

# Effects of CPAP therapy withdrawal on exhaled breath pattern in obstructive sleep apnoea

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## **KEY MESSAGES**

### **What is the key question?**

The question was brought up whether a disease specific breath profile of obstructive sleep apnoea (OSA) giving insight into the systemic and metabolic consequences of OSA can be detected by the novel approach of breath analysis using untargeted real-time mass spectrometry.

### **What is the bottom line?**

This randomized controlled trial is the first study providing data on metabolic breathprints using untargeted real-time mass spectrometry in OSA and shows that real-time breath analysis by mass spectrometry allows accurate detection of untreated OSA within minutes.

### **Why read on?**

The findings of this study provide a basis for future research using the non-invasive approach of breath analysis allowing the identification of specific diseases such as OSA, novel disease mediators or markers that may serve as both diagnostic and therapeutic targets, and could be translated into clinical practice.

### **Conclusion for Twitter feed (120 characters)**

Analysis of exhaled breath by real-time mass spectrometry allows rapid non-invasive identification of untreated OSA with high accuracy.

## **ABSTRACT**

**Background.** Obstructive sleep apnea (OSA) is highly prevalent and associated with cardiovascular and metabolic changes. OSA is usually diagnosed by polysomnography which is time-consuming and provides little information on the patient's phenotype thus limiting a personalized treatment approach. Exhaled breath contains information on metabolism which can be analyzed by mass spectrometry within minutes. The objective of this study was to identify a breath-profile in OSA recurrence by use of secondary-electrospray-ionization-mass spectrometry (SESI-MS).

**Methods.** Patients with OSA effectively treated with continuous positive airway pressure (CPAP) were randomized to either withdraw treatment (subtherapeutic CPAP) or continue therapeutic CPAP for two weeks. Exhaled breath analysis by untargeted SESI-MS was performed at baseline and two weeks after randomization. The primary outcome was the change in exhaled molecular breath pattern. The trial is registered at ClinicalTrials.gov (NCT02050425).

**Results.** 30 patients with OSA were randomized and 26 completed the trial according to the protocol. CPAP withdrawal led to a recurrence of OSA (mean difference in change of oxygen desaturation index between groups +30.3/h; 95%CI:19.8/h,40.7/h,  $p<0.001$ ) which was accompanied by a significant change in 62 exhaled features (16 metabolites identified). The panel of discriminating mass-spectral features allowed differentiation between treated and untreated OSA with a sensitivity of 92.9% and a specificity of 84.6%.

**Conclusions.** Exhaled breath analysis by SESI-MS allows rapid and accurate detection of OSA recurrence. The technique has the potential to characterize an individual's metabolic response to OSA and thus makes a comprehensible phenotyping of OSA possible.

**Trial registration number:** NCT02050425.

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## INTRODUCTION

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

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 artefacts 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 apnoea and hypopnoea and thus prevents the pathophysiologic consequences of OSA. Short term CPAP therapy withdrawal, in patients hitherto compliant with CPAP, 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 apnoea results in altered

plasma metabolites.[8] The main drawback of traditional plasma-based metabolomics analyses is that these procedures are time-consuming and labour-intensive. Alternatively, the wealth of metabolic information contained in exhaled breath could be analysed using electronic sensors.[9 10] However, the poor chemical specificity of electronic sensors does not allow for structural elucidation of the molecules imparting such OSA-specific breathprints, limiting the usefulness of this technology. In this study, an approach combining the analytical power of mass spectrometry with the speed and non-invasiveness of electronic sensors was for the first time applied in OSA patients to provide a comprehensive analysis of exhaled breath metabolites in real-time. This technique, dubbed secondary electrospray ionisation-mass spectrometry (SESI-MS) [11-14], has shown promise in detecting a wide range of metabolites in breath and allows rapid screening of patients. [15 16]The objective of the study was to answer the question whether there is a disease specific profile of exhaled breath in patients with OSA applying untargeted mass spectrometry, which might be used to both confirm the diagnosis and also provide insight into the pathophysiology of OSA and underlying disease mechanisms on a metabolic level.

## **METHODS**

### **Trial design**

Randomised controlled parallel group trial.

### **Subjects**

Patients aged between 20 and 75 years with known moderate to severe OSA with an oxygen desaturation index (ODI) of  $>20/h$  at the time of diagnosis were eligible if they currently had an ODI  $>20/h$  ( $\geq 4\%$ -dips) during an ambulatory nocturnal pulse oximetry on the forth night off CPAP, and were effectively treated with CPAP for  $>12$  months [4 17] (supplementary file).

### **Sample size**

Sample size estimation was based on the assumption that a clinically relevant difference in ODI – reflecting the disease of interest – between therapeutic and subtherapeutic CPAP is  $15/h$  (SD12).[4 17] Based on this assumption, power calculation indicated that 28 patients were required in total to not miss a clinically relevant difference in ODI with a power of 90% ( $\alpha=0.05$ ) (supplementary file).

### **Patient evaluation and follow-up**

The study was conducted at the University Hospital Zurich and Swiss Federal Institute of Technology Zurich, Switzerland. The first patient was recruited in February 2014 and the last patient follow-up was completed in August 2014. OSA patients effectively treated with CPAP were randomly assigned (allocation-ratio 1:1) to one of the two treatment groups – either to continue therapeutic CPAP or to withdraw it by the use of a subtherapeutic device. Follow-up assessments were performed two weeks after randomisation. Allocation was determined by

unsorted envelopes. A member of the team not involved in patient enrolment and outcome assessment generated the allocation sequence and prepared the CPAP-devices. Patients remained blinded to allocation.

The study protocol was approved by the cantonal ethics committee of Zurich (KEK-ZH-Nr.2013-0536). The trial was conducted according to the Declaration of Helsinki and registered at ClinicalTrials.gov (NCT02050425). Written informed consent was obtained from participants before participation in the trial.

### **Sleep studies and CPAP devices**

Home sleep studies using the ApneaLink Plus-device (ResMed, San Diego, USA) were performed the night before the baseline and the follow-up breath-analysis. OSA severity was quantified by ODI ( $\geq 4\%$ -dips). Recurrence of OSA was defined as an ODI $>15$ /h in the follow-up sleep study (supplementary file).

### **Primary outcome**

The primary outcome was the change in exhaled breath pattern in response to CPAP withdrawal (exhaled breath pattern in OSA recurrence) and its diagnostic accuracy.

Real-time breath analysis was performed using secondary electrospray ionisation-mass spectrometry coupled to a quadrupole time-of-flight mass spectrometer (AB Sciex, Concord, Canada) (supplementary file).

### **Secondary outcome measures**

Secondary outcomes were changes in ODI, relationship between change in breath signal intensity and ODI, and the chemical identification of the compounds differentiating treated from untreated OSA (supplementary file).

## **Data analysis**

Changes within and between groups were analysed by dependent and independent t-test, respectively. In all cases, 5000 bootstrap samples were used to compute p-values. Subsequent estimate of the false discovery rate for multiple hypothesis testing was performed by computing q-values for each p-value as instructed by Storey.[18] Statistical significance level was set to  $\alpha < 0.05$ . Pearson's linear correlation coefficient and 95% bootstrap confidence intervals between changes in breath signal intensity and changes in ODI were computed. The most correlating features were subjected to principal component analysis (PCA). OSA prediction was accomplished in a leave-one-out-cross-validation (LOOCV) by feature selection and subsequent classification using a random forest classification algorithm.[19] The most discriminative features were used to compute hierarchical cluster analysis and multidimensional scaling of the proximity matrix.

Statistica V6.0 (StatSoft, Tulsa, USA) and Matlab (MathWorks, Natick, USA) were used for data analysis. Further details on data analysis procedures are described in the supplementary file.



## RESULTS

### Trial profile and patient characteristics

The study flow is shown in **figure 1**. 30 patients with moderate to severe OSA were randomly assigned to either continue therapeutic CPAP or to withdraw it by the use of a subtherapeutic CPAP device. 28/30 patients (14 in each treatment group) completed the study. One participant in each group was lost to follow-up: one patient randomised to therapeutic CPAP did not show up to the follow-up visit and withdrew from the study due to personal reasons, and one subject allocated to subtherapeutic CPAP was lost to follow up due to a relapse of a major depression requiring hospitalization. Two subjects did not adhere to the protocol: one participant randomised to therapeutic CPAP experienced difficulties handling the provided CPAP device and did not use it consistently thus having relevant OSA at follow-up, and one participant randomised to subtherapeutic CPAP manipulated his sham device. Thus the primary outcome was analysed in 26 patients who completed the trial per protocol. Baseline data are shown in **table 1**.

**Table 1: Baseline patient characteristics.**

|                                   | Therapeutic CPAP<br>(n = 13) | CPAP-withdrawal<br>(n = 13) |
|-----------------------------------|------------------------------|-----------------------------|
| Age, mean (SD), years             | 65.3±7.7                     | 67.6±6.5                    |
| Male sex, No (%)                  | 11 (85)                      | 9 (69)                      |
| BMI, mean (SD), kg/m <sup>2</sup> | 32.8±5.4                     | 34.4±6.2                    |
| Neck circumference, mean (SD), cm | 44.2±3.4                     | 40.2±3.4                    |
| Active smoker, No. (%)            | 1 (8)                        | 1 (8)                       |
| Former smoker, No. (%)            | 3 (23)                       | 8 (62)                      |
| Antihypertensive drugs, No. (%)   | 5 (38)                       | 9 (69)                      |

|   |             |             |
|---|-------------|-------------|
| <b>Statins, No. (%)</b>                             | 7 (54)      | 4 (31)      |
| <b>AHI at diagnosis, mean (SD), events per hour</b> | 55.3±22.6   | 45.8±16.4   |
| <b>ODI at diagnosis, mean (SD), events per hour</b> | 53.0±20.8   | 42.6±17.4   |
| <b>ESS at diagnosis, mean (SD), points</b>          | 13.0±2.9    | 12.3±4.8    |
| <b>AHI on CPAP, mean (SD), events per hour</b>      | 4.1±3.0     | 2.1±1.5     |
| <b>ESS on CPAP, mean (SD), points</b>               | 5.1±2.2     | 6.5±2.5     |
| <b>CPAP compliance, mean (SD), hh:mm</b>            | 06:50       | 07:08       |
| <b>FEV1/FVC, mean (SD)</b>                          | 0.80 (0.04) | 0.78 (0.02) |
| <b>FVC, mean (SD), % predicted</b>                  | 90 (9)      | 95 (16)     |
| <b>FEV1, mean (SD), % predicted</b>                 | 89 (13)     | 95 (16)     |

BMI = body mass index. AHI = apnoea-hypopnoea-index. ODI = oxygen desaturation-index. ESS = Epworth Sleepiness Scale. CPAP = continuous positive airway pressure. FEV1 = forced expiratory volume in 1 second. FVC = forced vital capacity.

### Effects of CPAP withdrawal on obstructive sleep apnoea

CPAP withdrawal was associated with return of OSA as evidenced by a significant increase in ODI (mean difference in change between groups, +30.3/h, 95%CI +19.8 to +40.7/h, P<0.001) (table 2).

**Table 2: Outcomes from sleep studies.**

| <b>therapeutic CPAP</b> | <b>subtherapeutic CPAP</b> | <b>treatment effect</b> |
|-------------------------|----------------------------|-------------------------|
| <b>(n=13)</b>           | <b>(n=13)</b>              |                         |

|            | baseline  | follow-up | baseline  | follow-up   | difference<br>in change | 95% CI          | p-value |
|------------|-----------|-----------|-----------|-------------|-------------------------|-----------------|---------|
| <b>ODI</b> | 4.3 (3.2) | 4.2 (1.7) | 3.9 (3.5) | 34.0 (16.3) | +30.3                   | +19.8,<br>+40.7 | <0.001  |

Data are presented as means (standard deviation). ODI = oxygen desaturation index (4% dips).

### Effects of CPAP withdrawal on exhaled breath

Exhaled breath pattern (primary outcome)

When compared to continuing CPAP, withdrawal of CPAP, and thus recurrence of OSA, was associated with altered exhaled breath patterns as determined by a statistically significant increase in 61 features and a decrease in one feature (**table E1**). **Figure 2a-e** shows one exemplary molecule which significantly increased in response to CPAP withdrawal. **Figure E3** shows additional examples of metabolites which allowed distinguishing between treated and untreated OSA. A blind prediction of OSA recurrence (defined as ODI >15/h) by a LOOCV using the 15 most discriminating exhaled features in each cross-validation, resulted in a sensitivity of 92.9% and specificity of 84.6% (**table 3** and ROC curve in **figure 3a**). Area under the ROC curve was 0.874 (95% CI = 0.612-1). For visualization of how these features discriminate both groups, **Figures 3b-c** shows a multidimensional scaling plot to the proximity matrix and a hierarchical cluster analysis.

**Table 3: Diagnostic accuracy of exhaled breath analysis in OSA**

| Predicted |        |       |
|-----------|--------|-------|
| OSA       | No OSA | total |
|           |        |       |

|           |        |    |    |    |
|-----------|--------|----|----|----|
| Condition | OSA    | 13 | 1  | 14 |
|           | No OSA | 2  | 11 | 13 |
|           | total  | 15 | 12 | 27 |

Confusion matrix of leave-one-out-cross-validation.

Summary of leave-one-out-cross-validation performance: Correct prediction rate 88.9%.

Sensitivity 92.9%. Specificity 84.6%. Positive predictive value 86.7%. Negative predictive value 91.7%. Positive likelihood 6.036. Negative likelihood 0.084. OSA = obstructive sleep apnoea defined by oxygen desaturation index  $\geq 15/h$ .

#### Chemical identification of exhaled breath compounds

A comprehensive structural elucidation strategy allowed chemical identification of 22 molecules in the breath of OSA patients (see for example benzothiazole in **figure 3d**). 16 of the 22 identified molecules were shown to significantly change in response to CPAP withdrawal. These molecules are listed in **table 4** (see also **table E1** and **table E3**). Most of the identified molecules stem from a cluster of chemical families (e.g. aldehydes). In addition, we found a strong association among the molecules within each chemical family (**figure 3e** and **figure E6**).

**Table 4: Identified exhaled metabolites changing in response to recurrence of OSA.**

| Breath signal features |              | Between-group changes (n = 26) |         |        |      |
|------------------------|--------------|--------------------------------|---------|--------|------|
| m/z                    | ID           | p-value                        | q-value | 95% CI |      |
| 69.0681                | Isoprene     | 0.020                          | 0.15    | 0.15   | 2.23 |
| 97.0622                | 2-ethylfuran | 0.022                          | 0.16    | 0.12   | 2.14 |

|          |                             |       |      |      |      |
|----------|-----------------------------|-------|------|------|------|
| 102.0888 | 2-pentenal                  | 0.002 | 0.15 | 0.55 | 2.55 |
| 109.0743 | Methylphenol                | 0.002 | 0.15 | 0.58 | 2.57 |
| 111.0782 | 2-propylfuran               | 0.003 | 0.15 | 0.47 | 2.40 |
| 118.0288 | Homocysteine thiolactone    | 0.019 | 0.15 | 0.14 | 2.08 |
| 123.0891 | Ethylphenol                 | 0.002 | 0.15 | 0.52 | 2.53 |
| 125.0930 | 2-Butylfuran                | 0.009 | 0.15 | 0.29 | 2.31 |
| 129.0885 | 4-hydroxy-2-heptenal        | 0.016 | 0.15 | 0.20 | 2.35 |
| 136.0193 | Benzothiazole               | 0.026 | 0.17 | 0.08 | 2.01 |
| 139.1090 | 2-pentylfuran               | 0.004 | 0.15 | 0.44 | 2.51 |
| 155.1522 | 2-decenal                   | 0.016 | 0.15 | 0.15 | 1.79 |
| 160.1298 | 4-hydroxy-2-octenal         | 0.019 | 0.15 | 0.17 | 2.38 |
| 166.0965 | Mevalonic acid              | 0.032 | 0.17 | 0.04 | 1.70 |
| 182.0068 | 2-(methylthio)benzothiazole | 0.020 | 0.15 | 0.13 | 1.99 |
| 196.1102 | Digitalose                  | 0.007 | 0.15 | 0.32 | 2.34 |

Of the 62 statistically significantly changing molecules, 16 were chemically identified. For more details (mean difference with confidence interval), see **table E5**.

#### Association of breath signals with sleep apnoea severity

In addition to identification of metabolites changing in response to CPAP withdrawal, those showing a significant association between changes in signal intensity and disease severity were identified. 54 features correlated significantly with changes in ODI as a measure of disease severity (see **table E2**), including all 16 identified metabolites constituting the disease-specific breath profile (see **figure 2f** and **figure E4**). The overall correlation of these

compounds with disease severity is visualized in **figure E5** by reducing the significantly correlating features to one dimension using principal component analysis (PCA).

## DISCUSSION

This is the first study applying untargeted mass spectrometry to extract information from the exhaled breath metabolome in OSA patients and paving the way for identifying a disease-specific breath profile. The use of the CPAP therapy withdrawal trial design enabled us to study OSA specific breath patterns in a randomised 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. symptomatic subjects or subjects with a pronounced increase in sympathetic activity or oxidative stress 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.[20 21] In contrast to blood and tissue specimens, exhaled breath carries easily accessible molecular markers, some of which are released along the blood-gas barrier and thus reflect changes in the blood and cell environment. It has been suggested that metabolites might move from different compartments into the blood and are then released via breath.[22]

Up to now there is only limited data in OSA derived from a small number of pilot studies on exhaled breath using electronic sensors.[9 10] These studies have shown good discriminatory accuracy in OSA. 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 used approach allows an immediate examination of

breath with unparalleled chemical selectivity that 1) delivers 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 [23]. This is critical especially in the case of a gaseous sample such as breath.

In line with preliminary related work, we found that recurrence of OSA has a distinct altered metabolic breath profile. The technology used in this study can determine such metabolic breath profiles and make it available for detection of the disease (**figure 3a-c**) within minutes (< 5 min/patient) (**figure 2a-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; **figure 1f**, **figure E4** and **figure E5**). 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 vascular risk, metabolic burden or comorbidities.

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 (**figure 3e** and **figure E6**). Most of the molecules identified here are known human metabolites related to different metabolic processes. For example a family of saturated aldehydes 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.[24] The closely related family of aldehydes found here reinforces the hypothesis of increased oxidative stress levels in OSA.[25] Isoprene, one of the identified discriminating molecules correlating with undecenal, is part of the mevalonic cycle (cholesterol synthesis).[26] Isoprene plays a role in sleep regulation and is increased in stressful conditions such as exercise, myocardial infarction, and increased cardiac output.[27-32] 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, and also play a role in lipid metabolism that is altered possibly because of oxidative stress in OSA.[33]

Intriguingly, the family of aldehydes strongly correlates with a series of furans (**figure 3e** and **figure E6**). Furans have been found in the breath of healthy subjects. [34] These compounds are related to smoking and are also thought to be secondary metabolites from the gut microbiome.[35 36] The number of former smokers in the CPAP withdrawal group was three fold higher than in the control group. However, this should not confound the finding of an increase in furans in the withdrawal group since it was only detected in response to CPAP withdrawal and was not present in the baseline breath analysis on therapeutic CPAP. The fact that only one participant in the withdrawal CPAP group was an active smoker suggests that the significant increase of alkyl furans in the CPAP withdrawal group is not be related to active smoking but possibly to an altered gut flora in response to OSA recurrence, as found in a previous study.[8] Similarly, gut microbiota metabolites have been reported to be altered in sleep deprived humans.[37] Along the same lines, intermittent hypoxia has been found to alter gut microbiota diversity in OSA mice models.[38] Moreover, we also found a family of phenols, including cresol, which is a unique bacterial metabolite from protein fermentation.[39] 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.[40] Another gut microbiome-related

identified compound is acetoin.[36] 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.[41]

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.[42] Interestingly, it has been found to be significantly increased in breath of patients with pulmonary arterial hypertension and cystic fibrosis.[43 44] It seems to correlate with pulmonary arterial pressure and pulmonary vascular resistance.[43] The fact that it is significantly increased following CPAP withdrawal suggests that apnoea related nocturnal hypoxemia indeed is associated with increased pulmonary arterial pressure. However, benzothiazole derivatives are widely used industrial products and have been reported to occur in the environment (e.g. drinking water).[45] The reason why these exogenous compounds appear to be significantly altered in pulmonary 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.[46] In summary, our data support the association of OSA with increased sympathetic activity, oxidative stress and perturbation of gut microbiota-host equilibrium.[7 25]

A possible limitation of our study is that the diagnostic accuracy of the OSA-specific exhaled breath pattern was not compared with polysomnography which is considered the gold

standard for diagnosis of OSA. Since this is a small sample of well characterized patients, generalizability to other OSA patients and a potential impact of comorbidities must be proven in future studies including more OSA patients. Another possible limitation of the study is that the role of potential confounders not related to the disease – e.g. increased caffeine intake because of increased sleepiness during the two week study period – cannot be determined. However, the applied short-term CPAP withdrawal model comparing treated and untreated OSA patients is less prone to general confounding factors that do not change in the short-term (e.g. BMI among many others). As to the correlations found between breath signals and disease severity, one cannot completely exclude the possibility that the correlations are due to differences between groups. Note nevertheless, the significant overlap between both populations. To avoid the anticipated influence of breathing manoeuvres on exhaled compounds[47] and to minimize artefacts[48], breath analysis was highly standardized including a constant exhalation pressure.

The exhaled compounds changing in response to CPAP withdrawal cannot be considered as disease-specific markers of OSA without caution before they are validated in a population of untreated OSA patients. Furthermore, in future studies, exhaled breath compounds should be compared with metabolomics of other body sources such as blood (hybrid metabolomics).[22] Nevertheless, the possibility of identifying exhaled metabolites in real-time and with high chemical selectivity is of general interest and makes this technique attractive for use in research and possibly in clinical practice. As such exhaled breath metabolomics can be applied for diagnostic goals, therapy guidance and monitoring, and to provide clues to underlying mechanisms.

In conclusion, real-time exhaled breath analysis by SESI-MS allows rapid and accurate detection of OSA recurrence. The technique has the potential to characterize an individual's

metabolic response to OSA and thus makes a comprehensible phenotyping of OSA possible. However, to draw definite conclusions on diagnostic accuracy, the findings of this study have to be validated in an untreated OSA population.

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## **Contributors**

Conception and design: MK, JRS, EIS, KEB, PMLS, RZ. Data acquisition: PMLS, EIS, LB, TG, DGG, NS, YNO. Analysis and interpretation of data: PMLS, EIS, DGG, MTG, YS. Drafting the article: EIS, PMLS, MK. Revising the article for important intellectual content and final approval: All authors.

## **Conflicts of interests**

All authors have completed the ICMJE uniform disclosure form at [www.icmje.org/coi\\_disclosure.pdf](http://www.icmje.org/coi_disclosure.pdf) and declare: no support from any organisation for the submitted work; no financial relationships with any organisations that might have an interest in the submitted work in the previous three years; no other relationships or activities that could appear to have influenced the submitted work.

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## **Trial registration number**

The trial is registered at [ClinicalTrials.gov](https://clinicaltrials.gov), registration number NCT02050425.

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## Figure Legends

### FIGURE 1. Consort diagram.

### FIGURE 2. Pentenal in exhaled breath.

Real-time breath analysis showing time traces of the signal intensity of one exemplary breath metabolite (pentenal) in a subject from the therapeutic CPAP and a subject from the CPAP-withdrawal group at baseline and at follow-up (**a-d**). The smoothed signals intensity traces increase during the exhalation cycles, providing good repeatability in signal intensity among the three to four replicate exhalations recorded within minutes. Signal intensity in the patient on therapeutic CPAP does not change from baseline (**a**) to follow-up (**b**) ( $7.4 \times 10^3$  to  $5.7 \times 10^3$  counts). In contrast, the patient undergoing CPAP withdrawal showed a signal increase from  $6 \times 10^3$  (**c**) counts at baseline to  $2.7 \times 10^4$  (**d**) counts at follow-up (i.e. 4.5 fold increase). Individual plots (**e**) of 2-pentenal in both groups ( $n = 26$ ) show significant breath compound enhancement. Correlation (**f**) between changes in breath signal intensity of pentenal and changes in oxygen desaturation index ( $n=28$ ) ( $r = 0.54$ ; 95% CI = 0.2-0.78;  $p < 0.003$ ).

### FIGURE 3. OSA prediction and metabolites identification.

(**a**) Receiver operating characteristic (ROC) curve obtained for OSA (i.e. ODI > 15/h) prediction ( $n = 27$ ); (**b**) Multidimensional scaling plot to the proximity matrix ( $n = 27$ ) computed using the most frequently selected features suggesting two distinct groups; (**c**) Similarly, hierarchical cluster analysis ( $n = 27$ ) visualizes the expected two distinct clusters; (**d**) head-to-tail fragmentation mass spectrum of benzothiazole found in breath (top) and the pure standard (bottom). (**e**) Visualization of the associations of the identified metabolites. Interconnected metabolites are based on their partial correlations (requiring  $p < 0.01$ ). 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.