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## Urinary proteomic profiling in severe obesity and obstructive sleep apnoea with CPAP treatment



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### ARTICLE INFO

#### Article history:

Received 23 January 2015

Received in revised form

23 June 2015

Accepted 24 June 2015

Available online 17 July 2015

#### Keywords:

Obstructive sleep apnoea

Severe obesity

Urinary proteomics

CPAP

### ABSTRACT

**Introduction:** Obstructive sleep apnoea (OSA) is common in obesity and is associated with cardiovascular and metabolic complications. Continuous positive airway pressure (CPAP) in OSA may lead to physiological changes reflected in the urinary proteome. The aim of this study was to characterise the urinary proteome in severely obese adult subjects with OSA who were receiving CPAP compared with severely obese subjects without OSA.

**Methods:** Severely obese subjects with and without OSA were recruited. Subjects with OSA were receiving CPAP. Body composition and blood pressure measurements were recorded. Urinary samples were analysed by Capillary Electrophoresis–Mass Spectrometry (CE–MS).

**Results:** Twenty-seven subjects with OSA-on-CPAP (age  $49 \pm 7$  years, BMI  $43 \pm 7$  kg/m<sup>2</sup>) and 25 controls without OSA (age  $52 \pm 9$  years, BMI  $39 \pm 4$  kg/m<sup>2</sup>) were studied. Age and BMI were not significantly different between groups. Mean CPAP use for OSA patients was  $14.5 \pm 1.0$  months. Metabolic syndrome was present in 14(52%) of those with OSA compared with 6(24%) of controls ( $p=0.039$ ). A urinary proteome comprising 15 peptides was identified showing differential expression between the groups ( $p < 0.01$ ). Although correction for multiple testing did not reach significance, sequences were determined for 8 peptides demonstrating origins from collagens, fibrinogen beta chain and T-cadherin that may be associated with underlying cardiovascular disease mechanisms in OSA.

**Conclusions:** The urinary proteome is compared in OSA with CPAP and without OSA in severe obesity. The effects of CPAP on OSA may lead to changes in the urinary peptides but further research work is needed to investigate the potential role for urinary proteomics in characterising urinary peptide profiles in OSA.

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Peer review under responsibility of Brazilian Association of Sleep.

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## 1. Introduction

In obstructive sleep apnoea (OSA), episodes of recurrent upper airway obstruction cause intermittent hypoxia, with frequent sleep arousals and fragmented sleep, resulting in excessive daytime hypersomnolence [1]. OSA has been increasing in prevalence, and it has been estimated that 13% of men and 6% of women between the ages of 30 and 70 years have moderate to severe forms of OSA [2]. This has been linked with the growing obesity prevalence as it is known that obesity is a risk factor for OSA. The prevalence of OSA in severe obesity has been estimated to be between 40% and 90% [3,4]. OSA is associated with hypertension, cardiovascular disease, type 2 diabetes and metabolic impairment [5].

It is known that OSA is independently associated with arterial hypertension and this may be related to repeated hypoxia and reoxygenation episodes that may cause ischaemia-reperfusion injury, as well as increased sympathetic activity, inflammation and oxidative stress [6]. The kidney has a role in blood pressure regulation and may be sensitive to the effects of intermittent hypoxia [7]. Nocturnal hypoxia may be associated with loss of kidney function [8], and a positive relationship has been observed between impaired glomerular filtration and desaturation frequency, suggesting an association between OSA and chronic kidney impairment [9]. Histological changes in renal glomeruli have been reported in severely obese patients with OSA [7], that may be related to pressor responses and endothelial dysfunction in OSA [10].

Capillary electrophoresis coupled to mass spectrometry (CE-MS) is a sensitive proteomic analytical technique that is robust with high reproducibility of results in an acceptable time frame [11]. In CE-MS, capillary zone electrophoresis is interfaced with high resolution mass spectrometry; electrophoretic separation of peptides is performed by CE according to charge and size, and the peptides are separated by application of high voltage and analysed in the mass spectrometer [11]. The accuracy, precision, selectivity, sensitivity, reproducibility, and stability of the CE-MS measurements have been previously demonstrated [12]. CE-MS has been widely used to study biomarker panels of urinary peptides for conditions such as coronary artery disease [13] and chronic kidney disease [14] that may indicate the presence of the relevant condition [15].

We have previously used CE-MS to characterise the urinary proteome in severe obesity with and without OSA, and to determine if there were significant differences in urinary peptide profiles that may potentially relate to OSA. 24 peptides were found to be differentially distributed between the two groups although these differences did not reach significance after correction for multiple testing. Sequences were determined for 8 peptides demonstrating origins from collagens and fibrinogen alpha [16]. The proteome in OSA prior to treatment may have been influenced by the effects of OSA potentially influencing the peptide panel and it was important to ascertain if there were changes in the urinary peptide panel with continuous positive airway pressure (CPAP) treatment.

CPAP therapy can improve glomerular hyperfiltration in OSA patients [17], perhaps by reducing renal renin-angiotension

system (RAS) activity, suggesting a potential effect of CPAP on kidney function [18]. The sensitivity of the kidney to repetitive intermittent hypoxia may influence the urinary proteome in OSA, and it is conceivable that the effects of CPAP treatment in OSA may lead to physiological changes reflected by the urinary proteome. Defining this in patients on CPAP may be useful as it would potentially reflect effects of CPAP on various mechanisms, such as RAS activity, oxidative stress, and endothelial dysfunction [19].

The use of urinary CE-MS analyses to study the urinary proteome in adult OSA and severe obesity with CPAP therapy has not been previously investigated. In this paper, we sought to investigate the hypothesis that the urinary peptide patterns were different between the two groups. To achieve this goal, we characterised the urinary proteome in severely obese subjects with OSA who were receiving CPAP compared to subjects without OSA.

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## 2. Methods

### 2.1. Research design

This was a case-control study comparing patients who were on effective CPAP treatment with subjects without OSA. The research involved patient recruitment and sampling, and subsequent CE-MS urinary proteome analyses.

### 2.2. Ethics statement

The study was approved by the research ethics committee (NRES 13/NW/0589) and was performed in accordance with the Declaration of Helsinki 2008. All patients gave written informed consent.

### 2.3. Participants

Severely obese patients from the weight management and sleep clinics at University Hospital Aintree were recruited from March 2012 to January 2013. Patients were selected according to the inclusion and exclusion criteria as assessed according to the clinical history, physical examination and analysis of the medical notes. At baseline, all subjects had spirometry testing and overnight polysomnography to confirm or refute the diagnosis of OSA. All subjects with OSA at baseline were previously untreated for their OSA and they were offered CPAP. All OSA subjects on CPAP and non-OSA subjects were followed-up from September 2013 to February 2014.

Patients were eligible if they were  $\geq 21$  years old and had body mass index (BMI)  $\geq 35$  kg/m<sup>2</sup>. Patients who were overweight or had mild obesity were not included because the majority of the patients were recruited from the hospital weight management service which manages patients with severe obesity. Exclusion criteria were patients with cardio-respiratory disease; current smokers or those with more than 10 pack years smoking history; kidney and liver disease; acute illnesses and pregnancy. Based on the exclusion criteria, we sought to control for potential confounders that may affect the interpretation of the urinary proteome including coronary artery disease, coexisting respiratory illness, renal

and liver disease, as well as other comorbidities. OSA patients who were not CPAP treatment or not compliant with their CPAP treatment were excluded from the analysis. Likewise, patients who had bariatric surgery were excluded from subsequent analysis as our aim was to study urinary proteome in subjects who were receiving CPAP treatment (CPAP  $\geq$  4 h per night) and non-OSA subjects.

## 2.4. Sample size

Although no previous studies evaluating the effect of CPAP on the adult urinary proteome using CE-MS in OSA and obesity have been performed, previous urinary proteomic work in paediatric OSA have investigated sample sizes of  $n=11$ –30 per group [20,21]. Therefore, we aimed for a minimum of 25 subjects per group.

## 2.5. Protocol

All patients attended a study visit day between 0800 and 1000 h and underwent a detailed history and physical examination. Body composition measurements, anthropometry, fasting blood and urine sampling and venous blood gases (to assess for hypercarbia) were performed. Daytime somnolence was assessed using an Epworth Sleepiness Scale Questionnaire (ESS) where a score  $>10$  indicated increased sleepiness. To ensure comparability and uniformity of assessments of patients, body composition, anthropometry and sampling were carried out at the same time at each study visit.

### 2.5.1. Blood pressure

Blood pressure was measured at the arm in a sitting position after a rest for at least 5 min at 1 min intervals between each measurement, using an oscillometric digital blood pressure monitor (HEM-705CP, Omron, Japan). The mean of three measurements was calculated.

### 2.5.2. Body measurements

All measurements were performed in triplicate. Weight and height were measured without shoes and with light clothing. Other measurements included neck circumference at the level of the laryngeal prominence; waist circumference midway between the lower rib and iliac crest; and hip circumference was measured horizontally over the widest part of the gluteal region. The tape measure was ensured to be snug and not compressing the skin, parallel to the floor with measurement at the end of a normal expiration. Body composition measurements used bioimpedance scales (TBF-521, TANITA, Tokyo, Japan). This method has been previously validated [22].

### 2.5.3. Biochemical measurements

Serum samples were collected using standard phlebotomy vials and immediately sent to the pathology laboratory for analysis in accordance with local protocol and standards. Serum biochemistry was measured using standard laboratory assays (Roche, UK). Blood gases were analysed with a Cobas Blood Gas Analyser (Roche, UK).

### 2.5.4. Metabolic syndrome

Subjects were assessed for metabolic syndrome according to the National Cholesterol Education Program (NCEP) guidelines [23]. Patients had metabolic syndrome if three or more risk factors were present: waist circumference (males  $>102$  cm; females  $>88$  cm), triglycerides  $\geq 1.7$  mmol/l, high density lipoprotein (HDL) cholesterol (males  $<1.04$  mmol/l; females  $<1.3$  mmol/l), blood pressure  $\geq 130/\geq 85$  mmHg, and fasting glucose  $\geq 6.1$  mmol/l.

### 2.5.5. Baseline sleep studies and spirometry

All subjects previously had overnight multichannel respiratory limited polysomnography (PSG) (Somnoscreen Digital PSG acquisition system, Version 2.0, SomnoMedics, Randeracker, Germany) and spirometry (Spiro Air LT system, Medisoft, Sorinnes, Belgium) to confirm or refute OSA. Apnoea was defined as a cessation of airflow for  $>10$  s. Hypopnoea was defined as a 50% reduction in airflow accompanied by a  $>4\%$  desaturation and a reduction in chest wall movement. OSA was diagnosed if the apnoea-hypopnoea index (AHI) was  $\geq 5$ .

### 2.5.6. CPAP therapy

Patients with OSA received standard CPAP therapy with S8/S9 Escape machines (ResMed, Abingdon, UK). CPAP compliance was based upon usage in hours per night (h/night) at the prescribed pressure. Compliance data was recorded by the CPAP machines and was downloaded using ResScan software (Version 4.2, ResMed, Abingdon, UK). This was assessed at each patient's most recent routine CPAP compliance/adherence clinic. Adequate compliance was defined as a usage time of  $>4$  h/night on  $>70\%$  of nights in the treatment group.

## 2.6. CE-MS materials and methods

### 2.6.1. Urine sample preparation

Spontaneously voided urine samples for CE-MS analyses were collected at the study visit using urine Monovettes (Sarstedt, Nümbrecht, Germany). Second void samples were collected at the same time each morning ( $\sim 09:00$  h) after an overnight fast for 8 h, at their study visit prior to the administration of any medication. The samples were stored at  $-80^\circ\text{C}$  in urine Monovettes until the sample preparation stage. Sample preparation for proteomic analysis was performed as previously described [24]. In this process, each 0.7 mL aliquot of urine was thawed immediately before use and diluted with 0.7 mL of 2 M urea, 10 mM  $\text{NH}_4\text{OH}$  containing 0.02% SDS. Each sample was ultrafiltered (2000 g, 60 min,  $4^\circ\text{C}$ ) to remove higher molecular mass proteins, such as albumin and immunoglobulin G using Centriscart ultracentrifugation filter devices (20 kDa MW) (Sartorius stedim Biotech, Goettingen, Germany) until 1.1 mL of filtrate was obtained. The filtrate from each sample was then applied onto separate PD-10 desalting columns (GE Healthcare, Uppsala, Sweden) equilibrated in 0.01%  $\text{NH}_4\text{OH}$  in HPLC-grade  $\text{H}_2\text{O}$  to decrease matrix effects by removing urea, electrolytes, and salts, and to enrich polypeptides present. Finally, all samples were lyophilised, and stored at  $4^\circ\text{C}$ .

### 2.6.2. CE-MS analysis

Samples were re-suspended in HPLC-grade H<sub>2</sub>O shortly before CE-MS analyses, as described [24]. CE-MS analysis was performed using a P/ACE MDQ capillary electrophoresis system (Beckman Coulter, Fullerton, USA) using a 90 cm 360 µm OD, 50 µm ID non-coated silica capillary with a tapered tip (New Objective, Woburn, USA) coupled to a microTOF MS (Bruker Daltonic, Bremen, Germany) [24]. A solution of 20% acetonitrile (Sigma-Aldrich, Taufkirchen, Germany) in HPLC-grade water with 0.94% formic acid (Sigma-Aldrich) was used as running buffer. The ESI sprayer (Agilent Technologies, Palo Alto, CA, USA) was grounded, and the ion spray interface potential was set between -4 and -4.5 kV. Data acquisition and MS acquisition methods were automatically controlled by the CE via contact-close-relays. For each sample, spectra were accumulated every 3 s, over a range of m/z 350–3000 over 60 min [24]. The accuracy, precision, selectivity, sensitivity, reproducibility, and stability of the CE-MS measurements have been previously demonstrated [12].

### 2.6.3. CE-MS data processing

Mass spectra were processed using MosaiquesVisu software (Mosaiques Diagnostics, Germany), including peak picking, deconvolution and deisotoping [25]. The software automatically examined all mass spectra from a CE-MS analysis for signals above the threshold (Signal-Noise-Ratio > 4). Only signals that were present in 3 consecutive spectra were accepted. The isotopic distribution was assessed and charge was assigned based on the isotopic distribution, using a matched filtering algorithm. This operation resulted in a list of signals defined by mass/charge, charge, migration time, and signal intensity (relative abundance defined by ion counts). This list was transformed into a dataset containing only mass, migration time, and signal intensity; signals that represent the same compound but with a different charge state were combined.

To allow for compilation and comparison of samples, normalised signal intensity was used as a measure of relative abundance. CE migration time and ion signal intensities of the samples were calibrated and normalised using internal polypeptide standards by linear regression [26]. ‘Housekeeping’ peptides that consistently appear in urine samples and are unaffected by disease states provided ideal reference mapping points for CE migration times [15]. Data sets were accepted only if they passed a strict quality control criteria: a minimum of 950 chromatographic features (mean number of features minus one standard deviation) must be detected with a minimal MS resolution of 8000 (required resolution to resolve ion signals with  $z \leq 6$ ) in a minimal migration time interval (the time window, in which separated signals can be detected) of 10 min. After calibration, the mean deviation of migration time (compared to reference standards) was below 0.35 min [24]. The resultant peak list included molecular mass (Da), normalised CE migration time (min) and normalised signal intensity for each peptide. All results were annotated into a Microsoft SQL database for statistical analysis.

Peptide profiles for OSA and non-OSA patients were compared for significant differences. Subsequently, the identity of the peptides was determined by matching against an

validated database that was previously developed using liquid chromatography-MS/MS analysis on a quadrupole time-of-flight mass spectrometer [15]. These human urinary peptide sequences can be accessed at ([http://mosaiques-diagnostics.de/diapatpcms/mosaiquescms/front\\_content.php?idcat=257](http://mosaiques-diagnostics.de/diapatpcms/mosaiquescms/front_content.php?idcat=257)).

### 2.7. Statistical analysis

Patient demographics were summarised by OSA group as means (SD) (or median (IQR) if non-normal) for continuous variables and frequencies (%) for categorical variables. Comparisons between groups were performed using a t-test for continuous variables which were normally distributed and a Mann-Whitney test for those that were not. Categorical variables were tested using a Chi-Square or Fishers Exact test depending on the expected frequencies. Assessment for normal distribution of patient data was by Shapiro-Wilks testing. Statistical significance for patient data was assigned at  $p < 0.05$ . Statistical analyses were carried out using SPSS version 20 (IBM Corp, Armonk, NY, USA).

For proteomics analyses, the non-parametric Wilcoxon test has been shown to be well-suited for proteomics data sets [27], and was used to determine significance in peptide abundance between the groups. Statistical significance was assigned at  $p \leq 0.01$ . Given the large number of possible peptides present, assessment of differences included an adjustment for multiple testing. Correction for multiple testing to account for false positives (false discovery rate) was performed using the Benjamini-Hochberg test.

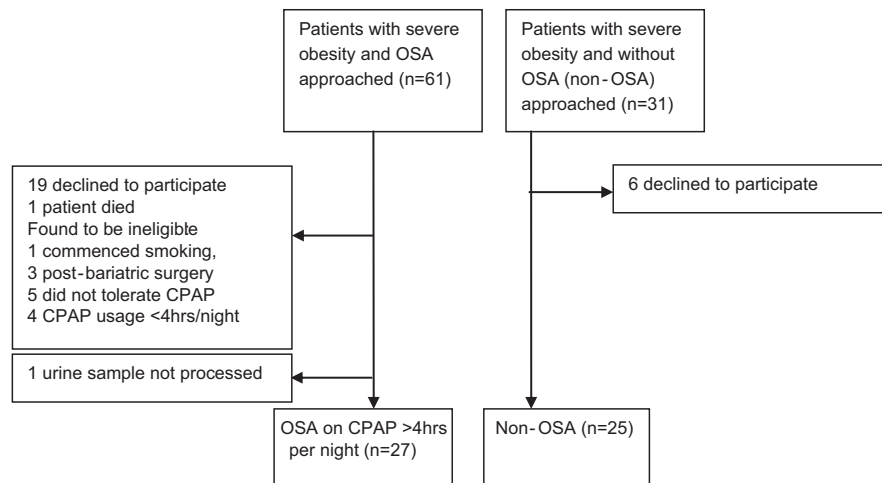
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## 3. Results

Fifty-three subjects who had been assessed at baseline were recruited. One urinary sample could not be analysed by CE-MS and therefore was excluded from the analysis. Therefore, 52 patients were entered into the study; 27 with OSA who were on CPAP and 25 without OSA (non-OSA) (Fig. 1). All subjects were of white European ethnicity. Patients attended their follow-up after a median of 15 (IQR 13,16) months. For patients with OSA, the mean CPAP use was 14.5 months (SD 1.0) and mean CPAP use per night was 4.6 h (SD 0.6). Mean CPAP pressure was 10 cmH<sub>2</sub>O (SD 1.0).

Clinical and biochemical parameters of the 52 patients according to those without OSA (non-OSA) and OSA-on-CPAP groups are presented in Table 1. The groups did not significantly differ in age, BMI and gender. More OSA subjects on CPAP had metabolic syndrome, particularly meeting the waist circumference, blood pressure, HDL and triglycerides components of the criteria. Although OSA subjects tended to have higher waist circumference, neck circumference, waist:hip ratio and body fat percentage by bioimpedance, these differences were not statistically significant. Smoking, diabetes, alcohol consumption and medication use were similar between groups.





**Fig. 1 – Study profile.**

### 3.1. Urine peptide results

#### 3.1.1. Peptide identification

We detected 1041 different peptides that were consistently found in more than 70% of the samples in at least one of the groups. The compiled urinary proteomic data from OSA on CPAP and non-OSA patients is shown in (Fig. 2). We found 15 peptides with significant unadjusted Wilcoxon  $p$ -values (all  $p \leq 0.01$ ) (Fig. 3). Although our initial data analysis showed a significant differential distribution in these peptides, it did not pass the more strict adjustment for multiple testing.

Sequences were determined for 8 peptides demonstrating origins mainly from collagen alpha chains. The 15 peptides are presented as in Table 2. The table contains the mass, the CE migration time, the peptide sequence, the name of the protein fragment, the SwissProt entry, the accession number, mean amplitude (ion signal intensity) and differential frequency of peptides within each group. Frequency was calculated based on the number of times each peptide was observed in each group. Mean amplitude was calculated based on the total signal intensity observed for each peptide divided by the number of subjects in each group.

## 4. Discussion

We used CE-MS to compare urinary profiles in severely obese adult subjects with OSA who were on effective CPAP and subjects without OSA at follow-up after a median of 15 months. To our knowledge, this is the first study to characterise the adult human urinary proteome in patients treated with CPAP and also to compare this in subjects without OSA. We were able to identify a urinary peptide pattern of 15 peptides that were candidates for differential distribution between the two groups with significant unadjusted Wilcoxon  $p$ -values, though these differences did not reach significance after correction for multiple testing. Nevertheless, the trends in the peptide panel characterise differences in the two groups studied. We identified sequences for 8 of these peptides that comprised breakdown products of collagen, as well as fibrinogen and a cadherin subtype. These

findings may represent the urinary proteome in OSA subjects on CPAP compared with subjects without OSA. The observed urinary peptide panel relating to CPAP-treated OSA and non-OSA groups suggests that there may be a potential role for CE-MS in the proteomic study of urinary profiles in OSA.

The findings from this study extend what is known about the urinary proteome in OSA and severe obesity. Using CE-MS, we previously observed a panel of peptides in a study of severely obese subjects with and without OSA [16]. In the present study, the characterised urinary proteome with CPAP comprised a similar make-up of collagens and a peptide related to fibrinogen. Additionally, one peptide for which no sequence data is as yet available was found to be present in the peptide panel in both studies. The nature of peptides that constitute both panels may relate to the underlying vascular changes present in OSA including sympathetic activation, oxidative stress and inflammation [6].

In this study, the majority of the identified peptides were fragments of collagen alpha-1 (I) and (III) in addition to fibrinogen beta chain and cadherin 13 (T-Cadherin) peptides. Fibrinogen beta chain has a role in fibrinogen synthesis and haemostasis, blood viscosity as a binding surface for proteins and as an extracellular matrix component, and may be associated with cardiovascular risk factors [28]. T-Cadherin is highly expressed in the vasculature including endothelial cells, has been implicated in the modulation of angiogenic activities and is associated with circulating levels of adiponectin that is important for vascular homeostasis. Additionally, links between T-cadherin and blood pressure, lipids, metabolic syndrome, type 2 diabetes and ischaemic stroke have been found in genome-wide association studies [29]. It should be noted that the mean amplitude of fibrinogen beta chain and cadherin-13 was lower in the CPAP-treated patients and it is possible that this may reflect decreased breakdown or differential expression with treatment.

The extracellular matrix contains a fibrillar collagen network that is regulated by fibroblasts and myofibroblasts. Changes in vasoactive peptides and proinflammatory cytokines, and activity of enzymes that process procollagen precursors to mature collagen including procollagen proteinases and collagen degradation enzymes (matrix

**Table 1 – Patient demographics were summarised by treated OSA and non-OSA groups as means (SD) (or median (IQR) if non-normal) for continuous variables and frequencies (%) for categorical variables. Significant p-values are highlighted in bold.**

Patient characteristics			
	Non-OSA (n=25)	OSA on CPAP (n=27)	p-Value
Age (years)	52 (9)	49 (7)	0.2252
Gender: male	13 (52%)	15 (56%)	0.7972
Smoked in past	6 (24%)	7 (26%)	0.8727
Alcohol intake: <8 units per week	17(68%)	19 (70%)	0.8532
BMI (kg/m <sup>2</sup> )	39(4)	43(7)	0.186
Neck circumference (cm)	42.1(4.3)	44.2 (4.6)	0.0916
Waist:hip ratio	0.94 (0.1)	0.98 (0.1)	0.2009
Body fat (%) TANTA scales	42.8 (8.8)	44.4 (8.4)	0.5029
Diabetes	5 (20%)	9 (33%)	0.2788
Hypertension	5 (20%)	6 (22%)	0.8446
BP-lowering medications	5 (20%)	6 (22%)	0.8446
Glucose-lowering therapy	5 (20%)	9 (33%)	0.2788
Lipid-lowering medications	8 (32%)	9 (33%)	0.9184
Metabolic syndrome	6 (24%)	14(52%)	<b>0.0391</b>
<b>Cardio-respiratory parameters</b>			
Systolic BP (mmHg)	128 (11)	129 (11)	0.9684
Diastolic BP (mmHg)	82 (8)	79 (7)	0.2276
Mean Arterial Pressure (mmHg)	100 (91,104)	98 (93,101)	0.161
Heart rate (beats per min):	71 (10)	74 (10)	0.2775
CPAP use per night (h)	–	4.6 (0.6)	–
CPAP pressures (cmH <sub>2</sub> O)	–	10 (1.0)	–
Epworth sleep score:	4 (3, 9)	3 (2, 11)	0.5015
O <sub>2</sub> saturations (%):	97 (96,99)	97 (96,98)	0.918
PCO <sub>2</sub> (kPa)	5.4 (5.1, 5.6)	5.3 (5.1, 5.5)	0.3882
AHI at baseline	2.4 (1.6, 3.7)	24.6 (14.0, 43.6)	<b>&lt;0.001</b>
<b>Metabolic parameters</b>			
Albumin Creatinine Ratio (mg/mmol)	0.4 (0.2, 0.5)	0.5 (0.3, 0.8)	0.1475
Creatinine (micromol/l)	77 (13)	78 (15)	0.8252
MDRD-GFR (mL/min/1.73 m <sup>2</sup> )	87 (13)	89 (15)	0.542
Low density lipoprotein (mmol/l)	2.7 (1.1)	2.6 (0.9)	0.7637
High density lipoprotein (mmol/l)	1.3 (0.4)	1.2 (0.3)	0.3228
HbA1c (mmol/mol)	37 (34, 40)	38 (35, 48)	0.1466
Total cholesterol (mmol/l)	4.7 (1.1)	4.6 (1.0)	0.7119
Fasting glucose (mmol/l)	5.4 (5.1, 5.9)	5.4 (4.7, 6.0)	0.8905
Thyroid stimulating hormone (mU/l)	2.2 (1.3)	2.1 (0.9)	0.9275
Triglycerides (mmol/l)	1.2 (0.8, 2.0)	1.6 (1.0,2.6)	0.1634
Bicarbonate (mmol/l)	24.0 (1.8)	24.2 (2.1)	0.6896
Highly sensitive C-Reactive Protein (hsCRP) (mg/L)	3.3 (1.1, 6.8)	3.0 (1.6, 5.8)	0.8189

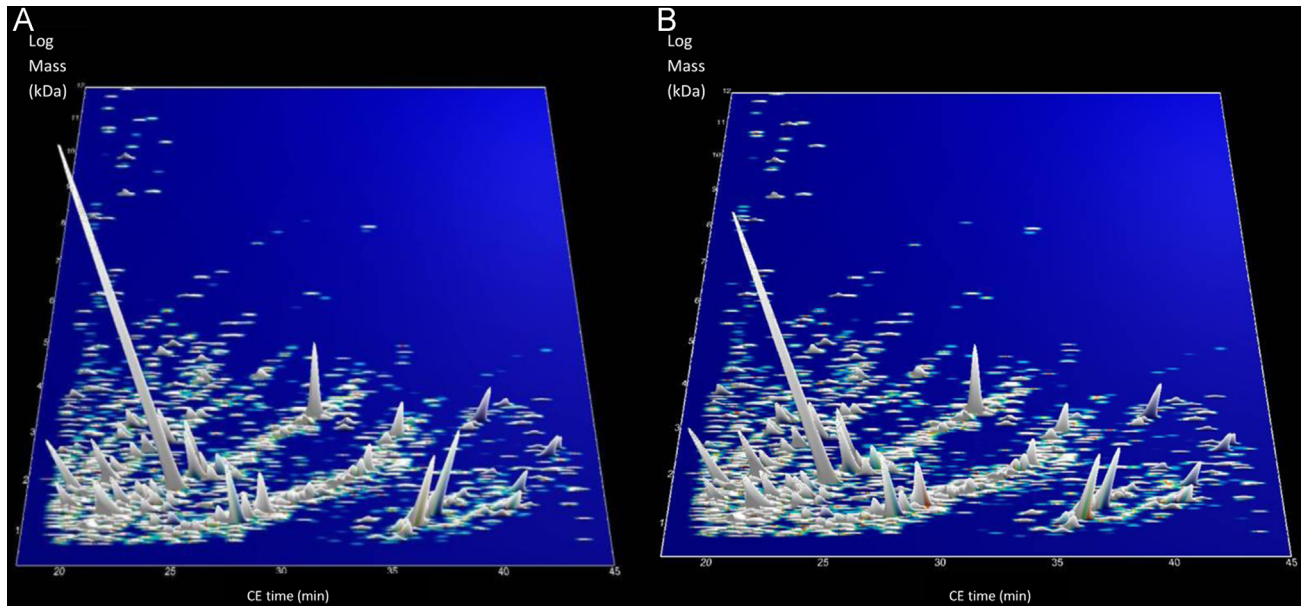
Abbreviations: AHI: Apnoea-Hypopnoea index, BMI: body mass index, BP: blood pressure, CPAP: continuous positive airway pressure, HbA1c: glycated haemoglobin, MDRD-GFR: Glomerular Filtration Rate using Modification of Diet in Renal Disease Equation, PCO<sub>2</sub>: partial pressure of carbon dioxide.

metalloproteinases) may account for the excretion of urinary collagen fragments [30]. Furthermore, it is known that alterations of the myocardial collagen matrix may occur in cardiovascular disease that may result in perturbations in vascular function [30]. In this context, it is known that OSA is associated with cardiovascular disease [31], and the peptides in the peptide panel may be a reflection of underlying cardiovascular changes associated with CPAP.

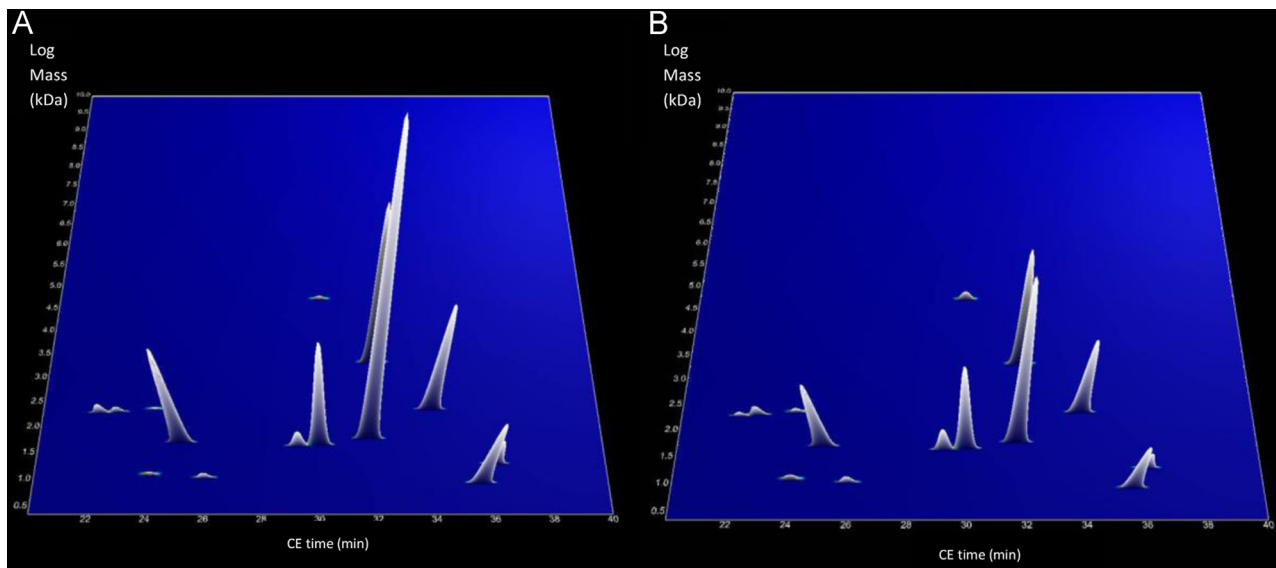
The mechanisms underlying the observed urinary peptide profile in this study remain unclear, but may be related to the treatment effects of CPAP on intermittent hypoxia and systemic hypertension. Post-translational modification of proteins has been postulated as a mechanism by which protein function is influenced by chronic intermittent hypoxia, and there is

evidence that this induces changes in the phosphorylation state of proteins associated with transcriptional activation, signal transduction pathways, and neurotransmitter synthesis [32]. Multiple lines of evidence underscore the importance of CPAP treatment in lowering BP in patients with severe OSA [33]. Additionally, OSA-mediated hypertension may be influenced by the effects of CPAP on reducing renin-angiotensin system activity with changes in renal perfusion [17,18]. It is conceivable that the treatment effects of CPAP on OSA disease progression may lead to the modification of proteins and changes in protease activity, and renal effects that may be reflected in the urinary proteome [15].

This study was limited by the lack of serial measurements of urine samples throughout the follow-up period and by



**Fig. 2** – Compiled 3-dimensional depiction of urinary peptide profile for non-OSA (A) and OSA-on-CPAP (B). The X axis represents CE migration time (min), Y-axis represents molecular mass (kDa, on a logarithmic scale), and the Z-axis represents mean signal intensity. (A) Non-OSA. (B) OSA-on-CPAP.



**Fig. 3** – 3-dimensional profile of 15 peptides that showed significant differences between non-OSA (A) and OSA-on-CPAP (B) groups. The X axis represents CE migration time (minutes), Y-axis represents molecular mass (kDa, on a logarithmic scale), and the Z-axis represents mean signal intensity. In order to demonstrate differences between the 15 peptides, **Figs. 2 and 3** are not of similar scale. (A) Non-OSA. (B) OSA-on-CPAP.

the presence of potential confounders such as metabolic syndrome and patient medications. It may be argued that the excretion of urinary peptides may vary during the day, because of physical activity, diet, or medications taken. We sought to control for this by sampling all participants at the same time each morning, at second void, after an overnight fast at their study visit prior to the administration of any medication. We envisaged that it was important for the urinary polypeptide panel to reflect ‘real-life’ clinical patients for it to be relevant and applicable. Therefore, controlling for

every potential confounding factor would not have been pragmatic and would have limited recruitment of potential participants. Repeat polysomnography was not conducted to prove if the AHI was sufficiently reduced on CPAP but the patients were managed according to local hospital protocol and were already established on their treatment at follow-up, with improved symptoms and ESS scores as assessed at their compliance/adherence clinic visits.

There were more patients in the CPAP-treated OSA group who had metabolic syndrome than non-OSA patients. This

**Table 2 – The table lists the peptide identification number (Peptide ID), experimental mass (in Da) and CE migration time (in min) for the 15 peptides in the OSA panel. For all sequence-identified peptides, the amino acid sequence, the name of the protein precursor and the amino acid positions within the protein's primary sequence (according to SwissProt) are presented. Frequency was based on the occurrence of each peptide within each group. Mean amplitude calculated based on the mean signal intensity of the peptide within each group.**

Peptide ID	Peptide mass (Da)	CE time (min)	Unadjusted Wilcoxon p-value	Area under the curve	Benjamini and Hochberg p-value	Sequence	Protein name	Start amino acid	Stop amino acid	SwissProt name	Accession number	Frequency OSA on CPAP	Mean amplitude OSA on CPAP (SD)	Frequency non-OSA	Mean amplitude non-OSA (SD)
61039	1813.715	31.69499	0.000532	0.77	0.499525	GmpGSp	Collagen alpha-1 (III) chain	537	555	CO3A1_HUMAN	gi124056490	1.0	1557 (656)	1.0	2616 (1196)
55756	1700.721	29.93699	0.00096	0.76	0.499525	GGpGSDGKpGPpG		1.0	913 (265)			1.0	1200 (292)		
130747	3359.578	31.89787	0.001473	0.75	0.511072	PpGADGQ FGAKGGEpGDAG AKGDAGPp GPAGPAGPpGPIG	Collagen alpha-1(I) chain	816	854	CO1A1_HUMAN	gi124056487	0.8	699 (487)	0.9	1237 (625)
167576	4759.125	29.94851	0.002237	0.73	0.582167		Collagen alpha-1(I) chain	800	810	CO1A1_HUMAN	gi124056487	0.7	69 (58)	0.3	24 (38)
15593	1066.478	25.95367	0.003683	0.72	0.645249	GDRGEpGPpGP						0.77	82 (88)	0.4	68 (205)
11249	980.2951	35.57253	0.004019	0.73	0.645249		Collagen alpha-1 (III) chain	180	198	CO3A1_HUMAN	gi124056490	0.8	529 (405)	1.0	808 (295)
54269	1675.708	29.20317	0.004713	0.72	0.645249	GpPGPpGTS GHpGSpGSpG						1.0	259 (87)	1.0	200 (112)
131294	3375.574	31.91691	0.005008	0.72	0.645249		Collagen alpha-1(I) chain	430	455	CO1A1_HUMAN	gi124056487	0.9	942 (569)	1.0	1311 (538)
90651	2385.054	33.94877	0.006009	0.72	0.645249							1.0	779 (399)	0.9	1168 (571)
88299	2339.085	22.66349	0.006386	0.72	0.645249	KGNSGEPGApGSK GDTGAKGGEpGPVG						0.8	102 (62)	0.6	63 (88)
87411	2321.165	22.05962	0.007984	0.70	0.645249	DKKREEAP SLRPAPPPIISGGGY	FGB protein (Fragment)					0.4	42 (69)	0.7	113 (125)
32874	1333.416	36.10802	0.008054	0.71	0.645249		Collagen alpha-1 (XXII) chain	1221	1232	COMA1_HUMAN	gi296434458	0.7	186 (155)	0.8	321 (184)
19969	1132.569	24.0363	0.008187	0.70	0.645249	PGKEGpGPQGP						0.7	49 (48)	0.4	20 (29)
91249	2401.078	24.0189	0.008734	0.70	0.645249		Cadherin-13					0.7	46 (50)	0.3	16 (26)
57584	1738.76	25.0428	0.009298	0.71	0.645249	FVHARTPHA EDmAEL						0.8	711 (477)	1.0	1115 (396)

SD: standard deviation. FGB: fibrinogen beta chain. OSA-on-CPAP: subjects with obstructive sleep apnoea receiving CPAP. Non-OSA: subjects without obstructive sleep apnoea.



was attributable to a combination of factors including waist circumference, blood pressure and lipid profiles. It would be difficult to separate metabolic syndrome from OSA in severe obesity as these conditions are closely associated. The incorporation of such factors in the discovery process may potentially lead to peptide profiles that may be more representative of patients with OSA in the severely obese population.

This study was limited by the relatively small sample size. Additionally, we did not directly compare the urine proteome in OSA patients directly before commencing CPAP treatment with findings after CPAP. However, it must be emphasised that this was foremost a discovery study, with the aim to use CE-MS to investigate urinary profiles in severely obese subjects with OSA who were receiving CPAP in relation to subjects without OSA. Our findings provide a novel insight into urinary peptide patterns in a well-characterised cohort of severely obese patients with OSA who received CPAP, and subjects without OSA. As this study was based on samples collected from patients at a specific point in time, a cause-and-effect relationship between the observed urinary proteome and CPAP itself cannot be established. Nevertheless, it would have been ethically questionable to perform a randomised placebo-CPAP controlled study comparable to our duration of CPAP use (>1 year) which is a strength of this study.

## 5. Conclusion

The findings from this study provide a unique insight of the urinary proteome in OSA with CPAP. The identified peptide panel includes collagen, cadherin and fibrinogen subtypes that may be related to mechanisms underlying cardiovascular disease in OSA. This may be associated with the treatment effects of CPAP on OSA disease progression influencing the expression of urinary peptides. There is a potential role for urinary proteomics in characterising urinary peptide profiles in OSA. Further work is still needed to investigate the use of proteomic methods in defining peptide profiles that reflect OSA and in the presence of CPAP that may potentially support CPAP treatment monitoring and progress or relating to the changes in cardiovascular risk profiles, potentially translating into better monitoring of OSA treatment and aid in the care of patients with sleep disorders.

## Funding

This research was supported by the University of Liverpool, St Helens and Knowsley Hospital and University Hospital Aintree, United Kingdom (Grant nos. JXR 10169 and JXR 10170).

## Conflict of interest statement

AT is employed by Mosaiques Diagnostics GmbH. HM is co-founder and co-owner of Mosaiques Diagnostics GmbH. All other authors have nothing to declare.

## Contribution

IS, RP, SC, ND, KH, JB, JW contributed to the design, analyses and conduct of the study. IS performed the CE-MS experiments. IS, JB, AT, AA, WM, HM contributed to the proteomic analyses. All authors contributed to the writing of the manuscript.

## Acknowledgements

We thank Dr. Stephen Embego, Tessa McKown (Sleep Laboratory); Stuart Wragg (Lung Function), Peter Thompson (Pathology Lab); Colin Armstrong (Liverpool John Moores University).

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