Volatolomics of breath as an emerging frontier in pulmonary arterial hypertension

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Volatile organic compounds: exhaled volatolomics analysis for noninvasive early diagnosis of PAH
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ABSTRACT There is accumulating evidence in support of the significant improvement in survival rates and clinical outcomes when pulmonary arterial hypertension (PAH) is diagnosed at early stages. Nevertheless, it remains a major clinical challenge and the outcomes are dependent on invasive right heart catheterisation.

Resulting from pathophysiological processes and detectable in exhaled breath, volatile organic compounds (VOCs) have been proposed as noninvasive biomarkers for PAH. Studies have confirmed significant alterations of the exhaled VOCs among PAH patients when compared to controls and/or patients with other respiratory diseases. This suggests exhaled breath analysis as a potential noninvasive medical application in the field of PAH.

In this article, we review and discuss the progress made so far in the field of exhaled volatolomics (the omics of VOCs) as a potential noninvasive diagnostics of PAH. In addition, we propose a model including possible biochemical pathways on the level of the remodelled artery, in which specific VOCs could be detectable in exhaled breath during the early phases of PAH. We debate the different analytical approaches used and recommend a diagram including a "bottom–top" strategy, from basic to translational studies, required for promoting the field.

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Pulmonary arterial hypertension

Pulmonary arterial hypertension (PAH) is a progressive cardiopulmonary disease characterised by extensive occlusion of small to mid-sized pulmonary arterioles. The gradual increase in mean pulmonary arterial pressure (mPAP) leads to the development of high resistance, leading eventually to right-sided heart failure and death [1–3]. PAH is a subset of precapillary group 1 pulmonary hypertension, defined by a resting mPAP $\geqslant$25 mmHg, pulmonary artery wedge pressure (P_{paw}) $\leqslant$15 mmHg, and pulmonary vascular resistance (PVR) >3 Wood units at right heart catheterisation [2, 4]. In Europe, prevalence of PAH ranges between 15 and 60 subjects per million population, and the annual incidence is 5–10 cases per million [1, 5]. When untreated, the mean survival is 2.8 years [2, 3, 6].

Vascular lesions associated with PAH are complex and take in all layers of the vessel wall. Cellular hypertrophy, hyperplasia, local inflammation, overproliferation and resistance to apoptosis, endothelial to mesenchymal cell transition, exaggerated migration and accumulation of extracellular matrix components in PAH are well documented, yet other essential pathomechanisms remain unclear [7–9]. Although a wide range of therapeutic agents have been established for the management of PAH, they fail to fully improve symptoms and survival. PAH remains an incurable disease, and lung transplantation continues to be the main treatment in severe cases [2, 6].

Importance of early detection

PAH is often diagnosed by right heart catheterisation at its advanced stages [10, 11], and is associated with high early mortality, despite therapeutic intervention [3, 12–14]. Although accumulating evidence supports that significant improvement in survival rates and clinical outcomes is achieved when PAH is diagnosed at early stages [3, 15], it continues to be challenging [12, 16–18]. This is mainly due to the clinically silent or unspecific early phase, as well as due to the controversy between haemodynamics and functionality [19]. Despite advances in the available tools for assessment of PAH, including Doppler echocardiography and lung function and exercise tests, their predictive value remains questionable. Biomarkers, such as brain natriuretic peptide (BNP) and N-terminal pro-BNP have been correlated with haemodynamics and mortality in PAH; however, their sensitivity can be low and is affected by kidney function [19]. On the whole, the primary definitive diagnosis is frequently delayed for as long as 2 years from the appearance of early symptoms, which also represents a significant challenge [20]. Accumulating evidence suggests that systematic testing, *i.e.* screening for PAH in high-risk populations, would promote early diagnosis and intervention, offering an opportunity to improve patient outcomes. Therefore, there is an unmet and urgent need for reliable noninvasive biomarkers for the early detection of PAH [19–21].

Exhaled volatolome as biomarkers

Volatile organic compounds (VOCs), produced constantly by physiological processes, are detectable at low concentrations in exhaled breath [22–26]. Pathophysiological processes affect their production and lead to changes in the VOC profile of exhaled breath, referred to as the exhaled volatolome. Thus, identification and quantification of the exhaled volatolome could provide informative noninvasive biomarkers [22–34]. Due to their proximity to the blood–air barrier, recognisable and easily detectable shifts in the exhaled volatolome are to be expected in respiratory diseases [24, 31, 35–40]. In these cases, VOCs do not circulate in the peripheral blood system, from which they might be metabolised or stored in fat compartments; rather, they are expressed almost immediately in exhaled breath, accompanying the pathophysiology [24, 31, 35]. Significant shifts in the exhaled volatolome have been reported in many respiratory diseases, including lung cancer [24, 25, 28, 31, 33], tuberculosis [41, 42], chronic obstructive pulmonary disease (COPD) [43, 44], asthma [45], pulmonary embolism [46], pneumonia and cystic fibrosis [47, 48].

Changes in the spectrum and/or concentrations of exhaled VOCs could be due to a variety of abnormal processes, reflecting alterations in the biochemistry of the blood. Oxidative stress, inflammation, unbalanced enzymatic activity, carbohydrate and lipid metabolism and other mechanisms are possible sources of disease-related VOCs [22–24, 28, 31, 32].

In PAH, changes in the exhaled volatolome could result from the pathophysiological process of remodelling that occurs at the level of the arterioles. However, compensatory mechanisms accompanying the development of PAH could be an additional source of volatolomic alterations. Anatomical and physiological changes in the lungs and heart and fluctuations in the pulmonary circulation could potentially create further alterations in the exhaled VOC profile and concentrations [8, 9]. Arterial remodelling in PAH, for example, is characterised by the overproliferation of endothelial cells and smooth muscle cells. Excessive proliferation could result in local hypoxia, leading to anaerobic metabolism by the cells [31, 49]. In the glycolytic pathway of energy production, ketones and alcohols are excessively produced and ultimately excreted in the exhaled breath [50]. Alternatively, enhanced proliferation demands greater metabolism of cholesterol, during which isoprene, for instance, is formed along the mevalonic pathway of

cholesterol synthesis in the cytosolic fraction [51]. Inflammation is another major hallmark of PAH. High levels of pro-inflammatory cytokines in lung samples [52, 53], as well as increased numbers of macrophages in plexiform lesions and perivascular inflammation of PAH patients' lungs have been documented [54–57]. Increased inflammatory activity could result in excessive production of reactive oxygen species (ROS), leading to local peroxidation of lipids and fatty acids. For example, alkanes (cyclic saturated hydrocarbons) are by-products of the peroxidation of polyunsaturated fatty acids [22, 23, 31, 51].

Based on known pathomechanisms on the vascular level, as well as experimental models in the field of VOCs, we have put together a hypothetic model of vascular remodelling as a potential source of VOCs; this model is presented in figure 1.

Exhaled volatolome as biomarkers in PAH

The complex pathological mechanisms involved in PAH, as described earlier, could be a source of a wide range of volatile biomarkers in the breath. Several studies have already indicated the presence of alterations in breath volatolome in PAH patients; Mansoor *et al.* [58] compared the composition of exhaled breath condensate of 27 idiopathic (I)PAH patients with 30 healthy controls. Two VOCs were uniquely identified among the control group, and 10 other VOCs were uniquely expressed among the patients' samples. Of the four VOCs that were common to both groups, 1-methyl-4-(1-methylethenyl)-benzene was significantly lower in IPAH patients compared to the controls. Furthermore, the concentrations of six VOCs were correlated with mPAP, PVR or P_{paw}. In a more qualitative approach, a pattern recognition algorithm was applied to the measured VOCs. The model discriminated between IPAH patients and controls with an accuracy of 75.4%. Although interesting, the identification of the statistically validated VOCs was based solely on a mass spectrometric analysis. This approach is lacking, due to a very similar spectrum of ionised fragments of several compounds, and therefore the results might not be reproducible. To overcome this obstacle, it is recommended that the identification is undertaken using the analysis of pure standards, confirming the identity of each VOC, as well as calibrating curves in order to estimate the absolute concentration of the VOCs, rather than the ion counts of each explored peak [22, 51]. On the other side, the IPAH group consisted of patients in advanced and severe stages of the disease (New York Heart Association (NYHA) classification 3 and 4), raising the crucial question of whether the reported differences in the volatolome would be valid at earlier stages of disease [58].

Cikach *et al.* [59] explored the exhaled volatolome of 31 PAH patients and 34 controls recruited in two phases (training and validation sets). Among the 21 VOCs explored, the concentrations of 2-nonene, 2-propanol, acetaldehyde, ammonia, ethanol and pentane were elevated in PAH patients compared with control subjects, whereas 1-decene and 1-octene were significantly lower in the same patients. Applying mathematical discriminant analysis to VOC concentrations, the model scored 86.1% accuracy in the

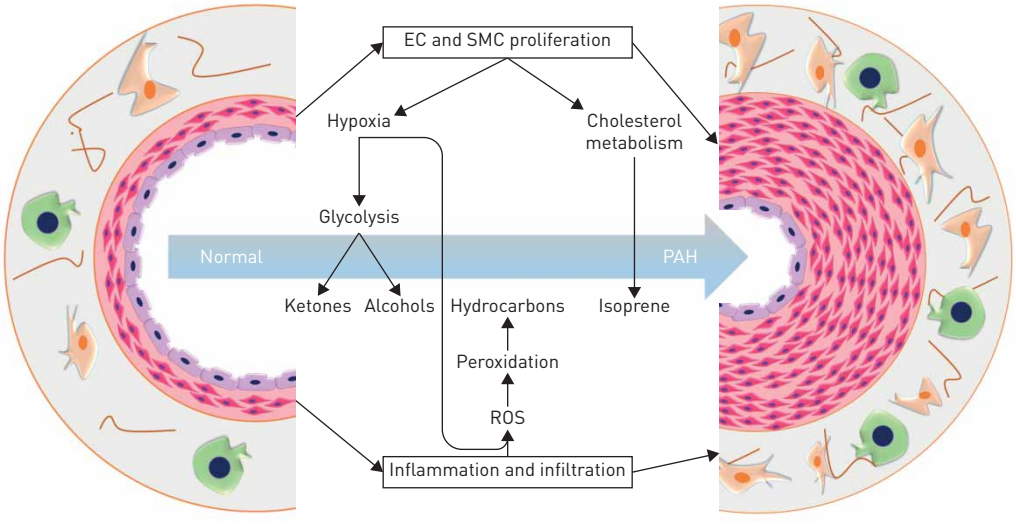

FIGURE 1 Hypothetical schema of possible sources of volatile organic compounds at the level of a remodelled artery in pulmonary arterial hypertension (PAH). Excessive proliferation associated with local hypoxia would promote glycolysis as an energy source, leading to the production of ketones and alcohols. In addition, cell proliferation demands high cholesterol metabolism that induces higher levels of isoprene. Production of reactive oxygen species (ROS) by inflammation leads to the oxidation of proteins and fatty acids, which release a wide range of hydrocarbons. EC: endothelial cell; SMC: smooth muscle cell.

training phase and 79.3% in the independent validation set. In addition, the concentration of exhaled ammonia was significantly associated with right atrial pressure and mixed venous oxygen saturation, after adjustment by age and sex, suggesting it as an independent breath biomarker for PAH. Conversely, blood ammonia levels were similar between the groups, and were not associated with breath concentration, nor any clinical feature. Thus, in the context of locally increased glutaminolysis [60] leading to hyperproliferative phenotype in PAH, the cells may generate more ammonia locally that is then expressed in exhaled breath, although not enough to affect whole blood concentration. These findings not only suggest a biomarker for the severity of PAH, but also attempt to link up the volatolome with the pathological mechanisms involved in PAH [59]. Nevertheless, ammonia has a major limitation as a disease-specific breath biomarker. This is because ammonia is reported as being raised in the exhaled breath of patients diagnosed with other diseases, including (but not only) kidney disease [61], inflammatory bowel diseases [62] and asthma [63], making it less reliable as a standalone test for PAH.

Mazzone *et al*. [64] enrolled 20 PAH patients as a control group to test the feasibility of a colorimetric sensor array for volatolome collective assessment to diagnose lung cancer. The results indicated that the collective breath volatolome of lung cancer patients differed from PAH, idiopathic pulmonary fibrosis, COPD and healthy controls. Once trained, the sensor array could discriminate between the diseases with a sensitivity of 73.3% and a specificity of 72.4%. The results were not influenced by sex, age, histology or smoking history [64]. Although innovative and easy to perform, in this qualitative approach the specific altered VOCs cannot be identified.

We have previously published a proof-of-concept in which an array of nanotechnology-based sensors for collective breath volatolome analysis was useful to detect and classify PAH. Using a different approach for breath analysis, we relied on the collective profile rather than specific alterations in the VOC concentrations in exhaled breath. Mimicking the sense of smell, an array of nonselective gas sensors was used to analyse breath samples of 22 PAH patients and 23 healthy controls. The signals obtained from the sensors after exposure were significantly different between samples from the two groups. A receiver operating characteristic-based classifier was obtained, discriminating between the samples with an accuracy of 92%. A second classifier was established to identify heritable PAH patients with mutations in the bone morphogenetic protein receptor type 2 (BMPR2) gene. The classifier successfully classified heritable PAH (n=7) compared to IPAH (n=15), scoring an overall accuracy of 87%. A third model indicated a correlation between the signals obtained from the sensor array and the severity of the patient's disease. With 91% accuracy, the samples were rated according to the NYHA classification, comparing the less severe cases (classes 1 and 2) and severe cases (class $\geqslant$3) [65]. Besides showing the high potential of qualitative breath analysis using nanosensor arrays to diagnose PAH, we showed that the breath volatolome is different in the mild stages than in more severe phase of the disease; the results also suggest that the pathological mechanisms of idiopathic and heritable PAH differ, as indicated by distinct breath volatolomes. However, the sample size in this case was relatively small, and further validation is required with an independent cohort [65]. Complementary quantitative gas chromatrography linked with mass spectometric analysis of 45 breath samples following the initial study confirmed significant differences in the concentrations of 10 breath VOCs associated with PAH, and subgroups that included the BMPR2 gene mutation PAH [66]. Currently, a clinical trial for validation (and expansion) is ongoing, with a goal of recruiting 400 volunteers, including IPAH patients, heritable PAH patients, high-risk subjects without PAH (asymptomatic relatives of familial PAH cases carrying a BMPR2 mutation) and chronic thromboembolic pulmonary hypertension patients (ClinicalTrials.gov identifier NCT02782026)

Together, these studies support the possibility that exhaled breath biomarkers could serve as a medical application in the detection and management of PAH.

Are exogenous VOCs actively involved in PAH?

Quite unexpectedly, when screening the VOCs already reported in association with PAH, it became apparent that some of the compounds identified were of exogenous origin. 1-methyl-4-(1-methylethenyl)-benzene, furan, tetrahydro-2,2,4,4-tetramethyl, benzaldehyde, *N*-ethyl-benzenamine, benzothiazole and others are not endogenously formed [58, 59, 66]. These compounds are considered pollutants, possibly due to exposure to cigarette smoke, air pollution and radiation [31]. Possibly, their high volatility and reactivity allow these compounds to leak into the cytoplasm of respiratory cells, leading to undesired and adverse effects, including peroxidative damage to enzymes and DNA. The accumulative damage of prolonged exposure is assumed to initiate and/or contribute to the pathogenesis of lung cancer [31].

Vascular remodelling in PAH shares analogous features with carcinogenesis. Pulmonary vascular lesions in PAH patients have cancer-like characteristics [7]. Resistance to cell death, a hyperproliferative phenotype, deregulation of cellular energetics, angiogenesis and tumour-promoting inflammation are common hallmarks of PAH and lung cancer [7]. Therefore, it is reasonable to link the adverse effects of inhalation

of pollutant VOCs to the initiation/progression of PAH. Montani *et al.* [67] ran a case–control study in which they put to the test the possible association of occupational exposure to organic solvents and the risk of pulmonary veno-occlusive disease (PVOD). In 33 PVOD and 65 PAH cases, PVOD was significantly associated with occupational exposure to organic solvents, particularly trichloroethylene (TCE) with an adjusted odds ratio of 8.2. For example, mice exposed to TCE developed lung injuries accompanied by increased oxidative stress, which is known to be involved in vascular remodelling [68, 69]. This suggests that volatile compounds from exogenous sources could directly contribute to the pathophysiology of respiratory and cardiovascular diseases.

Reproducibility and analytical approaches in breath volatolomics

One particular controversy found in published data is the wide variance and mismatch of results from different studies. For instance, in one case, the authors report that some of the VOCs were exclusively expressed by PAH patients, whereas others have shown that both patients and controls share the same profile of exhaled VOCs, but with differences in their concentrations [58, 59, 66]. In addition, the number and identity of the VOCs associated with PAH vary in the different studies. In all, it raises the question of the repeatability and reproducibility of breath analysis in general, and in PAH in particular. Nevertheless, this could be explained by different breath sampling procedures and analytical methods. Different sampling procedures and pre-concentration methods could result in the "capture" of different spectra of the expressed breath volatolomes [22]. In addition, each of the analytical instruments has a different sensitivity and limit of detection, and the small size and heterogeneity of the patient groups could preclude obtaining robust VOC biomarkers. Despite these differences, the overall take-home messages of the reports in the literature are similar, *i.e.* there are significant alterations in the breath volatolome in PAH.

In general, the search for uniquely expressed exhaled VOCs in a given disease has not yet been successful. Indeed, none of the recorded VOCs alone could assist in diagnosis [27, 33, 70–77]. For example, a large portion of the proposed breath biomarkers that have been suggested for PAH were also associated with other diseases, one such case being ammonia. This suggests that when two or more diseases share common pathological mechanisms, a similar range of exhaled VOCs could be affected. Conversely, the concentrations of part of the VOCs could be affected by more than one type of pathophysiology. Therefore, in a complex disease such as PAH involving multiple pathophysiological processes and several systems/organs, it is unlikely to find unique breath VOCs (absent in nonpatient controls and/or other diseases). Therefore, the collective volatolome assessment is potentially more realistic as a future medical application in the detection of PAH. As already reported, an intelligent artificial olfactory system consisting of arrays of nanotechnology-based sensors could be trained for the detection and classification of PAH [65, 66]. Learning from the mammalian sense of smell, cross-reactive (nonselective) sensors are used for the collective sensing of the VOC spectrum in breath [24, 29, 32, 78, 79]. Combined with pattern recognition algorithms, the collective, yet specific, breath-signature associated with PAH could be determined. Depending less on the concentration of each VOC, the collective analysis is more robust and resistant to possible confounding factors [24, 29, 32, 78, 79].

Perspective: volatolomics of the remodelled artery for early diagnosis

So far, all studies have explored volatolomic alterations (quantitatively and/or qualitatively) of PAH patients with established and advanced disease [58, 59, 64, 66]. At this stage of the disease, a variety of anatomical as well as physiological changes occur at the level of the lungs, heart, immune/inflammatory system and circulation [8, 9]. These compensatory and/or secondary mechanisms could also affect the exhaled volatolome. Therefore, it is unlikely that the overall changes in the volatolome described are representative of the early phases of PAH, *i.e.* the primary pathological mechanisms, at the vascular level. Furthermore, exhaled VOCs could be affected by unrelated mechanisms, medications, comorbidities and haemodynamic and cardiac effects [80, 81]. Therefore, it is doubtful that the VOCs that have been reported could assist in the early detection of PAH.

Arterial remodelling in the very early stages of PAH involves several molecular and cellular pathways that could affect the specific spectra of VOCs. This silent or vague phase can last up to 4 years before a formal diagnosis is made [20]. Therefore, we propose that the identification and targeting of these VOCs should be the first step towards early noninvasive diagnosis of PAH. To succeed in this challenge, we suggest two complementary approaches. First, clinical evaluation to further explore the volatolomic changes in PAH and its subsets, with a focus on high-risk subjects without known PAH (for example, familial cases of BMPR2 mutations). Periodic clinical, biological and volatolome assessment of this group could help to correlate the development of the disease with volatolomic changes, at its early phases. Once the disease is initiated leading to early haemodynamic consequences, the search should focus on the changes in exhaled breath volatolome prior to the symptomatic phase (figure 2).

Additionally, basic biological experiments run in parallel are needed, in which VOCs emitted directly by vascular cells, involved in the remodelling process, should be identified and investigated. To this end, lung specimens, for example from PAH patients after lung transplantation, could serve to establish microvascular endothelial and smooth muscle cell cultures. VOCs emitted by those cells could be identified and analysed quantitatively. *Ex vivo* in-culture expression of these VOCs is essentially linked to some pathological mechanisms at the vascular cell level, while unrelated to the haemodynamics or to other secondary mechanism associated with advanced stage PAH. Therefore, these VOCs could potentially represent "pure" vascular remodelling-specific biomarkers. We must also pinpoint the importance of the association between the production of specific VOCs and known or suspected metabolic pathways in PAH. Exposure of healthy vascular cells to stimuli, such as inflammation, hypoxia, ROS, excessive proliferation state/resistance to apoptosis and others could tell us how much the vascular cell volatolome is affected by each pathological process and the balance between them (figure 2).

Although animal models could be informative at this phase, very challenging aspects should be first addressed in order to benefit from such studies, as follows. 1) Animal models for pulmonary hypertension are mostly developed in small animals (induced in rats or mice using monocrotaline (MCT), exposure to hypoxia or Sugen-hypoxia) [82]. Thus, it is extremely challenging to collect a sufficient volume of exhaled breath (>500 mL per animal per test), taking into account their tidal volume [77]. 2) It remains unclear how much the mentioned models represent the actual disease occurring in humans, particularly in terms of chronological events and poor phenotyping [82, 83]. For example, the MCT model would produce features of severe pulmonary hypertension and heart failure within 3 weeks; however, due to MCT cytotoxicity it could also affect other unrelated organs. The MCT model is also suggested to cause pulmonary interstitial oedema, myocarditis and hepatic veno-occlusive disease that are not characteristics of human pulmonary hypertension [84]. As a consequence, these additional pathomechanisms would probably result in further unspecific volatolomic changes that could be mistakenly associated with pulmonary hypertension. Therefore, it would be very challenging to correlate the volatolomic changes in PAH patients and these animal models.

Overall, early-phase PAH volatile biomarkers could be identified and targeted for exhaled breath diagnostics in the future. It might help to find the origin of each of the VOCs, and possibly provide knowledge on the exact mechanisms leading to each of the volatolomic changes. Thus, monitoring these VOCs could be useful for PAH patient management, follow-up and monitoring.

Volatolomics in secondary PAH/pulmonary hypertension

It is admitted that IPAH is usually diagnosed in its advanced stages, due to the unclear and unspecific clinical presentation of its earliest stages. As already suggested in the literature, a noninvasive screening test would be very useful in these cases [19]. Therefore, a breath analysis based test could be a safe, easy to perform, rapid and repeatable screening tool that physicians could use as an indicator in the vague early

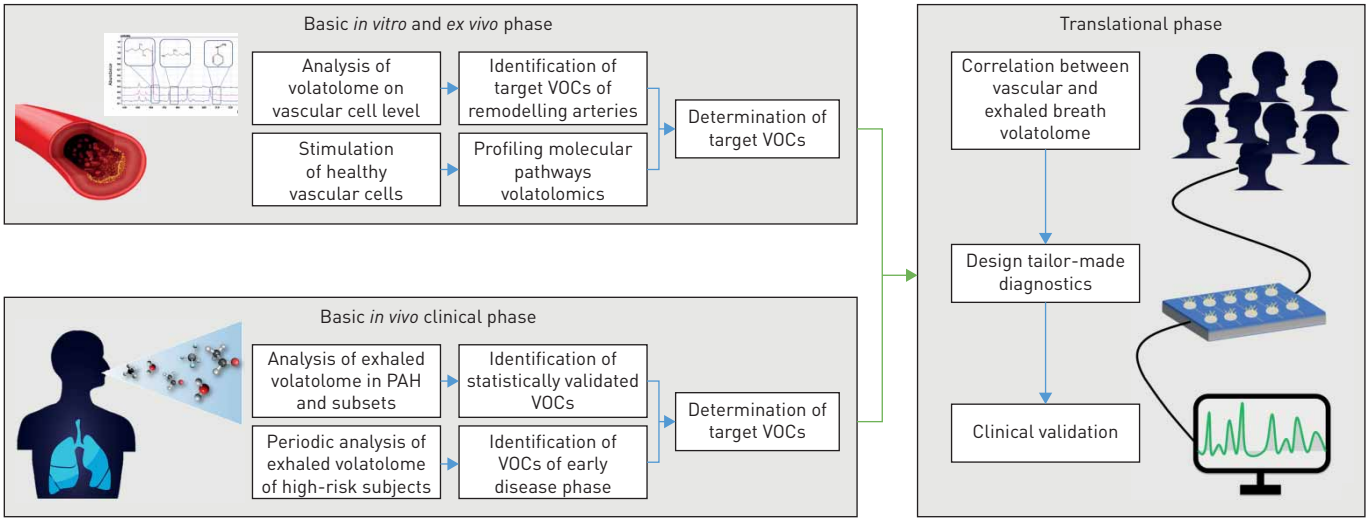

FIGURE 2 Schematic diagram of the suggested "bottom–top" experimental flowchart towards designated pulmonary arterial hypertension (PAH) diagnostics, based on exhaled volatolome analysis. An *ex vivo* phase to explore and determine volatile organic compounds (VOCs) at the vascular level as early-phase biomarkers accompanied by clinical studies to determine volatolome changes in exhaled breath of PAH patients. After identification of target VOCs found in early phases of the vascular phase and in the exhaled breath, a translational phase is required to establish a designated breath analyser for the early diagnosis of PAH.

phases. Therefore, in this context, focusing on the heritable group with BMPR2 mutation and their relatives would be a simplified strategy to determine the early volatolomic changes associated with PAH. As reported, 20–30% of this population will eventually develop PAH [85], and therefore periodic breath tests for this group could be a key factor in monitoring volatolomic alterations as soon as the arterial remodelling begins, indicating the need for right heart catheterisation.

As for more complex cases, such as PAH associated with connective tissue disease or HIV infection, or pulmonary hypertension secondary to heart disease or COPD, the basic research approach should be different. It is anticipated that due to the complexity of the pathophysiological process and comorbidities, the volatolomic patterns would be more complicated to understand. It remains unclear how the exhaled VOC profile would look, in the presence of two diseases (COPD and pulmonary hypertension, for example), since a portion of the VOCs could be affected by molecular pathways involved in each of the two diseases. Volatolomic changes associated with ("pure") COPD, for example, have been already published [43, 86], thus it will be necessary to evaluate subjects with secondary pulmonary hypertension, in a group of COPD patients, in order to pinpoint the specific volatolomic changes associated with the combination of both diseases. In fact, it is reasonable to anticipate that in these cases a third and specific volatolomic pattern would be found; representing the pathomechanism involved in both diseases, and would be useful for the classification of this specific group of patients. We suggest this strategy to be tested and evaluated for the classification, rather than detection, of pulmonary hypertension or PAH complex subclassifications.

Concluding remarks

With right heart catheterisation as the gold-standard diagnostic method, PAH is usually diagnosed in its advanced stages, negatively affecting clinical outcomes and survival rates. The exhaled volatolome is a database of information regarding cellular and molecular activity, and represents a unique window onto the pathological and metabolic pathways involved in PAH. Exhaled biomarkers could be useful in early diagnostics of PAH as noninvasive procedure. However, major basic and translational research is still required to determine the exhaled VOCs linked with the very early stages of the diseases before developing and training a simple breath analyser designated for early detection of PAH.

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