an incredibly complex mixture, composed of over 3500 VOCs (5).

Breath analysis relies on the premise that the VOC profile of breath is altered as a characteristic and specific response to underlying pathologic states, and that these VOC changes can be detected, measured, and used for disease diagnosis and monitoring. Exhaled breath metabolites reflect not only the metabolic activity of the respiratory tract and its microbiota, but also the metabolite changes occurring in distal parts of the body, as circulating volatile metabolites may diffuse into the alveolar space for exhalation. Of particular interest has been the noninvasive nature of breath analysis, which lends the ability to take repeated measurements with minimal stress or discomfort to the individual under investigation.

A wide range of breath sampling methods have been used for breath analysis. In general, the breath sampling strategy chosen depends on the target disease of interest and the environment in which the sample will be collected. “Offline” breath sampling approaches require transfer of breath metabolites and subsequent analysis by an instrument, which may be in a physically distinct location from the subject. In contrast, “online” breath analysis deploys on-site instruments in which the breath is analyzed in real time, directly by the instrument at the point-of-care. Offline sampling is currently the most widely implemented approach, with most investigators using gas chromatography–mass spectrometry (GC–MS) or two-dimensional gas chromatography–mass spectrometry (GC×GC–MS) for analysis. Breath gases must be preconcentrated before analysis, with thermal sorption (TD) tubes a common choice for preconcentration of VOCs. Online instrumentation has the advantage of continuous analysis (i.e., repeated measurements in short succession). Benchtop or laboratory-based online systems, such as ion mobility spectrometry (IMS), or selected ion flow tube–mass spectrometry and proton

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transfer reaction–mass spectrometry, are the most commonly used online technologies.

Exhaled breath is predominantly composed of nitrogen, oxygen, carbon dioxide, argon as well as water vapor. In contrast, the VOCs present in breath, including common metabolites such as isoprene, ethane, pentane, and acetone, are only found at trace levels [typically parts per million (ppmv) and lower]. While recognition of VOC patterns via human olfaction (smell) is naturally error-prone, highly sensitive analytical instruments are now available that permit reliable detection and identification of small volatile and semivolatile molecules. VOCs present in breath may originate from the environment, endogenous host metabolism, and the metabolic capacity of human-associated microbes, including not only pathogens of interest but also the endogenous microbiome of the upper and lower airways and the gut. Most of the identified VOCs in exhaled breath originate exogenously (6), but endogenous and microbial VOCs have greater clinical utility (7). Endogenous VOCs have the potential to provide an integrated view into the overall physiological state of an individual, while microbial VOCs may be of particular interest for pathogen identification. In this mini review, we describe the potential use of breath in clinical diagnosis of infectious diseases.

Respiratory Tract Infections

Many VOCs, including various alcohols, aldehydes, and terpenes, are known byproducts of microbial metabolism. Through mass spectrometric analysis of the headspace of cultured organisms, increasing evidence indicates that different microorganisms each possess distinct, characteristic VOC signatures (8). Because respiratory infections typically involve incursion of

pathogens into the airways, where host- or microbe-generated VOCs may be directly released into exhaled breath, breath VOC analysis has been extensively explored for diagnosis of respiratory infections (Fig. 1).

INFLUENZA

Influenza is a common respiratory viral infection caused by RNA viruses from the *Orthomyxoviridae* family (influenza A and B). Several diagnostic tests are available. The most common, so-called “rapid influenza diagnostic tests” detect viral proteins (antigens) and provide results at the point-of-care within approximately 10–15 min. While rapid and convenient, rapid influenza diagnostic tests lack the sensitivity of molecular influenza diagnostics and typically require nasopharyngeal sampling, which can be uncomfortable (9).

Influenza virus requires a host cell for metabolic activity. Because viral replication primarily occurs in the respiratory tract, VOCs exhaled from the lung can be expected to change significantly during influenza infection. In vitro studies have suggested that infection of host cells by influenza virus influences volatile production. For example, headspace VOCs distinguish cells infected with influenza A, compared to both uninfected cells and cells with viral-bacterial coinfection (10) with *Streptococcus pyogenes*. Similarly, Purcaro et al. (11) investigated the ability of volatile metabolites to discriminate between respiratory cells infected and uninfected with virus, in vitro. They performed VOC fingerprinting of respiratory syncytial virus and influenza A virus, finding marked differences in VOC profiles between uninfected, respiratory syncytial virus-infected, and influenza A-infected cells.

Evidence indicates that influenza A-associated changes in VOCs are likewise present in vivo. For example, exhaled breath profiles are altered following

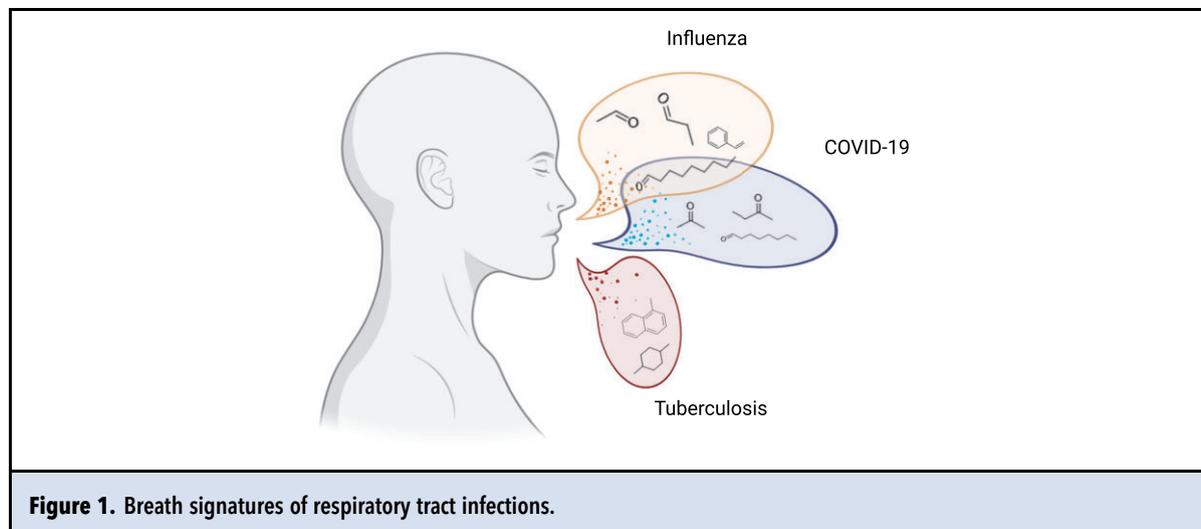


Figure 1. Breath signatures of respiratory tract infections.

intranasal live attenuated influenza A vaccination in humans (12). Live attenuated influenza A vaccination in healthy humans elicited a prompt and sustained increase in breath biomarkers of oxidative stress, in particular, the breath abundance of alkane derivatives (including 2,8-dimethyl-undecane) increases after 2 days, 7 days, and 14 days following vaccination. In similar studies, Mashir et al. (13) monitored the breath of 7 participants before and after vaccination with a monovalent (H1N1) live intranasal influenza vaccine. Exhaled nitric oxide and other exhaled breath volatiles were measured in 9 healthy healthcare workers. Nitric oxide, isoprene, and other uncharacterized volatiles were increased in the breath of vaccine recipients. Together, these studies strongly suggest that there are metabolic changes associated with the host response to influenza virus.

More recently, Traxler et al. (14) have provided additional evidence for influenza-associated breath VOC changes using the pig infection model. Breath VOC profiles of swine were analyzed longitudinally during a complete infection cycle with influenza A. Six VOCs were found to be related to disease progression: acetaldehyde, propanal, n-propyl acetate, methyl methacrylate, styrene, and 1,1-dipropoxypropane.

SARS-COV-2

SARS-CoV-2, the causative agent of the current COVID-19 pandemic, is a member of the β coronavirus family. SARS-CoV-2 is transmitted by respiratory droplets and aerosols that are released during infection (15). There are 2 primary methods for clinical diagnosis of SARS-CoV-2 infection: (a) molecular tests and antigen-based tests that quantify the amount of viral ribonucleic acid (RNA) or viral protein, respectively, in respiratory swabs; and (b) serological tests that exploit the production of virus-specific antibodies. Molecular testing requires well-equipped laboratories with a high biosafety level and skilled laboratory technicians, while antigen-based tests have suboptimal performance characteristics. Serological tests are easy to perform but require blood draws, and they are limited in clinical utility for diagnosis of acute infection, as antibodies may not yet be present early in the disease course.

Initial studies show promise for breath-based diagnosis of SARS-CoV-2 infection. In independent observational prevalence studies at Edinburgh (UK) and Dortmund (Germany), Ruzkiewicz et al. (16) recruited adult patients with COVID-19 symptoms at the time of hospital presentation. Breath samples were collected and analyzed using near-patient gas chromatography–IMS, while SARS-CoV-2 infection was established by quantitative reverse transcription PCR of respiratory samples. Multivariate analysis identified aldehydes (ethanal, octanal), ketones (acetone, butanone), and methanol as discriminating COVID-19 from other

conditions. An unidentified feature with significant predictive power for severity/death was isolated in Edinburgh, while the aldehyde heptanal had predictive characteristics in the Dortmund cohort. Differentiation of patients with COVID-19 from non-COVID-19 was possible with 80% and 81.5% accuracy in Edinburgh and Dortmund, respectively. In a similar study with a pediatric cohort (median age = 11 years for SARS-CoV-2 positive and 15 years for SARS-CoV-2 negative), Berna et al. (17) identified related aldehydes (octanal, heptanal, and nonanal) as significantly increased in the breath of children with SARS-CoV-2 infection. Aldehydes are a subproduct of the destruction of the cell membrane as a result of oxidative stress; reactive oxygen species may be generated by various types of inflammatory, immune, and structural cell in the airways. In this study, pediatric SARS-CoV-2 infection did not lead to changes in other breath biomarkers, such as acetone and 2-butanone, that were highly characteristic of COVID-19 in adults (16). The work of Berna et al. (17) provides compelling evidence that SARS-CoV-2 infection leads to characteristic VOC changes in the breath of children, as it does in adults. Compared to adults, children have a distinct immune response to SARS-CoV-2 infection and distinct clinical outcomes, with a markedly reduced likelihood of developing severe COVID-19. This physiological difference in response to viral infection may explain the differences in biomarkers enriched in the breath of adults with symptomatic COVID-19 compared to those in children.

Other researchers (18) have used real-time, online, proton transfer reaction time-of-flight mass spectrometry to perform metabolomic analysis of expired air from adults undergoing invasive mechanical ventilation in the intensive care unit, due to severe COVID-19 or non-COVID-19 acute respiratory distress syndrome (ARDS). This group found that the instrument differentiates between COVID-19 and non-COVID-19 ARDS with accuracy of 93% (sensitivity 90%; specificity 94%; area under the receiver operating characteristic curve 0.94–0.98, after cross-validation). The 4 most prominent breath volatile compounds in COVID-19 patients were methylpent-2-enal, 2,4-octadiene, 1-chloroheptane, and nonanal. Most notably, the aldehyde nonanal was previously reported as increased in patients with COVID-19 (16, 17). Interestingly, the authors also found that the VOC concentrations measured were not correlated with the severity of illness or viral load.

In related studies, Shan et al. (19) developed a breath device composed of a nanomaterial-based hybrid sensor array with multiplexed detection. The authors used the array in an exploratory clinical study in Wuhan, China, during March 2020 on healthy patients compared to those with COVID-19 or other lung infections. The training and test set data exhibited 94%

and 76% accuracy, respectively, in differentiating patients from controls as well as >90% accuracy in differentiating between patients with COVID-19 and patients with other lung infections.

More recently, other instrumental techniques such as IMS coupled with a multicapillary column (MCC–IMS) was used to evaluate nasal volatile profiles of individuals with SARS-CoV-2 or influenza infection versus uninfected controls, yielding highly accurate classification. The authors argue that as MCC–IMS could differentiate between SARS-CoV-2 and influenza A infections, different viruses may result in different host responses and, therefore, produce distinct IMS spectra fingerprints. Of note is that MCC–IMS does not provide chemical identification of peaks, unlike mass spectrometry. Independent replication is thus extremely challenging.

Finally, electronic nose devices have also been used to predict SARS-CoV-2 infection in individuals with and without symptoms presenting to a public test facility (20). In this study, the eNose sensors were combined into a composite biomarker with a ROC-area under the curve of 0.948. In 3 independent validation cohorts ($n = 3606$ in total), eNoses reliably excluded SARS-CoV-2 infection in 70%–75% of individuals, with a sensitivity ranging between 98% and 100%, and a specificity between 78% and 84%.

MYCOBACTERIUM TUBERCULOSIS

Infection with *Mycobacterium tuberculosis* (Mtb), the causative agent of tuberculosis (TB), is one of the leading causes of morbidity and mortality from infectious diseases worldwide, with an estimated 10 million new cases of active TB and 1.2 million deaths annually (21). As Mtb is a slow-growing organism, successful culture of Mtb from respiratory samples requires several weeks to confirm diagnosis and assess antibiotic resistance. Early diagnosis and treatment can prevent progressive illness and drug resistance, and therefore several studies have investigated the use of breath VOCs to diagnose TB using both human and animal models. For example, Philips et al. found a range of VOCs associated with TB. The most abundant were naphthalene, 1-methyl-,3-heptanone, methylcyclododecane, heptane, 2,2,4,6,6-pentamethyl-,benzene, 1-methyl-4-(1-methyl-ethyl)-, and cyclohexane,1,4-dimethyl-. Breath VOC markers were similar to those observed in vitro, including naphthalene,1-methyl- and cyclohexane,1,4-dimethyl- (22). The same authors found VOCs related to oxidative stress products (alkanes and alkane derivatives) and volatile metabolites such as cyclohexane and benzene derivatives, confirmed in larger international studies (226 symptomatic high-risk patients in the USA, Philippines, and UK) (23).

Beccaria et al. (24, 25) conducted 2 studies using comprehensive gas chromatography–time-of-flight mass spectrometry to diagnose active TB via breath in subjects with confirmed Mtb infection. Discriminatory volatiles yielded sensitivities of 0.82 and 1.00 and specificities of 0.92 and 0.60 in the training and test data, respectively. In a second study in Haiti, the same analytical technology was used in individuals with sexually transmitted infections as control group (25). Analysis using 3 random forest models generated a panel of 46 breath features. The 22 common features within each random forest model used were selected as a set that could distinguish between Mtb-infected and -uninfected populations. Eight out of 22 were tentatively identified and one of these (2-butyl-1-octanol) was identical to a candidate biomarker reported in previous studies on TB-associated breath VOCs (23).

In more recent studies, Bobak et al. (26) investigated whether exhaled breath from children has diagnostic utility in a pilot South African study of pediatric patients diagnosed with confirmed TB. The group found that 4 compounds in breath characterize children with a confirmed TB diagnosis from patients unlikely to have TB with an alternate lower respiratory tract infection (LRTI). These analytes comprised decane and 4-methyloctane, as well as 2 unconfirmed analytes. Machine learning accurately classified children with confirmed TB ($n = 10$) from children with another LRTI ($n = 10$) with a sensitivity of 80% and specificity of 100% observed across cross-validation folds.

Altogether, these studies strongly support that Mtb infection in both adults and children is characterized by distinct breath odor profiles. Further support for Mtb-associated breath volatiles comes from several studies in both murine and primate models of infection (27–29). However, the variability in VOC profiles in pediatric versus adult patients with Mtb, as well as between adult studies, highlights the need to standardize methods for breath collection and analysis to improve the ability to discern replicability.

PSEUDOMONAS AERUGINOSA

Pseudomonas aeruginosa is an important pulmonary pathogen, particularly in individuals with cystic fibrosis (CF), a genetic disease caused by mutations of the gene encoding the CF transmembrane regulator. Lungs of patients with CF may become colonized or infected with *P. aeruginosa*, with both short- and long-term impacts on pulmonary function and overall prognosis. Early diagnosis of *P. aeruginosa* in individuals with CF would be highly advantageous.

P. aeruginosa itself produces volatile compounds in culture that may be harnessed for breath-based diagnosis of colonization and/or infection. For example, Scott-Thomas et al. (30) measured 2-aminoacetophenone

(2-AA) by GC–MS in patients with CF as a volatile biomarker produced directly by *P. aeruginosa*. This volatile compound, which accounts for the characteristic “grape-like” odor of cultured *P. aeruginosa*, is consistently produced by *P. aeruginosa* strains and is detectable in the headspace of *P. aeruginosa* cultures by GC–MS and SPME–GC–MS (30–32). Significantly higher levels of 2-AA were found in exhaled breath of patients with CF when colonized with *P. aeruginosa*, while the concentration of this metabolite was below the detection limit in both control groups (healthy subjects and CF patients colonized with other bacteria species). A follow-up study revealed the presence of 2-AA in a variety of foods, suggesting that the consumption of such foods may lead to a false-positive breath test for 2-AA. In response, the authors recommended improved breath sampling procedures, in order to avoid this potential confounder (33). Cultured *P. aeruginosa* also commonly produces a different volatile, methyl thiocyanate (34). Methyl thiocyanate is also enriched in the breath of children with CF and *P. aeruginosa* colonization, in the range of 2–21 ppbv (median 7 ppbv) (34).

Untargeted VOC profiling of individuals with *P. aeruginosa* infections has also been performed to identify additional biomarkers of infection. For example, an ion mobility spectrometer coupled with a multicapillary column was used to examine the exhaled breath of individuals infected or colonized with *P. aeruginosa*, compared to healthy, nonsmoking control patients. The volatile profiles were markedly different between these groups, with positive and negative predictive values of 83% and 86%, respectively (35). Finally, breath volatiles were profiled in a cohort of children with and without CF (36). A distinct VOC profile consisting of 22 VOCs enabled 100% correct discrimination of children with CF. Furthermore, only 10 VOCs were required to predict *P. aeruginosa* culture positivity with high (92%) accuracy, based on C5–C16 hydrocarbons and *N*-methyl-2-methylpropylamine (36).

ASPERGILLUS FUMIGATUS

Aspergillus fumigatus is an opportunistic fungal pathogen that causes life-threatening invasive pulmonary infections [invasive aspergillosis (IA)] among immunocompromised patients. Symptoms of IA are nonspecific, and diagnosis of IA in its early stages remains difficult due to major limitations in diagnostic tests for IA, including radiologic imaging techniques, cultures, detection of fungal antigens in the serum and bronchoalveolar lavage (BAL) fluid, and *Aspergillus* polymerase chain reaction. A sensitive, rapid, and accurate diagnostic assay for invasive aspergillosis would be highly advantageous in clinical management of immunocompromised patients with suspected IA.

Like *P. aeruginosa*, *Aspergillus* spp. are also an excellent model for use of in vitro volatile profiling to identify VOCs that may be present during respiratory infection. In vitro studies of cultured *Aspergillus fumigatus* cultures have identified 2-pentylfuran (2-PF) as a candidate fungal volatile metabolite (37, 38). Interestingly, 2-PF was enriched in breath samples of individuals with CF with positive cultures for *Aspergillus*, but was not present in healthy controls, suggesting that 2-PF reflected the active metabolism of *A. fumigatus* in the airways. Similarly, Koo et al. (39) sought to identify volatile metabolites of *A. fumigatus* that might be useful for noninvasive diagnostic development. The authors prospectively collected breath samples from patients with suspected invasive fungal pneumonia and assessed whether they could discriminate patients with proven or probable IA from patients without aspergillosis. They identified distinctive terpene secondary metabolites in the volatile metabolome of the most common pathogenic *Aspergillus* species in vitro (monoterpenes camphene, α - and β -pinene, and limonene, and the sesquiterpene compounds α - and β -*trans*-bergamotene). Detection of α -*trans*-bergamotene, β -*trans*-bergamotene, a β -vatenene-like sesquiterpene, or *trans*-geranylacetone was diagnostic of IA with 94% sensitivity and 93% specificity. Interestingly, the sesquiterpene metabolites found in breath did not perfectly overlap with those found in vitro, and the biological functions of these and other fungal terpene metabolites remain an open question (40).

Other Infectious Diseases

Breath analysis is a natural area of investigation for respiratory infections, in which the respiratory tract itself comprises the host–pathogen interface. However, the alveoli of the lungs are a physiological site of gas exchange between the human host and the outside world. Volatile compounds produced anywhere in the body are likely to circulate in the bloodstream and partition down their concentration gradient into breath exhalate. For this reason, breath VOCs can be thought of as a noninvasive lens into the physiological condition of the entire body, including infections at distal body sites from the lungs. Here we present the examples of *Helicobacter pylori* gastrointestinal infection and *Plasmodium falciparum* malaria infection as evidence that nonrespiratory infections may also produce specific breath volatiles that may be used for disease diagnosis.

HELICOBACTER PYLORI

Helicobacter pylori is a globally important and incredibly common bacterial pathogen, infecting approximately 50% of the world population. While infections may be mild or asymptomatic, prolonged carriage can result in functional

dyspepsia or gastric cancer (41). While diagnosis is important to prevent long-term sequelae, the gold standard for *H. pylori* diagnosis is endoscopy with biopsy and histopathological examination of tissue, which remains largely infeasible in low- and middle-income settings worldwide.

In 1996, the Food and Drug Administration approved the ^{13}C -urea breath test (UBT) for diagnosis of *H. pylori*. The premise of the ^{13}C -UBT is a diagnostic increase in urease activity, which is otherwise low in the absence of infection. Bacterial urease converts orally administered urea to labeled $^{13}\text{CO}_2$, which is excreted from the body via breath. The test has proven to be useful in clinical management of suspected *H. pylori* infection, as well as in monitoring the response to antibiotic therapy. While there is substantial study-to-study variability in performance characteristics of the ^{13}C -UBT, a recent meta-analysis indicated a pooled sensitivity is >95% with a specificity of 93% (42). Due to the need for specialized equipment and infrastructure to manage radioactive material, the UBT is still limited in its global applicability. However, the UBT demonstrates many of the promised advantages of breath-based testing strategies, especially when compared to biopsy, including the noninvasive nature of the test and reduced sampling error that can otherwise lead to false negative results from biopsy of a disease with a nonuniform tissue distribution.

Similar breath tests include the hydrogen breath test (HBT), which was developed with the aim to quantify hydrogen producing bacteria that reside in the colon. An application of the use of HBT is in the detection of small intestinal bacterial overgrowth (SIBO) (43).

MALARIA

Plasmodium falciparum, the causative agent of severe human malaria, remains a critical global health concern, particularly for young infants and children who are uniquely susceptible to serious disease and death. Rapid diagnostic testing based on detection of the *P. falciparum* protein HRP has been transformative for malaria control, but it is limited by high false-positive results. In addition, HRP2-based testing is at risk due to emergence and spread of *P. falciparum* strains that no longer produce HRP2 (44). There is an urgent need for new malaria diagnostics, and the World Health Organization has declared this a key global health priority (45).

Interest in odor-based detection of malaria, a mosquito-transmitted infection, arose from the intriguing evidence that humans infected with *P. falciparum* may be more mosquito-attractive than uninfected humans (46, 47). In pursuit of *Plasmodium*-produced odorants, Kelly et al. (48) found that cultured *P. falciparum*-infected erythrocytes release the plant-like terpenes α -pinene and limonene, likely originating from the apicoplast of the parasite, an unusual organelle with a similar origin to the chloroplast. In a follow-up study, Schaber et al.

(49) investigated whether these terpenes were present in breath during natural human malaria, through analysis of breath samples from Malawian children with fever, with and without *P. falciparum* infection. Malaria infection was associated with global differences in breath VOC composition, with 6 breath volatiles found to be highly correlated with infection status: methyl undecane, dimethyl decane, trimethyl hexane, nonanal, isoprene, and tridecane. The only compound that had previously been associated with *Plasmodium* infection was nonanal (50). Although *Plasmodium*-infected individuals had decreased levels of skin-emitted nonanal when volatiles were sampled from the arm (50), skin-emitted nonanal was found increased in the feet of *Plasmodium*-infected individuals (51). Schaber et al. also found significantly increased breath levels of 2 terpenes, α -pinene and 3-carene, which are recognized by *A. gambiae* odorant receptors (48).

These findings in natural pediatric malaria infection are distinct from the previous breath metabolite findings reported by Berna et al. (52) from a population of naïve healthy adults undergoing controlled experimental human infection with *P. falciparum*. Berna et al. had previously reported several breath thioethers that served as biomarkers for *P. falciparum*. These compounds were not detected in the pediatric study, suggesting that parasite densities, parasite stage, or age or immune status of the host might induce a range of physiological changes in the human body that manifest in distinct breath and body odor profiles (49).

A summary of volatile organic compounds associated with infectious diseases found in breath and in vitro is shown in Table 1.

Conclusions and Perspectives

Breath has emerged as an attractive, noninvasive strategy to explore global metabolic changes during health and disease in humans. Modern analytical instruments (e.g., GC-MS and gas chromatography-IMS) now readily and reproducibly detect the low levels of analytes in the breath and facilitate biomarker discovery. Many challenges remain before breath analysis can become routine for clinical use. These challenges include standardization of breath sampling, analytical methods, and data analysis, as well as independent validation of candidate markers in distinct, diverse populations with appropriate clinical controls. The emergence of SARS-CoV-2 and the current COVID-19 pandemic may provide the necessary impetus to drive this standardization and advance the field. Recently, RADx Radical, a National Institutes of Health (NIH) initiative to accelerate SARS-CoV-2 diagnostics, has included active engagement of researchers to support the development of innovative diagnostic technologies, including breath-based diagnostics (53).

Table 1. Infectious diseases associated with breath and in vitro volatile organic compounds (VOCs).		
Disease	Volatiles	References
Respiratory tract infections		
Influenza	alkane derivatives (including 2,8-dimethyl-undecane) nitric oxide isoprene acetaldehyde propanal n-propyl acetate methyl methacrylate styrene 1,1-dipropoxypropane	(12-14)
SARS-CoV-2	ethanal acetone butanone methanol octanal heptanal nonanal methylpent-2-enal 2,4-octadiene 1-chloroheptane	(16-18)
<i>Mycobacterium tuberculosis</i>	naphthalene,1-methyl-3-heptanone methylcyclododecane heptane 2,2,4,6,6-pentamethyl- benzene 1-naphthalene, 1-methyl- 1-methyl-4-(1-methylethyl)- cyclohexane 1, 4-dimethyl- 2-butyl-1-octanol decane 4-methyloctane	(22, 23, 25, 26)
<i>Continued</i>		

Table 1. (continued)		
<i>Pseudomonas aeruginosa</i>	2-aminoacetophenone methyl thiocyanate C5-C16 hydrocarbons N-methyl-2-methylpropylamine	(30, 34, 36)
<i>Aspergillus fumigatus</i>	2-pentylfuran camphene α - and β -pinene limonene α - and β -trans-bergamotene	(37-39)
Other infectious diseases		
Malaria	terpenes α -pinene limonene methyl undecane dimethyl decane trimethyl hexane nonanal isoprene tridecane 3-carene thioethers	(48, 49, 51, 52)

NIH logistical and financial support, data coordination, and oversight will serve to facilitate data harmonization, promote standardization of breath analysis, and reproducibility across institutions.

Few studies so far have addressed the chemical and biological origin of the complex collection of VOCs found in human breath, many of which are not known metabolic byproducts of human metabolites. As noted before, there is strong evidence to indicate that several breath VOCs arise from microbial secondary metabolism (e.g., *Pseudomonas aeruginosa*, *Aspergillus fumigatus*, and *P. falciparum* malaria parasites), at least in the setting of colonization or infection. Many other breath VOCs, especially those that increase in abundance during viral infection, likely arise from the host response. Understanding the cellular and chemical origin of breath volatiles associated with disease states will be an important tool for predicting whether a given biomarker is likely to be specific to a given disease.

Of the infectious diseases discussed in this review, tuberculosis may be closest to clinical translation into a “TB breathalyzer.” Compelling and reproducible studies have compared human and animal breath data (24–28) paired with studies of in vitro cell culture (29). However, most in vivo and in vitro investigations on tuberculosis have deployed novel sensors, including biosensors such as African pouched rats (54) and analytical chemistry methods. An important step forward for clinical translation will be the unequivocal chemical identification of biomarkers.

Finally, as development of breath-based diagnostics progresses, investigators must continue to validate biomarker performance across the entire target patient population. Despite the importance of infections to child health and the often-distinct clinical immunological responses to infection in children compared to adults, children remain underrepresented in studies of breath biomarkers of infection. Including pediatric populations in biomarker discovery and validation studies is essential to develop breath-based diagnostics with wide applicability.

Nonstandard Abbreviations: VOCs, volatile organic compounds; TD, thermal sorption; IMS, ion mobility spectrometry; MCC–IMS,

IMS coupled with a multicapillary column; Mtb, Mycobacterium tuberculosis; TB, tuberculosis; CF, cystic fibrosis; UBT, 13C-urea breath test.

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