Breath analysis for rapid detection and phenotyping of obstructive sleep apnea

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Obstructive sleep apnea (OSA) is a highly prevalent sleep related breathing disorder associated with increased morbidity and mortality. OSA is usually diagnosed by in-laboratory polysomnography which is cumbersome for the patient, time-consuming and costly. In addition, such tests are uninformative of the implications of OSA at the molecular level. In this randomized controlled translational study, we deployed a mass spectrometry technique that allows instantaneous non-invasive analysis of exhaled metabolites. The investigation of exhaled metabolites of patients on therapeutic- and sub-therapeutic continuous positive airway pressure revealed that OSA leads to a significant alteration of metabolites involved in different metabolic pathways. This metabolic fingerprint not only allows for an accurate detection of OSA, but also provides insight into its pathophysiological consequences. This novel breath analysis technique paves the way to i) rapid diagnosis of OSA ii) understanding of disease mechanisms, and thus progress in developing novel treatments and individualizing therapy.

Obstructive sleep apnea (OSA) is a highly prevalent[1] and underdiagnosed[2] sleep-related breathing disorder commonly associated with daytime sleepiness, increased rate of accidents[3], vascular dysfunction and hypertension[4], adverse cardiovascular outcome[5,6], and diverse metabolic changes[7]. The major biological mechanisms underpinning the association between OSA and its systemic pathophysiologic consequences are thought to include apnea-related intermittent hypoxia leading to increased oxidative stress and increased sympathetic activity and arousal-induced reflex sympathetic activation[8].

The gold-standard diagnostic test for OSA is in-laboratory polysomnography. Alternatively, portable home monitoring devices can diagnose OSA with sufficient accuracy in subjects with a high pre-test probability for OSA. However, all sleep studies are time-consuming and costly, and sometimes inconclusive due to technical artifacts or impaired sleep in an unaccustomed environment. Moreover, usual measures of disease severity derived from sleep studies do not allow reliable differentiation between different phenotypes of OSA, i.e. cannot identify subjects who are susceptible to metabolic, neurologic and cardiovascular effects of OSA.

OSA can be effectively treated with nocturnal continuous positive airway pressure (CPAP) which abolishes apnea and hypopnea and thus prevents the pathophysiologic consequences of OSA. Short term CPAP therapy withdrawal, in patients hitherto compliant with CPAP for two weeks, can be applied to efficiently investigate the effects of OSA recurrence on physiology[4].

Exhaled breath contains biochemical information about metabolism and its alterations. Most of the numerous exhaled compounds in breath are not produced in the lungs but released along the blood-gas barrier during gas-exchange. Metabolic alterations are reflected in perturbed metabolic profiles that may be used to support the diagnosis of diseases such as OSA. This hypothesis is supported by studies suggesting that sleep apnea results in altered plasma metabolites[9]. Besides supporting the diagnostic process, traditional plasma-based metabolomics analyses deliver detailed biochemical information that allows insight into complex pathophysiological processes. The main drawback is that these procedures are time-consuming and labor-intensive. Alternatively, it may be possible to take advantage of the wealth of metabolic information contained in exhaled breath to rapidly identify OSA-related metabolic profiles using electronic sensors[10,11]. However, the poor chemical specificity of electronic sensors does not allow for structural elucidation of the molecules imparting such OSA-specific breathprints, limiting the applicability and usefulness of this technology. Here, we propose an approach combining the analytical power of mass spectrometry with the speed

and non-invasiveness of electronic sensors to provide a comprehensive analysis of exhaled breath metabolites in real-time. This technique, dubbed secondary electrospray ionization-mass spectrometry (SESI-MS), has shown promise in detecting a wide range of metabolites in breath[12,13].

The objective of the current study was to apply this novel technique in a clinical setting to establish whether there is a disease specific profile of exhaled breath in patients with OSA, which might be used to both confirm the diagnosis and also provide insight into the pathophysiology of OSA on a metabolic level.

RESULTS

Effects of CPAP withdrawal on obstructive sleep apnea severity

28 patients with moderate to severe OSA (anthropometric data shown in **Supplementary Table 1**) previously effectively treated with CPAP were randomized to either continue therapeutic CPAP, or to have it replaced with a sub-therapeutic device, thus withdrawing effective treatment for two weeks (**Supplementary Methods and Supplementary Fig. 1**). CPAP withdrawal was associated with return of OSA as evidenced by a significant increase in cyclic dips of oxygen saturation recorded by pulse oximetry. As a result, the mean difference between groups in oxygen desaturation index (ODI) at two weeks was +30.3/h; 95%CI +19.8 to +40.7/h; $p<0.001$.

Rapid breath mass spectrometric fingerprints in OSA patients

SESI-MS, a real-time technique, allows rapid screening of patients[14]. This is illustrated in **Figures 1a-d**, which show the signal intensity of one exemplary breath metabolite (pentenal) as a function of time. It shows the baseline and follow-up measurements of one subject from the therapeutic CPAP group (**Figs. 1a-b**) and the baseline and follow-up measurements of one subject from the CPAP-withdrawal group (**Figs. 1c-d**). The signals increase during the exhalation cycles, providing good repeatability in signal intensity among the replicates. Three or four replicate exhalations per patient were recorded in approximately three minutes. Signal intensity in the CPAP treated subject remained essentially unchanged ($7.4x10^3$ to $5.7\ x10^3$ counts; **Figs. 1a and 1b**, respectively), whereas the average signal in the subject having withdrawn CPAP, and thus experiencing a recurrence of OSA, increased significantly, from 6 $x10^3$ counts to $27x10^3$ (**Figs. 1c and 1c**, respectively).

CPAP withdrawal alters breath metabolite profiles

Further identification of the features that changed significantly after CPAP withdrawal was accomplished using hypothesis testing and controlling for the false discovery rate (**Supplementary Methods**). Between-groups comparisons revealed that at follow-up, 61 features significantly increased and 1 decreased in the CPAP-withdrawal group. In contrast, no significant change in breath signal intensity of these compounds was found in the therapeutic CPAP group (**Supplementary Table 2**). Pentenal was confirmed as one of the exhaled metabolites that remained unaltered on CPAP treatment, but increased significantly after CPAP withdrawal (**Fig. 1e**). **Supplementary Figure 2** shows additional examples of metabolites altered after CPAP withdrawal.

Association of breath signal intensities with sleep apnea severity

In addition to identifying metabolites changing upon CPAP withdrawal, we sought to determine whether disease severity may be mirrored by the concentration of some metabolites in breath. Fifty-four features correlated significantly with changes in ODI as a measure of disease severity. **Supplementary Table 3** lists the breath signals that showed a significant association between changes in breath signal intensity and ODI. Among them, pentenal correlated with disease severity ($r = 0.54$; 95% CI =0.2-0.78; $p < 0.003$). **Figure 1f** illustrates the association of changes in exhaled pentenal and ODI for the 28 study participants. Additional examples are shown in **Supplementary Figure 3.** The overall correlation of these compounds with disease severity can be visualized by reducing the significantly correlating features to one dimension using principal component analysis (PCA). **Supplementary Figure 4** displays the change (Δ) in ODI vs. the first principal component score ($r = 0.59$; $p < 0.001$).

OSA prediction based on breathprints

A blind prediction of OSA (defined as ODI > 15/h) based on the breath mass spectral fingerprints, was done by a leave-one-out-cross-validation (LOOCV). Predictions were computed using the top 15 most informative features, as selected by a random forest classification algorithm[15]. The LOOCV resulted in a sensitivity of 92.9% and specificity of 84.6% (confusion matrix and diagnostic accuracy are shown in **Supplementary Tables 4** and **S5**). **Figure 2a** shows the receiver operating characteristic (ROC) curve of the classification. The computed area under the curve was 0.874. **Supplementary Table 6** lists the features selected during the LOOCV. A multidimensional scaling plot to the proximity matrix using

the top 19 most frequently selected features suggests two distinct groups (**Fig. 2b**). Similarly, **Figure 2c** shows the resulting dendograms and a heat-map of a hierarchical cluster analysis. Two distinct clusters (i.e. "OSA" and "no OSA") are revealed.

Chemical identification of exhaled breath compounds

A comprehensive structural elucidation strategy of some of the most discriminative compounds was performed. A total of 22 compounds were chemically identified with different degrees of certainty (**Supplementary Table 7**). For example, **Figure 2d** shows a head-to-tail fragmentation mass spectrum of benzothiazole found in breath (top) and the pure standard (bottom). Additional fragmentation spectra are shown in **Supplementary Figure 6.**

To reveal any latent association across the identified compounds, the correlation of each metabolite with all other metabolites was computed. **Supplementary Figure 7** shows a heat-map of the correlation matrix for all the identified compounds (the 20 with the highest degree of confidence) in descending order of mean correlation from top to bottom. Note that the top ranked molecules are a series of furans and aldehydes. A further visualization of the connectivity network of the identified metabolites is shown in **Figure 2e**. Interconnected metabolites are based on their partial correlations (requiring $p < 0.01$).

DISCUSSION

This is the first study applying SESI-MS to extract information from the exhaled breath metabolome in OSA patients. The use of the CPAP therapy withdrawal design enabled us to study OSA specific breath patterns in a randomized controlled fashion, thus avoiding the possible biases of observational case-control studies.

The gold standard for OSA diagnosis, i.e. in-laboratory polysomnography, is technically demanding and time consuming. In addition, measures of OSA severity derived from sleep studies do not differentiate between distinct phenotypes of OSA, e.g. highly symptomatic subjects or subjects with a pronounced increase in sympathetic activity and thus increased vascular risk. For these reasons, a number of ongoing studies are attempting to define markers of sleep disorders at the protein and metabolic level, although much work remains to be done[16]. In contrast to other specimens, one advantage is that exhaled air carries easily accessible molecular markers, in particular in patients with lung and airway diseases. This possibility has been pursued by developing technological methods. For example, electronic noses have shown good discriminatory accuracy in OSA[17,18]. However, electronic noses do not allow chemical analysis, limiting their use in elucidating pathophysiological mechanisms

of diseases. State-of-the-art mass spectrometry has unparalleled chemical selectivity and sensitivity, however most available commercial instruments were developed for off-line analysis of liquid samples. Here we show that it is possible to take advantage of the powerful analytical capabilities of mass spectrometry, with minor hardware modifications, that make instantaneous analysis of gas-phase metabolites in breath feasible. The approach proposed here allows an immediate examination of breath with unparalleled chemical selectivity that 1) promises high diagnostic accuracy and at the same time 2) pinpoints the chemical structure of the exhaled molecules, with which a distinct metabolic fingerprint becomes recognizable. An additional natural advantage of examining metabolites *in vivo* is that any bias due to sample collection, storage and further manipulation is avoided. This is critical especially in the case of a gaseous sample such as breath.

In line with preliminary related work[17,18], we found that OSA has a distinct altered metabolic breath profile that can be read out and used for detection of the disease (**Fig. 2a**) within minutes (< 5 min/patient; **Figs. 1a-d**). Our data suggest that some metabolites are augmented in the breath of patients with OSA, and also correlate with disease severity (i.e. ODI; **Fig. 1f** and **Supplementary Figs. 3** and **4**). This suggests that these compounds may also be used to assess CPAP therapy effectiveness or compliance. Information derived from exhaled breath has the potential to characterize an individual's metabolic response to OSA which is important considering that to date, initiation of CPAP treatment mainly relies on symptoms and not on future vascular risk, metabolic burden or comorbid disease.

The fact that the most significant compounds identified belong to families of closely related molecules (e.g. a homologous series of aldehydes), provides high confidence in the overall quality of the data obtained and implies that they are not raised merely by chance. This confidence is strengthened by the fact that the series of compounds for a given family correlate significantly with each other (**Fig. 2e and Supplementary Fig. 7**). Most of the molecules identified here are known human metabolites related to different metabolic processes. For example, as shown in **Fig. 2e,** a family of saturated aldehydes (red frame) was significantly increased after CPAP withdrawal and correlate strongly with each other. Cell membrane lipid peroxidation leads to generation of various aldehydes. Subsequently, the released end-products may trigger different cellular events[19]. The closely related family of aldehydes found here reinforces the hypothesis of increased oxidative stress levels in OSA[20,21]. Isoprene, one of the identified discriminating molecules correlating with undecenal, is part of the mevalonic cycle (cholesterin synthesis)[22,23]. Isoprene plays a role in sleep

regulation[24-26] and is increased in stressful conditions such as exercise[27], myocardial infarction[28], and increased cardiac output[29]. Increased isoprene in the exhaled breath of OSA patients may be due to increased sympathetic activity. Furthermore, isoprenoids serve as a base frame for steroids and thus cortisol[30], and also play a role in lipid metabolism that is altered possibly because of oxidative stress in OSA[31].

Intriguingly, the family of aldehydes strongly correlates with a series of furans (**Fig. 2e and Supplementary Fig. 7**). Furans have been found in the breath[32] of healthy subjects. These compounds are related to smoking[32] and are also thought to be secondary metabolites from the gut microbiome[33]. The fact that only one participant in the withdrawal CPAP group was an active smoker suggests that the significant increase of alkyl furans in OSA may not be related to smoking but possibly to an altered gut flora, as found in a previous study[9]. Similarly, gut microbiota metabolites have been reported to be altered in sleep deprived humans[34]. Along the same lines, intermittent hypoxia has been found to alter gut microbiota diversity in OSA mice models[35]. Moreover, we also found a family of phenols, including cresol, which is a unique bacterial metabolite from protein fermentation[36]. These metabolites are toxic bacterial end-products that, in the case of patients with compromised renal function, have been found to accumulate in the circulation, resulting in increased levels measurable in blood[37]. Another gut microbiome-related identified compound is acetoin[33]. Thus our data reinforces the notion that the altered metabolism associated with OSA may perturb the metabolic interplay between the gut flora and its host. In this regard, our findings add to the hypothesis that there is an association between gut microbiome composition and clinical phenotype[38].

Another molecule found to be augmented in the breath of patients with OSA is benzothiazole and its closely related metabolite 2-(methylthio)benzothiazole. Benzothiazole has previously been reported in breath[39,40]. Interestingly, it has been found to be significantly increased in breath of patients with pulmonary arterial hypertension[17] and cystic fibrosis[41]. It seems to correlate with pulmonary arterial pressure and pulmonary vascular resistance[17]. The fact that it is significantly increased following CPAP withdrawal suggests that apnea related nocturnal hypoxemia indeed is associated with increased pulmonary arterial pressure. However, the fact that these sulfur compounds may be altered and ultimately exhaled in breath is puzzling. Benzothiazole derivatives are widely used industrial products and have been reported to occur in the environment (e.g. drinking water)[42]. The reason why these exogenous compounds appear to be significantly altered in pulmonary arterial hypertension, cystic fibrosis and OSA remains unknown. One hypothesis is that, as in the case of the toxic phenolic metabolites

produced by gut bacteria, OSA could be associated with an impaired capacity to detoxify endogenous as well as exogenous small molecules, leading to an accumulation in blood and hence increased levels in breath. Another sulfur-containing molecule identified in the current study was homocysteine thiolactone, which has been associated with pathological conditions; for example, plasma levels have been linked with the development and progression of vascular complications in diabetic patients[43]. In summary, our data support the association of OSA with increased sympathetic activity[8], oxidative stress[21] and perturbation of gut microbiota-host equilibrium.

In conclusion, the possibility of identifying exhaled metabolites in real-time and with high chemical selectivity, makes this technique attractive for use in research and clinical practice, i.e. to rapidly identify OSA and its specific phenotypes. These readily available metabolic signatures may be helpful in assessing disease severity, understanding mechanisms of disease and thus in individualizing therapy.

METHODS

Methods and any associated references are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

PMLS, EIS, LB, TG, DGG and NS performed the experiments. PMLS, EIS, DGG, MG analyzed and interpreted the data. EIS, YN, KEB, JRS, RZ and MK designed the study. PMLS, EIS and MK wrote the manuscript. All authors discussed the results and reviewed the manuscript critically.

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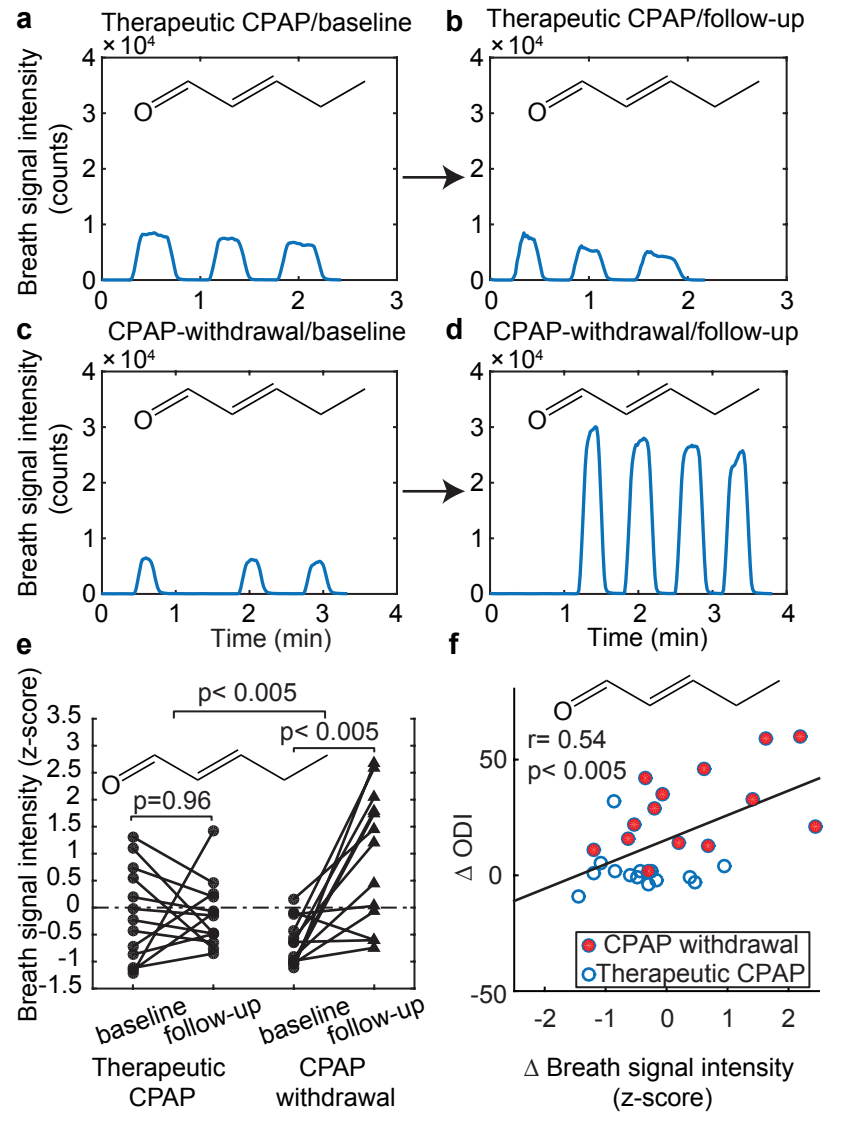
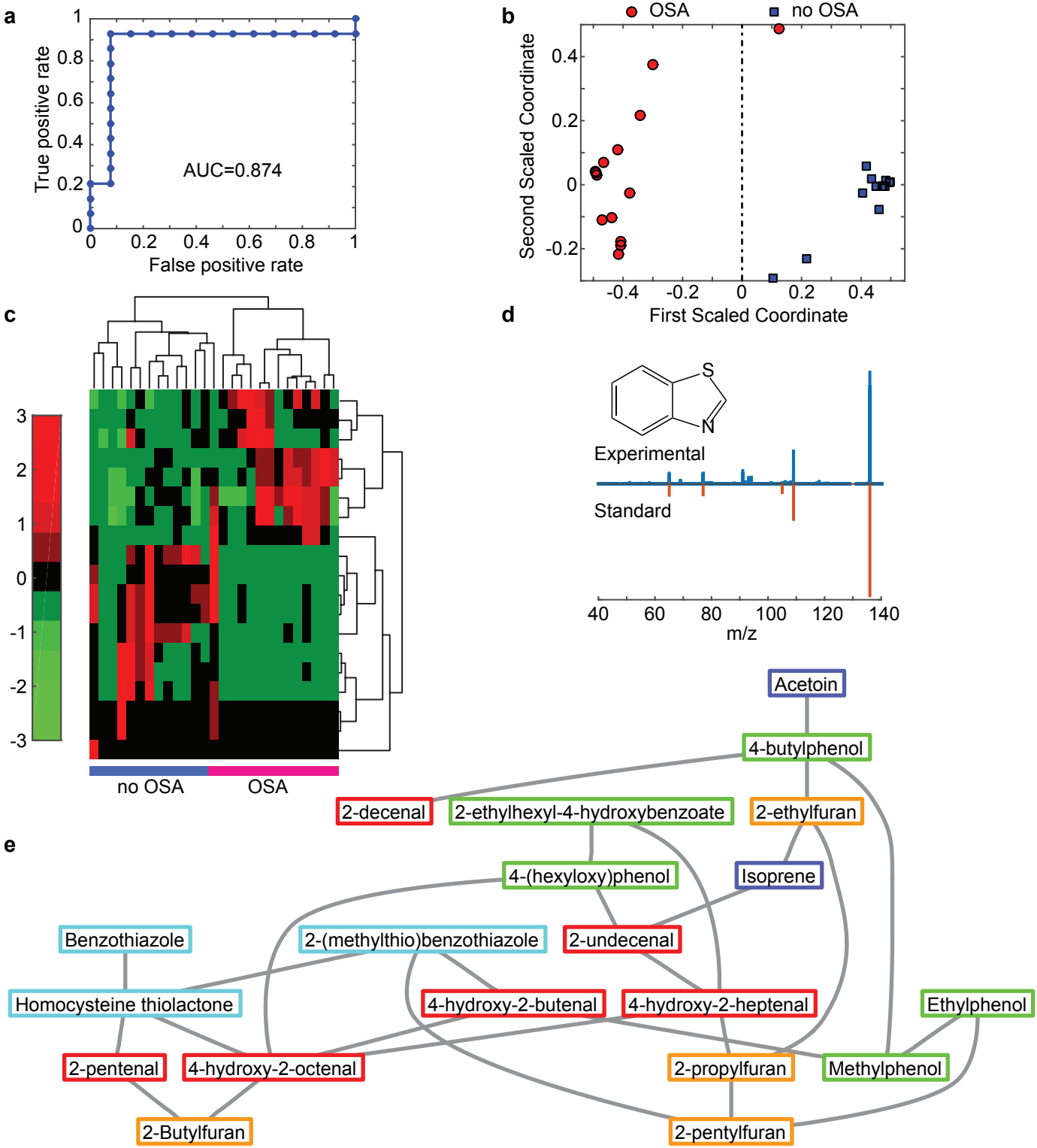
Figure Legends

Figure 1 CPAP withdrawal translates into altered exhaled metabolic profiles **(a-d)** Real-time breath analysis showing time traces of pentenal at baseline and follow-up in the therapeutic CPAP and the CPAP-withdrawal group at baseline and at follow-up. The peaks correspond to each exhalation. In approximately three minutes per subject, three to four replicate exhalations are recorded; **(e)** CPAP withdrawal leads to a significant increase of pentenal in breath (n = 26); **(f)** Correlation between changes in breath signal intensity and the oxygen desaturation index (as the measure of sleep apnea severity; n = 28).

Figure 2 OSA prediction and metabolites identification. **(a)** ROC curve obtained for OSA (i.e. ODI > 15/h) prediction (n = 27); **(b)** Multidimensional scaling plot to the proximity matrix (n = 27); **(c)** Hierarchical cluster analysis computed using the top most frequently selected features (n = 27); **(d)** head-to-tail fragmentation mass spectrum of benzothiazole found in breath (top) and the pure standard (bottom). **(e)** Visualization of the associations (based on partial correlation coefficients) of the identified metabolites. Compounds in red frames are a family of aldehydes; in yellow frames a series of furans; in green frames phenolic compounds; in light blue frames sulfur-containing compounds; in dark blue others.


a
Therapeutic CPAP/baseline
b
Therapeutic CPAP/follow-up
c
CPAP-withdrawal/baseline
d
CPAP-withdrawal/follow-up
Breath signal intensity (counts)
× 10^4
Time (min)
e
Breath signal intensity (z-score)
p< 0.005
p< 0.005
p=0.96
baseline
follow-up
baseline
follow-up
Therapeutic CPAP
CPAP withdrawal
f
r= 0.54
p< 0.005
Δ ODI
CPAP withdrawal
Therapeutic CPAP
Δ Breath signal intensity (z-score)


a
True positive rate
False positive rate
AUC=0.874
b
OSA
no OSA
Second Scaled Coordinate
First Scaled Coordinate
c
no OSA
OSA
d
Experimental
Standard
m/z
e
Acetoin
4-butylphenol
2-decenal
2-ethylhexyl-4-hydroxybenzoate
2-ethylfuran
4-(hexyloxy)phenol
Isoprene
Benzothiazole
2-(methylthio)benzothiazole
2-undecenal
Homocysteine thiolactone
4-hydroxy-2-butenal
4-hydroxy-2-heptenal
Ethylphenol
2-pentenal
4-hydroxy-2-octenal
2-propylfuran
Methylphenol
2-Butylfuran
2-pentylfuran