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## Metabolic effects of inhaled salbutamol determined by exhaled breath analysis

To cite this article: Martin T Gaugg *et al* 2017 *J. Breath Res.* 11 046004

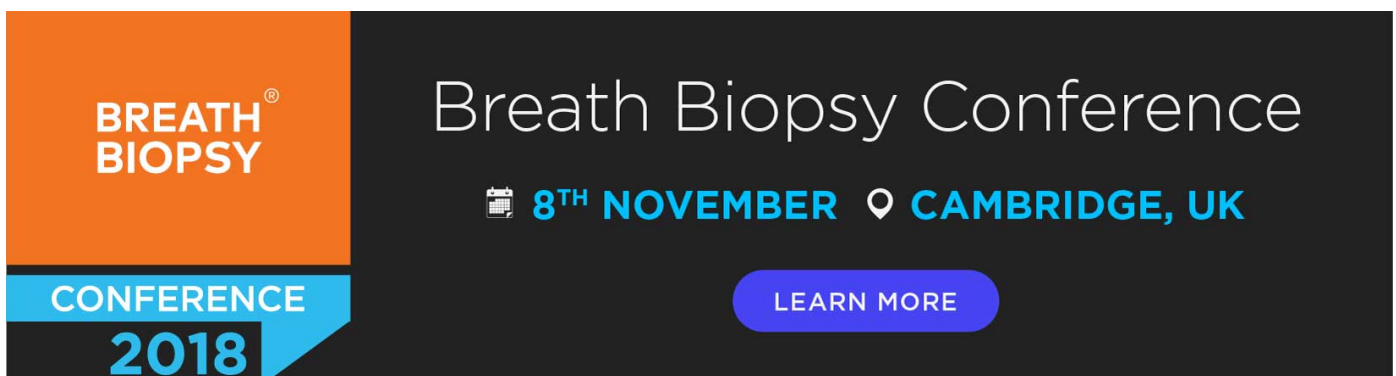
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## PAPER

## Metabolic effects of inhaled salbutamol determined by exhaled breath analysis

## OPEN ACCESS

RECEIVED  
22 April 2017REVISED  
28 June 2017ACCEPTED FOR PUBLICATION  
30 June 2017PUBLISHED  
13 September 2017

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We explore whether real-time breath analysis by high resolution mass spectrometry is suitable to monitor changes at the metabolic level due to inhaling bronchodilator medication. We compared the breath levels of metabolites in a group of patients ( $n = 50$ ) at baseline and 10 and 30 min after inhalation of 200  $\mu\text{g}$  salbutamol. The same procedure was performed with a group of controls ( $n = 48$ ) inhaling a placebo spray. A total of 131 mass spectral features were significantly altered as a result of inhaling medication, but not after inhaling placebo. We found that homologous series of chemical classes correlated strongly with each other, strengthening the notion that certain biochemical processes can be monitored. For example, a series of fatty acids was found to be increased after salbutamol intake, suggesting lipolysis stimulation. Peaks corresponding to salbutamol, its main metabolite salbutamol-4-O-sulfate and formoterol were found to be generally increased in patients inhaling the drugs on an as-needed basis, as compared to non-medicated volunteers. Overall, these results suggest such real-time breath analysis is a useful tool for non-invasive therapeutic drug monitoring.

**Introduction**

Inhaling drugs is an attractive administration route due to rapid absorption into the systemic circulation and low concentrations of drug-metabolizing enzymes in the lungs compared with the oral route [1]. In addition, it provides rapid local effects in the lung, minimizing systemic drug effects. For these reasons, inhaled medications have been used for many years for the treatment of respiratory diseases (i.e. lungs are the target organs), as well as more recently to treat systemic diseases (e.g. diabetes) [1, 2]. During drug development programs, optimal aerosol particle size is sought, to target the optimal site of deposition. Typically, clinical response (e.g. forced expiratory volume in 1 s (FEV<sub>1</sub>)) is assessed upon drug inhalation. Parallel determination of drug response at a molecular level is of course needed to fully understand mechanisms of action. Metabolic profiling of body fluids is a

possible approach to quantifying physiological response to inhaled medication [3, 4]. For example, a recent nuclear magnetic resonance-based serum and urine metabolomics study has mapped the overall metabolic changes after inhalation of budesonide and salbutamol in asthmatic children during acute exacerbation, concluding that seven metabolic pathways were altered as a result [5]. However, inhaled drugs tend to act quickly (e.g. hydrophobic molecules absorbed within seconds) [1], posing some challenges to the capture of highly dynamic responses to plasma- or urine-based metabolomics. In addition, sample collection [6] and manipulation [7, 8], as well as subjecting metabolites to harsh processes (e.g. heat leading to thermal degradation [9]) may lead to biased results. To overcome these issues, we propose that *in vivo*, real-time analysis of exhaled breath metabolites provides an attractive means to track response to inhaled medication rapidly and non-invasively.

**Table 1.** Anthropometric data of the participants in this study.

	Placebo ( <i>n</i> = 48)	Salbutamol ( <i>n</i> = 50)
Mean age ± SD (years)	41.8 ± 17.8	55.3 ± 14.2
Male/Female	22/26	28/22
COPD(GOLD1-2/GOLD3-4)/Asthma/Healthy	0/8/40	37(8/29)/13/0
Mean FEV1 pre ± SD (% pred)	96.2 ± 12.1	50.8 ± 25.1
Mean FVC pre ± SD (% pred)	101.5 ± 11.9	80.0 ± 20.6
Smokers/Non-smokers/Ex-smokers	9/33/6	8/13/29

Selected ion flow tube- and proton transfer reaction-mass spectrometry (SIFT-MS and PTR-MS) are two real-time techniques that have been widely used in a clinical context [10–12], although few examples can be found in the literature for monitoring therapeutic intervention [13–16].

To prove this novel concept, we used secondary electrospray ionization [17]-high resolution mass spectrometry (SESI-HRMS) in this work to study changes in endogenous metabolites in response to inhaled medication, in contrast to a group inhaling placebo. By using a placebo inhaler identical to the medication inhaler except for the active ingredient, we ruled out confounding factors such as contamination of the oral cavity or changes due to the inhalation maneuver. We chose to study the effect of salbutamol, which is a short-acting bronchodilator widely used in the treatment of obstructive lung diseases such as asthma and chronic obstructive pulmonary disease (COPD) [18].

## Methods

### Subjects

A total of 98 participants were included in this study: 48 inhaled placebo and 50 inhaled salbutamol. Of the 48 subjects inhaling placebo, eight suffered from asthma and 40 did not have any lung disease. Of the 50 patients inhaling salbutamol, 13 suffered from asthma and 37 suffered from moderate to severe COPD. The anthropometric data is shown in table 1. Participants were asked to refrain from eating, drinking (except water), chewing gum, smoking and brushing their teeth at least 1 h prior to measurement. The study was approved by the local ethical committee (PB\_2016-00421, KEK-ZH-Nr. 2015-0148, KEK-ZH-Nr. 2014-0088) and all subjects gave written, informed consent to participate.

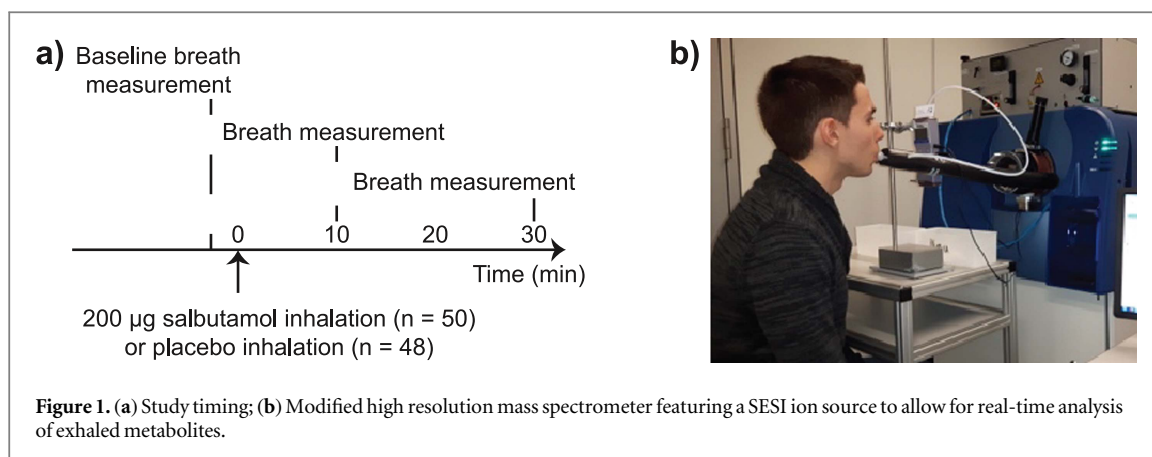
### Real-time breath measurements

Exhaled breath analysis was performed at baseline, 10 and 30 min after the inhalation of 200 µg salbutamol (*n* = 50) or placebo (*n* = 48). The placebo contained only norflurane. Figure 1(a) shows the timing used in the study. Figure 1(b) displays a picture of the modified mass spectrometer (Sciex Triple TOF 5600+) to allow breath analysis by SESI-HRMS. The breath analysis approach has been described in greater detail

elsewhere [19, 20]. In brief, the atmospheric pressure ion source of a quadrupole-time-of-flight mass spectrometer was replaced by a lab-built SESI chamber [21, 22]. A nano-electrospray of 0.1% formic acid in water was infused at ~200 nl min<sup>-1</sup> through a silica capillary (PicoTip emitter—O.D. 360 µm—I.D. 20 µm). The electrospray was operated in the cone-jet mode [23, 24] and its current (~100 nA) constantly monitored. In addition, a lens was used sporadically to visually inspect the Taylor cone [25]. The breath sampling tube connected to the SESI chamber consisted of stainless steel (6 mm I.D.), and was heated to 80 °C to prevent water condensation and adsorption of low-volatility metabolites onto the walls. In order to avoid confounding factors such as differences in exhalation maneuvers [26], the sampling protocol was standardized across all participants. The subjects would typically provide five–six replicate exhalations. Full exhalations were provided at a constant flow rate by keeping the pressure in the sampling line at 12 mbar (regulated by bio-feedback by the breathing subjects). Measurements were recorded in positive and negative ion mode, leading to mainly protonated and deprotonated species, respectively. The mass range in positive ion mode was 40–700 Da and in negative ion mode 40–450 Da. Accumulation time was set to 1 s.

### Compound identification

Identification of 10-hydroxydecanoic acid was performed using on-line MS/MS experiments as well as UHPLC-MS/MS experiments of exhaled breath condensate (EBC) and a standard. EBC was collected using a sampling device constructed in-house following the guidelines set by the ATS/ERS task force [27]. Two healthy, non-smoking subjects were asked to exhale into a glass cold trap. The collected EBC samples were pooled ( $V_{\text{total}} = 32$  ml), 0.5 ml were extracted and stored at 4 °C and the remainder lyophilized to dryness [28]. The residue was then reconstituted in a mixture of 142.5 µl of the previously aliquoted EBC, 7.5 µl methanol and acidified with 0.1% formic acid. This up-concentrated sample was directly subjected to UHPLC-HRMS/MS analysis. UHPLC separation was done on an ACQUITY UPLC system (Waters, MA, USA) with a C18 column (1.7 µm, 2.1 × 50 mm, Waters) with pre-column filter. The flow rate was set to 500 µl min<sup>-1</sup> using a binary mixture of solvent A (water with 0.1% formic acid) and solvent B (methanol



with 0.1% formic acid). The 10-hydroxydecanoic acid standard (Apollo Scientific Ltd) was prepared at a concentration of  $100 \text{ ng ml}^{-1}$  and analyzed with an injection volume of  $10 \mu\text{l}$ . A Triple TOF 5600 + (AB Sciex, Concord, ON, Canada) mass spectrometer was used to measure a mass range of 40–500 Da in negative ion mode, with 1–5 ppm mass accuracy. Collision energy was set at  $40 \pm 30 \text{ eV}$ .

### Data analysis

Raw \*.wiff (Sciex's proprietary format) data was converted into \*.mzXML format via MSConvert (Proteowizard) [29]. Each sample file was interpolated linearly and centroided, generating as a result a  $m/z$  list of 1208 peaks present in at least 40% of the samples. The mass spectra were normalized dividing by the sum of a set of 904 signals found to be present in 90% of our samples. We then computed the  $\log_2$  of the fold change (i.e. ratio between measurements after 10 min over baseline and after 30 min over baseline). Significant differences in exhaled metabolite levels in response to salbutamol, but not to placebo inhalation were sought. A paired two-tailed  $t$ -test followed by estimation of false discovery rate (FDR) in multiple comparisons was used for this purpose [30]. Statistical significance was set to  $\text{FDR} < 0.05$ . We further considered only the signals changing significantly after 10 and 30 min of salbutamol inhalation, but not changing significantly after placebo inhalation. Among the significant ones, we report here only those signals changing by at least  $\log_2(\text{fold change}) > 0.15$  after 10 or 30 min of inhaling salbutamol. The signals satisfying these conditions were subjected to an agglomerative hierarchical cluster tree (Ward method; Euclidean distance). In order to identify groups of signals showing similar behavior, we constructed agglomerative clusters using a cutoff Euclidean distance of 5. Finally, for the set of molecules that were chemically identified, we computed the correlation coefficients between pairs of variables for the group inhaling salbutamol.

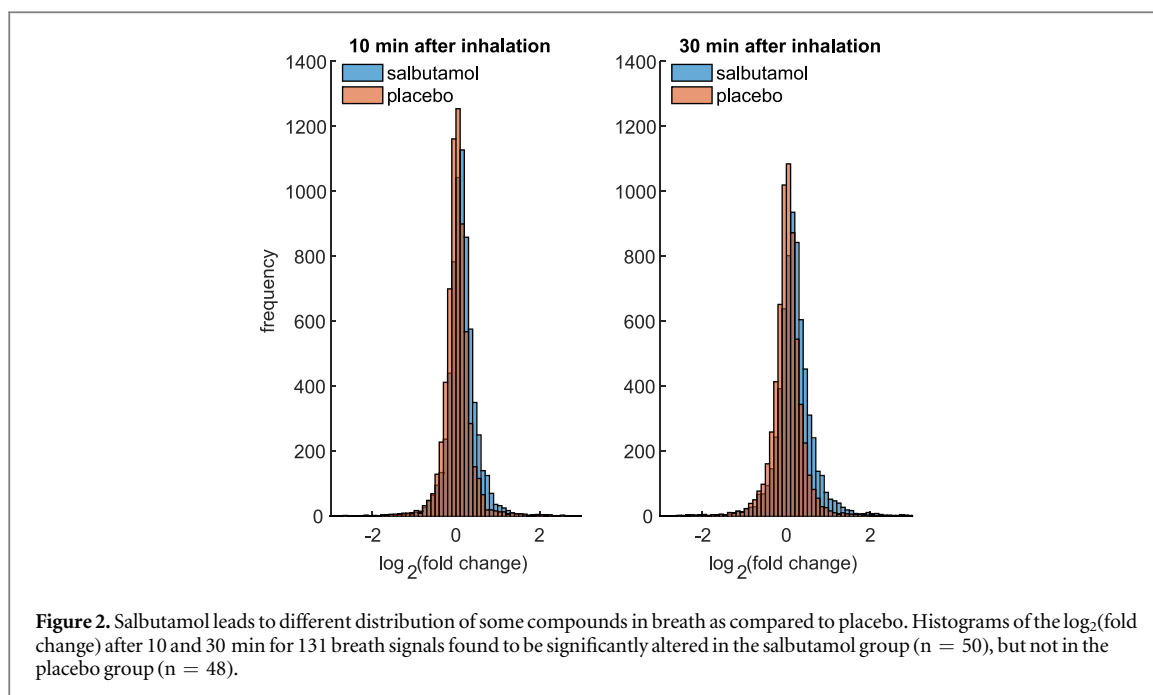
## Results and discussion

Monitoring of exhaled compounds to adjust inhaled medication dose to maximize efficacy and minimize toxicity is an attractive approach. For example, this concept has been implemented by measuring exhaled NO in asthmatic patients to titrate treatment with inhaled corticosteroids [31]. In the present work we have explored whether comprehensive real-time breath analysis can assist in a similar approach towards a more personalized therapeutic regime.

### Metabolites levels are altered after salbutamol inhalation, but not after placebo

Subjects' breath was analyzed, detecting as a result 1208 features (866 and 342 features in positive and negative mode, respectively) present in at least 40% of the samples. A total of 131 mass spectral features were found to be significantly altered in response to salbutamol inhalation, but not to placebo inhalation. Table S1, available online at [stacks.iop.org/JBR/11/046004/mmedia](http://stacks.iop.org/JBR/11/046004/mmedia) lists all of these along with their mean changes, 95% confidence intervals,  $p$ -values and FDR. Figure 2 shows the histograms of the  $\log_2(\text{fold change})$  for the 131 significantly altered ions after 10 and 30 min of inhalation of salbutamol. The distributions for the placebo group are fairly symmetrical around 0 (i.e. mean 10 min after placebo = 0.0254; mean 30 min after placebo = 0.0220). In contrast, the salbutamol distributions are flatter, and their means are displaced to the right (mean 10 min after salbutamol = 0.1458; mean 30 min after placebo = 0.1963), suggesting a general increase of these exhaled compounds.

Among 131 altered signals, 121 increased, while 10 signals were reduced after salbutamol inhalation, but did not change significantly in the placebo group. Figure 3 shows the breath-to-breath time trace for one selected compound ( $m/z$  259.1903;  $\text{C}_{14}\text{H}_{26}\text{O}_4$ ) for one subject before and after inhalation of placebo (a) and for a different subject inhaling salbutamol (b). The five–six replicate measurements within each time point show a satisfactory repeatability (4.8% average



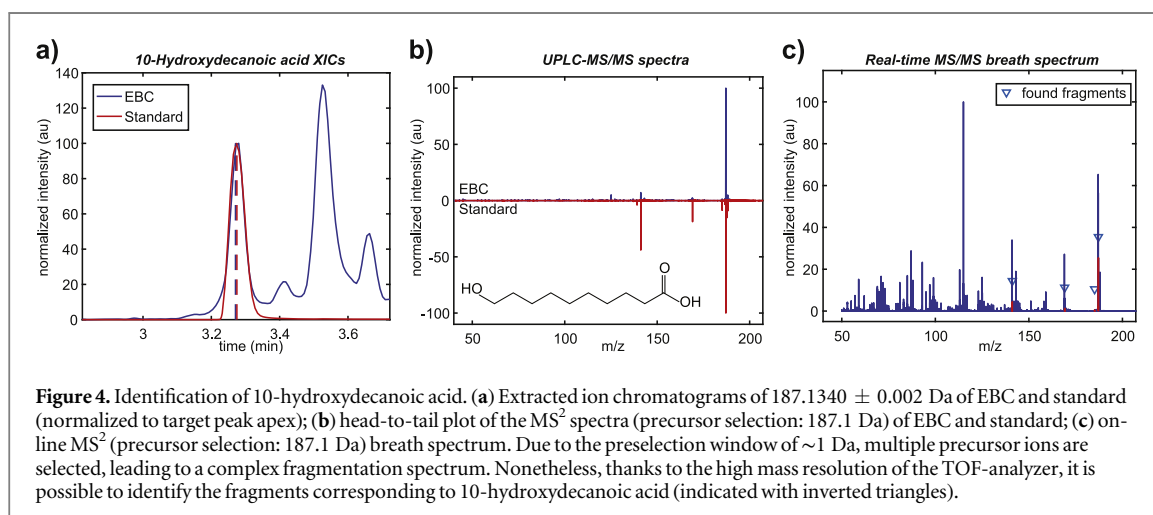
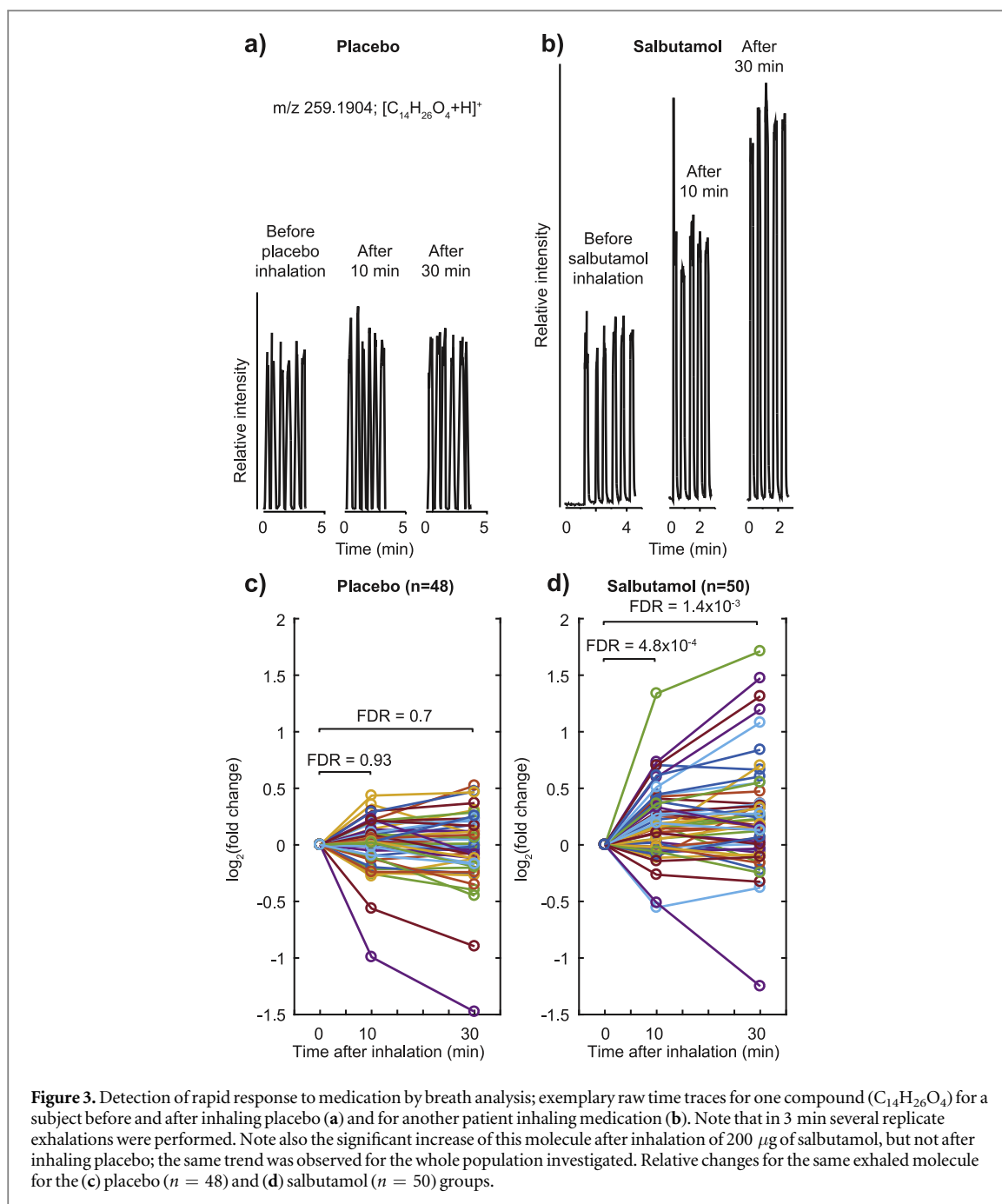
coefficient of variation). Clearly, the breath levels of this compound for this particular patient increased significantly shortly after salbutamol inhalation, and this change could be easily captured by on-line breath analysis. In contrast, the levels for the subject inhaling just placebo did not vary significantly. The overall picture of the trends for this compound for the placebo and medication groups is shown in figures 2(b) and (c), respectively. Mean  $\log_2(\text{fold change})$  for the placebo group after 10 and 30 min was 0.01 (95% CI =  $-0.06/0.07$ ; FDR = 0.93) and  $-0.02$  (95% CI =  $-0.12/0.07$ ; FDR = 0.7), respectively. In contrast, mean  $\log_2(\text{fold change})$  for the salbutamol group after 10 and 30 min was 0.19 (95% CI =  $0.10/0.28$ ; FDR =  $4.84 \times 10^{-4}$ ) and 0.26 (95% CI =  $0.12/0.4$ ; FDR =  $1.44 \times 10^{-3}$ ), respectively. However, despite the significant changes for this particular molecule (molecular formula  $C_{14}H_{26}O_4$ ) in the salbutamol group, there is clear inter-individual variability, with some subjects experiencing no changes at all. This seems to be in line with the known lack of response to medication for large parts of the population. For example, it has been estimated that a daunting 75%–96% do not respond to the top-ten grossing drugs in the market. Our data suggests that breath analysis has potential to contribute to the identification of sub-populations of responders and non-responders, at least for certain drugs such as bronchodilators.

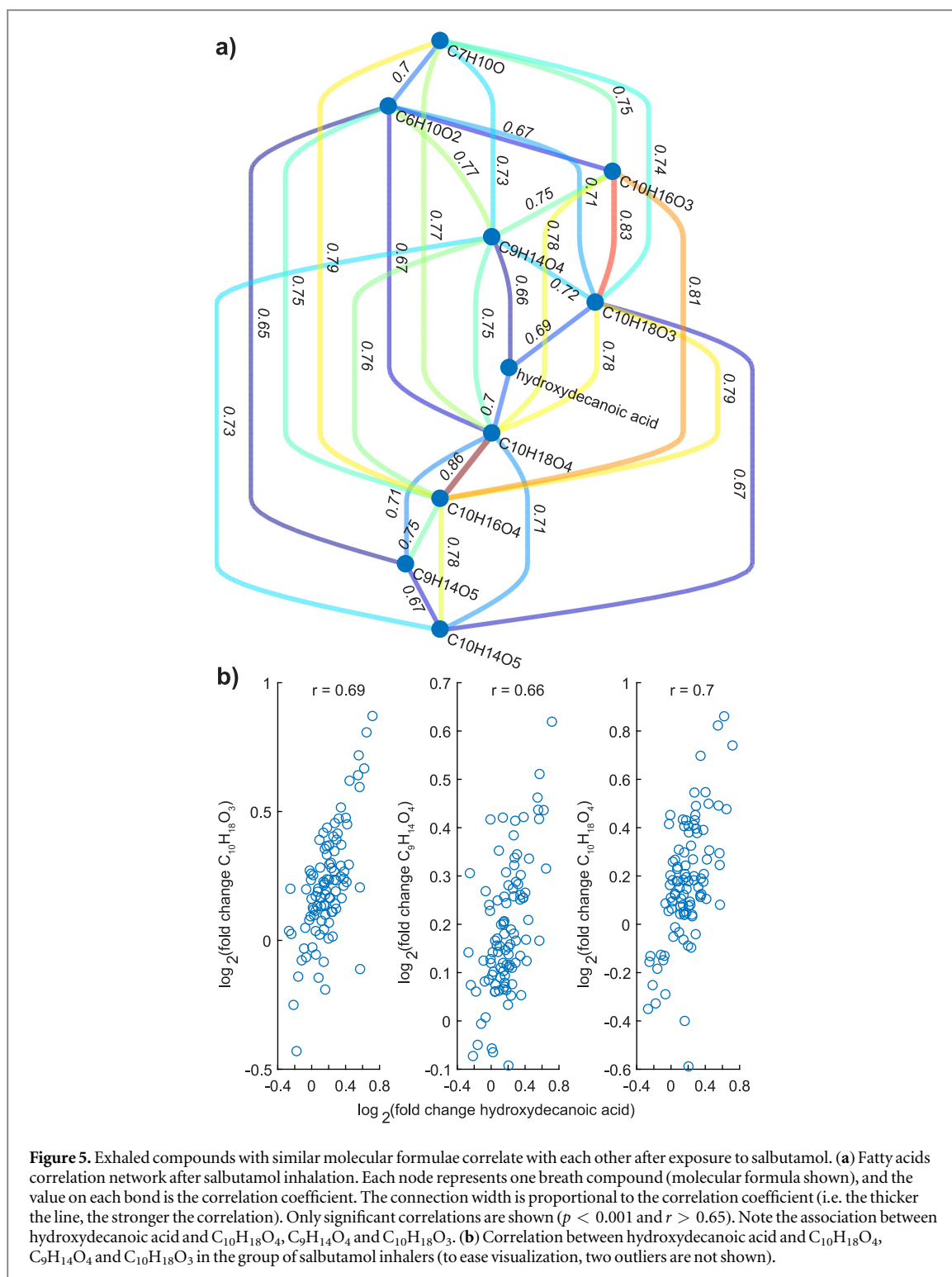
#### Metabolite associations and compound identification

We attempted the chemical identification of some of the exhaled metabolites, to provide a biochemical interpretation of our results. First, we computed an agglomerative hierarchical cluster tree (Ward method; Euclidean distance) and subsequently constructed

clusters from the hierarchical cluster tree using as cutoff a distance of 5. This resulted in 26 clusters of signals behaving similarly. The resulting clusters are listed in table S1. As a result, it became apparent that homologous series of closely related molecular formulae (and most likely compounds) tend to cluster together. This is a first indication that this real-time technique is capable of capturing metabolic cascades in response to medication. For example, cluster number 16 consisted of acetic, propionic and butyric acids, which were in all cases found to be increased after salbutamol inhalation. Similarly, heptanoic, octanoic and nonanoic acids [21] clustered together (number 22) and were found to increase after salbutamol inhalation. Another interesting series of compounds was cluster number 10. They all have similar molecular formulae and were detected in negative ion polarity. Compounds detected by SESI-MS operated in negative ion mode are dominated by deprotonated acids [21, 32]; thus, we hypothesize that these compounds are likely fatty acids. This was confirmed by deploying a comprehensive analytical strategy. Figure 4 shows experimental evidence indicating that the molecular formula  $C_{10}H_{20}O_3$  corresponded to 10-Hydroxydecanoic acid. Figure 4(a) shows the extracted ion chromatograms at  $m/z$  187.1339 in negative ion mode for EBC and the standard of 10-hydroxydecanoic acid. Figure 4(b) shows a head-to-tail MS/MS for EBC and the standard. The perfect match in retention time and fragmentation pattern provides strong evidence for 10-hydroxydecanoic acid in exhaled breath condensate. Figure 4(c) shows the fragmentation spectrum at  $m/z$  187.1339 during a real-time exhalation. The diagnostic fragments of this compound were indeed present, indicating that 10-hydroxydecanoic acid is present in exhaled in real-time and detected by



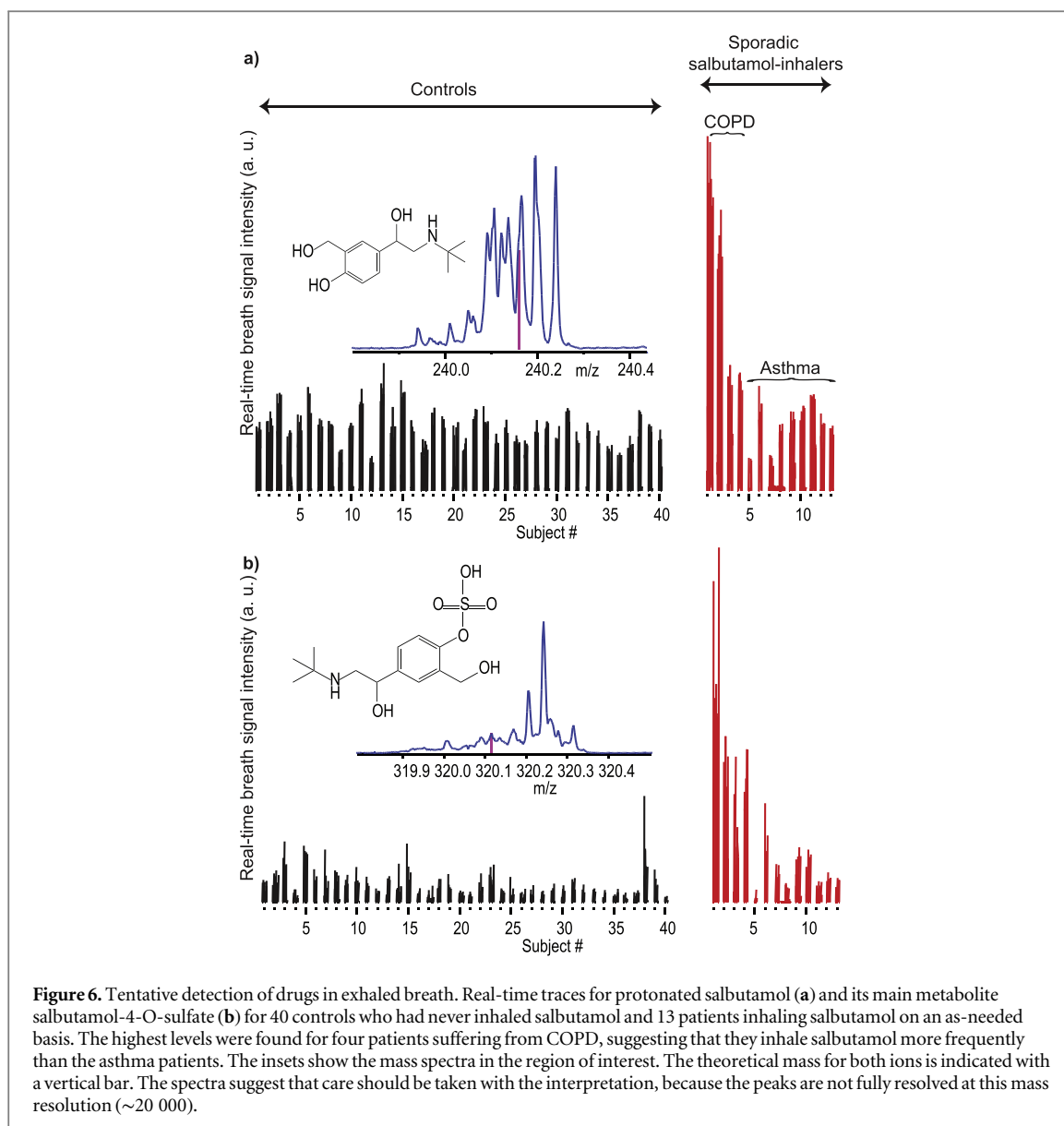




SESI-MS. However, it is obvious that other fragments are also present, because the isolation window of the quadrupole is 1 Da, leading to the fragmentation of all the species present at nominal mass 187 Da. It is also interesting to note that different isomers of 10-hydroxydecanoic acid were found in breath condensate (figure 4(a)). For example, the most abundant peak in the chromatogram at retention time  $\sim 3.55$  min, corresponds to 3-hydroxydecanoic acid (Gaugg *et al* in preparation), which is an intermediate

in fatty acid biosynthesis. It is therefore very likely that all different isomers are exhaled simultaneously.

To better understand the interactions of 10-hydroxydecanoic acid with the rest of the compounds in the same cluster we computed their correlations ( $r$ ) for the salbutamol group and plotted the correlation network. Figure 5(a) shows the results (only  $r > 0.65$  are shown). Overall, we found strong positive correlations among the different nodes (i.e. molecules). The strongest correlation ( $r = 0.86$ ) was between  $C_{10}H_{18}O_4$  and  $C_{10}H_{16}O_4$ . The former of these is



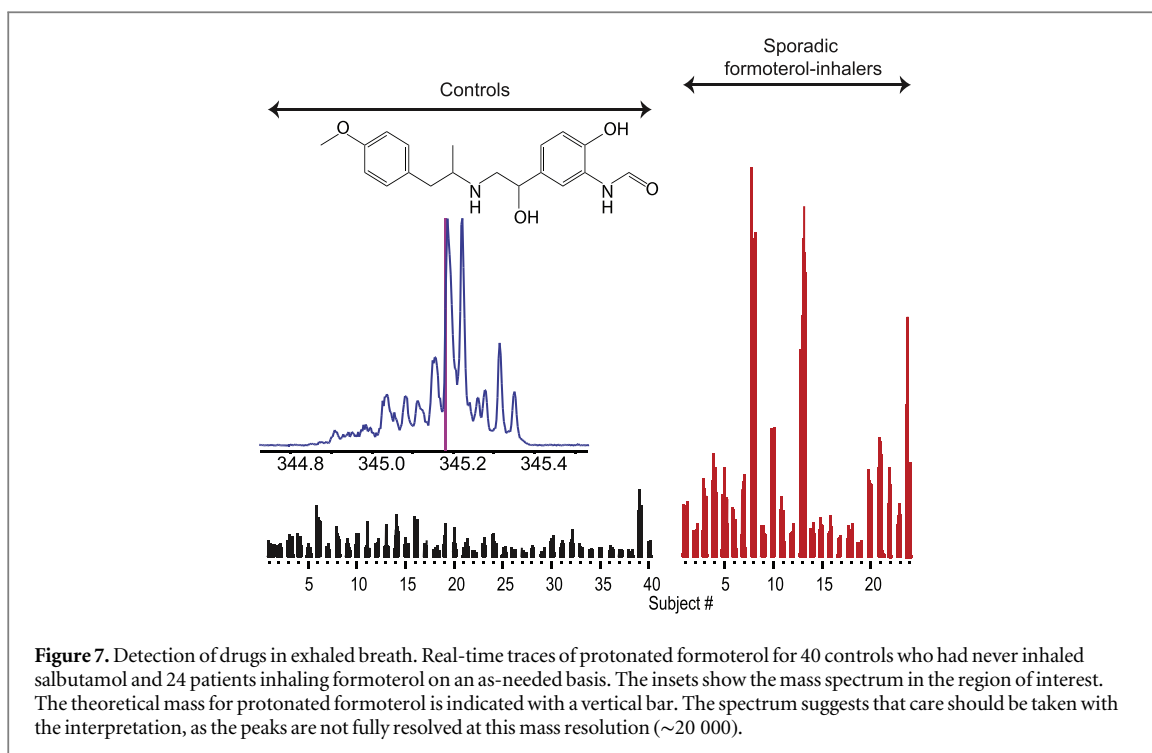
**Figure 6.** Tentative detection of drugs in exhaled breath. Real-time traces for protonated salbutamol (a) and its main metabolite salbutamol-4-O-sulfate (b) for 40 controls who had never inhaled salbutamol and 13 patients inhaling salbutamol on an as-needed basis. The highest levels were found for four patients suffering from COPD, suggesting that they inhale salbutamol more frequently than the asthma patients. The insets show the mass spectra in the region of interest. The theoretical mass for both ions is indicated with a vertical bar. The spectra suggest that care should be taken with the interpretation, because the peaks are not fully resolved at this mass resolution ( $\sim 20\,000$ ).

sebacic acid (Gaugg *et al* in preparation), and the latter an unsaturated form of it. As to the identified hydroxydecanoic acid, the network shows a correlation with sebacic acid ( $r = 0.7$ ),  $C_9H_{14}O_4$  ( $r = 0.66$ ) and  $C_{10}H_{18}O_3$  ( $r = 0.69$ ). The striking correlation between these compounds is demonstrated in figure 5(b), which shows the  $\log_2(\text{fold change})$  of hydroxydecanoic vs.  $\log_2(\text{fold change})$  for  $C_{10}H_{18}O_4$ ,  $C_9H_{14}O_4$  and  $C_{10}H_{18}O_3$ . While the complete network remains to be fully characterized, overall, our breath analysis data are consistent with previous studies that describe significant increase of non-esterified fatty acids [33], and in general with the lipolytic effects of beta-adrenergic agonists [34]. Moreover, these changes in fatty acid concentrations are known to happen very rapidly, in the order of minutes [35, 36], in line with our own observations here. Additional correlation networks are shown in figure S1.

### Drug detection

We have just discussed that SESI-HRMS seems to be sensitive and selective enough to capture significant changes of exhaled metabolites such as fatty acids shortly after salbutamol inhalation, in contrast to a placebo group. We argue that such information may provide a more comprehensive understanding of the mechanism of action of drugs allowing, for example, responders and non-responders to medication to be identified [37]. Obviously, monitoring of the administered drug itself would complement such metabolic information. In this regard, we have recently shown the potential of SESI-HRMS as a rapid and non-invasive platform to perform pharmacokinetic studies of injected drugs by measuring them *in vivo*, in mouse breath [38, 39]. Initially, we did not observe obvious changes in salbutamol levels above the background levels. One explanation could be that the chemical background in the  $m/z$  region where the salbutamol is expected (i.e.  $m/z\,240.1594$ ;  $[C_{13}H_{21}NO_3 + H]^+$ ) was





generally very crowded. Figure 6(a) shows representative mass spectral data at nominal mass 240 along with the chemical structure of salbutamol. For reference, the theoretical exact mass where protonated salbutamol would be expected is indicated with a vertical line. Clearly, even at mass resolution as high as  $\sim 20\,000$ , the separation capability is insufficient to discriminate salbutamol from other isobaric species [20, 40]. We nevertheless inspected the time traces for protonated salbutamol, as well as for its main metabolite, salbutamol-4-O-sulfate (figure 6(b);  $m/z$  320.1162;  $[\text{C}_{13}\text{H}_{21}\text{NO}_6\text{S} + \text{H}]^+$ ) [41]. Figure 6 shows the time traces for salbutamol (a) and salbutamol-4-O-sulfate (b) for the 40 healthy controls that had never inhaled salbutamol and 13 patients that according to their medical records inhale salbutamol on an as-needed basis. The first four of the latter were COPD patients, and nine suffered from asthma. Note that the traces shown correspond to the baseline measurement, when salbutamol was not yet inhaled. While we cannot give definite proof that these two mass spectral peaks indeed correspond to salbutamol and its main metabolite, the high correlation between the two sets of time traces suggests an association. Also, the intensity of the time traces seems in general higher in the patients, especially in the COPD group.

To further investigate whether common bronchodilators can be detected in breath by SESI-HRMS, we expanded our data analysis. In particular, according to their medical records, fourteen of the patients were inhaling formoterol on an as-needed basis. Formoterol is a long-acting beta2-agonist used in the management of asthma and COPD. Its chemical structure is shown in figure 7, along with a typical mass spectrum in the region of  $m/z$  345. The exact mass at

which this molecule would be expected in protonated form  $[\text{C}_{19}\text{H}_{24}\text{N}_2\text{O}_4 + \text{H}]^+$  is  $m/z$  345.1809, which is indicated in the mass spectrum by a vertical bar. As in the case of salbutamol, many isobaric species prevent us from clearly resolving the expected peak. However, similarly to salbutamol, the time traces tend to be higher in the patients using the medication on an as-needed basis as compared to the healthy controls who did not inhale any medication. Further investigations via UPLC-MS/MS analysis of EBC and higher resolution mass spectrometry are required to confirm that salbutamol and formoterol can be detected in breath. However, the evidence shown in previous studies where drugs such as ketamine (MW 237 Da) were unambiguously detected in breath and correlated with plasma levels [38, 39] and the data shown in figures 6 and 7 strongly supports the idea that SESI-HRMS is suitable to monitor drugs and their metabolites in breath. If this hypothesis is confirmed, it additionally opens attractive possibilities to screen for doping agents in breath of athletes. Note in this regard that all beta2-agonists are currently prohibited in and out of competition by the World Anti-Doping Agency [42].

While this work suggests that breath analysis could contribute to ongoing efforts to elucidate response mechanisms to therapeutic intervention and possibly drug monitoring, this study has—of course—limitations. For example, the unambiguous identity of the molecules significantly altered, precludes gaining further insights on reprogrammed metabolic pathways associated with salbutamol. In this regard, we cannot entirely exclude the possibility that some of the changes observed are actually due to bronchial dilation, resulting in improved gas exchange at the lung level.

## Conclusions

We aimed at exploring the concept of inhalational drug monitoring via real-time breath analysis. We demonstrated that analysis of exhaled breath by real-time mass spectrometry allows one to capture rapid metabolic changes induced by inhaled medication. We confirmed that the use of high resolution and high mass accuracy mass analyzers with MS/MS capabilities is crucial to determining the identity of some of these compounds. We noted that homologous series of compounds tended to correlate, suggesting that cascades of metabolic changes were monitored. For example, observed intensity changes of families of fatty acids are consistent with previous blood-based studies. Uncovering altered metabolic routes is likely to provide insights into the mechanisms by which activation of beta-adrenergic receptors lead to relaxation of smooth muscles in the airways. We also conclude that a mass resolution of  $\sim 20\,000$  is clearly insufficient to unambiguously detect the inhaled drugs investigated in this study. Our data suggests that higher resolving power may allow for the unambiguous detection of salbutamol and formoterol in breath. Overall, this study supports the notion that therapeutic drug monitoring is plausible via real-time breath analysis.

## Acknowledgments

This work was supported by grants from the Swiss National Science Foundation (CR23I2\_149617 and 32003B\_143365/1) and a Marie-Curie European Reintegration Grant (to PMLS) within the 7th European Community Framework Programme (276860).

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