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Physio-Metabolic Monitoring via Breath Employing Real-Time Mass Spectrometry: Importance, Challenges, Potentials, and Pitfalls

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Contents

Abstract A tiny fraction of our breath contains trace volatile organics of various chemical classes. Due to their endogenous and/or exogenous origins, these volatiles can denominate many intrinsic and extrinsic effects. Exhaled volatile profiles are super dynamic in nature and their expressions may change from seconds to years. Exhaled volatile concentrations largely depend on normal or abnormal fluctuations in physiological and metabolic attributes. Minute or pronounced alterations in cardiorespiratory and other bronchopulmonary gas-exchange parameters due to simple changes in respiratory patterns, routes, and rhythms, posture, expiratory/inspiratory flow, and upper-airway resistance can immediately affect volatile

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profiles. Similarly, the subject's age, gender, sexual orientation, metabolic state or status, diet, nutrition, therapy, lifestyle habits and habitats, menstrual phases, contraception, pregnancy, menopause, as well as any acute or chronic condition and comorbidities may cause transient or long-lasting differences in breath compositions. Applications of real-time mass spectrometric techniques along with alveolar sampling enabled us to frame fast occurring and continuous changes under diverse physio-metabolic conditions. Physio-metabolic conditions affected breath components more pronouncedly than the differential expression proposed as disease biomarkers in the literature. Investigations of such regulating factors helped us to develop the present state of the art for clinical breath sampling and analysis. Besides, assessments of ventilation and hemodynamics driven changes in exhaled volatiles have depicted potential for physio-metabolic monitoring. Longitudinal personalized analysis of breath profiles may offer unconventional path toward pathophysiological and therapeutic monitoring.

Keywords Pathophysiology · Biomarkers · Breath analysis · Metabolism · Monitoring · Omics · Physiology · Sampling · Standardization · Volatile organic compounds (VOCs)

Abbreviations

COVID-19	Coronavirus disease 2019
FeNO	Fractioned exhaled nitric oxide
IMS	Ion mobility spectrometry
ppbV	Parts per billion by volume
pptV	Parts per trillion by volume
PTR-ToF-MS	Proton transfer reaction–time of flight–mass spectrometry
SARS-CoV-2	Severe acute respiratory distress syndrome – coronavirus 2
SESI-MS	Secondary electrospray ionization–mass spectrometry
SIFT-MS	Selected ion flow-tube–mass spectrometry
VOCs	Volatile organic compounds

1 Introduction

A very tiny fraction (<1%) of our breath contains hundreds of volatile organic compounds (VOCs). These compounds belong to various substance classes and the tracible concentrations of most of these VOCs range between parts per billion and parts per trillion by volume (ppbV–pptV) levels [1]. Many of these volatiles are exogenous – meaning that those are accumulated from our habits, habitats, diet, lifestyle, therapy, etc. For instance, if we smoke a cigarette, we will exhale considerable amounts of acetonitrile and furan [2, 3]. Visiting a fuel station will source

benzene and toluene, whereas entry to a hospital may increase breath isopropanol and formaldehyde concentrations. If we drink orange juice, limonene will become abundant in our breath and a visit to the forest will do the same for alpha-pinene [4]. On the other hand, some other VOCs are potentially endogenous – meaning that those are produced within us via various physiological, metabolic, biochemical, systemic microbial, and/or pathophysiological processes at the cellular/organ levels [5, 6]. For example, acetone is known to originate from cellular and/or hepatic glycolysis and lipolysis [7]. Ammonia, dimethyl, and trimethylamine are linked to protein catabolism [8, 9]. Organosulfur such as dimethyl sulfide, methanethiol, and butanethiol are produced by anerobic methylation by the systemic microbiota of our lower gut [8, 10]. Nonetheless, some substances such as isopropanol, ethanol, acetic acid, acetaldehyde, and acrolein have mixed origins – means that they are sourced within and without. Irrespective of the origin (after being produced in vivo or being accumulated/stored from outside), all VOCs are transported via blood to our lungs, and thereafter, they are released during the bronchopulmonary gas-exchange process and thereby are found in breath. Therefore, profiling of these substances may offer non-invasive, rapid, repeatable, and beyond conventional insights into various systemic phenomena, events, or status [11].

While largely abundant breath gases, e.g., oxygen (O_2) and carbon dioxide (CO_2), are conventionally applied in human medicine for diagnosis and monitoring of certain conditions, nitric oxide (NO) is the only trace gas, which is vividly investigated for point-of-care (PoC) applicability [12–14]. As NO is produced within the proximal airways and inflammation facilitates its production, the fractioned exhaled NO (FeNO) is well attributed to allergic asthma [13, 15]. Nonetheless, the scenario is substantially different in case of other trace VOCs. Same substances were proposed in independent studies as biomarker for different diseases [15–17]. Despite many efforts to find, propose, or establish unique volatile (profiles or patterns) as disease- or event-specific biomarkers in different studies, none of those could pass the independent validation tests [18]. Consequently, the trace volatiles could not enter into routine clinical practice yet.

In fact, almost all the VOCs are present in everyone's breath, and if we simply measure or compare the exhaled compositions of diseased patients with that of an age- and gender-matched healthy cohort, it is very unlikely to come across any unique VOC profile or pattern [19, 20]. After decades of breath analysis, the focus has shifted toward the detection of changes in exhaled VOC concentrations rather than expecting a unique marker. Meanwhile, a series of systematic investigations of immediate, transient, and/or persistent physio-metabolic effects on VOC profiles (mainly from healthy human subjects) helped us to realize the extremely dynamic nature of breath volatiles [21–25]. Changes observed in these physio-metabolic studies turned out to be even more pronounced than those published as biomarkers for diagnosis (even for early detection!) in many cross-sectional (healthy vs. sick) studies. Real-time mapping of such dynamic nature under various conditions along with the available knowledge and fundamental understanding of human physiology, metabolism, and analytical chemistry are the indispensable prerequisites to eventually translate VOCs into clinical applications [5].

2 The Dynamic Nature of Exhaled VOCs

Exhaled VOC concentrations are affected via immediate or precedent extrinsic and intrinsic factors such as environment exposure, diet and lifestyle habits, healthy physiological (respiratory, hemodynamic) and metabolic attributes, systemic microbial activity, as well as any health condition and/or therapy [25–27]. Putative breath markers not only differ interindividually but also may change instantly or over time within the same individual. Even if we succeed to rule out and exclude various extrinsic factors and/or pathological effects, simple and daily life fluctuations in normal and healthy physiology (ventilation and hemodynamics) and/or metabolism may alter our VOC profiles significantly. Besides relying upon our physio-metabolic and health status, such changes and differences are also closely related to the potential origins, physicochemical characters, exhalation kinetics, and compartmental distributions of these VOCs. Thus, while thinking of the dynamic nature of the breath volatiles, a fundamental question does arise. How dynamic are these VOCs or how long does it take to change concentrations?

Well, the actual dynamic timeframe for VOC concentrations ranges from seconds to years.

VOC concentrations can change within seconds simply due to changes in our normal breathing patterns. For example, if we hold our breath for a few seconds, sudden and profound changes are observed after the breath holding phase [21, 28]. Such changes are substance specific and depend on the origin and physicochemical characters such as solubility, volatility, and blood-gas partition coefficients of the VOCs. Concentrations of substances with low aqueous solubility, e.g., isoprene, benzene, furan, and acetonitrile, will increase significantly due to perfusion-limited accumulation during breath holding. In contrast to that, compounds like acetone will remain almost constant due to high solubility – that will allow it to get absorbed within the surrounding lung tissue/compartments. On the other hand, oral microbiota-originated VOCs, e.g., hydrogen sulfide, will decrease via washout due to increased respiratory rate (physiological compensation) after breath holding. The physiological effects due to breath holding of 10–60 s by healthy adults are neutralized to baseline within 8–10 breaths.

VOC concentrations will change within minutes if we just switch our body positions [22]. For instance, if you are reading this book by sitting on a chair, and you suddenly switch to supine posture, your cardiac output and pulmonary perfusion will change immediately resulting in increased exhalation of isoprene and similar substances, whereas compounds like acetone and alike compounds will remain unaffected by hemodynamic effects.

VOC concentrations may reflect metabolic changes taking place within hours or throughout the day [29, 30]. If we continuously measure breath VOCs from early morning till evening, systemic changes will be observed on VOC exhalations. Intake of standard breakfast and lunch will cause systematic postprandial metabolic adaptation via hyperglycemia and corresponding oxidative stress, which will be reflected

in the time profiles of substances such as acetone, 2-propanol, pentanal, dimethyl sulfide, and isoprene.

Changes in VOC concentrations are reported to occur within days, weeks, and also throughout the month. While looking at the natural menstrual cycle in pre-menopausal adult women, exhaled VOC concentrations largely depend on different phases of the monthly endocrine regulation [31]. Endogenous substances such as ammonia, isoprene, acetone, and dimethyl sulfide closely mirror many well-known effects of the female sex hormones on various metabolic pathways [32–35]. Natural interplay between estrogen and progesterone levels at the period, follicular, ovulation, and luteal phases is reflected distinctly on VOC profiles. Such changes differed significantly in adults undertaking daily oral contraceptive pills (i.e., comprised of supplementary female sex hormones). Similarly, longitudinal changes in VOC concentrations take place throughout pregnancy [36, 37]. In pregnant women, various physio-metabolic effects such as gestational endocrine changes, embryonic development, fetal oxygen and nutrient demand-driven increase in cardiac output and respiratory rate, increased cholesterol biosynthesis, and altered breathing pattern via diaphragm upliftment and physiological hyperventilation cause progressive changes in VOC exhalations [37–40].

If we look into the healthy aging process, exhaled expressions of many endogenous and exogenous VOCs differ significantly, based on our biological age. Recent analysis of breath VOCs from a large cohort of healthy females aged between 7 and 80 years has depicted substance-specific changes in breath composition. Breath concentrations of endogenous aldehydes, alcohols, organosulfur, short-chain fatty acids, alkene, ketones, and exogenous nitriles, aromatics, and terpenes have indicated physio-metabolic milieu between endocrine homeostasis, oxidative stress, gut and pulmonary microbial diversity/activity, energy metabolism, and lifestyle habits [41–45].

Due to such dynamic nature, it is utterly critical to trace and translate the actual pathophysiological information from breath VOC expressions – as those are often overridden by the everlasting physio-metabolic effects. Continuous breath-resolved profiling of VOC concentrations under different physio-metabolic conditions may pave the path for framing the complex behavioral dynamics and exhalation kinetics of the potential VOC biomarkers in real time.

3 Mass Spectrometric Methods for Real-Time Breath Analysis

Despite the fact that gas-chromatography and mass spectrometry (GC-MS) has been applied for many years as the gold standard for trace gas analysis [46, 47] at low ppbV–pptV levels, punctual measurements could not provide the actual insight into the dynamic nature of breath VOCs. Further, unavoidable confounding factors related to offline mass spectrometry such as preconcentration steps, sample

collection (e.g., blowing into bags, mixed breath phases), sample storage time/conditions, and analysis time remained as everlasting challenges. Inception of end-tidal CO_2 controlled manual or automated breath sampling in glass syringes or in micro-extraction-based traps (e.g., needle-trap microextraction) enabled researchers to collect the alveolar fractions of VOCs, which represents the actual systemic/blood concentrations [48, 49]. Nevertheless, fast occurring changes (within seconds or a minute) remained untraceable via offline MS methods.

Development and application of real-time MS techniques, e.g., selected ion flow-tube (SIFT)-MS, proton transfer reaction (PTR)-quadrupole-MS, and PTR-time of flight (ToF)-MS [50], along with online end-tidal/alveolar sampling have eventually overcome various confounding influences [51–53]. In principle, a SIFT and a PTR use alike ionization principles based on various primary/reagent ions such as hydronium (H_3O^+), NO^+, or O_2^+. Both of the instruments allow switching between reagent ions according to diverse analytical requirements. Given the fact that most of the breath VOCs belong to the relatively lower mass range (<500 Da) and have higher proton affinity than water, in the field of breath analysis, soft ionization via H_3O^+ ion is desirable to have minimal fragmentation [21, 23].

PTR-ToF-MS, H_3O^+ ions are produced via cathode discharge on pure (99.99%) water vapor. After production they are pulsed to the next chamber (drift tube) where the proton transfer reaction takes place. The breath sample is introduced to this chamber to react with H_3O^+ ions. Based on the proton affinity, VOCs react with H_3O^+ ions and get protonated ($VOC + H_3O^+ \rightarrow VOCH^+ + H_2O$). After that, protonated VOCs are detected via a quadrupole-MS or a ToF-MS according to their mass/charge ratio. Introduction of a ToF allows us to achieve mass resolution of 1,000–4,500 m/Δm that can assign volatiles upon their measured mass and corresponding sum formula with high precision as well as enable isobaric separation of VOC masses [54–56]. Unlike GC-MS, no sample preparation, preconcentration steps, and storage are required. Application of constant inlet flows in side-stream mode (in order to avoid interference to the mainstream of breathing) can uniformly introduce samples to the drift tube and measure at high time resolution in milliseconds (ms). For instance, studies have demonstrated application of 200 ms in clinical environment to simultaneously measure rapid changes of VOC concentrations in the ambient air and in the exhaled breath of healthy subjects or ventilated patients [24, 57, 58]. Here, in every 200 ms a data point was recorded and, on each data point, all protonated VOCs were measured as per mass/charge ratio. Thus, the assignment of VOCs at the exhaled alveolar plateau is also possible in real time. Besides continuous measurements, assignment of inspiratory and end-tidal breath phases (via customized data processing algorithms) helps to date the alveolar fraction of VOCs in a breath-resolved manner [21, 54]. For instance, signal intensity of an endogenous and blood-borne VOC (e.g., acetone or isoprene – abundant in exhalation) can be used to denominate the expiratory and inspiratory phases of breath. Based on the area/mass range of interest, mass scale can be recalibrated in desired time intervals. For clinical breath studies, 21.0226 Th (H_3O^+-isotope), 29.9980 Th (NO^+), and 59.049 Th (protonated acetone) can be used for mass scale calibration because of their natural abundances in expired and inspired air [52].

Secondary electrospray ionization-mass spectrometry (SESI-MS) has enabled ambient ionization via nano-electrospray-driven positive (protonated) and negative (deprotonated) ions that collide with sample analytes within the gas phase [59]. Detection of relatively large, semi-volatile, and even nonvolatile molecules is plausible by integrating high-resolution mass spectrometers such as Orbitrap-MS [60]. Nevertheless, the identification of substances with higher molecular weights is extremely challenging – especially where internal standards are not available for such mass ranges [61]. SESI is at an early stage and offers vivid scope for further optimizations and advancements in order to bridge other downstream omics (e.g., metabolomics, proteomics, and lipidomics) with volatolomics and exhaled breathomics.

In contrast to real-time MS techniques for nontargeted screening of VOCs, simpler methods such as electronic noses (eNOSE) or differential ion mobility spectrometry (DMS/IMS) are suitable for targeted approaches [62]. Artificial olfaction is often conducted via chemical, nano-optical sensors as well as via customized laser-based spectroscopy methods. Despite chemical and nano-optical methods being relatively cheap, easy to use, adaptable (e.g., integration of certain gas sensing arrays as per analytical requirements), and PoC applicable [63], they suffer from many demerits. These methods do not allow an unequivocal substance identification due to limited selectivity and specificity but offer promising perspectives for PoC breath tests, once marker substances are defined. Susceptibility to matrix effects, e.g., humidity, temperature, and complex sample compositions, is also an important disadvantage for eNOSEs and IMS.

4 Physiological and Metabolic Effects on Breath Biomarkers

Soon as it was realized that "magic bullet" biomarkers may not exist and pursuing VOC concentration changes under various pathophysiological conditions is more important and realistic than looking for unique biomarkers, the research focus was imposed on framing the factors that are affecting VOC concentrations. Gradually it is realized that "we are our actual challenge/problem." Being human, our own physiology and metabolism affect our VOC profiles more critically than other external factors.

After being produced or stored in vivo, VOCs undergo various metabolic cascades (regulated by our enzyme systems at cellular, systemic microbial, and organ levels, e.g., liver, gut, and muscle) and larger hydrocarbons often break down into smaller molecules. Thereafter, VOCs are carried by blood and pass through other body compartments where they are distributed/redistributed further. For instance, lipophilic substances are absorbed within the fatty compartments [64]. The lung is our blood–gas interface and the alveolar gas-exchange process is largely denominated by pulmonary ventilation-perfusion (VQ) mechanism and the

distributions of blood flow and air in lung compartments. Consequently, VOC exchange is closely relying on the pulmonary ventilation/perfusion (V/Q) ratios, i.e., primarily regulated by cardiac output and minute ventilation [26, 65–67]. After being released in alveolar air, VOCs are further distributed/redistributed within alveolar compartments (due to collateral ventilation between fused alveoli), undergo dilution via airway dead space and substances (e.g., NO and acetone) originating from airway epithelium, and are taken up via extra-alveolar exchange [24, 67]. Mechanisms such as pre-alveolar absorption and post-alveolar revalorization are also playing a crucial role in VOC modifications. Therefore, it is important to achieve a steady state of physio-metabolic interplay, while reproducible breath samples can be collected under minimal and systematic influence from subject's own physiology and metabolism.

Minute muscle movements during sleep or vigorous muscle activity under exercise are well known to reflect physio-metabolic effects on breath VOC profiles in real time [68–71]. The anerobic threshold under exhaustive exercise (e.g., by following step-wise and incremental ramp protocols) can be determined by means of VOC-based modeling of lactate threshold and ventilatory threshold. Besides the changes during movements, VOC profiles are also affected by normal physio-metabolic effects at rest.

In addition to the aforementioned effects from altered breathing patterns (e.g., breath holding) and/or posture, simple changes in breathing route during breath sampling can also cause substance-specific changes in breath composition at rest. For instance, if you are breathing in and out via mouth and suddenly (even unconsciously) switch to nasal breathing, immediate changes will take place in exhaled VOC concentrations [25]. Substances originating from the nasal cavity bacteria (e.g., methyl-propyl sulfide) will occur and substances originating from the oral cavity (e.g., H_2S and allyl-methyl sulfide) will immediately reduce in concentrations. Effects will be also seen on substances regulated by ventilation and hemodynamics. For example, switching from nasal to oral breathing will significantly reduce isoprene concentrations. Isoprene is negatively correlated to minute ventilation. Therefore, bypassing the nasal cavity dead space (i.e., 70–80 ml) will increase the minute ventilation at oral breathing and thereby will reduce isoprene exhalation.

Similarly, if we simply blow our breath into bags or canisters via small straws (i.e., <1 cm of diameter), the uncontrolled upper-airway resistance from the small breathing orifice will immediately affect our exhaled constituents [72, 73]. Reduction of the breathing mouthpiece diameter has shown substance-specific effects. Such effects depend largely on the breathing resistance-driven changes in respiratory and hemodynamic parameters [72]. Alveolar eliminations of VOCs with relatively higher volatility are increased due to airway resistance-driven negative intrathoracic pressure (at inspiration), which instantly alters the alveolar diffusion gradient and respiratory mechanics.

Spontaneously breathing human subjects (even if healthy) start to hyperventilate once they are asked to breath normally via a mouthpiece or mask [74]. Application of paced breathing (i.e., metronome-controlled via sound beats or via visual guide) can be used for breath sampling in order to keep subjects within the normal

respiratory rate of 10–14 breaths/min. Researchers observed that the intra- and interindividual ventilatory variations in exhaled VOCs increase significantly during paced breathing. On the other hand, switching between spontaneous and paced breathing causes immediate changes in exhaled concentration and variations of endogenous and blood-borne VOCs [75]. Such changes depended on minute ventilation and CO_2 exhalation. Any conscious and voluntary effort of breathing induces autonomic control and momentarily overwrites the natural automatic control of breathing by our respiratory center. Paced breathing, therefore, induces autonomic function that hampers the normal inspiratory:expiratory (I:E) ratio of ~1:2 and increases the minute ventilation. Switching to spontaneous breathing gradually resumes automatic control of breathing and thereby neutralizes the minute ventilation and associated ventilatory variations in CO_2 and VOC exhalations.

Moreover, simple changes in exhalation time and expiratory flow may cause profound effects on VOC exhalations [24, 76]. For instance, if we normally expire our expiratory reserve volume (i.e., maximum exhalation), substances like isoprene, furan, dimethyl sulfide, and allyl-methyl sulfide will increase immediately by mirroring the end-tidal CO_2 profile. This happens mainly due to the change in alveolar slope of exhalation and increased blood-gas contact time which facilitate collateral exchange of gases and VOCs (with low aqueous solubility) between the alveolar compartments [24]. On the other hand, if we perform a forced expiration, those VOCs will decrease instantly due to dilution effects, whereas other substances like acetone will increase significantly as the decelerating flow of exhalation may induce bronchial contribution of such VOC with aqueous miscibility and high blood-gas partition coefficient.

Alongside the above-described immediate and transient effects during sampling, long-term effects and differences due to subject's age, gender, sexual aerosol and orientation, menstrual cycle, pregnancy, menopause, and circadian metabolic rhythms are important attributes for comparison of VOC expressions between cohorts [5, 31, 77, 78]. Such factors increase the overall heterogenicity and randomness in breath data and call for more fundamental investigations to address physio-metabolic crosstalk with VOC exhalations. As physio-metabolic effects are everlasting and unavoidable during breath sampling, a basic understanding of those beyond analytical effects is extremely important for interpreting observations pragmatically.

Therefore, no matter how sophisticated and high end the analytical instrument we may use, if we cannot collect a standardized breath sample, the obvious physio-metabolic effects at the time of sampling may induce unsupervised effects that are sufficient to mislead our clinical interpretations. During the last decade, the importance of standardization of breath sampling came into focus and several taskforces were formed by the *International Association of Breath Research (IABR)* to address the relevant factors and state of the art for clinical breath sampling and analysis [20, 79]. The following section will briefly summarize the lessons learned during the efforts for standardization including the dos and don'ts.

5 Standardization of Real-Time Sampling for Breath Analysis

Around 20 years ago, alveolar sampling came into consideration in order to represent systemic/blood concentrations of VOCs. Thus, confounding effects from mixed-alveolar (i.e., including anatomical dead space of airways, trachea, and mouth/nasal cavity) sampling were minimized significantly reduced. Certainly, the case will be different if airways are of potential interest of a study – such as in obstructive and restrictive tracheobronchial conditions.

During the last 10 years, continuous real-time monitoring of VOCs under various breathing maneuvers at rest have enabled us to knock out the key factors that are essential to collect reproducible breath samples with minimal physio-metabolic influences. Reliable sampling at rest in consciously breathing humans should consider the following crucial aspects:

- *Avoid muscle movements:* During sampling, subjects should maintain relaxed body postures (e.g., sitting or supine) without having any unnecessary muscle movement [22]. These mainly include voluntary or unmindful movements of limbs and other body parts [68]. Even minute and/or involuntary muscle movements must be recorded for data interpretations.
- *Alveolar/end-tidal sampling:* As indicated earlier, if airways are not of the study interest, breath sampling must extract the alveolar phase of the breath [64, 80]. In case of continuous breath-resolved measurements, exhaled alveolar and inspiratory phases should be determined during data analysis for valid interpretations of systematic VOC concentrations.
- *Subjects breathing patterns*: Breathing pattern must remain as spontaneous, constant, and normal as possible during sampling [21]. That means parameters such as respiratory rate, minute ventilation, respiratory flow, and I:E ratio should remain constant throughout the sampling phase. Subjects should not perform prolonged (slow breathing with deeper inhalation and exhalation) or forced expiration or increased respiratory rate [24]. Unusual breathing pattern-driven physiological hyperventilation and cardiorespiratory fluctuations must be avoided to attain steady state of breathing. Prolongation of unusual respiratory rate-driven physiological hyper- and hypoventilation is well known to cause respiratory acidosis and alkalosis, which leads to change in plasma acid–base balance (pH) and affects VOC exhalation [81, 82].
- *Subject's posture*: A particular posture (e.g., sitting or lying on back) must be maintained in order to avoid effects from hemodynamic fluctuations and pulmonary distribution of ventilation and blood flow [22, 83]. If the breath from a sick patient is sampled while he or she was at supine position, during follow-up the same supine posture must be maintained (even if the patient is recovered and can sit on a chair) for valid comparisons of pathophysiological effects beyond normal physiological noise (i.e., posture-driven differences). This is also true for

cross-sectional comparisons between healthy vs. sick cohorts. Sampling should be executed at the same posture in both cohorts.

- *Subject's breathing route*: Either oral or nasal breathing must be maintained without any unconscious switching between both [25]. This is to avoid ventilatory fluctuations and dead space ventilation and to avoid sudden contributions from oral or nasal cavity flora.
- *Applied (instrumental/analytical) upper-airway resistance against breathing*: Any obstruction to the mainstream of breathing must be avoided. In order to overcome unsupervised upper-airway resistance-driven change in pulmonary diffusion gradients and respiratory mechanics, smaller breathing mouthpiece should not be used. The diameter of the breathing mouthpiece should range between 1.5 and 2.0 cm [72]. In case of unavoidable infection safety mandates (e.g., for SARS-CoV-2 and similarly contagious pathogens), mainstream viral/bacterial filters are applied to stop respiratory viral/bacterial transmission to room air [84]. In such cases, effects due to instrumental resistance must be accounted for while evaluating breath data.
- *Precedent effects from wearing face masks*: Researchers have demonstrated pronounced side effects of wearing medical face masks (e.g., COVID-19 protective surgical and FFP2 masks) on respiratory-hemodynamic parameters and exhaled VOC concentrations, at rest. Physiological effects from precedent mask wearing may cause significant hyperventilation (especially in older adults, aged $>$60 years) as a respiratory compensation process [85]. As such effects may last for minutes to hours based on subject's age and/or health condition, observed breath compositions must account for mask-related effects (where relevant). It is reasonable to allow such subjects to sit without mask for at least 15–30 min prior to breath sampling, in order to minimize precedent physiological effects.
- *Subject's ventilatory variations*: Respiratory rhythms must be meticulously controlled in order to sample breath with minimal ventilatory variations. A recent study has reported that if at least a minute of paced breathing is applied (with fixed respiratory rate of 10–12/min) and then switch to spontaneous breathing, ventilatory variations tend to reduce significantly and attain a steady state after the 2 min of spontaneous breathing and third minute is suitable for collecting/considering reproducible sample without physiological fluctuations [75].

Besides the above-indicated sampling conditions, analysis and interpretation of exhaled VOC markers should incorporate effects from acute ambient conditions and also due to long-term effects from subject's personal attributes. These are mainly associated with subject's age, gender, diet, habits, environment, and lifestyle [5]. Studies have proposed that application of fasting or certain standard diet prior to breath sampling may reduce effects from food intake [86–88]. Nevertheless, such pre-selection is far from the real-life situation and any screening scenario or nontargeted approach [89]. Furthermore, fasting may cause hypoglycemic adaptation and metabolic compensation effects that may induce inseparable effects onto the volatile metabolites [29, 90]. Therefore, it is rather reasonable to carefully consider the attributes from diet, lifestyle habits (e.g., smoking, drinking, nutrition

supplement, and oral contraception), therapy, acute or chronic condition/comorbidity, and living environment as questionnaires during recruitment and retrospectively account for any suspected effects. Most importantly, simultaneous measurements of VOC concentrations in the inspiratory ambient air are extremely important to rule out acute effects.

6 Applications and Potentials of Physio-Metabolic Monitoring in Breath Analysis

Despite the fact that breath research is still in its infancy, the above-mentioned knowhows reflect a steady development toward a state of the art for clinical breath sampling. Nevertheless, the list is rather exhaustive and a lot more effort and time toward standardization of unequivocal confounders related to breath analysis has to be invested.

In principle, a disease/pathophysiological condition is nothing but a disturbed physio-metabolic state. Exhaled VOC profiles may provide rapid information on in vivo physiological or metabolic processes as the time span between marker production and exhalation of VOCs is short. Available knowledge of physiology and metabolism must be translated into disease-driven pathophysiology, for realizing effects on breath compositions. Assessments of physio-metabolic interplay can bridge the gap between our analytical and clinical expertise. Therefore, real-time breathomics holds great promise toward non-invasive monitoring of physiology, metabolism, diseases, and therapy. Screening of a large number of population (e.g., at COVID-19 test center) is also feasible via real-time breathomics. Physio-metabolic and pathobiological effects induced by SARS-CoV-2 and other respiratory pathogens are well addressed in recent studies in hundreds of subjects [89]. Framing of systemic physio-metabolic effects also helped to optimize experimental setups and methods for safe breath analysis and patient monitoring under high safety conditions/mandated at this very time of the global pandemic [84].

As real-time breath analysis can rapidly deliver results directly at the point of care it is especially attractive for personalized monitoring in patients. No risk is imposed on the patient even if the analysis is done frequently or continuously. Physio-metabolic monitoring can be used to follow up substances that were administered to the patient, such as volatile or intravenous anesthetics [26, 47, 91]. Moreover, VOCs enabled continuous monitoring and immediate recognition of therapeutic efforts in intensive care unit patients [57, 58]. Individual monitoring of selected breath VOCs facilitates recognition of metabolic transition without any delay. These findings encourage more research with respect to therapeutic monitoring, longitudinal studies, and follow-up of patients. Besides there is large scope for metabolic monitoring of aging, related life events, and health conditions such as menopause, oxidative stress, endocrine changes, and energy homeostasis. Monitoring of VOC changes during menstrual cycle and pregnancy may reflect phases of healthy natural

cycles, gestation, or any complications based on continuous changes in exhalation profiles [31, 36]. Similarly, physio-metabolic monitoring is applicable to physical fitness tests, exercise training, and various applications in sports science/medicine [70]. Non-invasive assessment of anaerobic threshold, exercise capacity, combined diet, and isotopically labeled substrate interventions are of significant interest. As the putative endogenous origin of most of the VOCs is largely debatable/uncertain and a recent study has even disqualified the long-believed metabolic origin of the second most abundant VOC (i.e., isoprene) in our breath [36], physio-metabolic monitoring under labeled substrate intervention may offer unique insights into the downstream denominators of VOCs and thereby indicate the way to their true systemic sources.

7 Conclusions and Perspective

The dynamic nature of breath VOCs offers a complex but comprehensive spectrum of immediate, transient, and chronic aspects. As breath biomarkers may provide unique and immediate physio-metabolic information on the whole-body status, new insights into normal and pathological processes may be achieved. A fundamental understanding of substance's origins, physicochemical properties, and potential regulating factors such as physiology, metabolism, microbiome, nutrition, lifestyle, and pre-exposure is essential to perceive the VOC expressions case or individual wise. If such knowledge is integrated with state-of-the-art advances in sampling and analytical techniques, observed changes or difference in VOC concentrations may translate actual effects from pathobiological and clinical conditions.

Surprisingly, normal physiology and metabolism-driven changes and variations in VOC concentrations observed in follow-up measurements (where subjects were used as his or her own control) were more pronounced than those reported as unique biomarkers in many published cross-sectional studies. Therefore, cross-sectional comparisons between healthy and ill subjects, in relation to screening or early detection of diseases via breath analysis, are far from our current abilities. In perspective, longitudinal assessments of ventilation and hemodynamics driven changes in breath compositions have depicted excellent potential for physio-metabolic monitoring. Continuous and personalized analysis of breath profiles may serve as an unconventional window for monitoring disease progression and response to therapy that could become a cornerstone toward individualized medicine and therapy.

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